PHYTOCHEMICAL AND ENZYME SUPPLEMENTATION OF BROILER CHICKEN DIETS AND THE EFFECTS ON INTESTINAL MICROFLORA, NUTRIENT UTILISATION AND PERFORMANCE

BY

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Abstract

The objective of the work in this thesis was the evaluation of a range of plants and their extracts for dietary inclusion at low levels in growing broilers. These supplements were also tested in combination with a carbohydrase, a supplement normally added to cereal-based poultry diets to reduce the anti-nutrient effects of non-starch polysaccharides. The effects of feeding these supplements on growth performance, intestinal microflora composition, nutrient digestibility, ileal morphology and the caecal volatile fatty acid (VFA) profile was measured in the birds. Organoleptic analysis was carried out on meat produced from the birds in the third experiment.

A number of herbs and their essential oils (EO) were investigated, including *Thymus vulgaris* L. (thyme), *Origanum majorana* L. (marjoram), *O. vulgare* subsp. *hirtum* (oregano), *Achillea millefolium* L. (yarrow), *Rosmarinus officinalis* L. (rosemary) and *Allium sativum* L. (garlic). Three commercial condensed tannins (CT) were also tested, namely *Vitis vinifera* L. (grapeseed), *Vaccinium macrocarpon* L. (cranberry) and *Acacia mollissima* L. (mimosa).

In the first experiment, the different herb and essential oil supplements had both positive and negative effects on broiler performance. Differences were observed between birds fed the various plant species, and also in the form of plant supplementation used. Generally, thyme oil and yarrow herb dietary supplements had the most positive effects on performance, while oregano herb and yarrow EO were least suitable for dietary inclusion. At 28 days, the birds fed diets with thyme EO had an improved body mass (BM), compared to those fed diets with thyme herb, oregano EO and herb, and rosemary and yarrow EO supplements. Over the study period, the birds with thyme EO had an improved weight gain, compared to those fed the control diets, but the average feed consumption was not increased accordingly. The diets with thyme EO were consumed in greater quantities than the diets with yarrow and oregano EO. No treatment effects were observed on either the intestinal microflora populations or on nutrient digestibility. When compared to the other treatments, the reduced sialic acid concentration in the excreta of those birds fed diets with oregano herb and rosemary EO may have indicated an inhibition of endogenous losses. The primary reason for any treatment differences was considered to be variations in terpene compositions within the plant supplements.

The second experiment was carried out using thyme EO, as it had previously shown positive effects on growth performance, and a thyme EO with a high antimicrobial activity was chosen. This thyme EO was fed in combination with a carbohydrase, to test for the presence of synergistic or antagonistic interactions. In isolation, the carbohydrase improved BM, weight gain, feed consumption and FCR over the first 21 days, which resulted in an overall improvement in performance over the study period. However, thyme EO in the diets required an adaptation period, with linear decreases in feed consumption over the first 14 days. There were no interactions between the supplements in their effects on intestinal microflora populations, where the thyme EO reduced caecal coliforms but the carbohydrase had no effect. However, the results for nutrient digestibility suggested that commercial levels of carbohydrase with thyme oil inclusion at 1 g kg^{-1} produced optimal effects in the birds. The birds on this treatment had the highest coefficients of DM and OM digestibility, energy and nitrogen metabolisabilities, and also the highest AME. The carbohydrase was responsible for most of these improvements, but thyme EO inclusion increased dietary AME to a greater extent than was provided by the supplied gross energy. Carbohydrase increased some amino acid digestibility coefficients, and reduced the heights of intestinal villi and crypt depths in the ileum. Dietary thyme EO inclusion increased caecal lactic acid concentrations at 42 days, whereas the carbohydrase reduced acetic, lactic, n-butyric and total VFA concentrations at 19 days. The thyme EO therefore has some potential to exert a protective effect in the intestine, perhaps by a selective antimicrobial action. Both these supplements could be added together in wheat-based diets of an average quality to improve dietary digestibility.

The third growth experiment indicated both positive and negative effects associated with feeding various herbs, garlic and CT in diets with carbohydrase. The birds fed diets with garlic had an increased BM in the first 7 days, compared to those fed the other treatments. Average weight gains were increased in the birds fed diets with garlic, compared to all other treatments except the controls. Between study days 8-21, diets with garlic were consumed in greatest quantities, while those with mimosa were consumed least. The diets with mimosa CT reduced broiler performance over the first 21 days, while those fed with cranberry and grapeseed CT maintained a level of performance equivalent to the birds fed the control treatment during this period. The composition of a supplement may need to be changed over time to optimise its effect. Dietary cranberry inclusion reduced the coefficients of digestibility of DM and OM in broilers at 21 days, compared to those fed

the remaining diets, except for those with yarrow herb. The birds fed diets with cranberry had the lowest digestibility of N. No treatment effects were observed on energy digestibility at either 21 or 42 days. However, the birds fed garlic supplements had the highest coefficients of N digestibility, compared to those fed diets with rosemary, cranberry and mimosa CT. Generally, the birds fed diets with garlic had the highest coefficients of amino acid digestibility at 21 days, and were equivalent to those birds on the control treatment. The birds fed diets with garlic had a higher amino acid digestibility than those fed with rosemary, mimosa and cranberry supplements. The lowest amino acid digestibility overall was found in the birds fed diets with cranberry CT, but this was not significant when compared to those fed diets with rosemary and mimosa CT. VFA concentrations changed with time, and there were various minor treatment effects. An organoleptic assessment indicated that feeding garlic produced changes in meat properties, which were not necessarily detrimental to product flavour. A more comprehensive screening procedure may be required before including CT in diets, but the absence of negative effects in digestibility with grapeseed may indicate its suitability for dietary inclusion.

The work in this thesis supports the hypothesis that certain plants or their extracts have the ability to modify performance in growing broilers, either positively or negatively depending on the supplement chosen. This effect occurs primarily by changing the nutritional demands or growth in the microflora, which may in turn improve dietary utilisation by the birds. Optimal effects can be achieved by the combined inclusion of a carbohydrase with plant extracts, and should be further tested with different enzymes.

Declaration

This thesis has been composed by the author and has not been presented in any previous application for a degree. The work, of which this thesis is a record, was done by myself, and all sources of information have been specifically noted by means of appropriate references or acknowledgements.

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List of Abbreviations

AA	Amino acids
ADE	Apparent digestible energy
ADEn	Apparent digestible energy corrected to nitrogen equilibrium
ADMD	Coefficient of apparent dry matter digestibility
AND	Coefficient of apparent nitrogen digestibility
AGP	Antimicrobial growth promoter
AME	Apparent metabolisable energy
AMEn	Apparent metabolisable energy corrected to nitrogen equilibrium
AMN	Coefficient of the apparent metabolisability of nitrogen
ANOVA	Analysis of variance
BM	Body mass
CP	Crude protein
CT	Condensed tannins
DM	Dry matter
DOMD	Coefficient of apparent digestibility of organic matter in the dry matter
EO	Essential oil (s)
EU	European Union
FCE	Feed conversion efficiency
FCR	Feed conversion ratio
GC	Gas chromatography
GCMS	Gas chromatography-mass spectrometry
GE	Gross energy
GIT	Gastro-intestinal tract
HPLC	High performance liquid chromatography
HT	Hydrolysable tannins
LAB	Lactic acid bacteria
LSD	Least significant difference
ME	Metabolisable Energy
MRSA	Methicillin-resistant *Staphylococcus aureus*
N	Nitrogen
NSP	Non-starch polysaccharides
OM	Organic matter
PCA	Principal components analysis
s.e.d.	Standard error of the difference between means
SEM	Standard error of the mean
VFA	Volatile fatty acids
VRE	Vancomycin-Resistant Enterococci

This thesis is dedicated to the memory of my late father, Samuel (Sam), who showed me how to be the very best that I could be.. and led the way that I should follow after.

Science strives constantly to investigate natural substances. The centuries old trust in nature thus finds its scientific base belatedly - since just the most recent investigations have shown that something unexpected, something useful, is hidden behind the old herb wisdom.

BERGWEIN, K. (1968) Effective plant substances in cosmetics.
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"Let your food be your medicine; let your medicine be your food."

(HIPPOCRATES)

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CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction: The development of antimicrobials and their use in agriculture

Antimicrobials have been defined as 'substances produced by micro-organisms that kill or inhibit other micro-organisms' (SMAC, 1998). Generally produced as secondary metabolites, antimicrobials may be sourced naturally or synthetically and incorporate antibiotic drugs and some antiviral and anti-fungal agents. Antimicrobials were first used therapeutically in humans in the 1930's, with the discovery of penicillin. They were first introduced experimentally into agriculture as an alternative treatment for bovine mastitis during World War II. Their commercial potential for use in growth promotion was discovered by chance in 1949 (Stokstad *et al.*, 1949). Antimicrobial growth promoters (AGP) have been approved for use in US agriculture since 1949, becoming standard commercial practice in 1951. In 1953, they were introduced into agriculture within the UK. The continuous development of different antimicrobial classes greatly expanded their use world-wide between 1950 and 1960. Within poultry rearing, antimicrobial usage was the single most influential factor that enabled agricultural intensification. Intensification allowed a greater number of birds to be housed within one location, which in turn enabled the costs of production to be decreased. The larger numbers increased the potential for disease problems, but the inclusion of antimicrobials in the diets improved disease prevention and allowed a more rapid disease control, by suppressing the activity of the intestinal microflora. Several generations of antimicrobials have been used since they were first introduced, as bacterial resistance development is a continuously evolving process. Currently, antimicrobials for use in UK agriculture for all purposes are now licensed centrally within the European Union.

LITERATURE REVIEW

1.2 History of concerns relating to the use of antimicrobials in agriculture

The first concerns regarding antimicrobial use in agriculture and the development of bacterial drug resistance were expressed in the early 1960's, about ten years after their introduction. By the end of the 1960's, both the Netherthorpe Committee (1962) and the Swann Committee report (1969) had publicly voiced their serious concerns and suggested caution in usage and methods to restrict antimicrobial use. The Swann report was unsuccessful in restricting the use of antimicrobial drugs for growth promotion, but it did serve to highlight awareness to

what would become a crucial issue in future years. Perhaps most importantly, the Swann report failed because there was no appreciation of the scale of the problems with regards the scale of development of bacterial resistance to antimicrobial drugs at this time. Modern day antimicrobials are "last resort drugs" in human medicine, and thus the development of bacterial resistance against these agents in humans is potentially life threatening. High selection pressures lead to the emergence of resistant bacteria, which are widely spread throughout animal species and the environment (Tollefson *et al.*, 1998). These selection pressures may be created directly against pathogenic bacteria and indirectly within the normal commensal microflora by antimicrobials, depending on usage policies (Tollefson *et al.*, 1998).

1.3 Benefits and risks of antimicrobial use in agriculture

Antimicrobials may be used either for therapy or for growth promotion. When used as growth promoters, the antimicrobials are fed at between 0-200g active substance $tonne^{-1}$ of animal feed. The presence of AGP in the feed has been reported to keep the mass or size of the animals or birds as uniform as possible, by deriving the maximum possible benefit from the food and reducing the waste excreted (Harvey & Mason, 1998). The cost of including an antimicrobial in animal diets has reduced over the years, both nutritionally or for medical purposes (Rosen, 1995). This has led to a greater efficiency in the cost of meat production, with the associated benefits in the increased efficiency of feed utilisation. The addition of an AGP to an animal feed can add 4-5% to the weight of the animal, without increasing the amount of food consumed (WHO, 1997), which may be either due to physiological effects or to the prevention of both sub-clinical and clinical diseases. After comparing 12,153 trials since 1948, a recent review has indicated that tetracycline AGP's have affected productive performance in 72% of these trials (Rosen, 1996), although the paper does not give an indication of the extent of either positive or negative responses. Rosen (1995) indicates that there has been a general trend for a decrease in the animals' response to AGP supplementation over the years, and a greater response generally in monogastrics as a result of starter rather than finisher in-feed supplementation. Antimicrobial usage in animal feed has previously been associated with increased growth in pigs (Stahly *et al.*, 1980; Cromwell *et al.*, 1996), with additive effects observed in growth rates between the antimicrobial and a copper supplement when both were supplied in diets. Antimicrobials have also increased growth rates in chickens, where supplemented diets allowed the body mass of the birds to be higher at 22 days of age (Bunyan *et al.*, 1977). In a comparison of a number of trials with 14 different

antimicrobials, Rosen (1995) stated that these additives stimulate feed intake in pigs and turkeys, but not in broilers or laying hens. However, mean improvements in live weight gains/feed conversion of +8.1/-4.8% (pigs) and +3.6/-2.2% (turkeys) were shown, compared with +3.6/-3.4% (broilers) and +2.8/-2.7% (laying hens) respectively (Rosen, 1995). Therefore, antimicrobials appear to encourage a better use of the diet provided.

The antimicrobial salinomycin has been associated with a decreased pathogen load in cases of necrotic enteritis in pigs, when used at the concentrations required for the purposes of growth promotion (Kyriakis *et al.*, 1996). Zinc bacitracin was reported to improve growth rates and feed conversion in broiler chickens, with an additive effect in the presence of salinomycin (Engberg *et al.*, 2000). The use of salinomycin decreased the numbers of ileal coliform bacteria in broilers (Engberg *et al.*, 2000), and an additive effect was observed when both these antimicrobials were used together. Fuller & Turvey (1971) proposed that a healthy gut to achieve maximum growth rate should depend on an early colonisation of the crop with lactobacilli, and suggested that antimicrobials may inhibit the growth of micro-organisms that would interfere with development of the lactobacilli. The action of various AGP used in pig and poultry diets are described (**Table 1.1**). These effects are not observed consistently in trials and may be influential in considering the variation in responses to antimicrobial supplementation over the years.

Apart from their uses in growth promotion, antimicrobials are applied therapeutically at high-risk times of infection during the growth period in order to control disease or infections, for example necrotic enteritis, *colisepticaemia*, salmonellosis, mycoplasma, scouring or necrotic dermatitis (*Staphylococcus aureus*). Nutritional stressors may occur when there are dietary changes, such as weaning in pigs or calves, leading to a short-term upset in the balance of the gut microflora, and may result in scouring. Stress may also occur as a result of changing management practices, such as a changed environment, or the mixing of animals within different age or social groups. Antimicrobial application at therapeutic doses over a longer term may have a more serious adverse effect. In rats and mice, high dosages of 5-nitrimidazole have resulted in carcinogenic effects, caused by microbial nitroreductases, which act to reduce the nitro-group fraction of the antimicrobial (Schmid & Schmid, 1999). The development of acidosis in dogs and humans has been reported with the use of enrofloxacin as a therapeutic treatment (Tras *et al.*, 2001). The composition of the intestinal microflora in the

animal may be changed by feeding oral antimicrobials, due to the death of various populations of gut bacteria (Linton & Hinton, 1987). The resulting unbalanced microfloral populations may select for bacteria resistant to these oral antimicrobials through competition for available niches within the gut (Linton & Hinton, 1987). When antimicrobials were not used as a routine practice on organic farms, there was no evidence of any decreased susceptibility to these drugs by bacteria (Aarestrup, 1995).

Table 1.1 *A summary of the action of AGP antimicrobials used in monogastric diets taken from research carried out over 40 years*

Microbiological	*Nutritional*	*Metabolic*	*Physiological*
Beneficial bacteria (+)	Energy retention (+)	Ammonia production (-)	Gut transit time (-)
Adverse bacteria (-)	Gut energy loss (-)	Toxic amine production (-)	Gut wall diameter (-)
Transferable resistance (+-0)	Nitrogen retention (+)	Alpha-toxin (-)	Gut wall length (-)
Competition for nutrients with gut flora (-)	Limiting amino acid supply (+)	Mitochondrial fatty acid oxidation (-)	Gut wall weight (-)
Gut floral nutrient synthesis (+)	Vitamin absorption (+)	Bacterial cell wall synthesis (-)	Gut absorptive capacity (+)
Clostridium perfringens (-)	Trace element absorption (+)	Bacterial DNA synthesis (-)	Feed intake (+-0)
Pathogenic *E. coli* (-)	Fatty acid absorption (+)	Bacterial protein synthesis (-)	Faecal moisture (-)
Pathogenic streptococci (-)	Glucose absorption (+)	Faecal fat excretion (-)	Mucosal cell turnover (-)
Beneficial lactobacilli (+)	Calcium absorption (+)	Liver protein synthesis (+)	Stress (-)
Beneficial *E. coli* (+)	Plasma nutrients (+)	Gut alkaline phosphatase (+)	
Debilitation of pathogens (+)		Gut urease (-)	

Taken from (Rosen, 1995), based on a survey of 12,000 trials using AGP in pigs & poultry.
(+) =increase, (-)=decrease, (0)=no change.

Small amounts of antimicrobial drug residues may be absorbed from the intestine, and may be present in meat, milk or eggs (Saucier, 1999), but residues tend to be difficult to detect as a result of the low concentrations involved in the tissue. According to Paige *et al.* (1999), tissue drug residues are left mainly after the absorption of the antimicrobial classes penicillin, streptomycin, tetracycline, sulfamethazine, gentamycin and neomycin. However, there is a suggestion that the absorption of some antimicrobials, such as streptomycin and kanamycin, from the chick intestine may be very poor, when they were observed in high concentrations within the caeca (Jeffries *et al.*, 1977). The animal may also metabolise and absorb antimicrobials from the caeca or other organs. The tetracycline penicillin exerts its action in the crop of broiler chicks, and afterwards is extensively metabolised and absorbed (Jeffries *et al.*, 1977). In a number of studies, the effects of absorbable tetracyclines on nutrition were

compared to those with zinc bacitracin usage, which is not absorbed from the intestinal tract. In these studies, zinc bacitracin showed much less variability in responses such as feed conversion, but not in weight gain, than the tetracyclines (Rosen, 1996). Thus, the variation in responses to antimicrobials may be due to whether they are metabolised or not and the extent of this metabolism. As a consequence, each antimicrobial product will act differently.

When they are given antimicrobials, domestic animals require a withdrawal period before slaughter in order that the drugs can clear the body system. This period is set at 7 days in poultry. There are also maximum residue limits (MRL's) established within animal tissues for antimicrobial drugs, as the effects of drug residues in animal tissues are unclear. It is difficult to prove that there is a direct relationship between adverse health conditions in humans and the consumption of meat containing antimicrobial drug residues. It has been suggested that the ill-effects of drug residues in humans includes; allergic reactions or carcinogenic effects, between 6-66% of tissue and organ developmental defects, bacterial pathogen disease susceptibility and microbial changes in the metabolism of circulatory hormones, vitamins and bile acids (Paige *et al.*, 1999; NRC, 1999). The problem of drug residues in poultry has recently come under increased scrutiny, after the discovery of residues of the coccidiostat lasalocid within eggs. The British Egg Industry Council (BEIC) have banned lasalocid from laying hen diets completely from August 2004, and prohibited the use of coccidiostats in laying hens over 12 weeks of age if eggs from these hens are to obtain the Lion quality trademark (Cruickshank, 2004).

When used for the purposes of growth promotion, antimicrobials are assumed to have a sub-lethal effect on bacteria, so they may be able to grow but not reproduce. However, if the effect of an antimicrobial is partial but not absolute, this may still create a selective pressure when the antibiotic is continually present in the gut (Salyers, 1999). Before 1997, avoparcin was the main AGP used in poultry diets. Avoparcin has now been implicated in transferring glycopeptide resistance to the human antimicrobial vancomycin, through the intestine, by means of the development of a resistant pool of enterococci (Klare *et al.*, 1995; Aarestrup 1995; Bager *et al.*, 1997; WHO, 1997). Resistant bacteria are then passed on to humans through contamination of the animal carcass with either faecal material or gut contents at slaughter and from animal to animal through close direct contact (Linton & Hinton, 1987; Witte, 2000b). Antimicrobials used for the purposes of growth promotion in animals have

been linked in their mode of action or their mode of resistance development to several human antibiotic drugs. Resistance transfer, or the development of cross-resistance, occurs when the same mutations are observed in bacteria in different animal species. The development of antimicrobial resistance in bacteria within hospitals has had significant repercussions throughout the veterinary, agricultural, horticultural and medical sectors. Antimicrobial usage within these areas has been severely restricted in order to stop the spread of antimicrobial resistant bacteria and multi-drug resistant bacteria in humans.

Antimicrobials used therapeutically for both veterinary and medical purposes have been associated with a greater risk of bacterial resistance transfer than those used as feed additives (Mulmattathil *et al.*, 2000), especially when there is a lack of information about previous treatment history, or when treatment policies are misused. In hospitals, resistant pathogens of importance now include Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE), β-lactamase producing Gram-negative bacilli including Klebsiella and Enterobacter, multi-drug resistant *Mycobacterium tuberculosis*, fluconazole-resistant *Candida* species and *S. aureus* strains with reduced susceptibility to vancomycin (Weber, 1999). A reawakening of concerns about the safety of using drugs for growth promotion and therapy in food animals took place after they were used to control outbreaks of disease in calves during the 1980's. As a result of these outbreaks, Sweden banned the use of all AGP in agriculture at this stage. In the rest of Europe, the bans on most agricultural AGP have been carried out primarily as a precautionary measure, to protect the antibiotics used in human medicine. However, with the current interest in organic foods and the development of natural products, a new consumer focus has awakened on the somewhat controversial subject of feeding drugs to animals, which means they are unlikely to return to favour.

1.4 The current situation in agriculture regarding antimicrobial usage

In the mid 1980's, Sweden was the first country to ban all agricultural AGP, and the country has been free from their use since this time. In the 1990's, the debate over the possibility of resistance development through drug use in medicine and the risks posed by the agricultural use of AGP intensified (WHO, 1997; WHO, 1998; SMAC, 1998). The links between the use of AGP's and the development of human resistance towards bacteria have been denied, mainly from those directly advocating antimicrobial use. Denmark banned avoparcin from use in all animal diets in 1995 to preserve the efficacy of the human antibiotic vancomycin, with the rest

of Europe, including the UK, following suit in July 1997. In January 1998, the permission to use virginiamycin as an AGP was withdrawn by Denmark in all animal species. These developments and the increased public and government interest eventually led to a ban on 4 out of the 8 remaining AGP products used in agriculture throughout the European Union on the first day of July, 1999; namely virginiamycin, zinc bacitracin, tylosin phosphate and spiramycin. Tylosin phosphate and spiramycin were removed from use as AGP, because they were also used therapeutically (SMAC, 1998). The streptogramin drug virginiamycin is a combination of quinupristin and dalfopristin, which is a combination antimicrobial used clinically in the USA for VRE, MRSA, and multi-drug pneumonia- and meningitis-related infections in humans. However, enterococci resistant to quinupristin/dalfopristin could well be present in animals before this drug combination can be used in humans, which may well limit its effectiveness as a new medical magic bullet.

Coccidiostats are drugs used to control outbreaks of *Eimeria* spp., which occur as 9 different species of unicellular organisms, and are relatively ubiquitous in poultry. During the period 1993-98, the use of coccidiostats decreased in the UK apart from a small rise in 1997 after the banning of avoparcin (VMD, 2000). Although these drugs are not regarded as being related to the antimicrobials used in human therapy, they still remain as a synthetic drug presence in animal feeds and have some antimicrobial properties. The data for the quantity of AGP agents used since 1999 is unreliable, but recent statistics suggest that only a small decrease in their use has occurred, with 43 tonnes sold as growth promoters in the UK in 2001, compared to 46 tonnes in 1998 (VMD, 2003). Currently, there are 4 legal drugs available to include in poultry diets either as AGP or anticoccidials, but their future existence in agriculture is uncertain. Many commercial enterprises have either stopped their use of antimicrobials altogether, or have increased their usage of these limited few. It is generally expected that the remaining four; avilamycin and flavomycin (bambermycin) antimicrobials, along with the coccidiostats monensin and salinomycin will be withdrawn by 2006 in the EU and no new feed additives will be permitted in poultry diets (McCartney, 2002). However, as the price of poultry production in the EU does not match that of some other countries using antimicrobials and with different welfare standards, there has since been a relaxation of the ban on antimicrobials. Certain antimicrobials may now be prescribed, but only those unrelated to the drugs used in humans, and only by a qualified veterinary surgeon (Cruickshank, 2002). In Norway, the avoparcin ban in 1995 caused poultry producers to switch to using narasin, a new ionophore

coccidistat, as a therapeutic agent to treat or prevent necrotic enteritis (NE). This drug is more or less used exclusively in Norway, with the additional use of only very small quantities of monensin-sodium, salinomycin and the antimicrobial amoxycillin, one of the penicillins (Grave *et al.*, 2004). These authors reported no rise in the incidence of NE in Norway since narasin was registered and its use in diets began.

1.5 Antimicrobial resistance and the influences of geography, environment and animals

Antimicrobial-resistant and multiple-antibiotic-resistant bacteria have been isolated in a wide range of natural environments (Ottolenghi & Hamparian, 1987; Sabry *et al.*, 1997; Weber, 1999). Areas of virtually non-existent human or animal activity are nearly impossible to find, and the extent of resistance has increased enormously with antimicrobial misuse and overuse. One of the problems of current farming practice involves the challenge of what to do with the excreta or effluent, which may leach into groundwater. In an examination of 250 samples of coliform bacteria from rural, untreated water supplies in the US, 87% of these were resistant to at least one of the 16 antimicrobials tested, and were predominantly resistant to novobiocin, cephalothin and ampicillin (McKeon *et al.*, 1995). Around 60% of these coliforms were resistant to multiple antibiotics, and isolates of *E. coli* could transfer resistance *in vitro* (McKeon *et al.*, 1995). Animal processing units represent another source of contamination of the environment with antimicrobial resistant bacteria. Poultry processing units represent one example of a point source of contamination, which may lead to the entry and recycling of resistant bacteria into the environment within closed natural systems, such as the treated human water supply (Mulamattathill *et al.*, 2000). This will certainly lead to human antibiotic selection pressures. Links between the incidence of antimicrobial resistance and bacterial tolerance to toxic heavy metals have also been reported in polluted seawater, with the majority of bacterial isolates being resistant to both factors through plasmid DNA transfer (Sabry *et al.*, 1997). Thus, the impact of environmental pollution may also have an effect on the resistance of bacteria to antimicrobials.

The range of animal species developing bacterial resistance to antimicrobials within a variety of environments is extremely diverse. In a study on 946 strains of *Enterobacteriaceae* with 32 antimicrobial agents and combinations of these antimicrobial agents between 1993 and 1997, resistant *Enterobacteriaceae* strains, and strains with multiple resistance to various antimicrobials have been isolated from wild Australian mammal populations (Sherley *et al.*,

2000). Although completely removed from the intensive situation of European agriculture, even environments such as this are not free from antimicrobial resistance development. Sherley *et al.* (2000) proposed that a resistance-free environment would be almost impossible to detect. Until vaccines were recently developed to replace the use of antimicrobials, the development of bacterial resistance was also a significant problem in aquaculture, except in the development of new fish species, and when used in therapeutic emergencies (Alderman & Hastings, 1998). The close proximity of contact between humans and companion or working animals may also lead to an increased resistance transfer potential (Normand *et al.*, 2000a), which has been suggested to extend to exotic pets such as ornamental fish species (Alderman & Hastings, 1998). A similarity was observed between pieces of DNA with mutations which confer a bacterial adaptation, or R-plasmids, isolated from both domestic pets and humans (Davies & Stewart, 1978; Anderson *et al.*, 1975). This may suggest that the two populations could transfer antibiotic resistance between them or have similar sources of resistance development (Davies & Stewart, 1978; Anderson *et al.*, 1975). In plants, one of the most well known examples of stable bacterial resistance development is the onset of crown gall tumour disease after some sections of plasmid DNA transfer in *Agrobacterium tumefaciens* (Currier & Nester, 1976; Chilton *et al.*, 1977), designated the *chv*A and *chv*B regions (Douglas *et al.*, 1985). Similarities between the virulence traits of bacterial plasmids in crown gall disease and various types of animal viral diseases are suggested, due to the insertion of stable bacterial gene plasmids into the transformed cells (Chilton *et al.*, 1977). The development of resistance in *E. coli* has been associated with age in pigs, with greater resistance development in the microflora of young nursery and post-weaning animals, which may be due to the extra antimicrobial requirements of the immature gut and an unstable immune status (Mathew *et al.*, 1999). The levels of commensal and pathogenic faecal bacteria released into environments such as estuarine water have been a cause for some concern, in terms of the different bacterial pathogen survival rates (Pettibone *et al.*, 1987) and also from environmental bacteria recycling. Pettibone *et al.* (1987) found the greatest survival of *Enterococcus faecium* from recovered water samples, and good survival of *Enterococcus* and *Streptococcus* spp. compared with *Escherichia coli*, but no difference in the survival rates of antimicrobial resistant and sensitive bacteria in samples isolated from estuarine water. Seawater represents a very complex medium due to variations in chemical composition and physical properties, tides and time of sampling, but also because it acts as a bacterial collection sink for bacterial contaminated waste from the land. A comparison of any link between the development of

human and companion animal resistance to antimicrobials is made very difficult, due to the distinct lack of information from the medical sector in the UK.

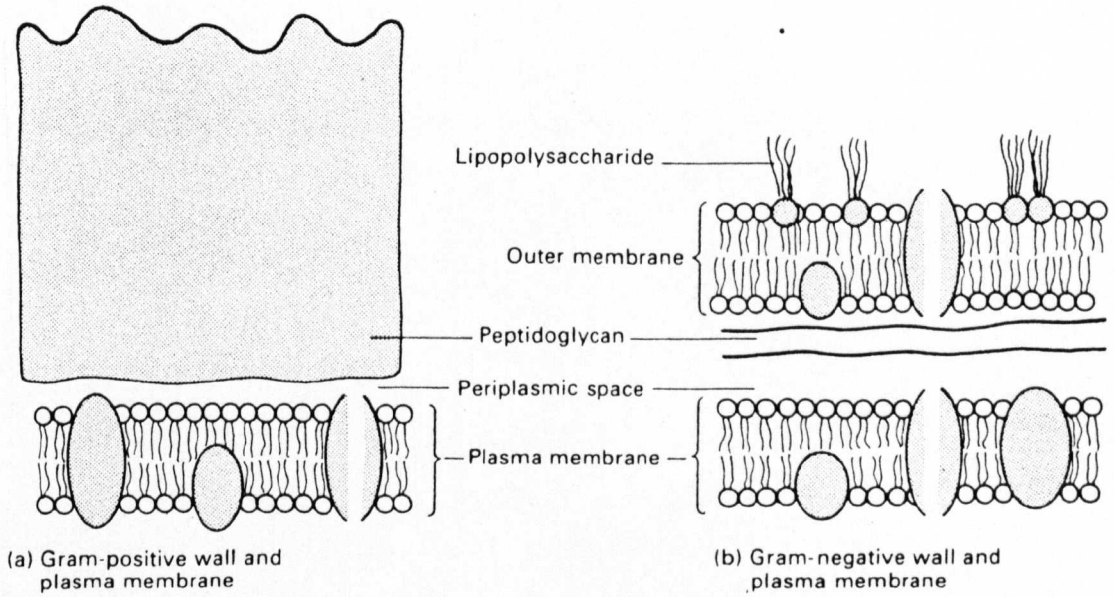
1.6 Seasonal variations in detection of multiple resistant and resistant bacteria

Several studies have indicated that the ability to recover resistant and non-resistant bacteria at sample sites will vary with the season of year. A study on wastewater sludge destined for farmland application in Ohio showed increased Salmonella isolates in the first quarter of the year (Ottolenghi & Hamparian, 1987). Similar results were reported for recovery of *Campylobacter* spp. from various locations in the gastro-intestinal tract of poultry (Wallace *et al.*, 1997). Grave *et al.* (2004) reported that the highest requirement for therapeutic treatment of necrotic enteritis in Norwegian broilers occurs in the period between October and December. However, no clear seasonal effects in multiple antimicrobial-resistant bacteria were isolated from the effluent of a chicken processing plant in Africa, from a closed water system in the study of Mulamattathil *et al.*, (2000). Mulamattathil *et al.* (2000) observed 273 faecal coliform isolates, reporting that 93% of these were resistant to one or more of the 8 antimicrobials tested. These observations would suggest that an increased application of antimicrobials at certain times of the year in most environments may predispose a greater risk of resistance transfer in humans.

1.7 Types of bacteria and their attachment to intestinal surfaces

There are two types of bacteria, which have been classified as being either Gram-negative or Gram-positive, based on differences in their cellular composition. Differentiation between the two types is based on the Gram-stain technique developed by Christian Gram in 1884. The Gram reaction stains peptidoglycan within the cell wall blue or purple, due to its ability to hold an insoluble crystal violet/iodine complex. All bacteria are composed of peptidoglycan, which provides rigidity to their structures, but it is organised differently in the two bacterial types. The mode of action of synthetic antimicrobials against bacteria takes several forms, including the inhibition of peptidoglycan synthesis by vancomycin in Gram-positive bacteria, and the inhibition of the formation of cross-linking in bacterial peptidoglycan by the penicillins (Rogers *et al.*, 1980), thus preventing bacterial cell wall formation. Generally, most antimicrobials act on some aspect of the peptidoglycan component. Gram-positive cells have thicker cell walls, whereas Gram-negative cells have thinner walls without enough peptidoglycan to hold the stain complex, so they must be counter-stained with a red dye such

as carbol fuschin. Gram-positive bacteria are generally considered more sensitive to antibiotics and also to natural alternatives than Gram-negatives, because of the presence of this thick outer bacterial cell wall (Russell, 1991), instead of the cell envelope in Gram-negatives.



Source: Cadogan & Hanks, (1995)

Figure 1.1 *The structure of Gram-positive and Gram-negative bacterial cell walls*

The Gram-positive bacterium has a cell wall varying from 30-80 nm in thickness, which lacks structure. Cell wall synthesis is not linked with protein or nucleic acid synthesis, other than in the formation of cell wall biosynthetic enzymes and a limited number of amino acids and nucleotides. In contrast, the Gram-negative bacterial cell envelope structure is mechanically weaker, but much more complex. The Gram-negative bacterial envelope has at least 4 layers. From the bacterial cytoplasm, the first layer is a thin cytoplasmic membrane of around 2-10 nm, which is composed of 3 sub-layers. It then contains only a few layers of peptidoglycan, compared with up to 40 in the Gram-positive bacterium. After the peptidoglycan, a structure-less "gap" zone occurs, which is around 3-4 nm in diameter. This is followed by an outer membrane, composed of phospholipids and proteins with a thickness of around 7.5-8 nm. The outer membrane is also composed of several layers, one of which is a layer of lipopolysaccharide. The lipopolysaccharide layer in Gram-negative bacteria is hydrophilic, and acts as an effective barrier against attack by bile salts, digestive enzymes, many antibiotics

and to protect the cell against phagocytosis (Nikaido & Vaara, 1985). However, the lipopolysaccharide layer also contains a hydrophobic region known as lipid A, which is anchored to the outer membrane, permitting the entry of low molecular weight hydrophilic molecules. The number and thickness of layers within the cell envelope vary in different Gram-negative bacteria.

Due to the presence of the cell envelope, high levels of antibiotic resistance can be built up in Gram-negative bacteria, as small amounts of antibiotic that can penetrate the outer membrane are quickly overcome by the cell defence mechanisms. The lipopolysaccharide component of Gram-negative bacteria contains channels known as porins, which are filled with water (Nikaido & Vaara, 1985). It also contains outer membrane proteins, which allow small hydrophilic substances to be transported through to the bacterial cell, such as nutrients and antimicrobials (Nikaido & Vaara, 1985). If small hydrophobic substances like terpenes contain a hydroxyl group, they may be able to form associations with water molecules and therefore be transported into the cell, but this needs to be established. Multiple types of porins are present in enteric bacteria (Nikaido & Vaara, 1985).

The intestinal mucosa in animals is generally covered by a mucus gel, which is composed of glycoproteins and glycolipids (Freter, 1981). Virulent bacteria attach by a process involving several complex stages, which are described below, and the process may also involve other factors such as surface-ligand binding (Freter, 1981):

- chemotactic attraction of motile bacteria to the mucus gel (intestinal surface)
- bacterial penetration of and entrapment within the mucus gel, either passively or promoted actively, by bacterial motility and chemotaxis
- adhesion to receptors in the mucus gel or to mucosa-associated layers of the microflora
- adhesion to the epithelial cell surface
- multiplication of the mucosa-associated bacteria

Sub-lethal concentrations of penicillin G and streptomycin antimicrobials have been shown to restrict the adhesive properties of bacteria such as *Streptococcus pyogenes* or *E. coli* to the intestine, without affecting bacterial growth *in vitro*, by interfering in the synthesis of protein subunits used in attachment (Beachey *et al.*, 1981).

1.8 Antimicrobial resistance and its importance in disease development

Bacterial resistance is generally either controlled genetically, or is acquired through the mutation or transfer of genetic material from other cells (Russell, 1991). For either animals or humans, the microbiological safety of food depends on how easy it is to transfer antibiotic-resistant bacteria from one host to another as the food passes down through the intestinal tract. Improved medical therapy has increased the survival potential of immuno-compromised patients with infections such as diabetes and HIV, as well as cancer patients and the elderly, but these individuals have a weaker immune system and are also much more sensitive to other infections, such as food-borne pathogens. The control of *Staphylococcus aureus* was first achieved in the early 20th century in surgical procedures, but there is no control today for methicillin-resistant *S. aureus* (MRSA) if vancomycin becomes ineffective. The current mismanagement of financial and staff resources within the National Health Service in the UK may have led to a reduction in hygiene standards within the NHS hospitals in the UK, and outbreaks of multiple-drug resistant bacteria such are MRSA are becoming increasingly common.

Shoemaker *et al.* (2001) found evidence of resistance transfer between *Bacteroides* spp and other Gram-positive bacterial species on conjugative transposons, and on the *tetQ*, *ermF* and *ermG* genes in the human colonic microflora, for both antimicrobial and non-antimicrobial treated patients. The intestinal tract represents a high-risk area for transfer of antimicrobial resistance, due to its large surface area, high microbial load and high water content. Klare *et al.* (1995) discovered glycopeptide-resistant enterococci in broiler chicken carcasses entering hospitals and proposed that hospital patients may be infected via their food chain due to their reduced immune status, which may not necessarily be observed in healthy individuals. The genetic material contained within resistant enterococci has a high potential for resistance transfer to other medically important Gram-positive agents such as *Staphylococcus* spp., and may be partly responsible for MRSA. It was recently reported that around 90% of broiler chickens in the UK were infected with Campylobacter (Poulter, 2000). However, one in eight cases of Campylobacter poisoning in humans cannot be treated with the antimicrobial Ciprofloxacin because the bacterium had developed resistance to Enrofloxacin, which is similar and was previously given to chickens (Poulter, 2000). Several bacteria that cause food-poisoning outbreaks in humans are also normal components of the intestinal microflora in domestic animals. Tollefson *et al.* (1998) has suggested that the increased frequency of

food-poisoning outbreaks may be due to the transfer of resistant genes between bacteria. Selection for bacterial resistance has occurred for Salmonellosis *via Salmonella typhimurium* DT104 infections and also Enterotoxin-producing *E. coli* infections by means of genetic transfer on plasmids and transposons (Saucier, 1999). Additionally, food poisoning bacteria which were previously resistant to quinolone drugs, such as *Salmonella* spp. and *Campylobacter jejuni,* have been known to transfer resistance across to the fluoroquinolones (WHO, 1997). The fluoroquinolones were introduced to replace quinolones as AGP, and this is known as the development of cross-resistance between antimicrobials.

Enterococcus spp. are thought to be able to develop resistance very easily to most antibiotics used in food animals, as resistance is commonly carried on one or a few genes leading to amino acid substitutions (White *et al.*, 2000). The use of broad-spectrum drugs in animals and humans has already selected indirectly for multiple-drug-resistant *E. coli.* A large carrier population of animals resistant to most antibiotic drugs can therefore be a significant threat to both human and animal populations. In the UK, animals acting as disease carriers when travelling to markets have wreaked nation wide devastation as recently as 2001, with the Foot & Mouth disease epidemic. Although the causative agent for this disease is a virus, the close contacts between animals and the additional environmental transmission of the disease resulted in the slaughter of millions of cattle and sheep, alongside a national biosecurity policy and a ban against selling the meat produced in European markets. This represents the level of chaos that could also ensue with the outbreak of a disease, which could not be controlled by antimicrobial drugs. Widespread genes coding for resistance are almost impossible to trace in the environment, except by introducing a genetically labelled source (Witte, 2000a). It is therefore vital that release of these genes is minimised and a population of healthy immuno-competent animals, which do not act as a resistance gene pool, can be maintained. The Europe-wide ban on most agricultural AGP agents represents governmental attempts to stop bacterial resistance selection pressure in domestic animals and limit the further spread of antimicrobial resistance.

1.9 Mode of resistance development to antimicrobial drugs

Bacteria acquire resistance as a result of wide selection pressures and extensive drug use, and the process of resistance development is constantly evolving to new and existing drugs. Resistance development is affected by bacterial taxonomic species, geographical location and

the type of antibiotic used (Sherley *et al.*, 2000). The rate of resistance development depends on the antimicrobial usage level. An increased development of bacterial resistance was observed on farms with high rather than low antimicrobial usage policies (Mathew *et al.*, 1999). However, the bacterial resistance levels on farms with a low antimicrobial usage policy remained constant over time (Mathew *et al.*, 1999). The development of resistance can also be very rapid. Resistance to the widely used antimicrobial, nalidixic acid, was initially observed in 1994 for various strains of *Salmonella typhimurium* in poultry other than DT104 (Malorny *et al.*, 1999). However, by 1997, *S. typhimurium* resistance had increased to levels of 72.7% for strains other than DT104 and to 16.1% for strains of DT104 in chickens, along with levels of 78.3% resistance for DT104 strains in turkeys (Davies *et al.*, 1999). Malorny *et al.* (1999) also predicted an increase in resistant *S. typhimurium* DT104 strains, after a sharp increase in resistance was reported in one year when enrofloxacin was used therapeutically to treat a DT104 outbreak in cattle. The high level and speed of resistance development mean that the antimicrobial nalidixic acid is longer used for therapy. Problems may also occur between different types of antibiotics, in that resistance towards one antimicrobial agent may reduce the effectiveness of another if the two have a similar mode of action. Fluoroquinolone antimicrobials, first introduced in medicine in the mid 1980's, are related to nalidixic acid and are used to treat life-threatening pathogenic bacterial infections by inhibiting bacterial DNA gyrase and topoisomerase IV enzymes. Some fluoroquinolones, such as flumequine, enrofloxacin and oxolinic acid are used therapeutically in veterinary medicine. Increased resistance to nalidixic acid and also to enrofloxacin by nalidixic-acid resistant *S. typhimurium* DT104 has been observed in Germany between 1986-98 across all species of animals, and especially for poultry since 1994 (Malorny *et al.*, 1999). The development of cross-resistance between quinolone and fluoroquinolone antimicrobials has been rapid in Saudi Arabian poultry by means of similar DNA gyrase A mutations (Bazile-Pham-Khac *et al.*, 1996), mutations which were previously associated with rapidly increased resistance against *E. coli* in quinolones. Direct comparisons between different studies are difficult because of variations in the environments, methodology, antimicrobials used and experimental designs. It is also possible that differences in the numbers of resistant bacteria between successive years may be due to the treatment rotations with various antimicrobials. All the studies undertaken recommend prudent antimicrobial usage to maximise the lifespan of their therapeutic effects.

1.10 Implications of these actions for the poultry meat industry

The contribution of the poultry meat industry towards food consumption has been outstanding in the last half-century. Chicken is the UK's most popular retail meat product today, forming an important dietary source of cheap but high quality protein. The industry continues to develop more rapidly than all others in meat production, in terms of genetics, nutrition and physiology. With the world population currently increasing at 1.33%, or 78 million people per year (McQueen, 2000), there will soon be substantial demands on the planet's food production capacity, which the poultry industry is well placed to help satisfy. In 1998, the UK produced 1.953 million tonnes of poultry meat from 5.9 million tonnes meat of total (all species) production (VMD, 2000). Veterinary drugs are an important component in modern intensified agriculture. Profit margins in the highly intensified poultry and pig industries are tiny for each animal, but are based on the flock or herd as a whole. The poultry and pig industries together use about 80% of all agricultural antibiotics. However, variations do exist between countries in their drug use. In 1998, about 10-17% of 522 tonnes total active antimicrobials sold in UK agriculture were used for growth promotion (VMD, 2000), with the remainder used in disease therapy. In contrast, the Netherlands fed twice as many AGP, using 250-300 tonnes of active ingredient, representing 50% of the total antimicrobial usage (van den Bogaard & Stobberingh, 2000). Some countries such as Sweden do not use antimicrobials at all. Thus, there is a large difference between European countries with regards to their usage policies. Individual species consumption or growth promotion values alone cannot be obtained for German food animals (Malorny *et al.*, 1999) or animals in the UK, as all growth promoters and 68-72% of therapeutic antimicrobials were licensed for use in several species (VMD, 2000). During the period between 1993 and 1998, poultry in the UK were mainly fed diets containing tetracyclines, representing around 42-50% of total usage of feed antimicrobials (VMD, 2000). Sulphonamides and β-lactams (including penicillin) in the UK were also administered to poultry, mainly within medicated feedstuffs (VMD, 2000), along with therapeutic agents such as fluoroquinolones. The regulation, licensing and supply of antimicrobials, both for therapy and as AGP's also require reorganisation and change, in order that levels of resistance can be monitored effectively. If licensing and registration of antimicrobials and other feed additives is carried out centrally within the EU, this should allow a standardised pattern of use to be developed for each country.

1.11 Development of resistance monitoring systems

Evidence regarding the transfer of resistance in bacteria of various origins is increasing, but the development of resistance monitoring in various environments is slower. Several countries have recently established committees, to collect information on the occurrence and spread of the development and spread of resistance for both pathogenic and commensal bacteria, in comparison with the patterns of antimicrobial use (Gnanou & Sanders, 2000; Bager, 2000; Follet, 2000; Moreno *et al.*, 2000; Coffman, 2000). The Danish programme, DANMAP, looks at microbial resistance levels in all animals at slaughter, and also in foodstuffs. Antimicrobial resistance data is monitored for several reasons, including the importance for public health, and the long-term relevance of any interventions, such as the withdrawal of permission to use certain antimicrobials. Unfortunately, the resistance monitoring programs vary between countries and thus direct comparisons between such studies are very difficult. There are also shortfalls in the information available, concerning drugs that are used in more than one species of animal, and whether AGP are considered separately from those used as therapeutic drugs between countries. Thus, a standardised program is required.

1.12 Prevalence of antimicrobial resistance

Not much is known about the stability or prevalence of resistant bacteria in the environment. Committees monitoring antimicrobials and resistance development have only recently been established, so this information may not be available for some time. Once introduced and established, resistant populations may be stable and thus may not require any further selective antibiotic pressure (Andersen *et al.*, 1975). However, natural antimicrobial resistance also developed prior to the synthetic antimicrobial era and is specific to each environmental location (Sherley *et al.*, 2000). The potential effects of therapeutic antimicrobials in the environment and their bioavailability and persistence are reviewed comprehensively (Jjemba, 2002). Heuer *et al.* (2002) reported that the occurrence of VRE in broilers from Denmark was 74.3% in broiler houses previously exposed to avoparcin, although Denmark banned avoparcin in 1995. However, only 9.1% of broilers from organic flocks on free range farms were VRE-positive (Heuer *et al.*, 2002). There were no significant decreases in the proportion of flocks positive for VRE, in the five years after the ban on avoparcin, suggesting it is relatively stable in the environment (Heuer *et al.*, 2002). In clinical situations, such as a small animal clinic, there has generally been an increased prevalence of bacterial resistance to

certain antimicrobials (Normand *et al.*, 2000b). In dogs, an increased prevalence of resistance to common antimicrobials has been reported, and there was an increasing incidence of resistance to less commonly used antimicrobials when 867 isolates of *S. aureus* and 1339 isolates of *S. intermedius* were compared (Hoekstra & Paulton, 2002). Interestingly, in their study, Hoekstra & Paulton (2002) surveyed isolates taken from different locations within dogs, such as the nose, ear, skin, urine and throat and found that susceptibility to the antimicrobials they used differed according to the age, sex and site of sampling in the dog. Pillai *et al.* (1996) suggested that resistant bacteria may be stable in agricultural wastes within the environment, even without selective antibiotic pressure. The analysis of 752 samples from sewage sludge, farmyard manure and slurry sources in France showed that antimicrobial resistance in verotoxin-producing *E. coli* (VTEC) have increased in prevalence (Vernozy-Rozand *et al.*, 2002). VTEC are the strains of *E. coli* that have previously caused *E. coli* O157 outbreaks in humans. In hospital environments, Weber (1999) has suggested that a similar problem situation may exist for certain antimicrobials including the β-lactam antibiotics, and also vancomycin as regards the development of vancomycin-resistant Enterococci (VRE). It has clearly been demonstrated that human and agricultural pressures have increased the occurrence of resistant and multiple-drug resistant bacteria. There is a hypothesis that the direct use of antimicrobials is not the only factor contributing to the development of bacterial resistance, which may also be expressed through the spread of resistant strains or genes, evolution of new strains or the environmental amplification of existing strains (Sherley *et al.*, 2000). Despite this evidence to the contrary, there is a possibility that most mutated bacteria should not be as viable as their original counterparts, which may limit this survival rate and prevalence in different environments.

1.13 Substrates for bacterial growth in cereal diets for poultry and their implications

Carbohydrates represent between 60-70% of the content of poultry diets, and they mainly supply energy to the bird. The main cereals used in the EU for poultry feed are wheat and barley, but may also include maize. These cereals consist predominantly of polysaccharides, which are composed of cell wall (extracellular) and non-cell wall (intracellular) components. Intracellular carbohydrates provide the main energy storage source of the seed, and are predominantly based on starch and fructans. Wheat and barley have a variable feeding quality, due to their content of non-starch polysaccharides (NSP) in the cell walls of the grain, which are not readily digested by poultry. The cell wall polysaccharides of cereal

carbohydrates provide structural strength to the cell, and enclose the intracellular components. They are complex carbohydrates composed of celluloses and hemicelluloses. They may also include pectic substances and galactomannans, and up to 20% lignins and 10% proteins. Cellulose is a glucan, consisting of between 5-95% of the wall composition, and is composed of β-(1-4)-D-Glucose. It provides the main source of rigidity of the plant cell wall. Hemicellulose components contain heteroxylans and (1-3, 1-4)-β-D-glucans. Other cell wall polysaccharides capable of increasing intestinal viscosity are the galacturonans (pectic polysaccharides and pectins), arabinogalactans and galactomannans.

The solubility of the hemicellulose components in water depends on the number of side-chains within the molecules, where low numbers of side chains and the presence of covalent cross-links between the arabinoxylan molecules will limit their solubility. Linear polymers of (1-3, 1-4)-β-D-glucans are almost specific to cereals, and repeated β-(1-4) linkages can form hydrogen bonds. Thus, these NSP are hydrophilic compounds and can attract a large amount of water, but they are not soluble, which can strongly increase the viscosity of aqueous solutions. Within wheat, 5 carbon pentosan or arabinoxylan polymers predominate as the source of NSP. These arabinoxylan polymers are composed of xylose and arabinose sugars. In barley, the main sources of NSP are the glucans, which are polymers of glucose. Rye can only be used in small quantities for poultry with arabinoxylanase, because of its high pentosan content. (Carré, 2002)

1.14 The anti-nutritive effects of NSP for poultry

The content of NSP is variable within different wheat and barley grains. High concentrations of dietary NSP, such as the content of wheat pentosans within the cereal grains, lead to depressed growth and a reduced intestinal absorption of starch, protein and lipid (Choct & Annison, 1992). NSP also alter the gut function in poultry, mainly through changing the composition of the gut microflora and in increasing the viscosity of digesta within the gut (Choct & Annison, 1992). Reductions in broiler growth rate and feed conversion efficiency (FCE) have been directly associated with an increasing concentration of dietary NSP (Bedford & Classen, 1992). The increased viscosity associated with additional supplementation of NSP within the diet has affected the transit time taken for feed components to pass through the intestine in broilers (van der Klis *et al.*, 1993), and delayed the rate and absorptive efficiency of sugars in rats (Johnson *et al.*, 1984). When diets based on wheat and barley containing a

high concentration of NSP were fed to broilers, the relative size of the digestive tract was increased (Brenes *et al.*, 1993). The presence of arabinoxylan within the broiler intestine resulted in a substantial increase in the growth potential of the intestinal microflora and their activity (Bedford, 1996). An increased digesta viscosity results in less mixing within the broiler intestinal tract, altering the concentration of oxygen within the environment and providing an opportunity for the ileum to be invaded by obligate anaerobes, which compete with the bird for nutrients and may cause disease (Bedford, 1996; Apajalahti & Bedford, 2000). Some of the adverse effects of NSP are eliminated when the diet is supplemented with antimicrobials, increasing broiler performance and decreasing the numbers of ileal *C. perfringens* and the overall weight of the ileum (Campbell *et al.*, 1983b; Stutz & Lawton, 1984; Hofshagen & Kaldusdahl, 1992). The decrease in ileal weight may occur as a reduction in the intestinal immune response associated with decreases in the bacterial load. In the absence of AGP to control the overgrowth of intestinal microflora caused by a high dietary NSP concentration, the digestibility of nutrients fed to poultry may be compromised.

1.15 Development of the potential of natural antimicrobial systems

The regulations against the use of synthetic AGP in animal diets represent a considerable challenge to the animal scientist in the control of unwanted bacterial growth in the gut. The EU is forced into world market competition with countries that allow AGP usage, and has been faced with problems in keeping pace with world markets in terms of animal health and productivity. Chickens may well be in a situation of compromised welfare in terms of the increased risk of disease development in intensive situations since the withdrawal of most of the key AGP in 1997 & 1999. The antimicrobials avilamycin and bambermycin are the only legalised forms of medicated dietary protection the birds receive, apart from 2 coccidiostats, and their future use in the industry is uncertain. Poultry meat sourced from outside the EU may be associated with higher efficiencies of production, an unknown incidence of disease, and a higher dependency on the use of AGP. It is possible that the development of natural antimicrobial products or some other form of bioactive plant supplementation may have the potential to provide the level of protection previously provided by synthetic antimicrobials in intensively housed poultry.

1.16 Range of potential natural antimicrobials or bioactive compounds

Bacterial spores are generally resistant to antimicrobials, but the regenerated bacteria are susceptible. When the spores grow out in favourable conditions, the growth of bacteria may then lead to the production of toxins in the intestinal tract, such as in the case of *C. perfringens* associated NE. Essential oils and their phenolic terpene components are known to be active against a wide range of bacteria, including the Gram-negatives, due to their lipophilic natures (Deans & Ritchie, 1987; Sivropoulou *et al.*, 1996; Dorman & Deans, 2000). Dorman & Deans, (2000) also suggested that alcohols and aldehydes such as formaldehyde and glutaraldehyde possessed powerful antimicrobial activity. The terpene alcohols, farnesol, nerolidol and plauntol, have shown antimicrobial activity *in vitro* against *Staphylococcus aureus* (Inoue *et al.*, 2004). The use of natural compounds present in foodstuffs may also be suitable as sources of antimicrobials, such as lactoperoxidase in milk, avidin in eggs and also lysozymes (Banks *et al.*, 1986). It may be possible to supplement diets with plants from the *Allium* spp., which include garlic, onions and chives, which have long-established medicinal properties (Banks *et al.*, 1986; Dillon & Board, 1994). Alternatively, polyphenols such as condensed tannins and also the terpenes within herbs, spices and their associated essential oils may be used for their antimicrobial and antioxidant activities (Banks *et al.*, 1986; Dillon & Board, 1994). Non-food compounds, such as hydrogen peroxide or natural peptides such as defensins, cecropins or attacins from insects, may also prove beneficial if they are not toxic (Banks *et al.*, 1986).

For the purposes of therapy, the use of ginseng as a treatment for bovine mastitis caused by *Staphylococcus aureus* has had positive effects (Hu *et al.*, 2001). Various systems of food preservation in industry already use alternative antimicrobial products, some of which are described in Russell, (1991). In the baking industry, phenolic essential oils (EO) are used to control various fungi on bakery products (Nielsen & Rios, 2000; Suhr & Nielsen, 2003; Guynot *et al.*, 2003), and also in stored grain (Paster *et al.*, 1994). EO and herbal extracts are also used in the food industry to lengthen the time required for the microbial spoilage of meat products (Skandamis & Nychas, 2001; Dickens & Ingram, 2001). Gram-positive cocci of clinical significance recovered from human wounds have been isolated and controlled effectively by the natural antimicrobial properties of honey (Cooper *et al.*, 2002). Several studies have focussed on plants that are traditionally associated with medicinal properties from various herbal remedies. Some 50 medicinal plants from India have been assessed for their

antimicrobial activity *in vitro*, where 72% showed either antimicrobial or anti-fungal activity or both (Srinivasan *et al.*, 2001). Fifteen crude extracts of traditional Ethiopian medicinal plants containing punicalagin tannins exhibited activity *in vitro* against the growth of *Mycobacterium tuberculosis* (Asres *et al.*, 2001). Of fifteen Palestinian plants tested against various bacterial species *in vitro*, 8 showed unique activities against various bacteria (Essawi & Srour, 2000). The *in vitro* study of 45 Indian medicinal plants against some drug-resistant human pathogens isolated 40 plant extracts with antimicrobial activity against one or more of the test bacteria (Ahmad & Beg, 2001). Therefore, the scope for sourcing natural alternatives to antimicrobial drugs is tremendous. However, the concentrations for bacterial growth inhibition and bactericidal properties would potentially be much greater than those associated with food supplementation, and problems may still exist through supplement toxicity restrictions and potential residues of additives. This may well limit the usefulness of many compounds as growth enhancers or gut modulators.

1.17 History of human interest in phytochemicals

Human interest in aromatic and medicinal plant phytochemicals began with the spice trade, which was mainly for incense purposes and was well established by 2000BC. This trade route was widespread between Arabia and other cultural centres, such as those of the ancient Egyptians, Greeks, Babylonians, Chinese, Sumerians, Indians and Aborigines. Several ancient physicians of interest have written books detailing the beneficial medicinal properties of herbs, including Galen, Dioscorides and Avicenna (Bremness, 1991). Neutraceutical use in modern medicine and industry has been explored since the 18th century, and many important medicinal plant compounds have been discovered. Aspirin, quinine, strychnine and morphine are only a small number of these compounds. Scientific research into the therapeutic properties of EO began in the 1920's with the French cosmetic chemist, René-Maurice Gattefosse, who found that lavender oil was superior to chemical antiseptics in treating infected wounds. Unfortunately however, a considerable amount of the reference material may be misleading, as it is based on a combination of scientific analysis and historical folklore, and there is a general lack of objective scientifically based data to back up the claims for these substances.

To confirm the validity of the medicinal properties of these herbal plants, the German federal health agency (*Bundesgesungheitsamt*) set up 'Commission E' in 1978 to undertake the first comprehensive scientific study on herbal medicines. Their findings represent the most

accurate scientific body of knowledge on herb safety and efficacy today (Tyler, 1994). Since 1995, scientific literature on the medicinal properties of botanical supplements has grown rapidly in terms of both its quality and accuracy, but is far from comprehensive and at this stage is variable in terms of both the efficacy and quality. In their study on the quality of 52 herbal reference books, Chambliss *et al.* (2002) describe a lack of appropriate scientific references to back up the text, insufficient information about interactions between plant supplements and existing drugs, or between one plant supplement and another. There is also a lack of appropriate guidance relating to the safety and efficacy for use of herbal materials.

Research into phytochemical compounds, or neutraceuticals as they are commonly known in humans, has only begun in the poultry industry and should be fully investigated, with a view towards introducing safe, non-toxic bio-active compounds into poultry diets. A research programme has already started in Asia and India, where preparations of herbal mixtures with proven positive health and productivity benefits have been prepared (Cruickshank, 2001). Several trials have demonstrated encouraging but variable performances with natural blends of EO and herbs in poultry to date (Losa, 2001; Losa & Köhler, 2001; Tucker, 2002; Mitsch *et al.*, 2002 & 2004; Lee *et al.*, 2003a & b; Jamroz *et al.*, 2003), a garlic and onion blended product for poultry diets (Osman *et al.*, 2002) and also organic acids in poultry (van Kol, 2002). Natural blends of EO have also been studied as dietary supplements for ruminants (McEwan *et al.*, 2002a & b; McIntosh *et al.*, 2003; Molero *et al.*, 2004; Newbold *et al.*, 2004), showing effects on protein degradability and the activity of microbial populations.

It may be possible to incorporate natural products such as those described above in poultry diets in order to provide a welfare or growth benefit, and at the same time increase the life-span of antimicrobials for clinical use in both humans and animals. Synthetic antimicrobial preparations have proven extremely susceptible to resistance development, but a complex "cocktail" of bio-active chemicals, such as those found in EO and other plants, along with product rotation strategies, may prove more efficacious for future use (Banks *et al.*, 1986). It may potentially also be useful to combine bio-active phytochemical compounds along with recognised dietary supplements, or with each other, to exert an alternative effect to those previously observed with synthetic antimicrobials. For example, a combination of 11 herbs based on the traditional system of Chinese and Indian medicine, known as Ayurvedic medicine, were noted for their positive effects on bronchodilatory and anti-inflammatory

properties in poultry respiratory problems (Cruickshank, 2001). Herbal substances are thought to act by stimulation of the immune and cardiovascular systems, intestinal tract and various organ and cell wall linings (McCartney, 2002), and may therefore be beneficial to bird health.

1.18 Why are secondary metabolites present in the plant?

Primary photosynthetic metabolism in all plant species produces carbohydrates and in lesser quantities, simple sugars, amino acids and low molecular weight carboxylic acids (Geissman & Crout, 1969), all of which are products essential for plant growth. Secondary plant metabolites may then be produced from these primary building blocks by means of specifically controlled reactions, which are more complex in the higher plant species. In general terms, secondary metabolites incorporate alkaloids, phenolics, flavonoids and bioflavonoids, saponins, terpenes, mucilage, glycosides, some amino acids and sugars, low molecular weight hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones, phenylpropanoids, and nitrogen and sulphur compounds such as coumarins (Geissman & Crout, 1969; Gill, 1999; Dorman & Deans, 2000). These compounds are not central to the functioning of cellular metabolic activity, but many have other important roles in the plant. Tens of thousands of secondary plant chemicals have so far been classified and more are being discovered at a rate of about 1000 per year, although only 20-30% of higher plants have been studied (Wink, 1999). Secondary plant compounds may be formed in plants when optimal conditions are limiting and the plants are under stress. However, other secondary plant products, such as lignin, may be formed as part of the natural ageing process in the plant.

Secondary metabolites are normally formed in the plant from basic metabolic pathways, such as glycolysis, the Krebs cycle or the shikimate pathway, using very specific enzymes, and degraded by less specific β-glucosidase enzymes, esterases and hydrolases (Wink, 1999). Previously considered as waste products, little is known concerning the biological significance of secondary metabolites in plants, but they are associated with roles in plant hormones, mainly for functions of dormancy and growth (Templeton, 1969). Terpenes have been associated with the defence of plants from attack by herbivorous insects, where their aroma attracts natural insect predators (Dicke *et al.*, 1990; Rose *et al.*, 1998; Small, 1997), or alternatively acting as feeding deterrants by inhibiting growth, development or reproductive processes in insects. The presence of different terpene compositions in *Thymus vulgaris* L. plants have been observed to affect the extent by which the plants were eaten by slugs

(Gouyon *et al.,* 1983). Most secondary plant products are normally synthesised in the cytoplasm, the endoplasmic reticulum or in cell organelles of the plant. Once formed, lipophilic secondary plant products are stored in resin ducts, glandular hairs, trichomes, thylakoid membranes, laticifers or on the cuticle, whereas water soluble compounds are usually stored in the vacuole (Wink, 1999). Their potential for use as medicinal antimicrobials has also been rated previously. In an *in vitro* study on 254 secondary plant chemicals, oxides were found to have the greatest activity against phytopathogenic bacteria, followed by ketones, acids, aldehydes, alcohols, esters, ethers, phenols, acetals, hydrocarbons and lactones (Maruzzella *et al.*, 1963). The bioactive potential of triterpenoid saponins, including a description of their anti-inflammatory, antimicrobial and analgesic properties, is given in the review of Mahato *et al.,* (1988).

1.19 Terpenes as plant secondary metabolites

Used industrially since the 18th century, terpenes and EO are involved today in the manufacture of pharmaceuticals, perfumes, cosmetics, in distilling, and also as aroma and flavour enhancers in foods (Cabo *et al.*, 1987). They are also found in narcotics, preservatives, vitamin supplements, soaps, paints and pigments (Mann *et al.*, 1994). Naturally occurring herbal terpenes are generally regarded as non-toxic in nature and are found in all parts of the higher plant, as well as in mosses, liverworts, algae and lichens. Within a five year period, the number of known terpene compounds identified has risen from 15,000 (Mann *et al.*, 1994) to over 22,000 (Wink, 1999), and the number is increasing still. Terpenes form the largest and most diverse secondary metabolite group, including mono-, sesqui-, di-, tri-, tetra- and poly-terpenoid compounds (Templeton, 1969), being classified into these subgroups according to the number of isopentane (5 carbon) "isoprene" units contained. In herbs, EO and oleoresins (semi-solid residues after the solvent extraction of EO), terpenes are mainly present as monoterpenes (C10), sesquiterpenes (C15) and may also include diterpenes (C20), and these lower molecular weight compounds are liquids at room temperature (Geissman & Crout, 1969; Templeton, 1969). The monoterpenes are usually quite volatile and are responsible for most of the dominant characteristic aroma of herbs, such as the mints in the family Lamiaceae. However, the sesquiterpenes are less volatile and tend to subtly modify the dominant aroma. Terpenes and steroid compounds share a common biosynthesis in the plant, along with carotenoids, steroids and polyisoprenoids (Mann *et al.*, 1994), utilising acetate and mevalonate as intermediates mainly by the acetate-mevalonate pathway (Charai *et al.*, 1996),

but they may also utilise the non-mevalonate pathway (McCaskill & Croteau, 1998). Terpenes may occur as a combination of stereoisomers, with one predominating in the plant, which could explain variations in their biological activity.

1.19.1 Metabolic pathway involved in the synthesis of terpenes

The biosynthesis of terpenes is a process where several enzyme-dependent steps are completely stereospecific. This process initially depends on the formation of isopentyl pyrophosphate (IPP) by phosphorylation and decarboxylation of mevalonic acid, and then a subsequent isomerisation to dimethylallyl pyrophosphate (DMAPP) (**Figure 1.2**; Moss, 1971). Addition of further IPP units to the DMAPP compound will produce a series of prenyl pyrophosphates including farnesyl and geranyl pyrophosphates, which will eventually form mainly mono- and sesqui-terpenes, as well as some acyclic terpenes (Moss, 1971). These acyclic terpenes have an irregular nature, and are formed if the main compounds are oxidised or reduced (Moss, 1971). Burbott & Loomis (1967) suggested that the site of monoterpene formation and metabolism in plants was isolated from both oxygen and most carbon substrates. The plant cells producing mevalonic acid, the necessary requirement for terpene biosynthesis, may therefore receive only certain metabolites such as sucrose, and terpene formation may be dependent on the substrate metabolites received (Burbott & Loomis, 1967).

1.19.2 Metabolic turnover and other influences on the terpene content in plants

Changes in the incorporation of a ^{14}C radioactive label within the plant, without a change in the overall numbers of monoterpenes, have been incorporated over time, suggesting that these terpenes were metabolised within the plant (Burbott & Loomis, 1969). This would suggest that the terpene composition was dynamic, where some of the terpenes are converted into others on a continuous basis. Peppermint monoterpenes may be involved in the recycling of plant energy and carbon sources from the leaves into the rhizome, by their degradation into acetyl CoA and incorporation into phytosterols and acyl lipids (Burbott & Loomis, 1969; Croteau *et al.*, 1987).

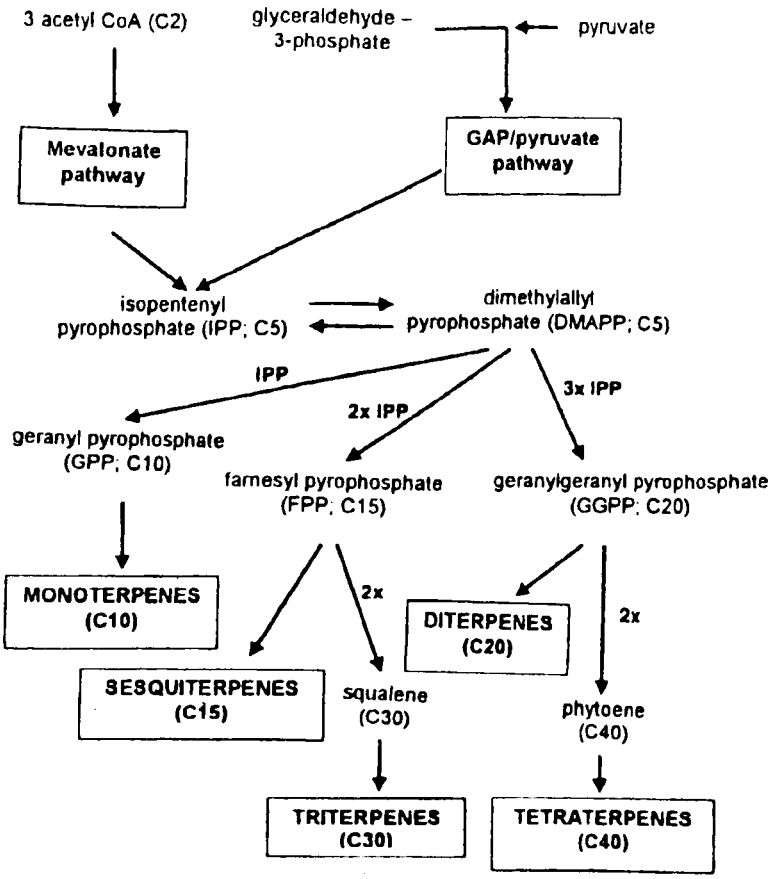


Figure 1.2 *Synthesis of the various classes of terpenes.*
IPP=isopentyl pyrophosphate; DMAPP=dimethylallylpyrophosphate; GPP= geranyl pyrophosphate; FPP= farnesyl pyrophosphate; GGPP=geranylgeranylpyrophosphate
(Adapted from Wink, 1999)

The monoterpene content of plants such as oregano may be indirectly influenced by the vegetative growth stages of the plant, with higher monoterpene contents in younger leaves (Kokkini & Vokou, 1989), which will therefore affect the EO yield and composition. It has been suggested that the lower monoterpene contents in older sage leaves are a result of terpene catabolism, where energy and carbon is recycled by the plant (Croteau *et al.*, 1987). Young leaves are most suitable for gathering between May and August (Greenhalgh, 1980), reaching peak monoterpene concentrations just prior to flowering (Burbott & Loomis, 1969). The oxidation or reduction state of the respiratory coenzymes of peppermint (*Mentha piperita* L.), which are determined by the rates of photosynthesis and respiration, may in turn influence the redox state of the monoterpenes (Burbott & Loomis, 1967). Burbott & Loomis, (1967) observed that 14hrs of daylength increased the yields of both the EO and the monoterpenes, and lowered the reducing potential of secretory cells within the peppermint. This would explain why most EO are produced in the Mediterranean and in warm countries with long

daylight hours. In a comparison of the EO content of several Greek subspecies, *Origanum vulgare* subsp. *hirtum* was shown to be positively influenced by the hotter temperatures and longer sunshine of the Mediterranean climate (Kokkini *et al.*, 1994). This resulted in greater numbers of oil glands and also amounts of EO produced, whereas plants from northern areas of the country with its continental climate had few oil glands and lower oil yield (Kokkini *et al.*, 1994). These studies may provide some explanation for the variable terpene content found within medicinal plant species, and thus the variable quality of plant sources of phytochemicals. The relatively low yield of active terpenes within some medicinal plant species may represent a potential problem in their usage as dietary supplements for poultry.

It may be possible to increase the levels of active constituents in medicinal plants, which may in turn reduce the cost of their inclusion within poultry diets. Supplementation of trace elements into the growing environment of these plants may be one method. An increase in the available trace element concentrations in the soil, especially chromium, manganese, iron and europium, increased the phenolics content and EO yield by 25% overall in Greek *Thymus capitatus* Hoffm. and Link. plants (Kanias & Loukis, 1987). There are also possibilities of improving the content of desirable secondary plant products by using genetic engineering to modify the plant biosynthetic pathways (Damiani & Arcioni, 1991; Lewinsohn, 1996), or by using plant cell culture and hairy root technology to enhance secondary metabolite levels (Hamill, 1987).

1.20 Plant herbs and their essential oils

The world trade in herbal supplements for humans currently exceeds $15 billion, with around $7 billion of this trade in Europe alone (Svoboda, pers. comm, 2002). This market is growing at a rate of about 15% per year. For the EO industry specifically, the world trade is around $US700 million and the market for this industry is growing at about 15% every year, due to the consumer-led trend for "natural" foods and alternative medicines and cosmetics (Svoboda, pers. comm., 2002). Herbs have been defined as 'crude drugs of vegetable origin utilised for the treatment of disease states, often of a chronic nature, or to attain or maintain a condition of improved health' (Tyler, 1994). They are often described as nutritional supplements and also as drugs, and it may be only the concentration required for their use that underlines this difference. When used as medicinal drugs, herbs are considerably more dilute than their synthetic counterparts, and the concentration at which they are used may determine their

therapeutic effects. EO have been defined as plant essences, or easily evaporated benzene or terpene derivatives that impart taste and aroma (Armstrong, 2001; Svoboda & Svoboda, 2000). There are two groups of EO, classified by their biogenetic origins. The most common are the terpene EO, and the second group of EO are based on the aromatic compounds derived from phenylpropane. The EO are mixtures of hydrocarbons or oxygenated derivatives of hydrocarbons, and may include such compounds as; terpenes, alcohols, esters, aldehydes, ketones, phenols, ethers and peroxides (Small, 1997). Although the EO may incorporate most of the chemicals within a plant, some non-volatile compounds known as "pungent or bitter principles" are normally found in viscous plant exudates or oleoresins left after extraction (Small, 1997). These may stimulate the production of digestive juices, along with secretion in the gall bladder and also peristalsis (Small, 1997). This stimulation of digestive secretions may be either a positive or negative effect, and should be studied in poultry.

The variations in demand and supply of the products within the world market for herbs and EO, which are generally grown in economically unstable countries, results in a "cobweb" economic cycle of high price fluctuations for these products (Greenhalgh, 1979; Essential Oils & Aromas, 1998). The yield and concentration of the active ingredients in drug plants are dependent on climate and soil conditions, the degree of plant maturity, the process of drying the plant and also the conditions of storage of the dried plant (Greenhalgh, 1979). In the plant, medicinal products can be extracted from the bark, roots, resin, wood, stems, leaves, flowers and fruits (Bremness, 1991). The processes of extraction of these natural compounds are complex and expensive. EO's are a mixture of a large number of individual chemical components. On average, it is expected that one EO will contain about 100 of these, including mainly terpenes, alcohols, esters, aldehydes, ketones and phenols, which give a synergistic effect in their mode of action (Worwood, 1990). It may be possible to enhance the effect of two or more oils if they are mixed together in the correct proportions, a technique that may prove beneficial in future medicinal use. However, it is equally possible that mixing EO will suppress the activity of bioactive compounds, so any mixing of bioactive components will require research before they can be used practically.

1.21 Quality control of herbs and essential oils

Quality control of both production processes and plant material represents something of a problem in the herb and EO industry. For the last few centuries, the EO industry has been

based in Grasse, in southern France, which is an influential region for fragrances and perfumes (Bremness, 1991). With the dropping of international trade barriers and globalisation, quality control standards are set by affluent Western countries which lack actual oil production, while the oils are being produced by less economically developed ones, who then have no say in the quality control process (Ibanez, 1999). The most stringent quality controls are imposed in the USA (Greenhalgh, 1979), but generally quality control varies between countries and between herbs, although cleanliness, colour, flavour, aroma and the time of harvesting are the most important quality considerations when buying (Greenhalgh, 1980). Large-scale cropping, as opposed to the gathering of wild herbs, could aid the consistency of product quality as well as weeding and disease control, as the demand increases. The medicinal properties of the oils have not previously been of any particular economic importance, as oils were distilled mainly for the perfumery industry. As a result, speciality combinations of less expensive oils have been prepared to simulate or change fragrance properties. The pharmacological quality in terms of the chemical composition of bioactive constituents is required to assess the efficacy of the EO or herb as a medicinal supplement. The desired terpene composition is usually requested directly from the supplier, and then an independent analysis is performed on the oil compositional quality. The chemical quality of an EO may be changed if a producer adds synthetic terpene analogues to boost this fraction in the composition in order to meet specific customer requirements for price and quality. Such practices were not previously important when EO were used for fragrance purposes. It has been recommended that new international standards be developed for the presence of residual solvents such as benzene or pesticides within the EO (Ibanez, 1999). Solvents such as these may be carcinogenic and would therefore be detrimental to animal and human health. The addition of synthetic terpene analogues to EO were suggested to be the reason for the recent allergenicity and sensitivity complaints in Europe, in oils used for aromatherapy in humans (33rd Essential oil workshop, Lisbon, 2002, pers. com.). However, the supplementation of EO into animal diets at low concentrations should not cause the same problems as oils directly applied onto human skin. The quality of the EO may also be reduced by dilution with a carrier vegetable oil base, which would cause it to leave a residue after evaporation. It is therefore essential to buy from a reputable supplier, and the oil price should differ with respect to the different types of EO, as some are harder to extract or are less readily obtainable than others. Bearing this in mind, it would appear that completely different standards should be set for the purposes of fragrance and pharmacological use of EO.

1.22 The antimicrobial action of essential oils and plant herbs

Phenolic compounds may potentially be suitable for the job of preventing the regeneration of bacterial spores and killing bacteria in the intestinal tract (Russell *et al.*, 1991). The EO of thyme and oregano have a similar mode of action, and are composed primarily of dominant phenolic monoterpenes such as thymol and carvacrol in various proportions (Daferera *et al.*, 2000). These terpenes are believed to be primarily responsible for the high antimicrobial activity of these oils (Cosentino *et al.*, 1999). Active terpene components include thymol, linalol, 1,8-cineole, chamazulene and carvacrol, in a range of proportions depending on the herb or EO. It has been suggested that active components such as thymol and carvacrol may produce an additive effect antimicrobially (Lambert *et al.*, 2001). Thymol, carvacrol and the terpene alcohols farnesol, nerolidol and plauntol, act by altering the cell membrane of bacteria (Helander *et al.*, 1998; Hertrampf, 2001; Inoue *et al.*, 2004). This increases the entry of water into the cell and release of potassium ions from the cell, which in turn increases cellular imbalance and decreases the osmotic integrity, eventually leading to the lysis or death of the cell (Helander *et al.*, 1998; Hertrampf, 2001; Inoue *et al.*, 2004). Inoue *et al.* (2004) demonstrated that the initial rate of potassium ion leakage and the amount of ions leaked were useful indicators of the extent of the antibacterial activity of these compounds. In a study into the antimicrobial properties of plant aliphatic α,β-unsaturated aldehydes (known as the alkenals) against phosphatidylcholine liposomes *in vitro*, these alkenals exerted a damaging effect against the lipid fraction of the plasma membrane, which increased its permeability and caused the leakage of cell contents (Trombetta *et al.*, 2002). Trombetta *et al.* (2002) observed that the antimicrobial activity of alkenals depended on the increasing length of its carbon chain, the content of the alkyl hydrophobic region and the presence of a double bond, with the presence of the double bond being the most important factor. Although some phenolics are perceived as being astringent compounds, the domestic chicken has only a moderately well developed sense of taste, with 24 taste buds, when compared to 15,000 in the pig (Kare & Rogers, 1976). The addition of these compounds in very low quantities within the bulk of the diet should reduce any problems associated with their astringent nature, and the effects *in vitro* suggest that these compounds could be utilized to manipulate the intestinal microflora of poultry.

1.23 Secretory structures and production of essential oils in the plant

The secretion of EO occurs in gland cells of the plant and involves the enzymically controlled discharge of substances. These substances include a range of salts, waxes, latex, fats, flavonoids, gums, resins, sugars, oleoresins and EO onto the plant exterior surface (exotropic secretion) or into specialized interior compartments (endotropic secretion) (Svoboda & Svoboda, 2000). The rate and amount of oil produced by the plant is dependent on the plant subspecies, along with the number and size of its secretory glands, with the largest secretory glands producing the most oil (Kokkini *et al.*, 1994). The types of secretory structures involved in EO production are generally a characteristic of the plant family and therefore may be used in plant identification.

Most plant species in the family Labiatae synthesize EO in glandular trichomes, which are cells formed from modified epidermal hairs on the surface of the plant leaf, stem or flower. These modified cells occur at a very early stage of plant development, and are formed by the separation or disintegration of parenchymal cells to leave intracellular spaces, known as lumina or lacuna. Externally, an impenetrable waxy cuticle normally encloses the surface of each glandular trichome head. EO are produced by sub-cuticular secretory cells, which are attached by a single basal stem cell to the epidermis. The EO then diffuse outwards from the centre of the plant towards its external surface. The cuticle ruptures to release the EO when sufficient concentration has accumulated. In fully mature glands, EO production may require a high investment of energy from ATP, as mitochondria are present in high numbers (Svoboda & Svoboda, 2000). In *Origanum vulgare* ssp. *hirtum*, the secretory glands are composed of 1 basal and 1 stalk cell surrounded by a secretory trichome head of 4 central and 8-10 peripheral cells (Kokkini *et al.*, 1994). These secretory glands are detached from the outer part of the cell wall by a cuticle and contain the EO (Kokkini *et al.*, 1994). *Achillea millefolium* L. produces azulene (the precursor to the sesquiterpene chamazulene) in the secretory trichomes of the ray and disk florets and also on the plant leaves (Svoboda & Svoboda, 2000). A fully mature trichome in *A. millefolium* L. is comprised of 10 cells, including 1 pair each of basal and stalk cells, and 3 pairs of glandular cells (Svoboda & Svoboda, 2000).

Figure 1.3 *Glandular trichome on the flower of Achillea millefolium L. (yarrow) [SEMx700]*
(Photograph reproduced by kind permission of K. Svoboda)

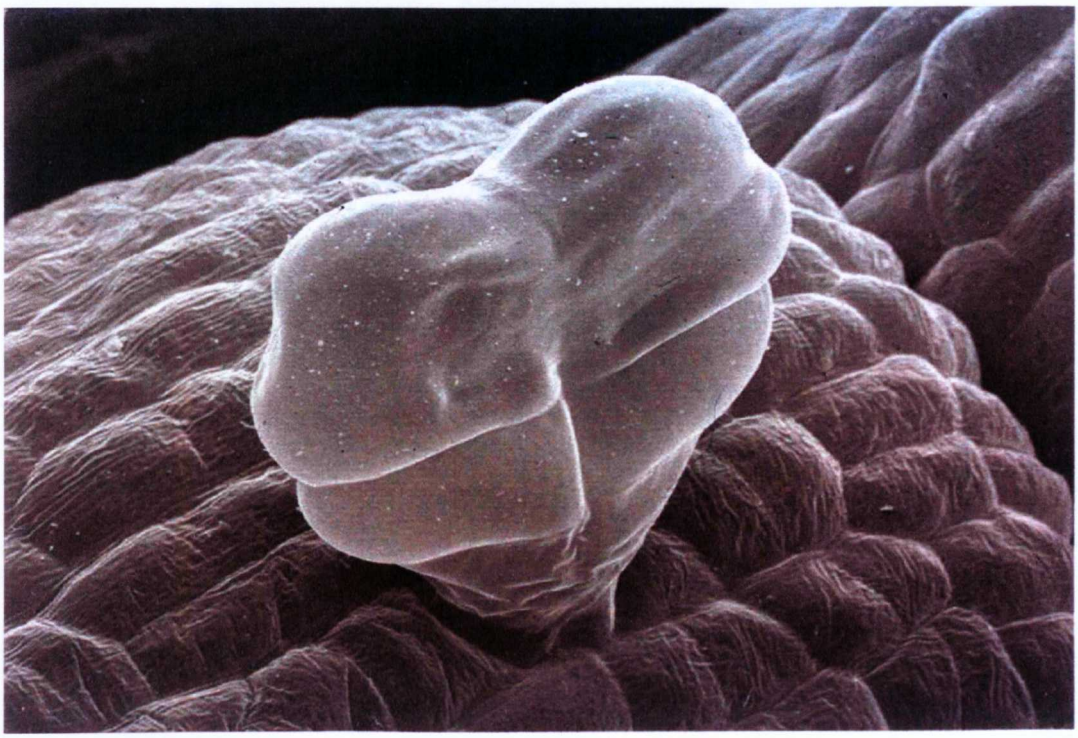


Figure 1.4 *Glandular trichome on the leaf of Origanum vulgare L. (oregano), with ruptured cuticle, revealing the individual secretory cells inside [SEMx1230]*
(Photograph reproduced by kind permission of K. Svoboda)

Figure 1.5 *Cross section of leaf of Rosmarinus officinalis L. (rosemary), revealing sessile and stalked glandular trichomes (cryo-SEM) [x615]*
(Photograph reproduced by kind permission of K. Svoboda)

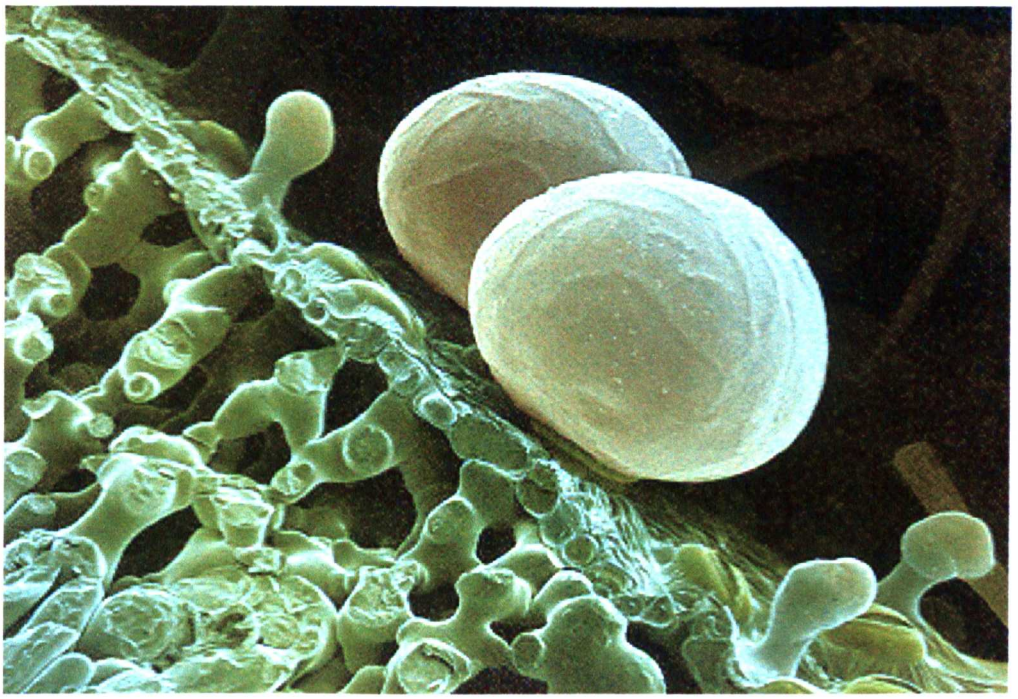
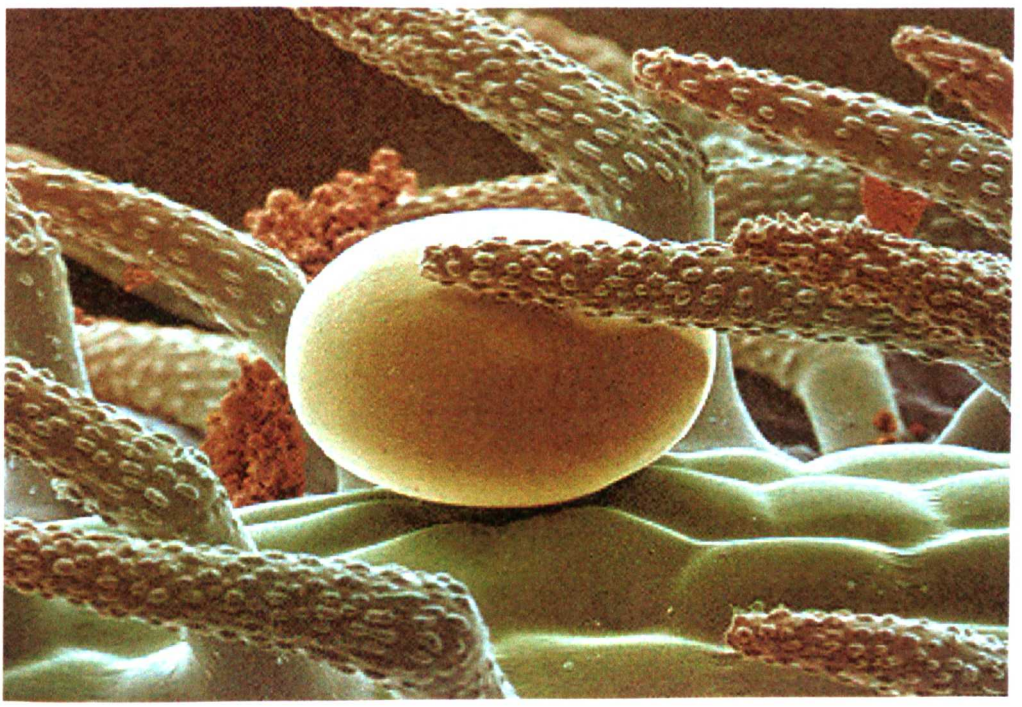


Figure 1.6 *Glandular trichomes and non-glandular hairs on the stem of Thymus vulgaris L. (Thyme) [SEM x1230]*
(Photograph reproduced by kind permission of K. Svoboda)



Steam distillation is the most common method of extraction of the EO from plants. Other methods include solvent extraction, expression, enfleurage and maceration, depending on the plant species. The methods involved for the extraction are time-consuming, labour-intensive and require expert use of complex equipment and large quantities of plant material. The terpene compounds are normally identified using gas chromatography (GC) and mass spectrometry (GC-MS), but solid-phase microextraction coupled with GC-MS can also be used (SPME-GC-MS) (Rohloff *et al.*, 2000). The drying process in herb preservation involves reducing the moisture within the harvested herb to a maximum of 5-10% by continuous heating at a temperature of 40°C, which will allow the herb to retain most of its original colour and terpene content, while minimising spoilage (Greenhalgh, 1980).

1.24 Monoterpene containing plants and their properties

The following section describes the monoterpene plants used in the work reported in this thesis, giving a brief explanation of their botanical and bioactive properties. In general, the plants used in the present studies originate from the mint family (Labiatae or Lamiaceae), the onion family (Alliaceae) and the sunflower family (Compositae). A detailed description of the variability in the composition of terpenes for each plant used; namely thyme, marjoram, rosemary, oregano and yarrow plants is found in **Appendix 1** (after Lawrence, 1976-1994).

1.25 THYME

1.25.1 Botanical description and origin

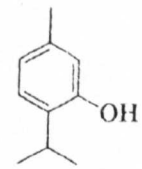
Thymus vulgaris L. is one of about 350 Thymus species in the mint family Labiatae and is a native of the western Mediterranean, but can also found growing wild, in rocky areas and on grassland throughout Eurasia, in Egypt (Worwood, 1990; Bown, 1998) and in North America (Armstrong, 2001). Its name comes from the Greek "thymon", meaning courage, and extracts of the plant may have been drunk before battle to provide strength. It is cultivated in Germany, France, Hungary and other countries (Stary & Jirásek, 1973). The genus Thymus incorporates a wide variety of evergreen, aromatic, woody-based perennials and shrubs. Known as culinary or common thyme, *Thymus vulgaris* L. is a semi-shrub growing from 30-45 cm in height with linear to oval leaves and bearing small, cylindrical flowers ranging from white to purple between May to October (Bown, 1998).

1.25.2 Economic and traditional medicinal reasons for cultivation

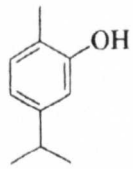
Thyme has been used as a culinary herb in flavouring game and fish, stews, butters, cheeses, onions, tomatoes, stuffings and pickles. It has been noted for its strong smell and relatively pleasant flavour (MAFF, 1980). Common thyme is used for the treatment of whooping cough, warts, rheumatism, acne, neuralgia and fatigue in aromatherapy (Worwood, 1990) along with digestive and respiratory diseases, gargles, toothpastes and mouthwashes due to its disinfectant properties (Starý & Jirásek, 1973; Armstrong, 2001; Bremness, 1991). When applied to the trachea, thymol and carvacrol have relaxant properties, and thyme extracts cause an increase in secretion of bronchial mucus (Duke, 1985). Thyme is a reputed folk remedy for anaemia, asthma, bad breath, bronchitis, bruises, callosities, cancer, catarrh, colds, colic, cough, cramps, diabetes, diarrhoea, dysmenorrhea, fever, flatulence, gastritis, gastroenteritis, gingivitis, gout, headache, indigestion, nerves, neuralgia, sciatica, sclerosis, snakebite, sore throat, sprains, tumours, warts, whooping cough and worms (Duke, 1985). The burning of thyme is supposed to repel insects, it is used as a fungicide to treat mildew, and it is also an ingredient in toothpaste. It is also reported to stimulate the production of white blood corpuscles to resist infection (Bremness, 1991).

1.25.3 Chemical composition

Thymus vulgaris L. or thyme plants contain between 0.5-2.5% of EO and up to 7% tannins, bitter principles and flavones (Starý & Jirásek, 1973; Dewick, 2002). Duke (1985) reports that triterpenoid saponins, flavones, ursolic acid (1.5%), caffeic acid, tannins and resins occur in the herb. The main constituents of thyme EO are primarily thymol and/or carvacrol terpene hydrocarbons, depending on the chemotype of the plant. These terpenes are primarily thymol or carvacrol (at around 40% composition), *p*-cymene, terpinene-4-ol and linalol (Duke, 1985; Dewick, 2002). Chemically, the plant will contain about 9 g protein, 7.5 g fat, 64 g total carbohydrate, 19 g fibre, 12 g ash, 1890 mg Ca, 201 mg P, 124 mg Fe, 220 mg Mg, 55 mg Na, 814 mg K, 6 mg Zn, 3800 IU vitamin A, 0.5 mg thiamine, 0.4 mg riboflavin, 5 mg niacin, 0 g cholesterol and 163 mg phytosterols in 100g DM (Duke, 1985). However, the composition of thyme EO is extremely variable, due to its complex genetic polymorphism and the varying environmental conditions where the plants are grown. De Feo *et al.* (2003) reported that the terpene composition in the EO of *T. spinulosus* was related more to soil composition than altitude above sea level, with high monoterpene contents in thyme grown on calcareous soils and high sesquiterpene contents on siliceous soils (**Table 1.2**).



Thymol



Carvacrol

Figure 1.7 *Common or garden thyme (Thymus vulgaris L.)*

Adapted from Small, (1997)

Table 1.2 *Variation in terpene fractions and composition in samples of T. spinulosus essential oil grown under varying soil compositions and altitude above sea level*

Terpene composition (% of total oil)				
	Environmental composition			
Type of terpenes identified	*Oil sample 1 Calcareous (1140m)*	*Oil sample 2 Calcareous (1060m)*	*Oil sample 3 Siliceous (1140m)*	*Oil sample 4 Siliceous (1060m)*
Monoterpenes	58.5	44.0	19.5	35.6
Oxygenated monoterpenes	10.7 (8)	3.4 (4)	12.0 (7)	8.0 (9)
Sesquiterpenes	32.3	49.0	69.4	58.1
Oxygenated sesquiterpenes	2.6 (5)	11.6 (5)	19.4 (7)	13.3 (7)
Phenols	0.7	0.0	1.1	1.0
Total no. of components	53.0	40.0	50.0	56.0
Total % oil identified	93.3	97.2	93.4	95.9

The figures in brackets describe the numbers of compounds identified of each terpene type
Each plant sampled was grown under varying conditions of soil composition and altitude as outlined in the table.
(Adapted from De Feo *et al.*, 2003)

1.25.4 Evidence for the bioactive significance of the plant and its essential oil

Strong bacteriostatic or bactericidal properties against a range of bacteria have been noted for thyme EO *in vitro* (Smith-Palmer *et al.*, 1998; Dorman & Deans, 2000; Aureli *et al.*, 1992, De Feo *et al.*, 2003; Bagamboula *et al.*, 2004). The antioxidative nature of thyme EO has also been proven *in vitro* (Deighton *et al.*, 1993), and the plant has shown antifungal properties *in vitro* (Daferera *et al.*, 2000). In their study on the *in vitro* antibacterial properties of various herbs, Tabak *et al.* (1996) reported the complete inhibition of bacterial growth with thyme extracts at a concentration between 3.5-4.5 mg ml^{-1}. This gave the thyme extract a higher antibacterial activity than various common antibiotics including nalidixic acid, but lower than that of ampicillin, tetracycline or erythromycin (Tabak *et al.* 1996). De Feo *et al.* (2003) also suggested that the high antimicrobial potential of the EO of *T. spinulosus* compared to antibiotics such as gentamycin and tetracycline is due to its chemical composition, but noted that these plants represented a different chemotype than most plants within the *Thymus* spp. Additionally, thyme EO may have potential in the treatment of antibiotic resistant bacterial infections, as antimicrobial activity *in vitro* has been demonstrated against antibiotic-resistant bacteria such as *Pseudomonas aeruginosa, Enterobacter aerogenes* and MRSA (Nascimento *et al.*, 2000; Nostro *et al.*, 2003).

1.26 ROSEMARY

1.26.1 Botanical description and origin

The genus Rosmarinus is also part of the mint family Labiatae and contains a number of species and varieties, including *Rosmarinus officinalis* L. The plant is native to the rocky limestone places, dry scrub and open woodlands of the Mediterranean. It is also found growing wild in other countries including Spain, France, Yugoslavia, Italy, North Africa and Japan (Worwood, 1990; Starý & Jirásek, 1973) and has been introduced to Britain and North America (Stobart, 1970). *Rosmarinus officinalis* or rosemary grows to around 2m in height, and is a dense bushy evergreen shrub with dark, straight, highly aromatic, leathery leaves. The plant flowers in spring to early summer, producing either white or pale to deep purple tubular flowers (Bown, 1998).

1.26.2 Economic and traditional medicinal reasons for cultivation

As well as being an important culinary herb plant, the EO of rosemary acts both as a stimulant and on muscular tissues (Starý & Jirásek, 1973). It is said to increase the flow of gastric

secretions and blood circulation (Starý & Jirásek, 1973; Bremness, 1991) and is used in aromatherapy against upset stomach and externally for blood circulation problems by increasing the circulatory flow (Armstrong, 2001). Historically, both the herb and the EO of rosemary have been used as emmanagogues (to stimulate menstrual flow) and as abortifacients (Tyler, 1993). In humans, it is used today in aromatherapy to treat arthritis, rheumatism of muscles and joints, headaches and migraines, coughs, influenza, depression, fatigue and memory loss (Worwood, 1990). It is also used as an antiseptic gargle and in mouthwashes in humans (Bremness, 1991). In the therapeutic uses of aromatherapy, the use of an EO is highly dependent on its chemotype. The 'camphor' type is used for stimulation of the circulation and as a muscle relaxant, the 'cineole' type for antibacterial and anti-fungal properties, and finally the 'verbenone' type as an endocrine system regulator (Anon, 1996). Economically, the EO of rosemary is used in wine, perfumes, shampoos, soaps, deodorants, hair tonics to prevent baldness, in cosmetics and also as insect repellents (Duke, 1985).

1.26.3 Chemical composition

The flowers and leaves of rosemary contain about 1-2% of EO, as well as tannins, with an aromatic flavour reminiscent of camphor (Starý & Jirásek, 1973). The EO of the plant is credited with most of its bioactive properties. Wolski *et al.* (2000) determined that the EO content for rosemary was around 1.5-2%, with an average content of 1.78%. The leaves also contain saponin, tannin, ursolic acid, carnosic acid, amyrins, betulin and rosmarinic acid (Duke, 1985). Rosemary contains many flavonoids such as diosmetin, diosmin and apigenin, along with phenolic acids including caffeic, chlorogenic, noechlorogenic and rosmarinic acids (Tyler, 1993; Wolski *et al.*, 2000). Of the EO constituents, the major terpene compounds identified and their % composition include cineole (15-45%), α-pinene (10-25%), camphor (10-25%) and β-pinene (8%) (Dewick, 2002). There are 3 chemotypes of rosemary EO. The first is based on the camphor, α- and β-pinene and borneol components and known as the camphor type (at about 30% camphor), the second type is based on 1,8-cineole and linalol (between 40-55% 1,8-cineole) and the third is based on verbenone (at 15-40% verbenone) (Wolski *et al.*, 2000). Generally, 1,8-cineole and linalol produce a sweeter and more pleasant taste, when compared to α- and β-pinenes, camphor & borneol, which produce a harsher woody taste (Svoboda, 2003, pers. comm.). On a dry matter basis, 100 g of the rosemary plant contains about 5g protein, 15g fat, 64g carbohydrate, 18g fibre, 6g ash, 1280mg Ca, 70mg P, 29mg Fe, 220mg Mg, 50mg Na, 955mg K, 3mg Zn, 3128 IU vitamin A, 0.5mg thiamine, 1mg

niacin and 61mg ascorbic acid (Duke, 1985).

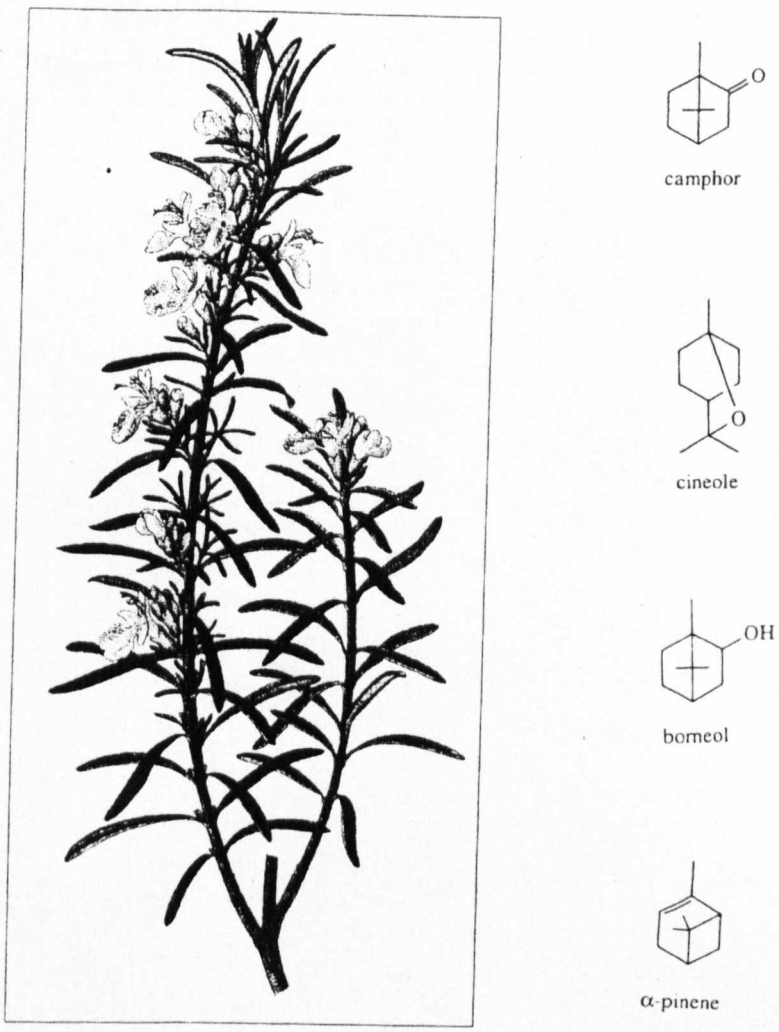


Figure 1.8 *Rosmarinus officinalis L.*

Adapted from Small, (1997)

1.26.4 Evidence for the bioactive significance of the plant and its essential oil

Rosemary is not widely used for its bioactive antimicrobial properties and as a phytomedicinal its significance is largely unknown. However, the antimicrobial properties of the EO of rosemary (Narasimha Rao & Nigam, 1970; Deans & Ritchie, 1987; Pandit & Shelef, 1994; Smith-Palmer *et al.*, 1998; Hammer *et al.*, 1999) and antioxidant properties of rosemary extract (Gűntsperger *et al.*, 1998; Dorman *et al.*, 2003) have been described. Tyler (1993) indicated that oral doses of rosemary EO are not safe at therapeutic levels, as it irritates the stomach, kidneys and intestines. However, it was still approved for internal use by the German authorities based on the recommendations of Commission E (Tyler, 1993). When fed

in the diets of laying hens, there was no antioxidant effect of rosemary EO in eggs, compared to the highly antioxidative effect of α-tocopherol acetate (Galobart *et al.*, 2001). Rosemary extract was found to increase the intracellular accumulation of common synthetic therapeutic antimicrobials in drug-resistant human breast cancer cells, by modulating the activity of the transmembrane transport pump P-glycoprotein (Plouzek *et al.*, 1999). Thus, it was considered to have potential in cancer therapy.

1.27 OREGANO

1.27.1 Botanical description and origin

The genus Origanum is a member of the family Labiatae and contains about 20 species of evergreen or semi-evergreen shrubs. Its name originates from the Greek "oros gonos", or joy of the mountain. *Origanum vulgare* L. grows in dry and sunny conditions, being distributed throughout hillsides, meadows and woodlands from the lowlands to the mountains of Mediterranean Europe and the Middle East, Iran and Southwest Asia. Oregano is a rhizomatous perennial growing to about 45 cm in height, with dark-green, rounded to oval leaves and dense clusters of white to pink-purple flowers mixed with purplish bracts from mid summer to autumn (Bown, 1998). It has been introduced into the Far East and North America.

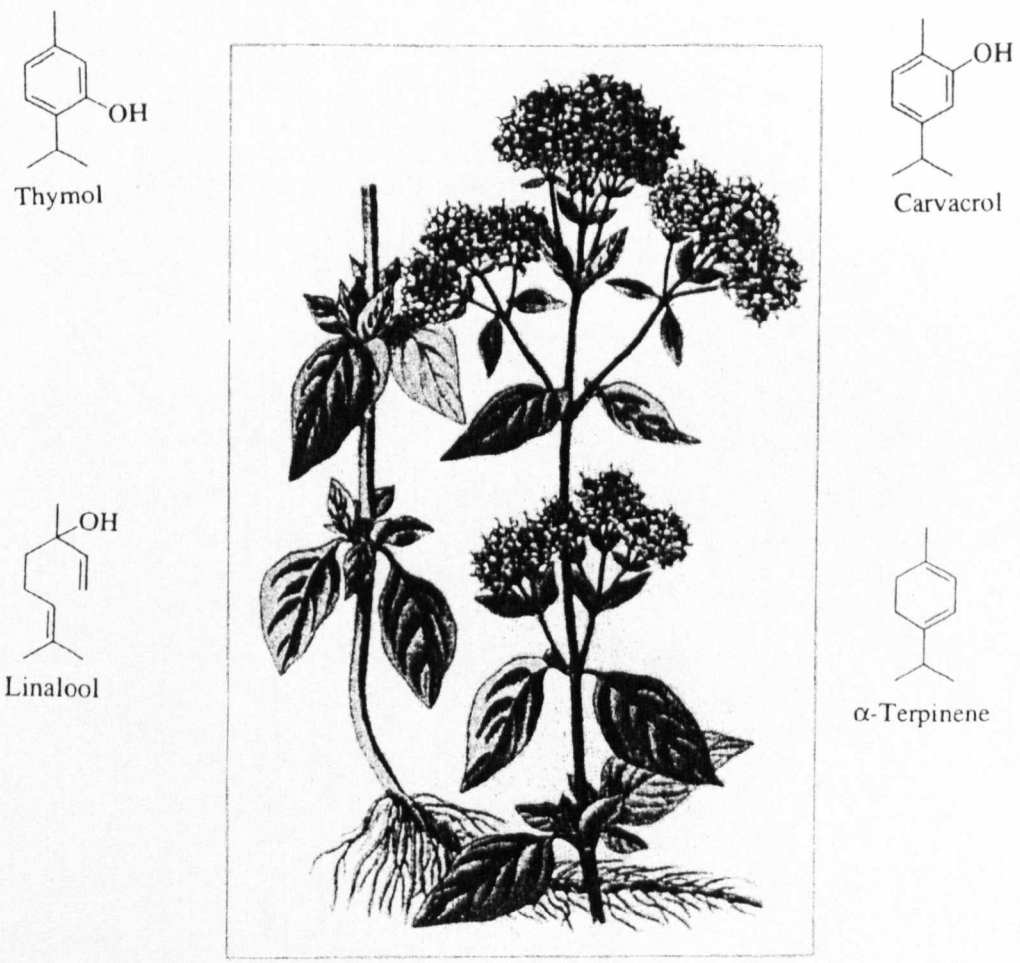
1.27.2 Economic and traditional medicinal reasons for cultivation

The flowering shoots are the parts collected for their pharmacological properties. Commercial dried oregano is cultivated and produced from several species, including *O. vulgare* and *O. vulgare* subsp. *hirtum*. It is used in the food industry as flavouring, especially in Italian cooking for pizzas, salads and meat, and in the cosmetic industry in soaps and other products. Traditionally, oregano is used to aid digestion and for stomach and gallbladder disorders (Bremness, 1991), as it is believed to have anti-spasmodic effects, with anti-inflammatory action and disinfectant properties. It has also been used externally for swellings and as an inhalant or gargle in treating heavy coughs and respiratory tract diseases (Bremness, 1991).

1.27.3 Chemical composition

The oregano plant contains EO, bitter principles and tannins (Starý & Jirásek, 1973). The EO is a yellowish-red to dark-brownish-red liquid, depending on its terpene composition. The species is not homogenous, and has two recognised predominant chemotypes, based on either thymol or carvacrol. The plant *O. vulgare* ssp. *vulgare* commonly has a low EO yield, at

<0.3% (Kokkini & Vokkou, 1989), which may rule out its use for economic reasons. In contrast, the EO content of the *O. vulgare* ssp. *hirtum* is very rich, varying from 1.8-8.2% (Kokkini & Vokou, 1989). The chemical properties of oregano EO can be characterised in terms of its carvacrol and thymol content, which vary between the different subspecies, and are especially variable in *O. vulgare* subsp. *hirtum,* with carvacrol ranging between 2.4-95% of composition (Kokkini & Vokou, 1989; Kokkini *et al.*, 1994). The antioxidant properties of oregano are generally due to the presence of rosmarinic and caffeic acids, and also to the glycosides within the plant, which release volatile compounds by acid or enzymic hydrolysis (Milos *et al.*, 2000). The best type of oregano is normally considered to be "Greek" oregano, which commonly has high thymol and carvacrol. Over a range of *O. vulgare* ssp. *hirtum* plants, the thymol content varied from 7.0-52.6%, and carvacrol somewhere between 0.12-56.6%, with their precursors *p*-cymene and γ-terpinene in lower quantities to make up the bulk of the EO composition (Sivropoulou *et al.*, 1996; Russo *et al.*, 1998).



Adapted from Small, (1997)

Figure 1.9 *Origanum vulgare L.*

The EO of oregano may also contain an intermediate mixture of these two phenolic compounds and their precursors, *p*-cymene and γ-terpinene (Russo *et al.*, 1998; **Table 1.3**). A typical EO of oregano from Greece contains high phenols at 45.2% thymol, 33.1% carvacrol, 5.54% γ-terpinene and 7.35% *p*-cymene (Adam *et al.*, 1998). The analysis of a Northern Iranian *O. vulgare* ssp. *viride* revealed a low proportion of monoterpenes such as thymol and carvacrol, and a much higher proportion (44%) of sesquiterpene components such as β-caryophyllene, caryophyllene oxide and germacrene (Afsharypuor *et al.*, 1997). The variability in the terpene composition of the *O. vulgare* plant is extensive.

Table 1.3 *The dominant chemotypes within Origanum vulgare essential oils and their relative composition, based on a comparison of 24 samples from different areas of Greece*

Compositional variation in chemotypes of the essential oil of O. vulgare				
Component class	*Thymol*	*Thymol/Carvacrol*	*Carvacrol*	*Carvacrol/Thymol*
Phenols	49	47	61	57
Monoterpene hydrocarbons	38	39	25	29
Oxygenated monoterpenes	8	8	7	8
Sesquiterpenes	4	5	3	8
Others	Trace	Trace	Trace	Trace

The information in the table is based on a comparison of 24 oil samples from different areas of Greece (Adapted from Russo *et al.*, 1998)

1.27.4 Evidence for the bioactive significance of the plant and its essential oil

Oregano has been shown to possess antioxidant properties *in vitro* (Deighton *et al.*, 1993; Hammer *et al.*, 1999; Dorman *et al.*, 2003) and in chicken meat (Botsoglou *et al.*, 2002; Young *et al.*, 2003; Papageorgiou *et al.*, 2003). It also has antimicrobial properties against both Gram-negative and Gram-positive bacteria *in vitro* (Sivropoulou *et al.*, 1996; Hammer *et al.*, 1999; Sagdic, 2003), anti-fungal properties *in vitro* (Azzouz & Bullerman, 1982; Paster *et al.*, 1994; Adam *et al.*, 1998; Daferera *et al.*, 2003). Oregano has also been reported to have anticoccidial properties in chickens (Gill, 1999). Thymoquinone and other minor chemical components have been considered to produce a significant portion of the antioxidant effects, in combination with or separate to those associated with thymol and carvacrol (Milos *et al.* 2000). When infecting epidermal cells with *Trychophyton rubrum*, the precursor to ringworm, which is associated with lesion development, thyme EO was reported to have no potential activity for causing mutations in epidermal cells (Adam *et al.*, 1998). However, Adam *et al.* (1998) suggested that a long period of treatment might be required for this to take place.

1.28 MARJORAM

1.28.1 Botanical description and origin

A member of the family Labiatae and also the genus Origanum, *Origanum majorana* L. originates in the area from Libya and Egypt through Arabia to India, and is also found in Southwest Africa. In the Mediterranean regions it has been cultivated for centuries and is now accepted as a naturalised perennial. The plant *O. majorana,* known as marjoram or sweet marjoram, is an erect, bushy, aromatic shrub, which may be annual or biennial. Each plant grows up to 80 cm in height, with oval, grey-green leaves and pink or white sprays of tubular flowers in early to late summer (Bown, 1999). It is cultivated today in the Mediterranean, along with Germany, Hungary, the Czech Republic, Asia and North America.

1.28.2 Economic and traditional medicinal reasons for cultivation

Marjoram has a sweet and scented flavour and is used as one of the most important culinary herbs to add flavour to meat dishes when used in small amounts. It is recommended that this plant should not be used in pregnancy, due to its anti-spasmodic properties (Bremness, 1991). However, it is noted for its digestion-aiding properties when used to treat intestinal colic, diarrhoea, flatulence and digestive upsets; it stimulates gastric juice production and is also applied externally to treat persistent wounds (Bremness, 1991; Starý & Jirásek, 1973).

1.28.3 Chemical composition

The bioactive principles in Marjoram are found in the aerial plant parts, which are harvested in June or July before flowering occurs. The plant comprises up to 2% EO, which is fairly constant in composition, and also contains bitter substances and mucilages (Starý & Jirásek, 1973). Sarer *et al.* (1982) examined the chemical composition of *O. majorana* EO found growing wild in Turkey, concluding that the EO contained 12.6% monoterpene hydrocarbons, 18.4% oxygenated monoterpenes and 69.0% phenols. Likewise, Granger *et al.* (1975) examined *O. majorana* and concluded that the EO existed in 2 chemotypes, based predominantly on either cis-sabinene hydrate or terpinene-4-ol. Both are bio-genetically related, so there is a central theme for the chemical basis of marjoram EO.

1.28.4 Evidence for the bioactive significance of the plant and its essential oil

The antimicrobial activity of the EO of marjoram has been described *in vitro* (Duke, 1985; Deans & Ritchie, 1987; Charai *et al.*, 1996; Smith-Palmer *et al.*, 1998; Hammer *et al.*, 1999;

Mejlholm & Dalgaard, 2002), along with its anti-fungal activity (Daferera *et al.*, 2000 & 2003).

Figure 1.10 *Origanum majorana L.*

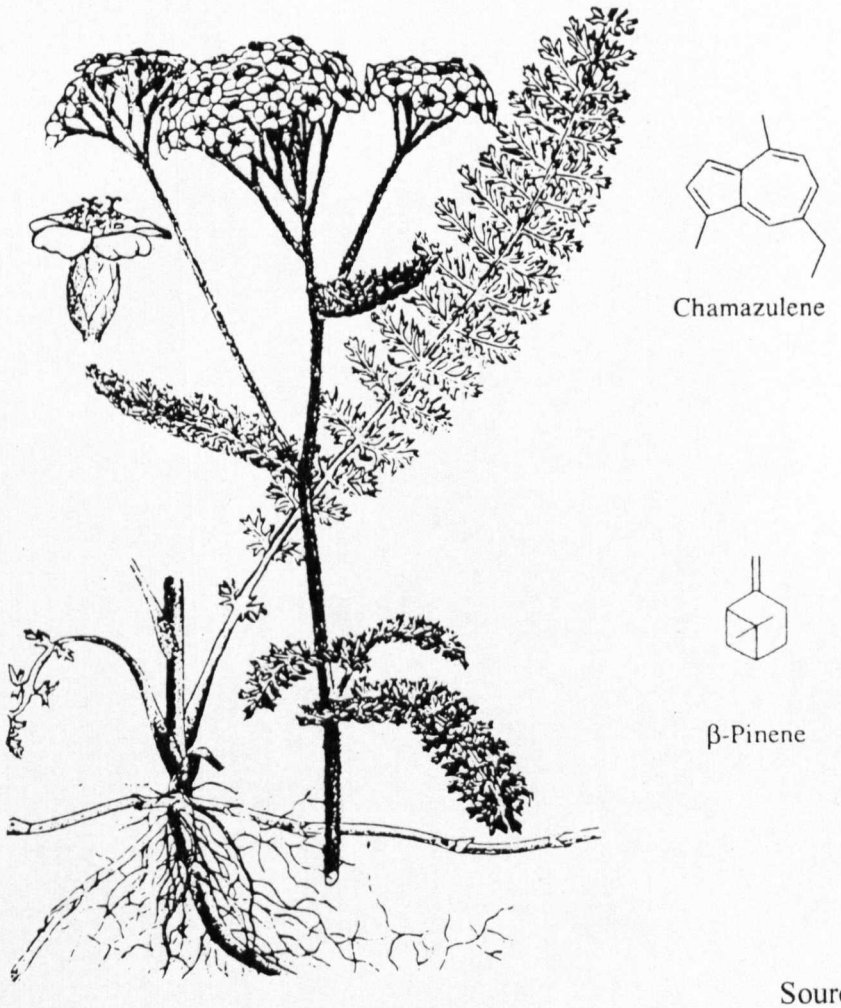
Adapted from Small, (1997)

1.29 YARROW

1.29.1 Botanical description and origin

Belonging to the plant family Compositae/Asteraceae or daisy family, *Achillea millefolium* L. is a perennial closely related to the chamomiles. Yarrow is also known as woundwort, knight's milfoil, devil's nettle, old man's pepper or thousand weed. It originates from grassland habitats such as meadows, hedgerows and woodland clearings in Britain, along with temperate parts of Europe and western Asia. The plant has also been introduced to North America, Australia and New Zealand. Its scientific name originates from the Greek legendary hero Achilles, in the battle of Troy, who was said to have cured wounded soldiers with the

plant (Slavik, 1974; Armstrong, 2001). Yarrow grows from 30-60 cm in height, forming a carpet by means of rhizome production, with erect stems, linear, divided dark-green leaves and bearing a flattened inflorescence between June and September of tiny white, cream or pale pink flowers (Bown, 1999). The plant has flowers characteristic of the Compositae family, with disc flowers in the centre and ray flowers or "petals" around the rim. The use of locally grown material in studies, and the polymorphic genetic nature of the plants makes it difficult to distinguish the plant species used (Chandler *et al.*, 1982), thus the presence of taxonomy details in the scientific literature are very important.



Source: Duke, (1985)

Figure 1.11 *Yarrow plant (Achillea millefolium L.)*

1.29.2 Economic and traditional medicinal reasons for cultivation

Traditionally, yarrow has been used as a folk remedy to treat digestive disorders, cramps, various skin conditions and minor infections. Yarrow has been reputed to stimulate the production of gastric secretions, stimulate appetite, soothe coughs, cure ulcerous wounds and

rashes and act as a haemostatic to stem internal bleeding (Slavik, 1974) and external wounds (Stobart, 1970; Mitich 1990). It is also noted for its beneficial effects on blood circulation and anti-inflammatory properties, leading to its use in treatment of rashes and gum inflammation (Starý & Jirásek, 1973; Tyler, 1993). Duke (1985) mentioned that yarrow is used as an herbal tea, in herbal tobaccos and also as a hair rinse or shampoo to prevent baldness. Extracts are used in bath preparations to calm the skin, and the plant is also used to flavour bitters and vermouth (Duke, 1985). The yarrow plant is a component in the drugs used in conventional medicine to treat hepatitis, and it is also used as a tea to relieve depression (Mitich, 1990). The plant is being investigated as a larvicide and for anti-tumour activity (Mitich, 1990). An extensive range of medicinal uses for yarrow have been reported in humans, including the treatment of bruises, sprains, swollen tissues, fevers, dysentery, diarrhoea, wounds, rashes and as an anaesthetic (Chandler *et al.*, 1982a). It is also used as an anti-spasmodic agent for treating menstrual cramps (Tyler, 1993). Chandler *et al.* (1982a) also provides an extensive list of the non-medical and cosmetic uses of the plant. Slavik (1974) reports that yarrow added to animal fodder aids digestion, but recommends the use of only limited quantities of young shoots.

1.29.3 Chemical composition

Yarrow is closely related to the chamomiles, both botanically and chemically, and the properties of the EO are mainly due to the azulene derivatives contained within (Tyler, 1993). The aerial parts of the plant are used for medicinal purposes, and the plant contains flavones, tannins, and has an EO content of about 0.5% (Starý & Jirásek, 1973). Over 120 components have been characterised in yarrow plants, and many others have not yet been determined (Chandler *et al.*, 1982b). Chemically, 100g of the yarrow plant contains about 13-14.5 g of protein, 2-4 g fat, 72 g total carbohydrate, 20-24 g fibre, 10-12.5 g ash, 1,330 mg Ca and 360 mg P on a dry matter basis (Duke, 1985). The EO contains sesquiterpene lactones, which are precursors of the azulenes and chamazulene, and the plant also contains flavonoids and alkaloids such as achilleine, sterols and triterpenes, which determine its biological activity (Chandler *et al.*, 1982b). Other compounds, including the monoterpenes camphor, menthol and eugenol, as well as salicylic acid and sterols will also have bioactivity (Chandler *et al.*, 1982 a & b). The EO also contains linoleic-, oleic, cerotic-, myristic- and palmitic acids, as well the amino acids alanine, glutamic acid, histidine, leucine and lysine (Duke, 1985). Candan *et al.* (2003) found eucalyptol, camphor, α-terpineol, β-pinene and borneol as the

principal terpene components of their yarrow EO. The yield of EO, although generally low, varies with the developmental stage of the plant, from 0.13% in the vegetative stage to 0.34% when the plants are in full bloom (Rohloff *et al.*, 2000). The terpene content is also associated with the plant developmental stage. Flowering yarrow plants yield mainly monoterpenes (about 80%), with 1,8-cineole, sabinene and *trans*-sabinene hydrate the most dominant components in both flowers and leaves (Figueiredo *et al.*, 1992). Yarrow plants in the vegetative stage consist mainly of sesquiterpenes (92%) such as germacrene-D, which has around 65% composition within the plant (Figueiredo *et al.*, 1992). The increased mono- and sesqui-terpene concentration when more mature plants are harvested has also been described (Rohloff *et al.*, 2000). It has also been suggested that the pharmacological action of yarrow may be dependent on its concentration of the sterols, including β-sitosterol and stigmasterol, campestrol and cholesterol, along with the triterpenes α-amyrin, β-amyrin, taraxasterol and pseudotaraxasterol (Chandler *et al.*, 1982b). Many plants are bred for their high concentration of chamazulene, which is the most active therapeutic component of the EO. It is this component that gives the EO its characteristic dark blue or occasionally greenish-olive colour.

1.29.4 Evidence for the bioactive significance of the plant and its essential oil

In their comprehensive review, Chandler *et al.* (1982 a & b) discuss the chemical composition of yarrow in terms of its biological activities and the traditional uses of the plant. However, Chandler *et al.* (1982a) also mention that there are several genotypes of *A. millefolium*, and extensive variations in the concentrations of its active constituents. In general, antimicrobial properties are not normally reported for yarrow. Water- and ether-extracts of yarrow were observed to have a limited antimicrobial activity against *Staphylococcus aureus* (Bishop & MacDonald, 1951), but the concentration of the active plant substance in the extract was not measured. Antimicrobial and antioxidant activity has been reported for the EO and also for methanol extracts of yarrow (Candan *et al.*, 2003), but no antimicrobial activity was reported for a yarrow extract on antimicrobial-resistant bacteria (Nascimento *et al.*, 2000).

1.30 GARLIC

1.30.1 Botanical description and origin

A member of the family Liliaceae, the garlic plant (*Allium sativum* L.) is a bulbous perennial originating in central Asia, being introduced into Mediterranean Europe in Roman times. Today, it is grown in warm places globally. Growing to a height of 30-90cm, the plant has

linear, grey-green leaves and white clusters or umbels of bell-shaped flowers in the summer (Bown, 1999). The bulb is mounted on a flattened base and surrounded by a white, papery coat (Bruneton, 1999). The white to mauve/pink-skinned bulb is harvested in July or August and is composed of several smaller cloves. Garlic can be obtained as bulbs, capsules, in various fresh and water or powder extracts, and also as an EO. All of these are obtained from the bulb or root of the plant.

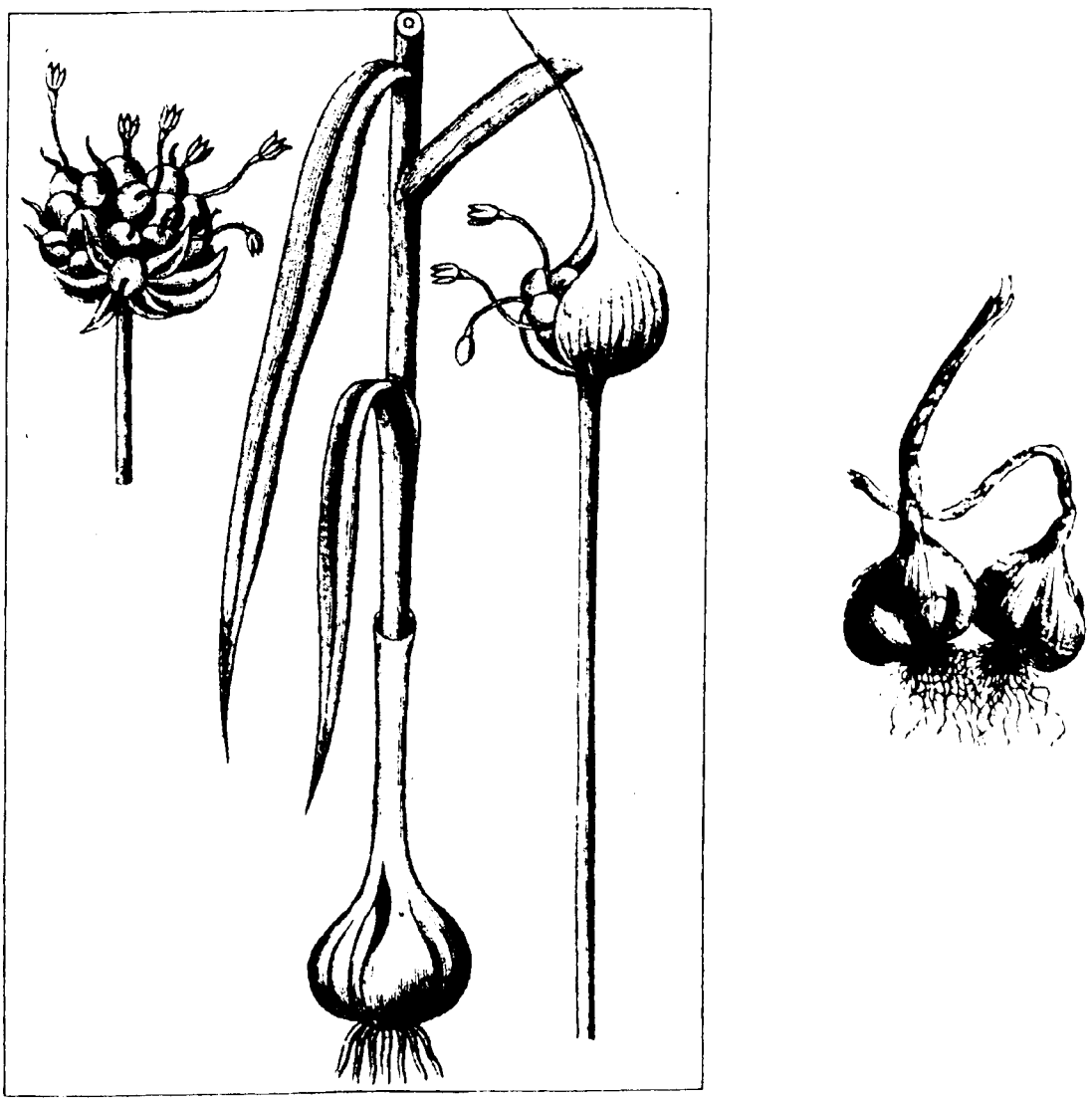
1.30.2 Economic and medicinal reasons for cultivation

Known as the "stinking rose" by the Romans, Garlic has been used for at least 5000 years as a medicinal herb and is known as a "cure for all ills". It originates in Asia and can also be found in the Mediterranean. Garlic is used globally as a seasoning for all meat and game dishes, shellfish and most vegetables. The high content of the sulphur and anti-clotting compounds found in garlic, that are also formulated into synthetic high blood pressure medications, support its use medically against hypertension, atherosclerosis, as a circulatory stimulant and for lowering cholesterol (Starý & Jirásek, 1973; Bremness; 1991; Armstrong, 2001). It can also be used as a general stimulant and for the treatment of flatulence, to aid digestion, intestinal catarrh, common colds and bronchitis in humans (Starý & Jirásek, 1973; Stobart, 1970). The German Commission E monograph states that garlic can be used in the diet of hyperlipidemic patients (Bruneton, 1999). Garlic has also a strong mythical relevance, where the bulbs are valued as aphrodisiacs and for their ability to ward off demons, vampires and witches (Tyler, 1993).

1.30.3 Chemical composition

The predominant active ingredient in garlic is a diallyl disulphide compound known as allicin, with a complex chemistry. The plant contains 0.1-0.5% of a strongly smelling EO, consisting of a variety of organic sulphur compounds, vitamins A, B & C and hormones (Starý & Jirásek, 1973; Amagase *et al.*, 2001). Garlic has been classed both as a sulphated terpene and a sulphoxide (Cowan, 1999). Bruneton (1999) states that garlic contains fructans, saponins (including the furostanol glycosides sativin, proto-erubin B and others) and also an array of sulphur-containing compounds. The compound that is usually considered to be the principal antimicrobial compound, allicin, is not present in the intact garlic bulb. When the garlic bulb is damaged, alliin (S-allyl-L-(+)-cysteine sulfoxide) is converted to pyruvate and 2-propenesulfenic acid by the action of the enzyme alliinase (S-alkyl-L-cysteine lyase)

(Bruneton, 1999; Amagase *et al.*, 2001). The compound 2-propenesulfenic acid is then converted immediately into allicin (allyl-2-propene thiosulfinate), ajoene and dithiin sulphide compounds, which are highly unstable and are quickly converted to other compounds (Amagase *et al.*, 2001). Oxidation of allicin in the air leads to the production of 1,7-dithiaocta-4,5-diene or diallyldisulphide and other sulphurous compounds, which are the main constituents of the EO of garlic (Bruneton, 1999). Analysis by HPLC shows the presence of thiosulphinate compounds based on around 80-90% allicin as the main component of the EO, namely 6Z- and 6E-ajoenes (4,5,9-trithiadodeca-1,6,11-trien-9-S-oxide) and also vinylthiines based on propenethial in alcoholic garlic extracts (Bruneton, 1999).



Adapted from Small, (1997)

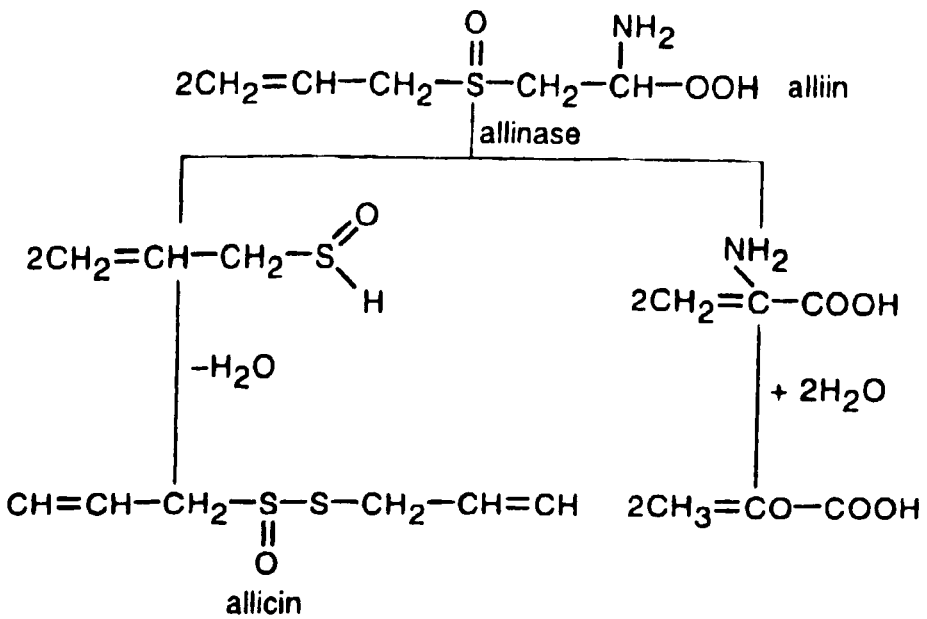
Figure 1.12 *Garlic (Allium sativum L.)*

The alliin component, as found in the undamaged bulb, has no antimicrobial properties. Most of the beneficial health properties of the plant are thought to be due to the component allicin, which gives the plant its characteristic smell, but garlic contains a range of active antimicrobial substances, including ajoene. Many active preparations of garlic also do not contain allicin (Freeman & Kodera, 1995), which can be attributed to its highly unstable nature and rapid conversion to other products. However, allicin is regarded as being responsible for the antimicrobial activity against Gram-positive and Gram-negative organisms (Tyler, 1993). Freeman & Kodera (1995) stated that allicin cannot be responsible for any bioactive effects of garlic as an *in vivo* supplement, and that it should not be the appropriate marker compound for all garlic products. The exposure of dehydrated garlic powder to simulated stomach fluids resulted in a 99% loss in allicin, and allicin also disappeared within minutes after its addition to blood (Freeman & Kodera, 1995). However, the condensation products formed from allicin may still be bioactive. The primary sulphur constituents in whole, intact garlic are the γ-glutamyl-S-alk(en)yl-L-cysteines and the S-alk-(en)yl-L-cysteine sulphoxides, which include alliin, as well as a small amount of S-allylcysteine (Amagase *et al.*, 2001). Amagase *et al.* (2001) mentions the stability of S-allylcysteine and suggests that it may be responsible for several pharmacological effects. The storage of garlic bulbs at cool temperatures will cause alliin to accumulate naturally, and on average, a garlic bulb will contain up to 0.9% γ-glutamylcysteines and up to 1.8% alliin (Amagase *et al.*, 2001). Garlic is normally standardised by its content of the alliin component, but this may not be an altogether true representation of its bioactive content, as this compound is so rapidly oxidised. Garlic cloves contain around 61.3 g water in each 100g (Duke, 1985). On a dry matter basis, 100g of garlic cloves contain about 6 g protein, 0.2 g fat, 31 g total carbohydrate, 1.5 g fibre, 1.5 g ash, 29 mg Ca, 202 mg P, 1.5 mg Fe, 19 mg Na, 529 mg K, 0.25 mg thiamine, 0.1 mg riboflavin, 0.5 mg niacin and 15 mg ascorbic acid (Duke, 1985).

1.30.4 Evidence for the bioactive significance of the plant and its essential oil

The antimicrobial effects of garlic have been well noted (Smith-Palmer *et al.*, 1998; Rees *et al.*, 1993; Jonkers *et al.*, 1999 a & b; Cowan, 1999; Benkeblia, 2004). Its anti-fungal (Azzouz & Bullerman, 1982; Rees *et al.*, 1993; Nielsen & Rios, 2000) and anti-viral properties (Rees *et al.*, 1993) have also been demonstrated. Garlic has also been shown to have antimicrobial properties against vancomycin-resistant enterococci (Jonkers *et al.*, 1999b). It has an anti-

fungal effect against *Candida albicans* (Lemar *et al.*, 2002), which these authors attributed mainly to the activity of allicin. Garlic is also well known for its antioxidant effects (Azzouz & Bullerman, 1982; Wei & Lau, 1998; Yin & Cheng, 1998a) and has been shown to have applications as an anticancer agent in mice (Kang *et al.*, 2001) and humans (Durak *et al.*, 2003). Ajoene has also been shown to have both antimicrobial (O'Gara *et al.*, 2000) and anti-fungal (Pai & Platt, 1995; Yin & Cheng, 1998b) activity. Lactic acid bacteria, which are considered to be beneficial microfloral species, have been reported to have significantly higher minimum inhibitory concentrations (MIC) in the presence of garlic *in vitro*, at between 12.5-40 mg ml^{-1} compared to most other enteric micro-organisms at between 0.8-3.3 mg ml^{-1} (Rees *et al.*, 1993). A total of 26 *Leuconostoc mesenteroides* subsp. *mesenteroides* bacteria resistant to the antimicrobial activity of garlic were isolated from the surface of the peeled bulbs (Kyung *et al.*, 1996). This meant that these bacteria were resistant to the damaging effects of thiol inhibitors (similar to allicin) and other chemical reactions in garlic (Kyung *et al.*, 1996).



Source: CAST, (1998)

Figure 1.13 *Biosynthetic reaction of allicin and structure of the main active compound*

1.31 TANNINIFEROUS SUBSTANCES AND CONDENSED TANNINS

Tannins are a group of naturally occurring polyphenolic substances from plants, which are capable of tanning leather or precipitating gelatin from isolation, a property known as astringency. In the plant, they are anti-feedants, protecting the plant from attack by herbivores and insects, but they also act as chemical signals in plant pollination, flowering and in

symbiotic relationships such as nitrogen fixation (Mann *et al.*, 1994). Tannins enable the plant to resist pathogenic fungi and moulds. They have molecular weights ranging from 500 to 3000, are soluble in water and are found in almost every plant part, including the bark, wood, roots, leaves and fruits (Mann *et al.*, 1994). Tannins form only a small part of the phenolic compounds present in plants, and are divided into two distinct groups – hydrolysable (HT) and condensed tannins (CT). The remaining phenolic compounds are simple phenols, phenolic acids, lignins and lignans (Cheeke, 1998).

Hydrolysable tannins consist of a carbohydrate moiety, which contains hydroxyl groups esterified to either gallic acid or m-digallic acid (gallotannins), or alternatively to hexahydroxydiphenic acid (ellagitannins). The CT or proanthocyanidins are the most numerous plant tannins and they are arranged as polymers within the plant (**Figure 1.14**). They are formed from flavonoid molecules, and consist of oligomers or polymers of the flavan-3-ols (catechins) and also flavanol residues, which may degrade acids to produce red-brown coloured anthocyanidins. The polymers of flavan-3-ols are linked through an inter-flavan carbon bond that cannot be hydrolysed (Reed, 1995). The main CT sources are fruits (grape, apple, plum, peach, pear) and also legume seeds (such as broad beans), and they are also responsible for the astringent taste of beverages (red wine, cider, tea, cocoa and beer) (Scalbert *et al.*, 2000b).

1.31.1 Biological effects of tannins

Tannins are able to combine with proteins, cellulose, hemicellulose and pectin to form stable complexes (Mangan, 1988). Their astringent properties are attributed to the high affinity of polyphenols with salivary proteins, which reduces the lubricating efficiency of the saliva and is dependent on the degree of polymerisation and hydroxylation of the phenol and the pH and ionic strength of the medium (Gawel, 1998). Tannins form associations with protein molecules, by means of hydrogen bonds between the phenolic hydroxyl groups and the peptide groups of proteins (Cheeke, 1998) and also by hydrophobic associations and covalent bonding. Condensed tannins also form complexes with fibre, or with protein bound to the fibre fraction (Rittner & Reed, 1992). This ability to complex with proteins is utilised in the tanning of animal hides to make leather, a process that has been carried out for centuries.

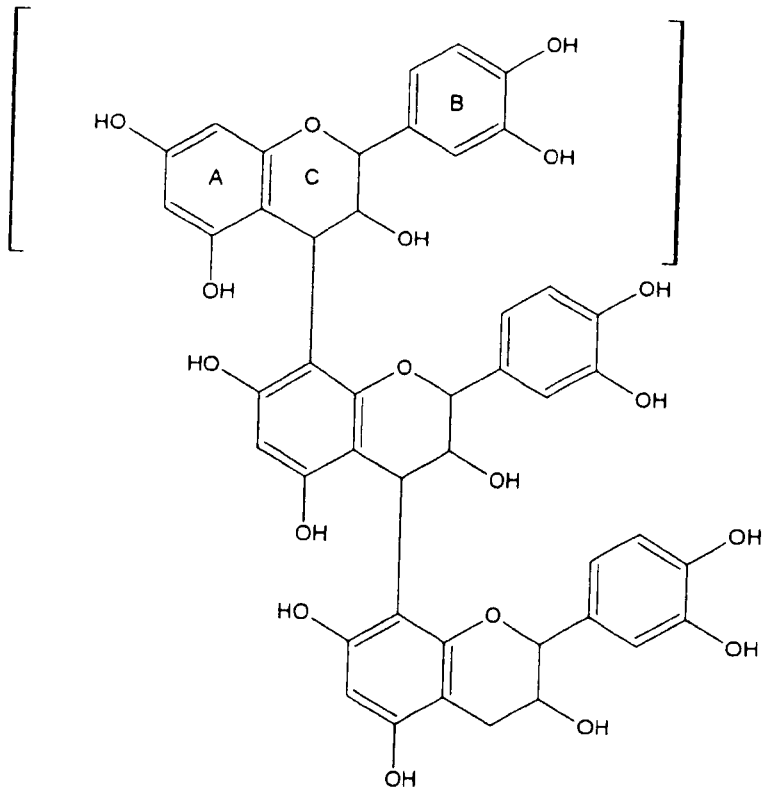


Figure 1.14 *Structure of a condensed tannin or proanthocyanidin*

Tannins have a positive effect in ruminants, where the intake of CT in forage has been associated with the reduction of bloat. When fed at restricted levels, tannin-protein complexes are also associated with preventing microbial protein degradation in the rumen, which then results in an increase in the availability of amino acids to the animal in the small intestine (Waghorn *et al.*, 1987; Wang *et al.*, 1994). The formation of tannin-protein complexes are dependent on pH (McLeod, 1974), where the complex is stable and insoluble at pH 3.5-7.0, but dissociates at a pH below 3.5 (Jones & Mangan, 1977). However, forage tannins may also either have no effect on ruminant growth (Terrill *et al.*, 1992) or have a negative effect on voluntary intake at high concentrations (Barry & Duncan, 1984; Barry & Manley, 1986; Waghorn & Shelton, 1995). There is no standard technique to measure the tannin content in a material, and thus these studies in the literature cannot be compared directly.

Purified CT may be suitable for inclusion into poultry diets, but as they contain large numbers of hydroxyl groups, this may still give them the ability to complex with proteins. At the higher molecular weight range, CT are suggested to be less able to complex with protein molecules, due to their reduced ability to align themselves with the molecules, and as a result

their astringent properties decrease (Mangan, 1988). However, the consumption of mixtures of various types of phenolics, and both HT and CT, are often associated with a reduction in growth and in nitrogen retention by chicks (Dale *et al.*, 1980; Elkin *et al.*, 1990; Ahmed *et al.*, 1991; Mahmood & Smithard, 1993; Majumdar & Moudgal, 1994). Mixtures of phenolics have also been associated with reduced weight gain in rats (Tebib *et al.*, 1996) and starch digestibility and metabolisable energy in chicks (Yapar & Clandinin, 1972; Lacassagne *et al.*, 1988). These negative effects may be due to increased microbial protein synthesis such as that observed in ruminants, an increased loss of endogenous nitrogen and the increased secretion of salivary glycoproteins (Reed, 1995). The precipitation of plant proteins by tannins has been observed to vary depending on the type of tannin used (Perez-Maldonaldo *et al.*, 1995). This precipitation was not observed for non-plant proteins and may be due to differences in the type of tannin or its structure (Perez-Maldonaldo *et al.*, 1995). Tannins may also reduce fibre digestibility by binding to bacterial enzymes, as well as forming indigestible complexes with cell wall carbohydrates in sheep (Barry & Manley, 1984). Tannins have a selective effect on endogenous enzymes also. When pigs were fed high tannin or low tannin containing faba bean CT in their diets, trypsin activity was reduced in the ileal digesta, but the activity of chymotrypsin was not affected (Jansman *et al.*, 1994). Jansman *et al.* (1993) reported that high levels of faba bean tannins in diets fed to piglets acted as anti-nutrients, decreasing the apparent digestibility of nutrients, especially that of proteins and amino acids. However, when feeding low levels of faba bean CT at 1 g kg^{-1}, these anti-nutritive effects were not observed in piglets (Jansman *et al.*, 1993). No depression in growth was reported after the inclusion of high CT sorghum meal in diets fed to ducks (Elkin *et al.*, 1990). Low concentrations of tannic acid at 100 mg day^{-1} in the diets of laying hens had no effect on feed intake in chicks (Majumdar & Moudgal, 1994). Flores *et al.*, (1994) also indicated that the effect of tannins depended on the amounts ingested. Small concentrations of CT in the diet have been suggested to be beneficial in ruminants (Terrill *et al.*, 1992) and in rats (Tebib *et al.*, 1996) in regulating the composition of the microflora. Many of the studies in the literature are based on forage or legume seed tannins, which may contain other compounds that may potentially cause some of these anti-nutritional effects, such as the glycosides vicine and convicine in faba beans. It is clear that research on the structure, type and content of CT included in diets will be important in determining their suitability for inclusion as bioactive compounds for poultry. For the purposes of this project, it was considered that purified CT extracts should be used to minimise any negative effects from other secondary plant compounds.

1.31.2 Medicinal uses of flavonoid compounds and condensed tannins

Condensed tannins form one category of flavonoid compounds. Flavonoid preparations have been used since the 1950's to treat problems of peripheral circulation. Propolis, a concentrated form of flavonoids, has been used for centuries to treat a wide variety of human conditions including headache, inflammation, allergy, cancer, viral infections, the common cold, bee stings and gastric and duodenal ulcers (Cook & Samman, 1995). Red wine, containing proanthocyanidins and high concentrations of flavonoids, is linked to a reduced risk of developing coronary heart disease, but due to its alcohol content also increases the risk of developing cirrhosis of the liver. Cranberry, containing both flavonoids and phenolic compounds, has been proven to prevent the development of urinary tract infections (Avorn *et al.*, 1994; Howell *et al.*, 1998; Foo *et al.*, 2000), by the prevention of bacterial adhesion to the walls of the liver.

1.31.3 Bioactive properties of condensed tannins

Many of the effects of using flavonoids pharmacologically have been linked to their known functions as strong antioxidants, free radical scavengers, metal chelators and their interactions with enzymes. Tannins are known for their antimicrobial (Makkar *et al.*, 1988; Bae *et al.*, 1993; Cowan, 1999), antioxidant (Serafini *et al.*, 1998; Yilmaz & Toledo, 2004), antiviral (White, 1957; Jassim & Naji, 2003), anti-inflammatory, anti-allergic and vasodilatory properties. They have been used as anthelmintics in ruminants to control nematode and worm populations in the gastrointestinal tract (Niezen *et al.*, 1993 & 1994; Butter *et al.*, 1998 & 2000). An increased concentration of procyanidin oligomers in the diet has been associated with a greater level of anti-ulcer activity in rats, by protecting the stomach lining against induced gastrointestinal damage (Saito *et al.*, 1998). Condensed tannins have been shown to be more effective in inhibiting the growth of Gram-positive rather than Gram-negative bacteria (Bae *et al.*, 1993; Jones *et al.*, 1994). The mode of their antimicrobial action may be related to the inactivation of microbial enzymes, adhesions, cell transport proteins or other compounds, as well as in the direct inactivation of microbial cells (Cowan, 1999). The activity of endoglucanase in rumen fluid was inhibited by the action of CT when studied *in vitro*, preventing it from digesting the cell walls within filter paper, which are plant cell walls (Bae *et al.*, 1993). Jones *et al.* (1994) observed an effect of CT from sainfoin plants, both in inhibiting microbial protease activity and in targeting the morphological structure of the bacterial cell wall. Simple phenolic acids have also been reported to inhibit both the

breakdown of cell walls and the association of bacteria with the cell walls of coastal bermudagrass (*Cynodon dactylon* L. Pers) and Italian ryegrass (*Lolium multiflorum* L.) when rumen fluid was cultured *in vitro* (Akin *et al.*, 1988). Rauha *et al.* (2000) observed a wide variability in the antimicrobial properties of Finnish tannin-containing plants *in vitro*, but found that quercetin, naringenin and flavone compounds were effective against all the tested bacteria. Mila & Scalbert (1994) observed that tannin polyphenols chelated any available iron, thus depriving it from *Erwinia chrysantemi in vitro*, and suggested this was one of the mechanisms of its antimicrobial action.

For the present project, extracted CT were obtained from three sources, mimosa extract or wattle extract (*Acacia mollissima* or *Acacia mearnsii*), which is a plant cultivated in Africa or Brazil, as well as cranberry extract (*Vaccinium macrocarpon* Ait) and grapeseed extract (*Vitis vinifera* L.). Mimosa represents mostly flavonoids, without the presence of other phenolic compounds. Cranberry contains predominantly flavonoids and phenolic acids, with some polysaccharide compounds. Grapeseed CT is considered an excellent source of polyphenolic tannins, mainly phenolic acids and flavonoids.

1.32 FERMENTABLE CARBOHYDATES

Intestinal microflora can also potentially be manipulated by "soluble fibres", or oligosaccharides, when these are present in the diet. One of the supplementary fermentable carbohydrates available is mannanoligosaccharide (MOS). Oligosaccharides are insoluble carbohydrates, which are not degraded by the digestive enzymes of human or animal hosts and therefore escape digestion and metabolism by the animal. MOS is one of a number of commercial products, and is formed after the addition of phosphorylated mannans to the components of yeast cell walls. Other fermentable carbohydrates such as fructooligosaccharides (FOS), xylo-oligosaccharides (XOS) and α-galactooligosaccharides can be extracted from various plants. Soluble carbohydrates can also be formed as a result of the controlled enzymic hydrolysis of polysaccharides or can be synthesized enzymatically from sugars such as sucrose. Fermentable carbohydrates cannot be broken down by the host enzymes in normal digestion, so they reach the lower intestine, where they provide a substrate for the growth of various species of microflora and affect the interaction of the microflora with the intestinal cells. Oligosaccharides are bifidogenic, supporting the growth of various bifidobacterial species, but not pathogenic or detrimental bacterial species such as *E. coli* or *C.*

perfringens, and are therefore known as prebiotic substances. Gibson & Roberfroid (1995) have defined prebiotics as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.

1.33 Other classes of important secondary plant compounds

Other compounds of importance in a consideration of the bioactive properties of plants are steroids, alkaloids and saponins. Some phytochemical components may have a specific effect, for example either antimicrobial or anti-fungal. However, any medicinal action of plant herbs occurs as a result of the synergy between the primary phytochemical component and other less abundant secondary phytochemical components. It is for this reason that the products are so diverse in their medicinal and biological properties.

1.33.1 Steroids

Steroids and terpenes are both produced by the same biosynthetic pathway. However, whilst steroids are widely synthesised by both plants and animals, the terpenes are produced mainly in plants. The sterols are a group of crystalline steroidal alcohols containing between 27 and 30 carbon atoms, where all possess a 3β-hydroxyl group and an endocyclic double bond (at position 5 or 6), along with a side chain, which is unsaturated and branched (Templeton, 1969). Cholesterol is the most well known steroid produced in mammals, where it acts as a structural component of biological membranes, and is the precursor of the bile acids, and the adrenocortical and sex hormones. Plant sterols differ from cholesterol by the presence of a methyl, methylene, ethyl or ethylidene group at carbon atom 24. The most abundant plant sterol is stigmasterol. Sapogenins are saponin aglycones, which are C27 alcohols with a spiroketal side chain and may also contain additional hydroxyl or carbonyl groups or double bonds. Around 50 sapogenins have been isolated. Hormones have been produced from plant steroids, such as the sapogenins diosgenin and hecogenin, and also cortisone and stigmasterol since World War II. However, steroids can be responsible for the strong toxicity of some poisonous plants. Steroidal glycosides, such as digitalis, have cardiac stimulation properties and strengthen the action of the heart, but can also cause cardiac arrest in higher doses. Other steroidal aglycones include digitoxenin in plants of the foxglove family.

1.33.2 Alkaloids

Alkaloids are defined as plant-produced compounds that contain nitrogen, or are "alkali-like". Their name is derived from the fact that they form salts when they react with acids. Synthesised from amino acids, there are several thousand alkaloids within plants, occurring in about 20% of the angiosperm species at around 0.5-1% dry plant weight. They are highly potent and many have important uses in medicine, such as morphine and atropine. They are usually based on a heterocyclic ring structure, and are sub-classified, based on the chemical nature of this heterocyclic ring. Often alkaloids can be toxic or addictive in nature, such as strychnine or cocaine. The most important groups clinically are the pyrrolizidine alkaloids, which cause irreversible liver damage, and also the piperidine alkaloids, which affect the central nervous system. As such, when considering plant herbs and EO for feeding to poultry, compounds containing high alkaloid contents have not been considered to be suitable for the present work. (Small, 1997; Cheeke, 1998)

1.33.3 Saponins

Saponins are glycosides, which are relatively common in plants of economic importance. They have either a steroidal or triterpenoid structure, are associated with a bitter taste and form a soapy lather when shaken with water. Saponins have both positive and negative effects in humans and animals, and are contained within forage legumes such as alfalfa. Saponins in plants are well known as potent blood poisons, as they cause the haemolysis of blood cells. On a more positive note, *Yucca shidigera* has been used as an alternative supplement for poultry diets, but did not produce a clear effect in comparison with an antimicrobial and also a probiotic supplement on performance, urease activity or on ammonia production in broiler chicks (Yeo & Kim, 1997).

1.34 Adverse effects of these secondary plant products

Not all of the properties of natural plants are beneficial or even safe, and as a result it is important to source any potential medicinal plants carefully. The Greek philosopher Socrates died after being poisoned by an herbal tea containing hemlock (Tyler, 1993). The concentration of the secondary plant component consumed is extremely important. Small (1997) describes the story of the enthusiastic herbalist Basil Brown in 1974, who consumed 45 litres of carrot juice in 10 days, which provided him with 10,000 times the daily requirement of vitamin A. At the end of these 10 days, he had bright yellow skin and was dead as a result

of acute damage to the liver. The amounts of herbs and spices used for flavouring are generally considered harmless, especially in a varied diet, but in some cases the herb or spice plant will be harmful if consumed in larger quantities or concentrations (Cowan, 1999). The adverse properties of some plants that pose a severe and sometimes fatal health risk versus the beneficial medicinal properties of plants are presented in the review by Lewis, (1982). There are a considerable number of terpene toxins, including the cardiac glycosides as described above and triterpene acids, but these are generally found in the higher terpenes and not as constituents of EO. However, some EO are irritants and can cause allergic reactions on the skin of humans. It is in the development of allergies that most adverse effects of the culinary herbs can be determined. Many higher terpenes, such as the limonoids and cucurbitacins are anti-feedants in ruminants, and can limit feed intake. The external application of garlic may cause blisters, inflammation or irritation of the skin in humans, while rosemary in higher doses can be toxic and can cause convulsion and delusions (Starý & Jirásek, 1973).

Gupta & Sandhu (1997) observed that a high molecular weight lectin from garlic, known as ASA_{110}, bound specifically to some cellular components of the gastro-intestinal mucosa in the brush border membrane, leading to the inhibition in food intake and arresting the weight gain of swiss albino rats. The negative effect of lectins can be removed by cooking, which suggests that these effects may be reduced if this compound is included in pelleted feeds. The review by Amagase *et al.* (2001) mentions several references dealing with the adverse effects associated with usage of garlic as a supplement. Although an anti-inflammatory EO, closely related to chamomile in composition, yarrow contains pollen and allergens, and it has been stressed that these compounds may cause contact dermatitis, anaphylaxis or hypersensitivity reactions (Tyler, 1993). Aromatherapists recommend that pregnant women avoid taking rosemary or yarrow medicinally, as rosemary may induce abortion and yarrow may cause allergies resulting in nasty rashes (Armstrong, 2001). Though not considered a toxic plant, yarrow has been quoted in the literature as having caused the rapid death of one calf after ingestion (Duke, 1985). Yarrow containing thujone should not be taken internally, as this compound is carcinogenic. The German Commission E did not find any adverse effects of the use of thyme herb or its flowers, or any interactions with other drugs, but they did not study the EO fraction (Armstrong, 2001). The EO of thyme was regarded to be quite poisonous, having caused dermatitis in dentists, and has been reported to cause severe inflammation and hyperaemia in bath preparations (Duke, 1985). It has been suggested that only the thyme

linalol chemotype should be used in aromatherapy for children, and then with great care (Worwood, 1990). However, the main adverse effects associated with the EO's are in the development of allergies, which are specific to the end-user.

The negative nutritional effects of decreasing the availability of carbohydate and protein are well known with the presence of tannins in the diets of non-ruminants. Cook & Samman (1996) mention that flavonoid molecules cause adverse effects such as bladder cancer in rats when fed at 2% in the diet, or the development of renal failure, haemolytic anaemia, hepatitis, fever and skin reactions in humans when fed at doses of 1-1.5 g day^{-1}. However, a dietary concentration of 2% represents a high concentration of tannin for monogastric animals. There are no reports of negative effects associated with feeding fermentable carbohydrates.

1.35 Legislative problems in the field of plant neutraceuticals

The laws and regulations covering the use of herbs and other plants as neutraceuticals or bioactive natural phytochemicals in humans are extremely complex and misleading. Unlike natural plant bioactives, synthetic pharmaceutical products can be produced at a fraction of the cost and controlling the amount of active ingredient is relatively easy. For these reasons, herbal products lost their favour as drugs and have been relegated from pharmaceutical outlets to health food shops. Out of a possible sample size of 350,000 herbal products for humans, the FDA (Food and Drug Administration, USA) commissioned a drug efficacy study in 1969, which looked at 420 products and found evidence of efficacy in only one quarter of these (Tyler, 1993). Most suppliers of botanical products do not claim that their product will have a direct influence on health, as this would require them to be registered as drugs in most countries. Instead, they are described as flavours or palatability enhancers that have 'beneficial effects'. Over-the-counter preparations of herbal remedies for humans are not regulated, resulting in herbal preparations of an uncertain purity, and the herb source may not be from the correct plant as labelled (Cowan, 1999). The laws governing the registration of feed supplements for animals are extensive, and depend on a consistency of the product or supplement, its toxicological effects and a wide range of other criteria. As a consequence, if these compounds are to be used in animal feedstuffs, the consumer should demand a test of the quality of the product before it is used in the diet.

1.36 The role of carbohydrases and their use in poultry diets

Enzymes have been used widely since the early 1980's in poultry diets. It is estimated that around 95% of the diets fed to poultry within the UK are supplemented with carbohydrases, which would mean that poultry consume about 62,000 tonnes of these enzymes annually under current dietary formulation policies (Acamovic, 2001). Enzymes are defined as organic catalysts, which speed up a reaction without being used up in the process. In order for a reaction to occur, the enzyme forms a reversible complex with the substrate of the reaction, decreasing the energy input for the reaction or speeding up the reaction by an order of about 10^6 (Mc Donald *et al.*, 1995). The enzyme-substrate complex is then broken down, yielding both the unchanged enzyme and the reaction product (Mc Donald *et al.*, 1995). Enzyme activity is affected by the relative concentrations of both the substrate and the enzyme and also by the presence of enzyme inhibitors, which may block the active site and prevent substrates binding to the enzyme. The concentration of hydrogen ions, or the relative acidity has an important effect on enzyme activity, and generally most reactions take place at around a pH of 6-7. The activity of an enzyme can also be doubled by each 10°C increase in temperature up to about 50°C, but after this the enzyme is destroyed or denatured. The binding action of enzymes to substrates is highly specific, following a "lock and key model".

Since the 1980's, the use of enzyme supplements have been associated with greater digestibility values of the diet from the same nutrients, increasing the Apparent Metabolizable Energy (AME) value and reducing the anti-nutritive action and intestinal viscosity of non-starch polysaccharides (Preston *et al.*, 2001; Scott & Boldajhi, 1997). The nutritive quality of wheat was found to be very variable in an examination of 16 wheat cultivars, depending greatly on the content of NSP, especially arabinoxylan (Steenfeldt, 2001). Milling quality wheats are generally of better quality and contain less NSP than their counterparts used in animal feeds (Steenfeldt, 2001). Enzymes are commonly used in animal diets based on wheat, barley and rye to decrease the effects of the NSP content of the cereal, but their effect is variable within birds due to variations in the substrate quality of the diet. A review of several papers suggested that enzyme use led to a 10% improvement on average in the efficiency of dietary and nutrient utilisation by poultry (Acamovic, 2001). However, in some cases, the inclusion of enzymes had no effect or may even have exerted an adverse effect on dietary utilisation in the birds (Acamovic, 2001). In general, the greater the complexity of the NSP or the greater its content within a diet, the greater the improvement that can be made with the use

of an enzyme in that diet and the less variable the flock performance as a result of supplementation. Inclusion of β-glucanases improved performance and digestibility in cockerels fed diets based on barley (Salih *et al.*, 1991) and also in broiler chicks fed diets based on naked oats (Cave *et al.*, 1990). Dietary pentosanase addition reversed the adverse effects of rye NSP in broilers, which were increased with higher dietary concentrations of the cereal (Bedford & Classen, 1992). The performance characteristics of broiler chickens were improved by pentosanase addition to rye-based diets, and the incidence of sticky droppings decreased (Pettersson & Aman, 1989). Adding xylanase to a wheat-based diet resulted in an improvement in the effects associated with a high dietary NSP content (Bedford & Classen, 1992; Choct *et al.*, 1995; Marron *et al.*, 2001). An enzyme presence within the diet appears to have a dual action on the intestinal microflora within poultry. Enzymes may reduce the presence of bacterial pathogens in the ileum and therefore increase the digestibility of starch and protein for the bird, and also increase the fermentable proportions of small sugar substrates from arabinoxylans and β-glucans by the caecal microflora (Bedford, 2000). An enzyme presence in wheat-based diets decreased the concentrations of starch-degrading bacteria within the caeca of turkey hens (Persia *et al.*, 2002), which then enabled an earlier and more complete and efficient dietary digestion by the birds. The addition of enzymes to a barley-based diet also reduced the relative weights of the intestinal organs (Brenes *et al.*, 1993). Enzymes have also decreased intestinal viscosity in broilers (Salih *et al.*, 1991; Crouch *et al.*, 1997; Marron *et al.*, 2001), laying hens (Mathlouthi *et al.*, 2003) and turkeys (Crouch *et al.*, 1997). Enzymes can reverse the effects of anti-nutrients such as lectins and trypsin inhibitors (Bedford, 1996). The enzyme may also liberate components from the cell wall that can be used nutritionally, which would otherwise be unavailable to the bird for digestion (Bedford & Schulze, 1998). The relative importance of enzymes in poultry diets, gained over the last two decades, require them to be considered along with phytochemicals for potential synergistic or antagonistic effects.

1.37 The digestive tract of the chicken

The chicken has a relatively simple monogastric digestive system, which is composed of the alimentary tract, liver and pancreas. These three components work together to allow the intake and storage of food, digestion and absorption processes, and finally the eventual elimination of waste materials. Feed is taken in *via* an arrow-shaped tongue, and forced back into the oesophagus, where it passes down into the alimentary tract. This is aided by a

swallowing mechanism, and the food particles are lubricated by mucous saliva to aid their passage to the crop. The alimentary tract is a tube extending from the mouth to the cloaca, the internal surface of which is lined with various associated mucous membranes, where the food passes along by peristaltic waves.

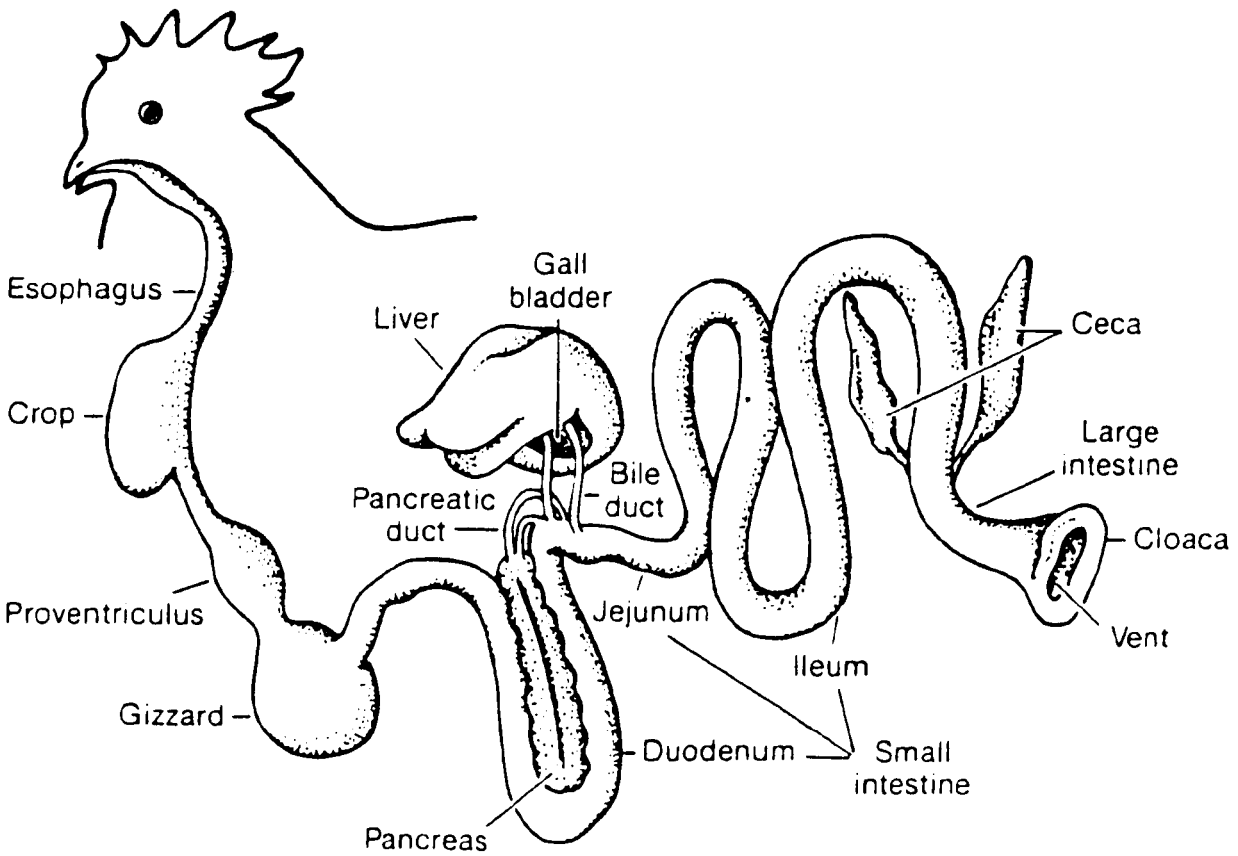
1.37.1 The crop and proventriculus

The crop is a pouch-like temporary storage organ in the oesophagus, where food is softened by water and saliva. Some microbial digestion also occurs here, resulting in the formation of lactic and acetic acids (McDonald *et al.*, 1995). The main sites in the alimentary tract associated with the microflora were observed to be the crop, ileum and caeca, and only Lactobacilli were associated closely with the walls of the crop (Fuller & Turvey, 1971). These Lactobacilli will ferment some carbohydrate, producing lactic acid. Fuller & Turvey (1971) suggested that the composition of the microflora in the crop could influence the health or nutrition of the bird. At the end of the oesophagus, the ingested feed passes to the proventriculus or glandular stomach, which secretes pepsinogen (pepsin) to aid in protein digestion. Hydrochloric acid is also secreted, which decreases the pH to about 2.5 and starts to break down the grain walls of the feed. Not much enzymatic digestion occurs in the proventriculus, but when the feed enters the gizzard, it is ground down by muscular contraction. The gizzard contains two pairs of thick, powerful muscles, which are protected from the acid conditions by a thick layer of mucus.

1.37.2 Small intestine

In chickens, the small intestine includes the duodenum, jejunum and ileum and is the main site of nutrient digestion and absorption. The small intestine contains villi, which are a series of projections increasing the surface area to aid the absorptive process. In adult birds, the small intestine can reach around 1.5 m in length. The duodenum occurs as a folded loop situated below the gizzard. In the centre of this fold is the pancreas, which secretes amylase, lipase and protease directly into the duodenum to aid in the breakdown of starches, fats and proteins, and also insulin to regulate carbohydrate metabolism. Bile is necessary for the emulsification of fats to help in their subsequent digestion by lipases. It is secreted in the liver and stored in the gallbladder, before being released when the gallbladder contracts as digesta enters the small intestine. At the end of the duodenum, pancreatic secretions and bile mix with the

digesta. The intestinal mucosa walls also secrete mucin, α-amylase, maltase, sucrase and proteolytic enzymes.



Source: Parkhurst & Mountney, (1988)

Figure 1.15 *Diagram of the digestive tract in the chicken*

1.37.3 Large intestine and microfloral activity

Poultry have a relatively short large intestine, measuring around 5-8 cm and beginning at the ileo-caecal junction, including both caeca and also the colon. The caeca can be up to 10-12 cm in length, are blind-ended and are mainly absorptive organs. Up to 1.6×10^{11} c.f.u. g^{-1} dry tissue (Salanitro *et al.*, 1978) of microflora can be found within the caeca and large intestine, which subsequently make these areas the main sites for microbial activity in the intestine. Microflora inhabit the mucosal epithelia, and are either directly bound to the epithelial cells or are contained within the mucous layers around the villi.

In the large intestine, the extent of microbial metabolism and the utilisation of the associated products by the birds are not well understood. The microflora may be involved in the synthesis and digestion of vitamins, as well as in the utilisation of nitrogenous and fibrous compounds. It is possible that birds may derive some benefit as a result of the activity of the caecal microflora, but there is only a short residence time for the digesta in the lower intestinal tract, and thus only a small window of opportunity for microfloral metabolism. Any energy derived in this way will be obtained less efficiently than when obtained as a direct product of digestion, as the microflora also utilise some of the dietary nutrients for energy. The main species of microflora in the poultry caeca are lactobacilli, streptococci, coliforms, bacteroides, clostridia and yeasts (Mc Donald *et al.*, 1995), and the caecal environment is usually relatively anaerobic. However, the type of microbial metabolism is dependent on the nature of the environment, the age and health status of the birds and also on the substrates available from the diet (Jozefiak *et al.*, 2004). An overgrowth of intestinal microflora has been associated with the presence of a high fibre or NSP content in diets (Bedford, 1996), which may allow anaerobic bacteria to colonise the ileum due to the refluxing action of the caeca, known as reverse peristalsis. In this situation, microfloral overgrowth of species such as *C. perfringens* has been associated with diseases such as necrotic enteritis (NE). Exogenous enzymes modify the composition of the microflora in the digestive tract, through their action on the NSP components of the diet. The main products of microbial fermentation are volatile fatty acids, but a range of amines and other nitrogenous compounds are produced, including uric acid.

The colon may serve to remove water and electrolytes from the digestive tract, but its function is not well understood in poultry (Turk, 1982). After the colon, waste material is then excreted through a single orifice, known as the cloaca. This waste material is predominantly uric acid, but also contains undigested food residues, digestive secretions, epithelial cells, bacteria and the products of microbial metabolism.

1.38 Background to this work

Over the last 50 years, AGP's in poultry diets have been associated with benefits in the control of disease and in the increased efficiency of utilisation of feed for growth of the birds, through controlling microfloral populations and their association with the immune response. Due to their enforced removal by the EU government, and the knowledge that the action of the

microflora can be either a positive or a negative one in poultry depending on its composition, it is important that more research is focussed on this area of poultry nutrition. Poultry diets contain variable amounts of NSP from wheat, barley and oats as the main cereal components, which act as a substrate for microfloral growth through fermentation. This may lead to a reduced digestion and dietary utilisation efficiency, as these compounds increase the water-holding capacity and viscosity of the digesta. It is important to observe secondary plant compounds to assess their effects *in vivo* and to elucidate their mechanisms of action. If enzymes and/or secondary plant compounds can be utilised together to improve dietary utilisation and the balance of the intestinal microflora, then they may potentially be significant in the improvement of both poultry health and the economic costs associated with feeding in the 21st century.

1.39 Aims and Objectives

This work aimed to follow a multi-factorial approach in assessing EO and herbs from a number of plant materials, namely thyme, rosemary, oregano, marjoram, yarrow and garlic, as well as grapeseed, mimosa and cranberry CT extracts, for their incorporation as bioactive supplements in poultry diets. The objective was to compare these compounds for their effects on growing bird performance. Additionally, the effects of these compounds were studied on dietary nutrient digestibility, the composition of the intestinal microflora species and the products of bacterial fermentation in the birds. The effect of feeding these compounds on the morphology of the small intestine was studied, in order to build up a complete picture of their overall action. An investigation into the interactions between thyme EO and a commercial xylanase was investigated, with the aim of assessing the potential of their combined presentation in poultry diets. Due to the extensive variability of composition of species for their bioactive products within the plant kingdom, each plant used was tested for the chemical composition of its primary phytochemicals, to ensure a sound basis for the reasons behind any bioactive effects. The results of these assessments are presented in the following chapters.

CHAPTER 2

2. GENERAL METHODOLOGY

2.1 Location of research facilities

All animal feeding studies were carried out at The Scottish Agricultural College, Ayr, in the Avian Science Research Centre. The work in this thesis is based on four animal experiments; one based in cage facilities and the other three on clean and used wood shavings as a litter substrate. Both the general laboratory and animal study methodology are presented here, and information specific to each feeding study is included in later chapters. The Animal Experiment Committee (AEC) assessed and approved each feeding study prior to the start of experimental work, for its ethical acceptability and the benefits to poultry research and bird welfare.

2.2 Laboratory experimental facilities

Laboratory work was completed using facilities based at the sites managed by the Scottish Agricultural College, in Aberdeen and Ayr. Microbiology analyses were carried out both at the Aberdeen and Ayr laboratory facilities. Most of the nutrient digestibility work was performed in Ayr, but High Performance Liquid Chromatography (HPLC) for amino acids (AA) and volatile fatty acids (VFA) were carried out in Aberdeen. Nitrogen analysis by the Dumas method was done at the Roslin Institute, Edinburgh. A description of the methodology for each analysis is presented either in the text or in the Appendices.

2.3 Screening and in vitro testing of plant supplements

2.3.1 Sources of essential oils tested against bacterial species

Twenty essential oils and aqueous extracts of plant herbs were screened for their antimicrobial properties prior to their use in feeding trials (**Table 2.1**). Oregano, marjoram, thyme, yarrow and rosemary dried herbs were purchased (Green City, Glasgow, UK) and the essential oils (EO) of these herbs distilled for 2 hours from plant material at SAC Ayr using Clevenger apparatus. The remaining 15 EO came from Turkey (Selcuk Universitesi, Konya, Turkey). All EO were stored in sealed glass containers and stored at temperatures <4°C until required for analysis.

Table 2.1 *Essential oils screened for their antimicrobial activity*

Essential oils screened for their antimicrobial activity	
Sample	*Plant*
Satureja hortensis	Savory
Origanum majorana	Marjoram
Thymus spicata	Thyme
Origanum vulgare	Oregano
Rosmarinus officinalis	Rosemary
Echinophora tenuifolia	-------
Mentha spicata	Spearmint
Myrtus communis	Myrtle
Laurus nobilis	Bay
Foeniculum vulgare	Fennel
Cumin cyminum	Cumin
Salvia fruticosa	Sage
-Leaves	Leaves of sage
-Leaves & branches	Leaves & branches of sage
-Fruit	Sage fruit
Origanum majorana	Marjoram
Origanum vulgare	Oregano
Thymus vulgaris	Thyme
Rosmarinus officinalis	Rosemary
Achillea millefolium	Yarrow

2.3.2 Bacterial strains used in the in vitro testing

Pure cultures of 10 bacterial species were obtained from food sources through the microbiology department of SAC Ayr on Isosensitest Agar slopes (CM 471, Oxoid, UK). These were *Listeria monocytogenes* NCTC 11994, *Clostridium perfringens* NCTC 8237, *Salmonella enteritidis* PT4, *Escherichia coli* NCTC 10418, *Enterococcus faecalis* NCTC 775, *Bacillus cereus* NCTC 7464, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aureus* NCTC 6571, *Yersinia enterocolitica* NCTC 10460 and *Campylobacter jejuni* NCTC 11322.

2.3.3 Distillation of the essential oils from plant herb material

The distillation of EO from the purchased herb material took place using British Pharmacopoeia distillation apparatus and methods following BS 4585 (1985). Further details of the method and apparatus can be found (**Appendix 2**). The quantity of EO collected was transferred into a glass vial with a teflon cap and stored at below 4°C until analysed by gas chromatography (GC).

2.3.4 In vitro testing of the antimicrobial activity of essential oils

Following the technique of Deans & Ritchie, (1987), a sterile loop was used to inoculate each pure bacterial culture into aerobically prepared isosensitest nutrient broth (CM 473, Oxoid, UK). The inoculated culture was grown aerobically for 3-4 hrs at 37°C, with the exception of *C. perfringens* and *C. jejuni* ssp., which were grown under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2). After this time, pour plates were then prepared for each species in duplicate by transferring 1 ml of inoculated broth containing 10^6 organisms into petri dishes, and adding about 20 ml of molten isosensitest agar (CM 471, Oxoid, UK) at a temperature of around 40-45°C. All plates were mixed evenly on a flat surface, rotating 5 times both clockwise and anticlockwise, followed by 5 forward and backward pushes and then a final rotation and then left to set. Two sterile antibiotic discs of 6 mm diameter (Whatman, Oxoid Ltd., UK) were placed onto the cooled agar surface and 10µl of each EO was then pipetted directly onto the sterile discs over the various bacterial cultures. After a period of 30 min, plates were incubated at 37°C for 24 hrs either aerobically or anaerobically, according to the bacteria grown. The diameter of each zone of inhibition in bacterial growth on the agar was measured with vernier calipers and this data was analysed in Microsoft Excel.

2.3.5 Analysis of condensed tannin content in the plant material

This analysis was carried out according to the method of Makkar, (2000), where condensed tannins (CT) are oxidatively removed from structural polymers in the plant into solution using butanol/HCl reagent. Plant herbal samples were obtained as dried herb leaves and milled to pass through a 1 mm sieve aperture (Retsch mill, Hahn, Germany), and the tannin samples were obtained as dry powders. About 200 mg of each sample (100 mg for tannin samples) was weighed accurately in quadruplicate into glass tubes and 10 ml of aqueous 70% acetone added. All tubes were then vortexed and then suspended in ice in an ultrasonic water bath for 20 mins, before being filtered through Whatman No. 1 filter paper. A 1 ml aliquot from each sample was transferred into the appropriate number of 100 ml round bottomed flasks. A volume of 6 ml butanol/HCl (95 ml butanol: 5 ml HCl) was added to duplicates of each sample in the flasks, along with a further 0.2 ml of ferric reagent to increase the sensitivity of the assay. The remaining duplicate tubes were prepared as a series of sample blanks for butanol/ferric reagent by adding 6 ml H_20/HCl (95 ml H_20: 5 ml HCl). All flasks were refluxed for one hour on an electromantle (Model EME6, Electrothermal, Southend, UK).

When cool, cuvettes of each sample were prepared and read at 550nm using a spectrophotometer (UVIKON-XL, Biotech Instruments, Hertfordshire). The content of CT in the sample was calculated using the following formula:

$$\text{Content of Condensed Tannin (g kg}^{-1}) = \frac{(\textit{Sample absorbance @ 550 nm } X\ 0.1565\ X\ 1000\ )}{\textit{Sample Weight}(g)}$$

(NB. 0.1565 is a reference standard for condensed tannin content (or the leucocyanidin equivalent, assuming that the extinction coefficient (E) of 1% leucocyanidin solution is 460, based on Porter *et al.*, (1986)). Samples were diluted in 70% acetone in order to achieve an assay absorbance value of <0.6@550nm.

2.3.6 Measurement of the activity of garlic by means of its Alliin content

The main active constituent responsible for the bioactive properties of garlic (allicin) is difficult to determine, as it is quickly oxidised and very unstable. The most widely used reference method for quality testing the biological activity of garlic is a determination of the inactive alliin precursor of allicin. The alliin content of the air-dried garlic used in the feeding trials was compared against the test organism *Candida albicans* NCYC 1470 by Interprise (Interprise Ltd., West Glamorgan, Wales), in an assay using a range of alliin standard concentrations and an internal reference standard with an activity of 100%. Further details of the methodology used can be found (**Appendix 5**).

2.4 General Methodology used in Animal Experimental Trials

2.4.1 Diet formulation and feed mixing

All rations used in the feeding trials were formulated as wheat/soya bean meal rations using a modified version of the Uneform package, originally developed in the University of New England, Armidale, Australia. When formulated, diets were mixed at SAC Ayr as a basal ration. Each plant supplement was pre-mixed into about 6 kg of the previously prepared basal dietary ration for about 5 mins, using an industrial food mixer (Model A200, Hobart Mfg. Co. Ltd., London). This concentrated carrier supplement was then mixed into the remaining basal ration to ensure an even dispersion of supplement throughout.

2.4.2 Housing and Environment

The conditions of housing and environment differed slightly for each experimental situation and are explained fully in the relevant chapters (See Chapters 3-5). All birds were housed in accordance with the recommendation codes for the welfare of livestock (MAFF, 1996).

2.4.3 Sampling and dissection of birds

All birds required for provision of tissue samples were randomly selected from the experimental pens and killed by an overdose injection of sodium pentobarbitone (euthatal). In each case, depending on the age and size of the birds, the pentobarbitone was administered by cardiac puncture or intravenously. Each bird was then dissected in turn and the intestinal package removed. The entire length of the ileum was removed and laid out in all birds. A protrusion of tissue known as Meckel's diverticulum was located, formed by the last remnants of the yolk sac and situated towards the base of the ileal length. Immediately posterior to Meckel's diverticulum in the direction of the caeca, a 5cm section was removed and stored in buffered formal saline for histological analysis. The contents of both caecae were removed and emptied into sterile containers and sealed; these were then frozen at -20°C for microbiological and volatile fatty acid analyses. The remaining ileal tissue contents were flushed into a petri dish using a 20ml syringe filled with water, and these samples frozen for the determination of digestibility coefficients.

2.5 Collection and preparation of dietary and excreta/ileal samples

Titanium dioxide (TiO_2; Roslin Nutrition, Edinburgh) was used in the diets as an indigestible dietary marker. During the animal experiments, diets containing 5g kg^{-1} TiO_2 were fed to the birds for 2 days prior to collection and on the day of collection to ensure titanium was present in the entire intestinal passage of the birds. Samples of all diets containing TiO_2 were taken and stored until required for analysis. On the day of tissue collection, excreta samples were collected on trays placed beneath the cages or in the pens. Trays were left overnight, and excreta placed in 140 mm diameter petri dishes and frozen at –20°C until required. The collected excreta or ileal samples were then freeze-dried (Rowett Research Institute, Aberdeen). When freeze-dried, the samples were ground through a 0.75 mm sieve aperture in a Retsch mill (Hahn, Germany), cleaning thoroughly between each treatment. The dietary samples were not freeze-dried, but were milled to the same particle size. After grinding, all feed samples were preserved by storing at –20°C prior to analysis. The freeze-dried samples were stored at room temperature.

2.5.1 Dry Matter (DM) and Organic Matter (OM) Analysis

The measurement of DM content for the collected samples standardised the weight measurements of feed and digesta samples throughout the digestibility analysis, as the various

samples were subjected to different storage and drying conditions. Analysis of the DM was carried out in accordance with BS 5766, Parts 1&8 (1983). Samples of the dietary materials were accurately weighed in duplicate (AM100, Mettler Instruments Ltd, High Wycombe) to around 3g onto previously dried and weighed porcelain or silica dishes. The digesta samples were measured out singly to 3g, as there were several replicates in each treatment. The samples were then dried at 80°C to a constant weight for around 24 hrs, before being removed into desiccators and left to cool to room temperature. Each cooled sample was removed in turn and the dry sample weight measured, which enabled the calculation of dry matter for the sample. All samples were then transferred to a muffle furnace and heated to 480°C for a period of 15 hours until only the ash remained. When cooled to room temperature, the samples were transferred to an oven preheated to 80°C for a period of 2-3 hours to evaporate any moisture accumulated during cooling and then placed in a dessicator for 30 min to re-equilibrate to room temperature. Each sample was weighed and the content of OM calculated from the ashed sample weight based on an intake of 1 kg DMweight.

2.5.2 Gross Energy (GE) content

Gross energy (GE) values were determined for both dietary and digesta samples by adiabatic bomb calorimetry, in accordance with BS 1016, Part 105 (1992). Each diet was analysed in duplicate and the digesta samples singly. In each testing day, samples were measured against a benzoic acid standard (VWR Ltd, UK) with a known heat capacity of 26.454 $MJ°C^{-1}$. These benzoic acid standards were the first samples analysed each day. All feed or digesta samples were accurately weighed to around 1g (AM100, Mettler Instruments Ltd, High Wycombe), pelleted for both sample compaction and ease of combustion and placed in nickel crucibles. At a fixed temperature between the range of 19-21°C, water was weighed into the steel water jacket to a final weight of 3 kg and then placed inside the bomb calorimeter (Autobomb, A. Gallenkamp & Co. Ltd, England). A 13 mm length of Nichrome fuse wire (VWR Ltd, UK) was inserted between the electrodes in the lid of the bomb casing, to complete the internal electrical circuit and allow firing of the bomb. A 10 cm length of thread was then doubled and tied around the fuse wire to connect it to the sample in the crucible. At this stage, the bomb casing was sealed and pressurised to 30 bars with pure O_2 (BOC Ltd, UK), before being placed inside the water bath and the calorimeter sealed. After leaving to mix for 5 minutes to allow equilibration of the water temperature, the initial temperature was recorded on a digital thermometer (R42/2, MGW Lauda, K⊕nigshofen). Each sample was ignited and the resulting

temperature increase measured after the length of time required for the water to reach its maximum temperature.

For each sample, the gross energy (GE) was then calculated using the following equation and corrected for dry matter before being used in calculations of nutrient digestibility:

$$\text{Sample GE} = \frac{([THC \times \Delta temp] - c)}{Weight}$$

Where: GE = Gross Energy (J g^{-1})

Δtemp = temperature rise (°C) of water in the calorimeter vessel from heat of sample combustion

weight = pellet weight of the sample material (g)

THC = Total heat capacity of bomb (around 10,800 J $°C^{-1}$ but slightly variable for each test day)

c = correction factor for the calorific value of the fuse wire and thread used (167 J)

2.5.3 Total nitrogen (N) content

All samples of diets and digesta were analysed at Roslin Nutrition, Edinburgh using the Dumas combustion method, to determine the total amount of N_2 present in each. Feed and excreta samples were weighed accurately to around 0.2g into small aluminium foil vials (Part 502-186-W, Leco, St. Joseph, Michigan, USA), sealed by folding to exclude air and then placed into the N_2 analyser (Leco FP-428/328, St. Louis, USA). Samples were firstly purged of any trapped atmospheric gases. The samples were then burnt in pure O_2 at 850°C and the gaseous products of combustion allowed to reach a homogenous mixture at a constant temperature and 975 mm pressure. The combustion products were then passed over hot copper to remove all O_2 and change nitrogen oxides to gaseous N_2. After removal of the water and carbon dioxide contained within the samples, the total N_2 content of the samples could then be used in the calculations of nutrient digestibility (Section 2.8).

2.5.4 Titanium Dioxide (TiO_2) analysis

The TiO_2 content of both diets and excreta was measured in accordance with an SAC standard protocol based on an adaptation of the method of Short *et al*, (1996). A standard 0.5 mg ml^{-1} TiO_2 solution was prepared by dissolving 250 mg of accurately weighed TiO_2 (Roslin Nutrition, Edinburgh, UK) with heating in 100 ml concentrated H_2SO_4 (VWR Ltd, UK). The resulting solution was carefully added with rinsings to 200 ml distilled H_2O in a 500 ml

volumetric flask, and a further 100 ml concentrated H_2SO_4 added. The cooled mixture was then diluted to 500 ml with distilled water. A solution of 7.4M H_2SO_4 was prepared by adding 400 ml concentrated H_2SO_4 to a 1 litre volumetric flask containing 400 ml distilled water and diluting to volume.

The digesta samples were accurately weighed in porcelain crucibles to around 0.12g and ashed in duplicate at 480°C for 13 hours, then transferred to a desiccator and analysed when cool. A quantity of 10 ml of 7.4M H_2SO_4 was added to the samples in tubes, and these were placed into a digestion block (Tecator ab, Sweden). The samples were heated overnight to a maximum temperature of about 140°C, to allow the complete digestion of TiO_2 within the inorganic fraction and evaporation of the water contained within the acid. The cooled solution was then filtered (Whatman 541 110mm, VWR Ltd, UK) and diluted to volume in 100 ml volumetric flasks, to which 20 ml 35% H_2O_2 (VWR Ltd, UK) had previously been added.

Eleven standards of known TiO_2 concentration (0-0.05 mg ml^{-1}) were prepared by pipetting 0,1,2,3,4,5,6,7,8,9 and 10 ml of standard TiO_2 solution into 100 ml volumetric flasks and making up to a total volume of 10 ml by addition of 7.4M H_2SO_4. A quantity of 20 ml 35% H_2O_2 solution was dispensed into each flask and each was made up to 100 ml using distilled water.

The samples were measured using a spectrophotometer (UVIKON-XL, Biotech Instruments, Herts.) at a wavelength of 410 nm. The standard sample without TiO_2 was read as the blank reference point, and a calibration curve was drawn up from the results. Titanium dioxide concentration of samples was calculated using linear regression analysis in Microsoft Excel.

2.5.5 Amino acid analysis by HPLC

Amino acids in feed and excreta samples were prepared at SAC Ayr and analysed by a standard HPLC method at SAC Aberdeen. All chemicals used to prepare the working or standard solutions were obtained from VWR Ltd., UK, unless stated otherwise, and details of the preparation of the standards and solutions used in the analysis are described (**Appendix 6**).

Around 50-75 mg of each sample was weighed accurately into screw-capped glass tubes for hydrolysis purposes. Feed samples were analysed in triplicate and digesta samples singly. A

5 ml volume of HCl 6N was dispensed into the tubes, which were then sonicated for 15 min. Each tube was flushed with gaseous N_2 (BOC Ltd., UK) for 30 seconds, dried with tissue and finally hydrolysed in a dry heating block for 24 hrs (Dri-Block DB-3, Techne, Cambridge, UK) at 110°C ± 1°C. The hydrolysed solutions were poured into 50 ml volumetric flasks when cool and diluted to volume, before mixing thoroughly and filtering (Whatman No. 2, VWR Ltd, UK) into 50 ml plastic bottles for storage. A 5 ml aliquot of sample was taken from each bottle and transferred into a 50 ml Quickfit round-bottomed flask, which also contained 0.5 ml internal standard (solution 6). The sample aliquots were evaporated to dryness under vacuum in a rotary evaporator, and the dry residue re-dissolved in 2.5 ml of 25mM acetic acid (solution 2). The solutions in acetic acid were stored until required in 20 ml plastic screw-topped bottles. A working standard for the assay was prepared by pipetting 0.5 ml amino acid standard mixture (Cat. No. AA-S-18, Sigma Chemicals, UK) along with 0.5 ml internal standard (solution 6) into a 50 ml Quickfit round-bottomed flask and evaporating to dryness as before, reconstituting with 2.5 ml acetic acid and storing in a 20 ml plastic bottle. All 20 ml bottles were stored until required at 0-4°C. For the purposes of HPLC, a 10µl sample volume was removed from each bottle using a micro syringe into glass vials (372/3400/50, VWR Ltd., UK) as required.

Samples were measured for their amino acid content by HPLC analysis (Dionex UK Ltd., Camberley) using an Alltech OPA-HR column (Alltech, Carnforth), with dimensions 4.6 X 150 mm. The column operated with a flow rate of 1.5 ml min^{-1} at a column temperature of 25°C, and the results were analysed using Genstat Release 5.

2.6 Calculation of Nutrient Digestibility

Incorporating titanium dioxide as a marker in the feeding trial diets allows the calculation of the coefficient of apparent digestibility of dry matter (ADMD) in that diet, based on a ratio of the proportion of the marker in the dietary material and in the excreta. This allows for the removal of dietary nutrients in the form of Dry Matter (DM). From this initial value, it is possible to estimate values for the digestibility parameters of other nutrients in the diet, such as the Apparent Metabolizable Energy (AME), nitrogen retention (Dig N content) and metabolised N (Metabolisable N content). Additionally, ratios or coefficients can also be calculated for apparent nitrogen digestibility or metabolisability (AND or AMN), apparent

digestibility of organic matter in the dry matter (DOMD) and apparent digestibilities for each amino acid (Sibbald, 1989). As titanium dioxide is indigestible, it must be assumed that:

$$TiO_2 \text{ (intake in feed)} = TiO_2 \text{ (excreted or out)}$$

However, there will be a proportional change in the concentration of the TiO_2 as nutrients are removed from the ileum. Thus:

$$[TiO_{2\,in}] = DM_{in} \text{ X } [TiO_{2\,feed}]$$

$$[TiO_{2\,out}] = DM_{out} \text{ X } [TiO_{2\,excreta}]$$

Where, DM_{in} = Dry Matter intake (g), DM_{out} = Dry Matter excreted (g), $[TiO_{2\,feed}]$ = Titanium Dioxide concentration within the feed (g kg^{-1} DM) and $[TiO_{2\,excreta}]$ = Titanium Dioxide concentration within the excreta (g kg^{-1} DM)

Titanium dioxide contents of the excreta or alternatively of the digesta can then be substituted into the equations depending on the nature of the samples being tested for digestibility.

$$\text{Apparent Dry Matter Digestibility (ADMD)} = \frac{[TiO_{2\,excreta}] - [TiO_{2\,feed}]}{[TiO_{2\,excreta}]}$$

Where: $[TiO_{2\,excreta}]$ = Titanium Dioxide concentration in excreta (g kg^{-1} DM), $[TiO_{2\,feed}]$ = Titanium Dioxide concentration in feed (g kg^{-1} DM)

$$\text{Dry Matter excreted} = \frac{[TiO_{2\,feed}]\ X\ Dry\ Matter\ intake}{[TiO_{2\,excreta}]}$$

This information can then be used in the following equations in order to calculate nutrient digestibility:

- Apparent Metabolizable Energy (AME) (MJ kg^{-1} DM) $= \frac{Energy_{in} - Energy_{out}}{DM_{in}}$

$$= \frac{(GE_{feed}\ X\ DM_{in}) - ((GE_{exc}\ X\ [TiO_{2\,feed}]) / [TiO_{2\,excreta}])}{DM_{in}}$$

where: GE_{feed} = Gross Energy content of feed (MJ kg^{-1} DM), DM_{in} = Dry Matter intake (g), $GE_{excreta}$ = Gross Energy content of excreta (MJ kg^{-1} DM)

AME is the energy available for metabolism by the animal, and refers to the difference between the energy value of a feed and the energy value of the faeces and urine, which are excreted together through the cloaca.

- Coefficient of Apparent Nitrogen Digestibility = $\frac{Nitrogen_{in} - Nitrogen_{out}}{Nitrogen_{in}}$

$$= \frac{([N_{feed}]\ X\ DM_{in}) - (([N_{excreta}]\ X\ [TiO_{2\ feed}])/[TiO_{2\ excreta}])}{([N_{feed}]\ X\ DM_{in})}$$

where: N_{feed} = Nitrogen content of feed, $N_{excreta}$ = Nitrogen content of excreta, DM_{in} = Dry Matter intake (g), DM_{out} = Dry Matter excreted (g)

- Digestibility of Organic Matter in Dry Matter = $\frac{Organic\ Matter_{in} - Organic\ Matter_{out}}{Organic\ Matter_{in}}$

$$= \frac{(OM_{feed}\ X\ DM_{in}) - ((OM_{excreta}\ X\ [TiO_{2\ feed}])/[TiO_{2\ excreta}])}{(OM_{feed}\ X\ DM_{in})}$$

where: OM_{feed} = proportion of organic matter in the feed, $OM_{excreta}$ = proportion of organic matter in the excreta, DM_{in} = Dry Matter intake

- Coefficient of Apparent Amino Acid Digestibility = $\frac{Amino\ Acid_{in} - Amino\ Acid_{out}}{Amino\ Acid_{in}}$

$$= \frac{([AA_{feed}]\ X\ DM_{in}) - (([AA_{excreta}]\ X\ [TiO_{2\ feed}])/[TiO_{2\ excreta}])}{([AA_{feed}]\ X\ DM_{in})}$$

where: [Amino Acid $_{feed}$] = Amino acid content of the feed (g kg^{-1} DM), [Amino Acid $_{excreta}$] = Amino acid content of the excreta (g kg^{-1} DM)

Apparent Metabolisable Energy can also be corrected for nitrogen equilibrium (AMEn), where no nitrogen is either in surplus or lost, by applying a correction factor of 36.5 kJ g^{-1} to adjust for catabolised nitrogen from the body (Sibbald, 1989). This correction should be carried out for studies on growing animals, as they retain nitrogen for the purposes of protein deposition, which will reduce the levels of urinary nitrogen excreted through body catabolism (McDonald, *et al.*, 1995; NRC 1994).

As the ileum forms the main site for the digestion and absorption of nutrients, calculations of nutrient digestibility based on the analysis of ileal samples are expressed as coefficients of

digestibility. Nutrient digestibility measurements based on excreta samples are considered as total tract digestibility, as they also include the metabolic activity of the intestinal microflora, and the results of these calculations are expressed as coefficients of metabolisability. These two terms are reflected in later results sections.

2.7 Sialic Acids content as a measure of endogenous cell loss

Sialic acid standard (N-acetylneuraminic acid {N-AM}) prepared from *Escherichia coli* was purchased (Sigma Chemicals, UK). A fresh 1 mM stock solution was prepared as required by accurately weighing around 0.0309g and dissolving this in 100ml distilled water, before storing at a temperature of <4°C until required. Other stock solutions prepared for the analysis are described (**Appendix 7**). A total of 7 N-AM standards were prepared daily in the laboratory, covering a range of concentrations between 0 and 0.03μmol. The standards were prepared in 4ml screw-capped glass tubes, where 0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 ml stock solution of N-AM were added to each tube in sequence. The tubes were then made up to a final volume of 0.5 ml, with the addition of 0.5, 0.45, 0.4, 0.35, 0.3, 0.25 and 0.2 ml distilled water.

Samples of digesta were analysed for their content of sialic or N-acetylneuraminic acid according to a modified version of the method of Jourdian *et al.*, (1971). A mass of 10-20 mg samples was weighed accurately (Sartorius Analytic A120S, Gottingen) into 4 ml screw-capped tubes (VWR Ltd., UK) and 0.5 ml distilled water was then added to each, to make the tube volumes equivalent to that of the standards. The tubes for both samples and standards were treated equally for the remainder of the assay. A volume of 0.1 ml periodic acid (0.04M) was added and the tubes mixed well, before the tubes were immediately placed in an ice bath for a period of 20 min, to allow the production of chromogens by oxidation of N-AN. With the tubes still standing in ice to slow the reaction, a volume of 1.25 ml resorcinol solution was added and the tube contents mixed well. The tubes remained in the ice bath for a further 5 min. After this time, the outer tube surfaces were dried using tissue and tubes were placed in a heating block (Dri-Block DB-3, Techne, Cambridge, UK) at 100°C for a period of 20 min. On removal, the tubes were instantly cooled in tap water and a volume of 1.25 ml tert-butyl alcohol pipetted into the tubes, mixing thoroughly to achieve a single-phase solution. The tubes were then set into a water bath at 37°C for a period of 3 min, to stabilise the colour achieved in the reaction before cooling to room temperature. The sample tubes were

centrifuged (Sorval RT600, Dupont, Stevenage) for 5 min at 1500*g* to remove solid particles from suspension. The supernatant from each tube was poured into plastic 1 ml macro-cuvettes (Makro, Greiner Labortechnik, Germany) and absorbances in the sample cuvettes were read at 630nm in a spectrophotometer (UVIKON-XL, Biotech Instruments, Herts, UK) connected to LabPower software (Biotech, Herts.). A standard curve prepared from the standard tubes was used to read the unknown sample concentrations in Microsoft Excel.

2.8 Analysis of volatile fatty acids by HPLC

An internal standard was prepared for the analysis by diluting 1 ml 2-ethyl-n-butyric acid (VWR Ltd., UK) in 100 ml distilled water in a volumetric flask with sonication. Additionally, metaphosphoric acid solution was prepared by diluting 21.5g metaphosphoric acid in 50 ml distilled water, before adding 2.2 ml concentrated sulphuric acid and diluting to volume in a 100 ml volumetric flask. Both these reagents were stored until the time of analysis.

Defrosted samples of caecal material were weighed accurately to 0.5g in screw-capped, sealable plastic tubes. A volume of 5 ml water was added to the samples, and the samples shaken well. All samples were left to stand until a homogenous solution was obtained and then were mixed using a whirlimixer. A 1 ml aliquot was drawn and placed into a microcentrifuge tube. To this aliquot, 0.2 ml metaphosphoric acid solution and 0.1 ml internal standard solution were added, and the microcentrifuge tubes were shaken well. The tubes were left to stand overnight at a temperature <4°C to allow maximum separation of the volatile fatty acid fraction. They were then centrifuged at 1740*g* (Sorvall RT600, Dupont, Stevenage, UK) for 15 minutes and then at 10,000*g* for 5 minutes (MSE Micro Centaur, Hampshire, UK). The supernatant was removed from each microcentrifuge tube and placed into an HPLC vial (VWR Ltd., UK), and the samples frozen before analysis.

Volatile fatty acids were analysed with reference to internal and external standards by HPLC using a Dionex ASI100 autosampler and P580 pump, detected using a Shodex SE61 refractive index detector and data was collected using a Dionex Chromeleon 6.20 data system (Dionex Ltd., Camberley, Sussex). Volatile fatty acids, lactic acid and ethanol were separated from the samples using a Supelco Supelcogel C-641H column (Supelco, Poole, Dorset), with dimensions 7.8 X 300 mm. The column had an injection volume of 80µl and an operating temperature of 57°C, operating with a flow rate of 0.45 ml min^{-1}.

2.9 Preparation of bacteriological media

The various microbial growth media used were each especially chosen for their selectivity. They were obtained as dehydrated media (Oxoid Ltd, UK), reconstituted according to the manufacturer's instructions and are described (**Table 2.2**). In each case, the powdered agar compound was weighed into a 500 ml Schott Duran bottle, and diluted to a volume of 500 ml in distilled water with mixing. When completed, the bottles of agar media were sterilised in an autoclave at 115°C for 20 min, before being cooled to 45°C in a preheated water bath until required for pouring. All supplements were removed from storage at <4°C about an hour before they were required, to allow acclimatisation to room temperature and the prevention of haemolysis in the case of blood supplements. Selective antibiotic supplements were reconstituted with 2 ml of sterile distilled water. Immediately before pouring, supplements were added to the agar one at a time in the water bath and the bottle mixed gently before pouring into petri dishes.

Table 2.2 *Details of the selective agar media and the selective supplements used*

Agar medium	*Bacterial selection*	*Supplement*	*Reference*
MacConkey Agar No.3	Coliform bacteria	None	Barnes & Goldberg, (1962)
De Man, Rogosa, Sharpe (MRS)	Lactic acid bacteria	None	De Man *et al.*, (1960)
Columbia blood agar (CBA)	Presence of haemolytic colony forming units	5% defibrinated sheep blood (1 vial or 25 ml)	Ellner *et al*, (1966)
Wilkins Chalgren	Anaerobe bacteria	5% defibrinated sheep blood (1 vial or 25 ml)	Wilkins & Chalgren, (1976)
Charcoal cefaperazone deoxycholate (CCDA)	*Campylobacter jejuni*	CCDA selective supplement (1 vial)	Hutchinson & Bolton, (1984)
Oleandomycin-polymyxin-sulphadiazine-perfringens (Perfringens OPSP)	*Clostridium perfringens*	OPSP antibiotic supplements A & B (1 vial of each)	Handford, (1974)

2.9.1 Pouring of sterile liquid agar media

All plates (9mm single vent, VWR Ltd., UK) were poured under continuous airflow and ultra violet light in a level cabinet to prevent contamination by settling of airborne bacteria on the agar surface. Each bottle of supplemented agar media was removed from the water bath, mixed gently *en route* and poured until the lower plate surface was just covered, a volume of around 15-20 ml. On setting of the media, each plate was opened to dry under continuous airflow for 30 min, to remove excess condensation, which may impair bacterial colony definition. All plates were inverted, sealed and stored until required at temperatures below 4°C. Any unused plates were disposed of after 4 weeks.

2.9.2 Maximum Recovery Diluent (MRD) preparation

Maximum Recovery Diluent (MRD) was used for serial dilutions of the digesta samples. Serial dilutions of 1 ml in 10 were prepared for each sample tested down to the 10^{-9} dilution, to give a total of 9 tubes in the dilution series. A mass of 9.5g MRD powder (CM733, Oxoid Ltd., UK) was weighed accurately in a fume cupboard and dissolved in 1 litre of distilled water using a magnetic stirrer. For the first serial dilution, 45 ml MRD was dispensed into a plastic screw-capped bottle. The remaining 7 dilutions were prepared by dispensing 9.2 ml MRD into metal-capped test tubes, after which all media was autoclaved for a period of 20 min at 115°C. After autoclaving, the final tube volume of 9.0 ±0.1 ml was checked by random sampling. All tubes were cooled to room temperature and stored at <4°C until required. Any unused sterile MRD was disposed of after 3 months.

2.9.3 Sample preparation

Fresh samples were obtained in sterile universal tubes (VWR Ltd., UK), and analysed either immediately or within 12-24 hr after collection. All digesta samples were tested according to the specifications of BS 5763 (1981). About 10g of faecal samples and 1g of caecal samples were obtained in these tubes. All samples were stored within packed ice inside a polystyrene box, to help prevent dehydration of the samples and reduce unwanted microbial activity. Analysis of trial samples took place both at SAC Aberdeen and at SAC Ayr. On arrival in the laboratory, samples were placed in the cold room below 4°C and individually removed for analysis. Each sample was taken in turn, mixed well and serially diluted in the MRD, before plating immediately onto the various agars to prevent loss of bacterial viability as far as possible. Due to the particulate nature of the material and the importance of the first dilution in the series, around 5g of each faecal sample was initially diluted in 45 ml MRD. For all the remaining dilutions, 1 ml of suspension was added to 9 ml of the MRD. Each stage of the dilution series in MRD was shaken well to create a liquid suspension as quickly as possible, before being transferred to the next tube.

2.9.4 Preparation, inoculation and incubation of plates

Plates were removed from cold storage on the evening prior to the experiment, and left to stabilise to room temperature. The samples were inoculated onto plates using several methods as described below (**Table 2.3**). Plates for coliform, lactic acid and anaerobic bacteria were inoculated by a drop method based on that described by Miles & Misra, (1938), where 2

replicate drops of 25 µl are used for each dilution, and three dilutions can be inoculated in duplicate on one plate. Perfringens OPSP plates were inoculated by the pour plate method to incorporate the bacteria inside the agar, while inoculation of Campylobacter plates was done by the spread plate method as the agar was black in colour and resulting colonies would be extremely difficult to spot. Anaerobic conditions were achieved in the study by the use of an anaerobic cabinet (Don Whitley MkIII), which was maintained at 39°C (10% H_2, 10% CO_2, 80% N_2). The microaerophilic conditions of not more than 5% oxygen necessary for Campylobacter growth were achieved using anaerobic jars with 1 sachet of Campygen (CN25, Oxoid Ltd., UK) per jar. Incubation conditions in the experiment were as shown in the table below.

Table 2.3 *Details of the incubation conditions required for each of the bacteria under test*

Test	*Agar type*	*Inoculation method*	*Incubation details*	*Incubation type*
Total coliforms	Mac Conkey no. 3	Drop	24 hr @ 39°C	Aerobic
Total lactic acid bacteria	MRS	Drop	48 hr @ 39°C	Anaerobic
Total anaerobes	Wilkins-Chalgren (+ 10% defibrinated horse blood)	Drop	48 hr @ 39°C	Anaerobic
Presumptive *C. perfringens*	Perfringens OPSP	Pour plate	24 hr @ 39°C	Anaerobic
Campylobacter jejuni	CCDA- Modified Preston	Spread plate	24 hr @ 37°C, then 24 hr @ 42°C	Microaerophilic
Haemolytic *E. coli*	Columbia Blood (+ 5% defibrinated sheep blood)	Single colonies	5 typical colonies subcultured from MacConkey plates. 24 hr @ 39°C	Aerobic

2.9.5 Identification and counting of colonies

After the incubation period, plates were removed and colonies counted at dilutions where numbers of about 10-30 colony forming units per gram or c.f.u. g^{-1} were observed on the drop plates and 30-100 c.f.u. g^{-1} on the others.

Bacterial counts were calculated using the following formula:

Bacterial Number = Plate count X Total volume (µl) X Dilution (eg. 1×10^5)

2.9.6 Confirmatory testing on bacteria

Several confirmatory tests were carried out on counted bacteria in order to confirm the presence of the desired micro-organisms.

Oxidase test

This test was carried out on 5 typical colonies in samples of Campylobacter on CCDA agar, to check for the production of hydrogen peroxide after catalysis of β-D-glucose by the enzyme glucose oxidase. Each colony was in turn removed with a sterile platinum loop, and inoculated onto a freshly prepared solution of a few drops of 1% tetramethyl-*p*-phenylene diamine dihydrochloride in distilled water on filter paper. Colonies turning dark blue/purple within 20 seconds after exposure indicated a positive test result. (Gerhardt *et al.*, 1981).

Catalase test

All types of suspected lactic acid bacteria grown on MRS at the counted dilution were tested for their reaction to catalase in order to confirm that these were not yeasts (Cowan & Steele, 1984; Gerhardt *et al.*, 1981). An immediate and effervescent reaction would indicate the presence of other substances including yeasts, as all lactic acid bacteria are catalase negative. After exposing the anaerobes to air for 30 min, each bacterial type was removed onto a glass microscope slide with a sterile loop and several drops of 3% Hydrogen peroxide added. The gas released should be oxygen. Suspect Campylobacter isolates were also transferred from CCDA plates and tested for their reaction to catalase.

Gram Stain

A Gram stain for morphology (Gerhardt *et al.*, 1981) was carried out on Campylobacter isolates from CCDA agar, along with coliform bacteria on MacConkey No.3 plates. Single colonies were removed and placed on a glass microscope slide, and gently heat-fixed over a bunsen burner. To distinguish between the presence or absence of an outer cell wall, the colony samples were successively stained with crystal violet and iodine solutions for one minute each, washing with water in between. Alcohol was added for 10 seconds to remove the complex associated with Gram negative cell walls and thus allow only these cells to be counter-stained for 1 minute with carbol fuschin solution. After drying, slides were examined under oil immersion and the structure and appearance of the cells noted.

Haemolysis

A representative sample of total coliform bacteria grown on MacConkey agar No.3 were tested for their haemolytic properties, as these were potentially more damaging than normal coliforms in the avian gut. At the counted dilution, 5 typical colonies were removed at random from plates with a sterile wire, and sub-cultured onto Columbia blood agar (CBA). The CBA agar was also streaked with the lowest dilution from MacConkey plates, by means of a sterile loop. Columbia blood agar plates were incubated at 39°C for 24 hr, and presence of blood haemolysis noted, indicated by a clear zone in the blood around the colony.

2.10 Histological analysis for morphology measurements

Tissues were obtained by the dissection of euthanased birds as mentioned previously (Section 2.4.3). These were then stored, sectioned, prepared and processed using an in-house standard protocol made up at SAC Ayr.

2.10.1 Preparation of histological fixative

On dissection, tissue samples are immersed in a fixative solution to preserve the cells and tissues both structurally and also by preventing putrefactive changes and autolysis of cells through inhibiting growth of moulds and bacteria and inactivation of lysosomal enzymes (Farmilo & Stead, 2002). Formal buffered saline solution was prepared as a fixative in a 9:1 ratio of buffered saline and formaldehyde. This solution was then buffered by addition of 3.5g sodium di-hydrogen phosphate and 6.5g di-sodium hydrogen phosphate (Merck Ltd., UK) per litre of fixative. At dissection, sections of proximal ileum (from Meckel's diverticulum towards the caeca) were placed in the buffered saline fixative and contents topped up after 24 hrs. This was done to ensure that the ileal sections were completely covered by fixative, with a ratio of about 10 times the fixative volume compared with the amount of ileal sample. Tissues were stored until required for processing.

2.10.2 Processing of tissue sections

Dehydration of tissue sections was done using a histokinette machine (Type E7326, British American Optical Co., Slough). The machine was used to immerse tissue samples in sequence over a 15h cycle. Tissues were suspended in each of a series of alcohol solutions for 30 min–2 hrs at each stage, to remove water contained within the tissue. When no water remained, alcohol was cleared from the tissues with xylene (Shandon Scientific Ltd., Cheshire) and

replaced with molten wax (Paraplast Plus, Monoject Scientific Ltd., Co. Kildare, Eire), necessary to prevent collapse and distortion of tissues during sectioning. Alcohol solutions for the histokinette were prepared using distilled water and installed in sequence as follows: 1 beaker each of 65%, 75%, 85% and pure industrial methylated spirit or IMS (95% alcohol) and 2 beakers of 100% pure alcohol (Hayman Ltd., Essex). The final beaker volume was 1200 ml, to ensure that solutions covered all tissues. A further 2 beakers of xylene were used, with the final solutions being two wax baths containing molten wax at 60°C.

A 3-5 mm length of ileal tissue for each sample was cut with a sharp blade, and placed into a small metal cassette with a label written in black biro pen. The cassette was closed with a tight-fitting lid. About 25-30 cassettes could be processed at any one time, and so samples were divided up into several groups as required. Cassettes containing the samples were placed into the basket on the first stage of the histokinette, and the cycle started. At the end of the cycle, sections were removed from the wax bath and immediately embedded in wax using the preparation workstation (Blockmaster III, Raymond A Lamb, London). Samples were removed from cassettes and placed in a segregated plastic tray, arranging with forceps heated to the same temperature as the wax so the sample lumen would be cut in cross-section. Wax was dispensed until the sample was covered, and then left to cool and solidify. Solid wax-covered samples were removed from trays, and mounted on a small wooden block using a hot spatula. Wax was trimmed from the samples before the cutting process took place.

2.10.3 Sectioning of tissues

Each section was cut to 5μm using a microtome (Model 1130/Biocut, Reichert Jung). A solution of 50% ethanol in distilled water was used to help straighten sections and remove wrinkles. Each section in turn was placed in a paraffin section mounting bath (Raymond A Lamb, London), preheated to 40°C and mounted on a glass microscope slide (Merck Ltd., UK). The slides were numbered with a diamond cutter pen and dried on a slide drying hotplate (Raymond A Lamb, London) in preparation for staining.

2.10.4 Staining of tissues

Using gloves, samples on the slides were stained with 5% Haemotoxylin and counter-stained with 1% Eosin (both VWR Ltd., UK). Haemotoxylin was used to stain the nuclear material

dark blue, while the Eosin was used to stain cytoplasm pink. The slides were placed in a slide holder and immersed in the various solutions using the following staining procedure.

1	Histoclear/ Xylene organic solvent	2 min
2	Pure alcohol	2 min
3	Industrial Methylated Spirit (IMS)	1 min
4	Distilled Water	1 min
5	Mayer's Haematoxylin	5 min
6	Running Water (from tap)	1 min
7	[1]Scott's solution (to sharpen the appearance of nuclear material and turn it blue)	10-20 sec
8	Water	5 min
9	Eosin (as a counterstain for non-nuclear material)	15 sec
10.	Running water (from tap)	15 sec
11.	Industrial methylated spirit (IMS)	10 sec
12.	Pure alcohol (ethanol)	1 min
13.	Pure alcohol (ethanol)	1 min
14.	Histosol /Xylene	30 sec
15.	Histosol /Xylene	30 sec
16.	Histosol /Xylene	30 sec

[1]Scotts solution - 3.5g sodium bicarbonate and 20g magnesium sulphate in 1 litre water

After the staining procedure, the slides were carefully blotted dry and mounted by placing DPX mounting medium (Agar Scientific Ltd., Stansted, Essex) onto a cover slip and arranging it over the top of the sections on the slide. The mounting medium was then left to set for 24 hrs until hard.

2.10.5 Measurement of tissue sections

Slides were examined by image analysis using a binocular stereomicroscope connected by camera to computer software (Bioscan Optimas, Version 3.01). For each treatment pen replicate, the measurement obtained was based on an average of 5 measurements around a section. Measurements on the gut section dimensions (perimeter, dimension of the ileal section and the diameter of the ileal lumen) were made under 6.3X magnification, whereas those on the internal surface of the intestine, namely villus length, mucosal wall diameter and crypt depth, were made under 16X magnification. Further details of these measurements are given in the respective experimental chapters.

CHAPTER 3

3. CAGE METABOLISM TRIAL TO ASSESS THE POTENTIAL OF FIVE HERBAL PLANTS AND THEIR ESSENTIAL OILS AS DIETARY SUPPLEMENTS FOR BROILER CHICKENS FROM 7-28 DAYS OF AGE

3.1 Introduction

The plant kingdom contains an extensive array of natural chemicals which have a wide variety of effects in animals, both beneficial and detrimental (Panter *et al.*, 2004; Wink *et al.*, 2004), depending on the compound used and its concentration. The recent removal of 5 widely used antimicrobial growth promoters (AGP) and the uncertain future of the remaining 2 increases the potential for bacterial disease in flocks, and will impact directly on the ability of the EU to remain competitive in global markets. It is therefore imperative to find natural substances conferring positive benefits on the health and potentially also on the productivity of intensively housed poultry.

Secondary plant compounds have been associated with natural herbal remedies for thousands of years and have the potential to be exploited for their medicinal, aromatic and flavouring properties. More complex in their development than synthetic antimicrobials, essential oils (EO) and herbs have a synergistic mode of action and may be metabolised within the body. They may therefore provide a more robust defence against bacterial attack, as their activity comes from a number of active components. Various terpenes have antimicrobial activity, and these terpenes form the primary phytochemical components of the herbs and EO (Charai *et al.*, 1996). All the herbs used in the present study are also used for culinary purposes, are considered to be non-toxic at the concentrations used, and are readily available at a relatively low price. Antimicrobial activity has been reported *in vitro* for the EO of *Thymus vulgaris* L., *Rosmarinus officinalis* L., *Origanum majorana* L. (Hammer *et al.*, 1999; Smith-Palmer *et al.*, 1998) and *O. vulgare* ssp. *hirtum* (Hammer *et al.*, 1999; Dorman & Deans, 2000). *Achillea millefolium* L. has been reported to reduce the effects of viruses and helminths *in vitro* (Cowan, 1999). However, outside these *in vitro* assessments, there is a lack of available literature to be found regarding the effects of these substances in live animals. Some industry trials have reported benefits of feeding these substances on productivity and growth. Addition of the product Crina® HC to diets, a natural additive based on a blend of EO, was shown to improve feed efficiency (FCE), reduce the digesta viscosity and also reduce feed intake by 7% in broiler trials (Francesch *et al.* 1999). In the study of Francesch *et al.* (1999), diets

supplemented with virginiamycin reduced feed intake by 3%. The supplement CRINA® POULTRY, based on EO, was reported to increase body mass (BM) in broilers, by reducing the intestinal colonisation of *C. perfringens* (Losa & Köhler, 2001; Sims *et al.*, 2004).

3.2 Aims of the experiment and Methodology

The experiment reported here was designed with the aim of assessing the bioactive potential of feeding various plant or EO supplements in the diets of broiler chickens. The effects of feeding their component terpenes as dietary supplements were considered in terms of the productive performance, the digestibility of the diet, the ileal morphology and on the populations of intestinal microflora in these birds. It was not known whether or not different forms of supplementation would produce the same effect *in vivo*, and thus this study also aimed to compare the effects of supplementation of both herb material and EO.

3.2.1 Sources of the supplements used in the feeding trial

The dietary supplements used in the study, namely *Thymus vulgaris* L., *Origanum vulgare* L. ssp. *hirtum*, *Origanum majorana* L. and *Rosmarinus officinalis* L., or alternatively thyme, oregano, marjoram and rosemary, were purchased as dried chopped leaves from Green City, Glasgow, UK. Seeds of *Achillea millefolium* L., also known as yarrow, were imported from Slovakia and grown at SAC Ayr. The yarrow plants were provided by Katja Svoboda, Department of Plant Biology, SAC Ayr. The chopped herb leaves were supplemented directly into the diets. EO from these dried plant herbs were distilled in the Department of Plant Biology, SAC Ayr, for 2 hours using British Standard distillation procedures (**Appendix 2**), by Val McFarlane, and these were also added directly into the diets during manufacture.

3.2.2 Housing and Environment

The birds were housed in a single room in one of 2 types of cages, although all treatment cages were identical within each experimental block. Of the 5 experimental blocks in the present experiment, 3 blocks housed birds in mobile cages with cup drinkers, and the other 2 blocks housed birds in fixed cages with nipple drinkers. Each cage was provided with a single feed trough. Heating was provided by a single gas brooder at one end of the room, set at the start of the experiment to 32°C at day-old, and then gradually decreased to give a final temperature of 21°C at 21 days. Supplementary heating was provided as required by mobile

butane gas heaters. The birds were provided with a standard broiler lighting pattern, with 1 hour of darkness following a period of 23 hours light, from the start to the finish of the study.

3.2.3 Experimental design

This study was carried out over a period of 28 days, between Feb-Mar 2001. In this experiment, 165 Ross 308 female broiler chicks were purchased at day-old and group reared on litter. Five cage replicates were assigned to one of 11 dietary treatments using a randomised block design, giving a total of 55 experimental cages. At 7 days of age, 3 chicks were randomly assigned to each of the treatment cages, where they remained for the study duration. An initial individual BM was measured on study day 7 and then again on days 14, 21 and 28. An allocation of feed suitable to bird requirements for each cage was calculated and weighed for each week in advance, with a weigh-back of uneaten feed conducted on days 14, 21 and 28. The BM and average feed consumption data per bird per week were then used to determine the productive growth response of the birds on each dietary treatment. Samples of each diet and also of the excreta from each cage were taken on study day 25 and retained for later laboratory analysis. On study day 28, 1 bird was randomly selected from each cage and euthanased using sodium pentobarbitone (euthatal) at a dose of 1 ml kg^{-1} body mass. Tissues for further analyses were obtained by dissection as explained in Section 2.4.3.

3.2.4 Diet formulation and nutritional composition

The diet was manufactured at SAC Ayr, and formulated to be marginal in energy and nutrients, in order to have slightly sub-optimal conditions for growth. However, the diets were balanced for energy and protein, and fed as closely as possible to match the requirements for birds of the age and genotype used in this study. The non-supplemented basal wheat/soya bean meal dietary ration in this study (**Table 3.1**) was formulated to be low in vitamin E at <50 mg kg^{-1} and was used as a control treatment. Each of the remaining 10 treatments were prepared by supplementing this control diet with either herbs or EO, thus making 11 treatment rations in total. All birds received the control diet from days 0-6 of the experiment, and from days 7-29 were fed either the control diet on its own or one of the 10 supplemented rations (**Table 3.2**). The plant herbs were incorporated directly on a weight for weight basis into the dry mix of the diet, and the EO were added into diets during mixing *via* the vegetable oil component of the ration. No in-feed antimicrobials, anticoccidial drugs or feed enzymes of any type were included in these diets.

Table 3.1 *Diet formulation and calculated chemical composition of the basal ration*

Feed Ingredient	*Amount in diet (g kg^{-1})*	*Calculated chemical composition*	*(g kg^{-1})*
Wheat	671.0	ME (MJ kg^{-1})	13.4
Soya bean meal	251.0	Crude Protein	201.6
Soya oil	35.0	Ether Extract/Fat	52.8
Monodicalcium phosphate	15.0	Crude Fibre	27.8
Limestone	15.0	Calcium	9.6
Sodium chloride	3.0	Phosphorus	7.5
Lysine	2.0	Lysine	12.7
Methionine	3.0	Methionine + Cysteine	8.9
Vit/ Min premix[1]	5.0		

Diets were additionally mixed with TiO_2 at 5 g kg^{-1} as a dietary marker and also with the herbal supplement.
[1]Supplied per kg diet: Vitamin A 12,000 IU, Vitamin D3 5000 IU, Vitamin E (as α-tocopherol) 50 mg, Vitamin K 3 mg, Folic acid 1mg, Nicotinic Acid 50 mg, Vitamin B1 (Thiamine) 2 mg, Vitamin B2 (Riboflavin) 7 mg, Vitamin B6 (Pyridoxine) 5 mg, Vitamin B12 15 µg, Biotin 200 µg, Calcium pantothenate 15 mg, Iodine 1mg, Molybdenum 0.5 mg, Selenium 200 µg, Cobalt 0.5 mg, Copper 10 mg, Iron 80 mg, Manganese 100 mg, Zinc 80 mg, Limestone 4.18 g

Table 3.2 *Details of study treatments and inclusion levels of herbs and essential oils*

Treatment	*Test Substance*	*Inclusion Level (g kg^{-1})*
1	Marjoram herb (*Origanum majorana* L.)	10
2	Marjoram EO	1
3	Oregano herb (*Origanum vulgare* L. ssp. *hirtum*)	10
4	Oregano EO	1
5	Rosemary herb (*Rosmarinus officinalis* L.)	10
6	Rosemary EO	1
7	Yarrow herb (*Achillea millefolium* L.)	10
8	Yarrow EO	1
9	Thyme herb (*Thymus vulgaris* L.)	10
10	Thyme EO	1
11	Control – no supplement	n/a

3.2.5 Statistical Analysis

This experiment was designed as a 5 X 2 factorial experiment, incorporating a non-supplemented control, to give a total of 11 treatments. The statistical analysis was carried out using GLM in Genstat Release 6 and Fishers Least Significant difference (L.S.D.) tests were used to separate means. To analyse the experiment factorially, the equation Cont/(herb_oil*type) was entered into the Genstat program. Thus, "Cont" identified either a control or herbal supplemented treatment, "herb_oil" identified the supplement as either a dried herb or an EO and "type" described the plant species supplemented. These criteria allowed a series of comparisons to be made on the pooled experimental data:

CON *vs* HERB_OIL – comparisons between the control treatment in relation to the pooled EO supplemented treatments and also pooled herb supplemented treatments
CON *vs* PLANT TYPE – comparison of the pooled data for each of the 5 plant species in relation to the control treatment
PLANT*HERB_OIL – all treatments against each other to look at the presence/absence of interactions

3.3 Results

This section includes the results from the *in vitro* analysis of the plant supplements, describing the antimicrobial activity of the EO and the condensed tannin (CT) contents within the herb samples. The compositional analysis of the diets is presented, followed by data describing bird performance characteristics, the intestinal bacteriology, as well as the dietary nutrient and amino acid (AA) digestibility coefficients. The experimental results also include the sialic acid concentration and the ileal morphology. As this experiment included large numbers of experimental variables, the statistical technique of principal component analysis (PCA) was used to confirm the results of the individual analyses of variance tests.

3.3.1 *In vitro assessment of the bioactive potential of the plant supplements*

The CT contents in the herb material were measured by the butanol/HCL method as described in section 2.3.5 and are presented (**Table 3.3**). The highest CT concentration was measured in oregano herb at 16 g kg^{-1}.

Table 3.3 *Content of condensed tannins measured in the herb material*

Plant supplement	*Condensed tannins (g kg^{-1})*
Marjoram	0.43
Oregano	15.95
Rosemary	0.85
Thyme	0.13
Yarrow	0.23

Results are expressed on a fresh weight basis due to the essential oil content of the material.
Each sample is based on an average of 2 measurements

The inhibition of bacterial growth by the EO used in the feeding trial is presented (**Table 3.4**). The EO from marjoram, thyme, oregano and rosemary plants were selected for use in the feeding trial, as they demonstrated the strongest inhibition of bacterial growth *in vitro*. The growth inhibition data for each bacterial species with the remaining 15 EO are shown (**Appendix 4**). *Campylobacter jejuni* failed to grow in the agar medium. This bacterium has particularly fastidious growth requirements, and the growth conditions may not have been sufficient. The partial inhibition of bacterial growth towards the outer edge of the zone of inhibition, shown by the presence of asterisks in Table 3.4, was also reported by Deans & Ritchie, (1987). The disappointing values for the yarrow EO may have been a reflection of the quality of the distilled EO, which had a light and watery consistency. Yarrow was selected for use in the feeding trial as it represented the only oil-producing plant capable of being grown

under local climatic conditions. The terpene composition for each EO was analysed by gas chromatography (GC) in the Department of Plant Biology, SAC Ayr, by Val McFarlane and Janice Hampson. Each EO was analysed by GC and the results compared against known terpene reference standards. The terpene composition in each EO is presented (**Appendix 3**).

Table 3.4 *Inhibition of the growth of 9 bacterial species in vitro using seeded Iso-sensitest nutrient agar by the essential oils used in the feeding trial*

	Area of inhibition (cm^2)				
Bacterial species	*Marjoram oil*	*Oregano oil*	*Thyme oil*	*Rosemary oil*	*Yarrow oil*
Listeria monocytogenes	0.47	1.47	2.82	*0.18	---
Clostridium perfringens	2.86	2.77	*1.23	2.94	---
Salmonella enteritidis	0.91	2.14	7.63	1.62	---
Escherichia coli	1.83	2.56	6.55	0.48	---
Enterococcus faecium	0.44	3.31	2.93	0.84	---
Bacillus cereus	*0.47	1.50	*1.72	*0.56	*0.22
Pseudomonas aeruginosa	0.34	0.36	*0.34	---	---
Staphylococcus aureus	1.34	2.16	2.64	0.63	0.16
Yersinia enterocolitica	1.52	2.77	5.02	1.09	0.31

Calculation of the area of growth inhibition was taken after exclusion of the paper impregnation disk (0.6 cm diameter). Each measurement is based on an average of 2 replicates.
* describes the presence of a second ring of bacterial inhibition located outside the measured inhibition zone, where it is possible that the growth of some strains were inhibited only partially.
--- refers to the absence of inhibition of bacterial growth.

3.3.2 Dietary nutrient composition

The results of the chemical analyses for determination of the dietary nutrient composition for each treatment ration as fed in the study (**Table 3.5**), and the amino acid (AA) composition within each dietary treatment as fed to the birds is presented (**Table 3.6**).

Table 3.5 *Composition of the dietary ration as measured by proximate analysis*

	Determined chemical composition of dietary treatments				
Treatment	*DM (g kg^{-1})*	*N (g kg^{-1} DM)*	*Ash (g kg^{-1} DM)*	*OM (g kg^{-1} DM)*	*GE (MJ kg^{-1} DM)*
Control	887	210	74	927	18.46
Marjoram herb	887	214	73	927	18.30
Oregano herb	885	210	69	931	18.53
Rosemary herb	889	198	70	931	18.40
Yarrow herb	885	212	64	936	18.51
Thyme herb	889	204	74	926	18.53
Marjoram oil	883	217	70	930	18.06
Oregano oil	887	208	65	935	18.49
Rosemary oil	890	214	68	932	18.40
Yarrow oil	884	198	67	934	18.63
Thyme oil	882	213	75	925	18.59

N=2 for each

Table 3.6 *Amino acids supplied in the treatment diets to broilers, as fed between 7-28 days of age*

	Composition of amino acids supplied by the treatment rations as fed (g kg^{-1} DM)										
	Diets with plant herbs @ 10 g kg^{-1}						*Diets with essential oils @1 g kg^{-1}*				
	Control	MH	OH	RH	YH	TH	MO	OO	RO	YO	TO
Alanine	7.43	7.25	7.38	7.19	7.45	7.47	7.42	7.74	7.71	7.77	7.64
Aspartic acid	16.70	16.16	16.13	15.45	15.93	16.79	16.57	17.40	17.39	16.47	16.65
Glutamic acid	39.40	38.71	38.63	37.44	38.05	40.76	38.62	40.16	39.42	39.21	40.25
Serine	9.55	9.34	9.45	8.49	8.53	9.65	9.10	9.67	9.47	9.05	9.34
Tyrosine	5.20	6.10	5.91	4.96	5.10	5.34	5.58	5.68	5.17	5.19	5.40
Σ Dispensible	**78.28**	**77.56**	**77.51**	**73.54**	**75.07**	**80.01**	**77.28**	**80.64**	**79.16**	**77.70**	**79.27**
Arginine	11.05	10.53	10.99	10.18	10.62	10.99	11.26	11.34	11.03	10.47	11.56
Glycine	6.47	6.28	6.27	6.32	6.22	6.38	6.54	6.48	6.56	6.65	6.66
Histidine	3.69	4.34	4.00	3.59	3.86	4.29	4.02	4.55	4.26	4.07	4.41
Isoleucine	6.66	7.18	6.90	6.59	7.15	6.64	7.42	7.50	6.58	6.83	7.31
Leucine	12.86	13.39	12.75	12.10	12.77	12.94	12.97	13.41	13.07	12.83	13.36
Lysine	10.57	11.05	9.52	9.34	10.03	10.37	10.60	10.80	10.49	10.32	10.65
Phenylalanine	8.50	9.00	9.06	8.04	8.42	8.53	8.92	9.21	8.54	8.44	8.75
Threonine	6.94	7.20	6.09	5.82	5.96	6.34	6.34	6.25	6.45	6.47	6.34
Valine	7.82	8.52	7.93	7.63	8.11	7.87	8.48	8.52	7.59	7.81	8.52
Σ Indispensible	**74.56**	**77.49**	**73.51**	**69.62**	**73.14**	**74.36**	**76.55**	**78.06**	**74.57**	**73.88**	**77.53**
Σ Total AA	**152.84**	**155.05**	**151.02**	**143.15**	**148.21**	**154.37**	**153.83**	**158.71**	**153.73**	**151.58**	**156.80**

Abbreviations in the table refer to the herb (H) or essential oil (O) supplemented treatments as fed to the birds for each of the plant species - Marjoram (M), Oregano (O), Rosemary (R), Yarrow (Y) and Thyme (T) plant species.
Each value is based on an average of 3 analysed samples.

The determined AA concentrations within each dietary treatment do not quite match the formulated levels, because methionine and the other sulphur AA were partially destroyed by the acid hydrolysis process, and neither proline nor hydroxyproline were measured. In both cases, the diets were observed to be nutritionally similar between treatments. This was expected, as a basal ration was used in the experiment.

3.3.3 Effect of the form of dietary terpene supplement on broiler performance

The basal diet used in this experiment was fed either without supplement, or supplemented with terpenes, either as plant herbs or their EO. The relationship of the type of supplement in broiler diets on the bird performance measures was shown (**Table 3.7**). No relationship was found between the performance measurements in the birds when either herbs or EO were fed as supplements, when compared to the non-supplemented control diets. As a result, only the summary data for the study is presented here.

Table 3.7 *Effect of dietary supplementation with herbs or oils on the average body mass (BM), feed intake, weight gain and feed conversion ratio (FCR) of broilers from 7-28 days*

Average broiler performance over the study in relation to the type of dietary supplement fed to the birds				
Supplement type	*Average BM @ Day 28 (g)*	*Average feed consumed (g bird^{-1} day^{-1})*	*Average weight gain (g bird^{-1} day^{-1})*	*Total FCR days 7-28 (feed gain^{-1})*
Control	727 *(29)*	53.06 *(3.72)*	30.86 *(1.18)*	1.712 *(0.068)*
Plant herb	723 *(19)*	51.99 *(1.54)*	30.63 *(0.93)*	1.702 *(0.028)*
Essential oil	714 *(22)*	51.07 *(1.55)*	30.23 *(1.04)*	1.703 *(0.034)*
s.e.d.	0.020	1.74	0.96	0.012
	NS	NS	NS	NS

Significant differences were expressed in each column by the presence of superscripts. NS (P>0.05)
Data are means (SEM), pooled over the plant species for the herb and oil supplemented treatments.

3.3.4 Effect of plant species on broiler productivity

The plant supplements produced an effect on body mass (BM) only after day 14, where the control diet, and diets including oregano and rosemary tended (P=0.083) to produce birds with lower BM than when fed the other supplements (**Table 3.8**). At day 14, birds fed on diets containing marjoram and thyme showed a 7.4% and 6.9% increase in BM relative to the control treatment birds, whereas those with oregano supplemented diets were decreased by 5.3% in BM. At day 21, birds fed diets with oregano and rosemary had a lower BM than those fed on thyme and marjoram (P<0.05), but were not significantly different from the control birds. At day 28, diets containing oregano produced the lowest BM in the birds when compared to those birds fed on diets containing thyme and marjoram (P=0.021). When

compared to control birds, the birds fed diets with thyme and marjoram supplements showed a 3.9% and 4.4% increase in BM, whereas those fed diets with oregano were decreased by 8.9%. This indicated that the beneficial effect of feeding the birds with thyme and marjoram decreased over time, but birds fed with oregano gradually became worse.

Table 3.8 *Effect of the various plant species on average BM in broilers when fed as dietary supplements between 7-28 days of age*

	Average body mass (BM) (g)			
Treatment	*Day 7*	*Day 14*	*Day 21*	*Day 28*
Control (no herb)	78.6 *(4.3)*	188 *(15)*	404^{abc} *(21)*	727^{ab} *(29)*
Marjoram	79.8 *(3.1)*	202 *(9)*	428^{ab} *(15)*	759^{a} *(20)*
Oregano	79.8 *(3.3)*	178 *(10)*	373^{c} *(19)*	662^{b} *(33)*
Rosemary	79.4 *(2.9)*	187 *(8)*	390^{bc} *(19)*	699^{ab} *(29)*
Yarrow	79.2 *(3.2)*	196 *(9)*	405^{abc} *(19)*	715^{ab} *(37)*
Thyme	78.0 *(3.5)*	201 *(7)*	430^{a} *(17)*	756^{a} *(33)*
s.e.d.	0.92	9.62	19.69	31.92
	NS	P=0.083	P=0.026	P=0.021

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM), pooled for each plant species over the herb and oil treatments.

No treatment differences were observed with respect to feeding the various plant supplements on broiler weight gain between days 7-14 (**Table 3.9**). Birds fed on diets with oregano showed a decrease in weight gain of 10.4%, whereas those with thyme and marjoram showed increased weight gains of 10.9% and 11.7% respectively, when compared to the control treatment. Thyme and marjoram supplemented diets resulted in the highest weight gains between days 15-21 (P=0.027), and also tended (P=0.069) to produce higher weight gain between days 22-28 in the birds, when compared to those fed with oregano. When compared to control treatment birds, diets with thyme and marjoram supplements increased broiler weight gain by 6.3 and 4.5% respectively, while diets with oregano decreased broiler weight gain by 9.4% between days 15-21. Diets containing rosemary resulted in a reduced weight gain compared to those with thyme between days 15-21 (P=0.027). Between 22-28 days, marjoram and thyme supplemented diets resulted in an increased weight gain in the birds of 2.6% and 0.8% respectively, when compared to the control treatment, whereas oregano in the diet reduced bird weight gain by 10.5%. Over the study period, birds fed diets supplemented with marjoram, thyme and the controls achieved higher weight gains when compared to birds fed on diets with oregano (P=0.021). When compared to the control treatment, birds fed diets with thyme and marjoram supplements had the greatest weight gains over the study period by 4.5% and 4.9% respectively, whereas those fed oregano supplements had a 10.1% reduction in

weight gain. The beneficial effect of thyme and marjoram on weight gain appeared to decline with increasing age, but oregano had a constantly detrimental effect throughout the study.

Table 3.9 *Effect of plant species when fed as dietary supplements to broilers on the average weight gain from 7-28 days*

	Average weight gain (g bird^{-1} day^{-1})			
Supplement	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control	15.7 *(1.6)*	30.8^{abc} *(1.1)*	46.2 *(1.4)*	30.9^{a} *(1.2)*
Marjoram	17.5 *(1.4)*	32.2^{ab} *(1.3)*	47.4 *(1.3)*	32.4^{a} *(1.1)*
Oregano	14.0 *(1.4)*	27.9^{c} *(1.5)*	41.3 *(2.3)*	27.8^{b} *(1.6)*
Rosemary	15.4 *(1.3)*	29.0^{bc} *(1.6)*	44.2 *(1.8)*	29.5^{ab} *(1.4)*
Yarrow	16.6 *(1.1)*	30.0^{abc} *(1.7)*	44.2 *(2.7)*	30.3^{ab} *(1.7)*
Thyme	17.4 *(0.9)*	32.8^{a} *(1.8)*	46.6 *(2.5)*	32.2^{a} *(1.6)*
s.e.d.	1.41	1.67	2.19	1.52
	NS	P=0.027	P=0.069	P=0.021

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data was tested at the p<0.05 level, but the largest treatment differences are significant to the level shown.
Data are means (SEM) pooled for each plant species over the herb and oil treatments.

Diets supplemented with thyme were most readily consumed, and diets with oregano consumed least from days 7-14 in the study (P=0.069; **Table 3.10**). When compared to the control treatment, diets with thyme and marjoram increased feed consumption between days 7-14 by 7.1% and 3.7% respectively, whereas consumption was decreased in diets with oregano by 13.3%. Thyme inclusion in the diet increased average feed consumption when compared to diets with oregano and rosemary supplements between days 15-21 (P≤0.01), while diets with marjoram supplements were preferred to those with oregano (P≤0.01). When compared to the birds fed the control treatment, diets with thyme and marjoram increased broiler feed consumption by 8.1% and 3.3% respectively, whereas those diets with oregano were consumed 12.5% less than the control diet. The control diet and the diets with marjoram and thyme supplements were all consumed more readily between days 22-28, compared to diets with oregano (P≤0.05). Thyme and marjoram supplements in diets increased broiler feed consumption by 1.1% and 1.2% during days 22-28 compared to the control treatment, but feed consumption was reduced by 12% in the birds fed diets with oregano. Throughout the study period, diets with thyme were eaten in greater quantities than those diets with rosemary or oregano (P=0.015), but were not significantly less consumed than the control diet. The diets with oregano were consumed less over the study when compared to the control diets and those diets with marjoram or yarrow (P=0.015). The diets with thyme and marjoram were consumed by 4.4% and 2.3% more, but those supplemented with oregano were consumed

12.4% less over the study period, when each was compared to the control diet. The initial increase in feed intake for the birds fed diets supplemented with thyme up to day 21 gradually became less obvious, whereas the feed intake was reduced in birds fed diets with oregano by 13% constantly throughout the study.

Table 3.10 *Effect of plant species included in broiler diets on the average feed consumption between 7-28 days*

	Average feed consumption (g bird^{-1} day^{-1})			
Supplement	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control (none)	29.8 *(3.4)*	48.8^{ab} *(3.4)*	80.5^{a} *(4.4)*	53.1^{ab} *(3.7)*
Marjoram	31.0 *(2.0)*	50.5^{ab} *(1.3)*	81.5^{a} *(1.3)*	54.3^{ab} *(1.4)*
Oregano	25.9 *(1.8)*	42.7^{c} *(2.6)*	70.8^{b} *(4.3)*	46.5^{c} *(2.8)*
Rosemary	27.2 *(2.5)*	45.8^{bc} *(2.7)*	74.8^{ab} *(3.9)*	49.3^{bc} *(3.0)*
Yarrow	30.3 *(1.2)*	48.6^{abc} *(2.1)*	77.7^{ab} *(3.2)*	52.2^{ab} *(2.0)*
Thyme	32.0 *(1.4)*	52.8^{a} *(2.6)*	81.4^{a} *(2.6)*	55.4^{a} *(1.8)*
s.e.d.	2.38	2.85	3.74	2.76
	P=0.069	P≤0.01	P≤0.05	P=0.015

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data was tested at the p<0.05 level, but the largest treatment differences are significant to the level shown.
Data are the means (SEM), pooled for each plant species over the herb and oil treatments.

The birds fed diets supplemented with rosemary had the lowest FCR values throughout the study, although there were no overall differences between the treatments (**Table 3.11**). The FCR values were poorer in the study, when compared to commercially fed birds. This data indicated a slightly changing pattern over the study weeks in relation to the effect of plant supplementation. There appeared to be an initial adaptation period required for the marjoram, thyme and yarrow supplements in diets, where the birds ate less, but during this same period the birds fed on oregano ate more. After this initial period, the FCR in birds was increased by inclusion of thyme and yarrow in the diets, due to a stimulated feed intake. However, the FCR in birds was reduced with the dietary inclusion of marjoram in the later stages of the study, which may indicate a more efficient use of the feed by the birds on this treatment. Throughout the study, birds fed diets with oregano had a reduced FCR compared to those on the control treatment, due to both reduced feed intake and weight gain.

Table 3.11 *Effect of plant species supplemented in diets on broiler FCR from 7-28 days*

	Feed conversion ratio in broilers (feed gain^{-1})			
Supplement	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control	1.90 *(0.06)*	1.58 *(0.08)*	1.74 *(0.07)*	1.71 *(0.07)*
Marjoram	1.84 *(0.16)*	1.58 *(0.05)*	1.73 *(0.04)*	1.69 *(0.05)*
Oregano	1.96 *(0.14)*	1.53 *(0.05)*	1.71 *(0.04)*	1.68 *(0.04)*
Rosemary	1.75 *(0.09)*	1.58 *(0.05)*	1.69 *(0.05)*	1.66 *(0.05)*
Yarrow	1.87 *(0.10)*	1.64 *(0.05)*	1.79 *(0.06)*	1.75 *(0.05)*
Thyme	1.87 *(0.10)*	1.63 *(0.07)*	1.77 *(0.06)*	1.74 *(0.06)*
s.e.d.	0.150	0.067	0.055	0.055
	NS	NS	NS	NS

NS (P>0.05)
Data are means (SEM), pooled for each plant species across the herb and oil treatments.

3.3.5 Effect of herbs or their essential oils as dietary supplements on broiler performance

The birds fed thyme EO and yarrow herb supplemented diets showed the greatest average BM during the study (**Table 3.12**). At day 14, there were no treatment differences on BM, but the thyme EO and yarrow herb supplemented diets increased BM in the birds by 11.2% and 10.1% respectively, when compared to the control treatment. At 21 days, BM was increased in the birds fed diets with thyme EO supplements, compared to those fed diets with rosemary and yarrow oils, as well as oregano EO and herb (P<0.01). At day 28, the birds fed diets supplemented with thyme EO had an improved BM, compared to those fed diets with thyme herb, oregano EO and herb, rosemary herb and yarrow EO supplements (P<0.01). Birds fed diets supplemented with yarrow herb achieved greater BM then those fed diets with oregano herb and rosemary EO (P<0.01; days 21 & 28) and also with yarrow EO (P<0.01; day 28). At day 28, the birds fed diets with oregano herb and rosemary EO had a decreased BM of 11.3% and 8.7% compared to those fed the control treatment. The BM of birds fed yarrow EO supplemented diets was 11.8% less than the control treatment at day 28, and these birds were the lightest. The positive increase in BM in birds fed on the thyme EO supplemented diets compared to the controls was relatively constant over each week of the study at 11-14%, whereas the positive effect of the yarrow herb diminished slightly with age. The birds fed rosemary EO supplemented diets showed a variable decrease in BM between 7.5-10% throughout the experiment, whereas the decrease in BM caused by inclusion of both the oregano herb and yarrow EO supplements became gradually worse throughout the experiment.

Table 3.12 *Effect of various herbs and essential oils supplemented in diets on average BM in broilers over study days 7-28*

	Average body mass (g)			
Treatment	*Day 7*	*Day 14*	*Day 21*	*Day 28*
Control	79 *(4)*	188 *(15)*	404^{bcde} *(21)*	727^{bcde} *(29)*
Marjoram herb	80 *(5)*	201 *(14)*	429^{abc} *(26)*	754^{abcd} *(34)*
Oregano herb	80 *(5)*	173 *(14)*	364^{de} *(30)*	645^{e} *(53)*
Rosemary herb	79 *(4)*	199 *(9)*	417^{abcde} *(14)*	734^{abcde} *(23)*
Yarrow herb	80 *(6)*	207 *(14)*	440^{ab} *(27)*	789^{ab} *(43)*
Thyme herb	77 *(6)*	193 *(10)*	397^{bcde} *(20)*	691^{cde} *(39)*
Marjoram oil	80 *(4)*	203 *(13)*	427^{abc} *(17)*	764^{abc} *(27)*
Oregano oil	79 *(5)*	183 *(16)*	382^{cde} *(26)*	680^{cde} *(44)*
Rosemary oil	80 *(4)*	174 *(13)*	363^{e} *(33)*	664^{de} *(52)*
Yarrow oil	78 *(4)*	184 *(10)*	371^{cde} *(19)*	641^{e} *(38)*
Thyme oil	79 *(5)*	209 *(9)*	464^{a} *(18)*	821^{a} *(35)*
s.e.d.	1.30	13.61	27.84	45.14
	NS	NS	P=0.009	P=0.001

Data was tested at the P<0.05 level, but the largest treatment differences were significant to the level shown. Significance is expressed in each column by the presence of non-identical superscripts. NS (P>0.05) Data are means (SEM) for 5 replicates of each treatment.

The birds fed diets containing thyme EO and yarrow herb consistently achieved the highest weight gains over the study period, whereas the birds fed diets supplemented with rosemary EO, oregano herb and yarrow EO achieved lowest weight gains (**Table 3.13**). Between 15-21 days, the highest gains were achieved by birds fed diets with thyme EO supplements compared to those fed diets with rosemary herb, oregano herb and EO, thyme herb supplemented diets and the non-supplemented controls (P=0.002). The lowest weight gains were observed in birds fed diets with rosemary and yarrow EO, compared to those fed diets with supplementary marjoram herb and EO, yarrow herb and thyme EO (P=0.002). Birds fed diets with yarrow herb were second only to those fed diets with thyme EO in terms of bird weight gain, and had higher weight gains than the birds fed with both oregano herb and EO supplements during days 15-21 (P=0.002). The birds fed diets with marjoram herb had higher weight gains than those including oregano herb between 15-21 days in the study (P=0.002). During days 22-28, the highest weight gains were observed with birds fed on diets containing thyme EO and yarrow herb, greater than those birds fed rosemary EO, oregano EO and herb and also thyme herb in their diets (P<0.001). The inclusion of yarrow EO in diets resulted in the lowest weight gains in the birds, compared with those fed diets including rosemary herb, marjoram EO or herb, yarrow herb or thyme EO or the control treatment between 22-28 days (P<0.001). The birds fed diets with marjoram EO also showed an improved weight gain, compared to those supplemented with oregano herb from 22-28 days (P<0.001). The weight

gains in birds fed diets with thyme EO were greater than those birds fed the non-supplemented controls over the study period (P=0.001). Birds fed on diets supplemented with thyme EO and yarrow herb had improved weight gains, compared to those fed diets with supplements of thyme herb, oregano EO and herb and also rosemary EO (P<0.001). The poorest weight gains were observed in birds fed diets with yarrow EO, when compared to those fed diets with thyme EO, marjoram EO and herb, rosemary herb and also yarrow herb (P=0.001). The birds fed diets supplemented with thyme EO showed consistent numerical increases of 18.3, 18.0 and 10.7% compared to the control treatment during the trial period, with an overall increase of 14.4% in weight gain. Likewise, the birds fed on diets with yarrow herb showed increased weight gains of 15.7, 8.3 and 7.8% when compared to the control treatment over the study period, which indicated a decreasing effect of dietary supplementation over time. The negative effect of rosemary EO supplementation in diets appeared to decrease over the study period, whereas that of oregano remained constant. The supplementation of yarrow EO in the diets resulted in birds with increasingly poor weight gains when compared to the control, of 13.5, 13.5 and 16.3% respectively over the study and an overall decrease of 13.2%, which illustrates the varying effects of each herbal supplement over time in the birds.

Table 3.13 *Effect of various herbs and essential oils supplemented in broiler diets on the average weight gain over study days 7-28*

	Average broiler weight gain (g bird^{-1} day^{-1})			
Treatment	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control	15.7 *(1.6)*	30.8^{bcde} *(1.1)*	46.2^{abc} *(1.4)*	30.9^{bcdef} *(1.2)*
Marjoram herb	17.3 *(2.3)*	32.4^{abc} *(2.0)*	46.5^{abc} *(1.1)*	32.1^{abcd} *(1.8)*
Oregano herb	13.3 *(2.4)*	27.4^{de} *(2.5)*	40.1^{cd} *(3.3)*	26.9^{ef} *(2.7)*
Rosemary herb	17.2 *(1.2)*	31.2^{bcde} *(1.0)*	45.3^{abc} *(1.6)*	31.2^{abcde} *(1.1)*
Yarrow herb	18.1 *(1.9)*	33.4^{ab} *(1.9)*	49.8^{a} *(2.3)*	33.8^{ab} *(2.0)*
Thyme herb	16.2 *(1.3)*	29.2^{bcde} *(2.1)*	42.0^{bcd} *(3.1)*	29.2^{cdef} *(2.0)*
Marjoram oil	17.6 *(1.9)*	32.0^{abcd} *(1.8)*	48.2^{ab} *(2.4)*	32.6^{abc} *(1.5)*
Oregano oil	14.8 *(1.8)*	28.4^{cde} *(1.8)*	42.5^{bcd} *(3.6)*	28.6^{cdef} *(2.0)*
Rosemary oil	13.6 *(2.1)*	26.9^{e} *(3.0)*	43.1^{bcd} *(3.3)*	27.9^{def} *(2.6)*
Yarrow oil	15.1 *(1.0)*	26.7^{e} *(2.0)*	38.6^{d} *(3.3)*	26.8^{f} *(1.8)*
Thyme oil	18.5 *(1.0)*	36.4^{a} *(1.9)*	51.1^{a} *(3.0)*	35.3^{a} *(1.8)*
s.e.d.	1.99	2.36	3.097	2.2
	NS	P=0.002	P<0.001	P=0.001

Data was tested at the P<0.05 significance level, but the largest differences are significant to the level shown.
Significant differences are expressed in each column by non-identical superscripts. NS (P>0.05)
Data are means (SEM) for 5 replicates of each treatment.

Thyme EO supplemented diets were most readily consumed throughout the study, whereas those diets with oregano herb and rosemary EO were less readily consumed (**Table 3.14**). Between days 15-21, diets supplemented with thyme EO, yarrow herb and also marjoram herb and EO were more readily consumed by the birds, when compared to those including oregano herb and rosemary EO supplements ($P<0.05$). The diets containing thyme EO were consumed in greater quantities, when compared to the control diets and those including thyme herb as well as yarrow and oregano EO ($P<0.05$). Thyme EO supplemented diets were also preferred from days 22-28 of the study, compared to those with thyme herb, oregano EO and yarrow EO supplements ($P=0.006$), although they were not significantly preferred to the controls. The birds fed diets with yarrow herb had greater consumption levels than those diets with yarrow EO ($P=0.006$). During days 22-28 of the study, the lowest values for consumption were observed for the diets containing rosemary EO and oregano herb, when compared to those diets with thyme EO, yarrow herb, marjoram EO and herb, rosemary herb and also the control diets ($P=0.006$). Over the study period (days 7-28), the diets with thyme EO were more readily consumed at 87.1 g $bird^{-1}$ day^{-1} when compared to those diets with yarrow EO (71.9 g) and oregano EO (74.0 g; $P<0.05$). However, they were not consumed in greater quantities compared to the control diets. During study days 7-28, the diets with lowest consumption were those containing oregano herb and rosemary EO, when compared to those with thyme EO, yarrow herb, marjoram EO and herb, rosemary herb supplemented diets and the control diets ($P<0.05$). When compared to the control diets, the diets supplemented with oregano decreased feed consumption in the birds by 14.0, 14.3 and 15.9% in each week, with an average decrease over the study of 15.1%. The diet with rosemary EO showed a large initial decrease in consumption of 19.9%, followed by 15.1 and 15.1% in the successive trial weeks, with an average decrease over the study of 16.0%, compared to the control diet. Yarrow EO supplemented diets showed progressively larger decreases in feed consumption by 0.6, 7.2 and 10.7% over the study period when compared to the controls, with an average decrease of 7.7%. The diets supplemented with thyme EO resulted in an average increase of 10.5% for each week over the study period, and diets with yarrow herb increased feed consumption on average by 4.5% in each study week when compared to the controls.

Table 3.14 *Effect of various herbs and essential oils supplemented in diets on the average feed consumption in broilers over study days 7-28*

	Average feed consumption (g bird^{-1} day^{-1})			
Treatment	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control	29.8 *(3.4)*	48.8^{bcd} *(3.4)*	80.5^{abc} *(4.4)*	53.1^{ab} *(3.7)*
Marjoram herb	29.2 *(3.3)*	50.5^{abc} *(2.6)*	80.7^{abc} *(2.5)*	53.5^{ab} *(2.8)*
Oregano herb	25.7 *(3.5)*	41.8^{d} *(4.9)*	67.7^{d} *(7.1)*	45.1^{c} *(5.1)*
Rosemary herb	30.6 *(1.7)*	50.1^{abcd} *(2.0)*	81.2^{abc} *(3.3)*	53.9^{ab} *(2.2)*
Yarrow herb	30.9 *(2.2)*	52.0^{ab} *(3.3)*	83.5^{ab} *(4.2)*	55.5^{ab} *(3.0)*
Thyme herb	32.0 *(2.7)*	48.4^{bcd} *(3.1)*	75.7^{bcd} *(3.3)*	52.0^{abc} *(2.7)*
Marjoram oil	32.7 *(2.6)*	50.5^{abc} *(1.1)*	82.3^{abc} *(1.0)*	55.2^{ab} *(0.9)*
Oregano oil	26.1 *(1.5)*	43.7^{cd} *(2.4)*	74.0^{bcd} *(5.2)*	47.9^{bc} *(2.8)*
Rosemary oil	23.9 *(4.5)*	41.5^{d} *(4.3)*	68.3^{d} *(6.1)*	44.6^{c} *(4.9)*
Yarrow oil	29.7 *(1.1)*	45.3^{bcd} *(1.9)*	71.9^{cd} *(3.5)*	49.0^{bc} *(2.0)*
Thyme oil	31.9 *(1.1)*	57.1^{a} *(3.5)*	87.1^{a} *(2.0)*	58.7^{a} *(1.5)*
s.e.d.	3.36	4.03	5.29	3.9
	NS	P≤0.05	P=0.006	P<0.05

Data was tested at the P<0.05 level, but the largest differences are significant to the level shown.
Data are means (SEM) of 5 replicates for each treatment.
Significant differences are expressed in each column by non-identical superscripts. NS (P>0.05)

Yarrow EO-supplemented diets resulted in the poorest FCR values in the birds during study days 22-28, when compared to those fed diets with rosemary EO, yarrow herb, oregano EO and herb, marjoram EO and herb, thyme EO and also the control diet (P=0.005; **Table 3.15**). The birds fed on diets with rosemary EO had a lower FCR than all the remaining diets, with the exception of those fed on the diet containing oregano herb during study days 22-28 (P=0.005). Overall, lower FCR values were obtained when the study birds were fed diets with rosemary EO, oregano herb and yarrow herb supplements, when compared to those fed diets containing rosemary and thyme herbs during days 22-28 (P=0.005). The birds fed diets with rosemary EO had the lowest FCR values, when compared to those birds fed diets including rosemary and thyme herbs over the study period (P<0.05). Over the study period the birds fed diets containing yarrow EO had highest values for FCR, compared to those fed diets with rosemary and thyme EO, as well as yarrow, oregano and marjoram herbs (P<0.05). When compared to the control birds, the birds fed oregano herb in the diet had an initial increase in the FCR of 8.7%, which was then decreased over the following weeks, resulting in an average decrease of 2.8% in FCR. The diets fed with yarrow EO consistently increased FCR values in broilers when compared to those fed the control diet, with an average increase of 7.7%. In contrast, diets supplemented with yarrow herb decreased FCR values in the broilers by 7.5, 1.8 and 3.7% in each week when compared to the controls, with an average decrease of 3.7% over the study. Thyme EO supplemented diets were shown to decrease the FCR values in broilers

by 8.2% initially, but after the first week of the study these birds were relatively similar to those fed the control diets. The diets including rosemary EO decreased the FCR values in broilers by 10.5, 2.0 and 8.8% in each study week when compared to the control diets, with an average decrease of 6.8% over the study period. It would appear from these results that an initial adaptation period of around 7 days may be necessary for any dietary supplementation with terpenes. This initial adaptation period may have either a positive or negative effect on the FCR values for the first study week depending on the type of plant and form of supplementation of the terpenes, which may then be reversed during the remainder of the study period.

Table 3.15 *Effect of various herbs and essential oils when fed as dietary supplements on the FCR values in broilers over study days 7-28*

	Average FCR (intake gain^{-1})			
Treatment	*7-14 days*	*15-21 days*	*22-28 days*	*7-28 days*
Control	1.90 *(0.06)*	1.58 *(0.08)*	1.74^{bc} *(0.07)*	1.71^{abc} *(0.07)*
Marjoram herb	1.72 *(0.10)*	1.57 *(0.06)*	1.73^{bc} *(0.04)*	1.67^{ab} *(0.04)*
Oregano herb	2.07 *(0.22)*	1.51 *(0.09)*	1.67^{ab} *(0.06)*	1.66^{ab} *(0.07)*
Rosemary herb	1.79 *(0.09)*	1.61 *(0.03)*	1.79^{cd} *(0.02)*	1.73^{bc} *(0.03)*
Yarrow herb	1.76 *(0.17)*	1.55 *(0.03)*	1.68^{b} *(0.03)*	1.65^{ab} *(0.04)*
Thyme herb	1.99 *(0.14)*	1.68 *(0.10)*	1.82^{cd} *(0.09)*	1.80^{bc} *(0.10)*
Marjoram oil	1.96 *(0.32)*	1.60 *(0.09)*	1.72^{bc} *(0.07)*	1.72^{ab} *(0.09)*
Oregano oil	1.85 *(0.20)*	1.54 *(0.03)*	1.75^{bc} *(0.05)*	1.69^{abc} *(0.04)*
Rosemary oil	1.70 *(0.15)*	1.55 *(0.09)*	1.59^{a} *(0.08)*	1.60^{a} *(0.08)*
Yarrow oil	1.98 *(0.10)*	1.72 *(0.08)*	1.89^{d} *(0.10)*	1.84^{c} *(0.07)*
Thyme oil	1.75 *(0.11)*	1.59 *(0.11)*	1.72^{bc} *(0.07)*	1.67^{ab} *(0.06)*
s.e.d.	0.212	0.095	0.077	0.078
	NS	NS	P=0.005	P<0.05

Data was tested at the P<0.05 significance level, but the largest treatment differences are significant to the level shown.
Significance is expressed in each column by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM) for 5 replicates of each treatment.

3.3.6 Effect of the inclusion of herbs on the main populations of intestinal microflora

The plant herbs used in the present study were studied for their effects on the main intestinal microflora populations and the populations of potentially pathogenic bacterial species in the birds. Unfortunately, it was not possible to study the effects of the EO on the microflora, due to time and sampling pressures. All samples were treated in the same manner, but the number of treatment replicates analysed was limited due to the transport of these samples and the necessity to analyse them within 30 hours from the time of collection. Both these factors will have affected the bacterial numbers in the samples. However, the count of total anaerobes was higher and that of total coliforms lower in caecal material when compared to faecal material

samples (P≤0.001, data not shown). This difference was expected, as the caeca constitutes the main microbial fermentation organ in poultry.

There were no treatment-associated differences in the counts when the main bacterial species of interest were cultured from either the faecal or caecal samples (**Table 3.16**). The data was highly variable, resulting in the higher SEM's observed, and the use of more treatment replicates may have decreased this variability. However, all the plant herb treatment groups had higher caecal lactic acid bacteria counts than coliform counts, indicating that the balance of the microflora within the birds was relatively healthy. Proportions of lactic acid bacteria (LAB) to intestinal coliforms provide a good indication of gut health (Fuller, 1977).

Table 3.16 *Bacterial population counts from samples of faecal and caecal material taken from broilers aged 28 days fed on diets supplemented with plant herbs*

Microfloral populations in samples from the intestinal contents of broilers (log_{10} c.f.u. g^{-1})								
	Coliforms		*Lactic Acid Bacteria*		*Total Anaerobes*		*C. perfringens*	
Treatment	Faecal	Caecal	Faecal	Caecal	Faecal	Caecal	Faecal	Caecal
Control	8.91	6.94	8.91	8.33	9.42	11.24	3.54	3.94
	(0.17)	*(0.65)*	*(0.09)*	*(0.31)*	*(0.20)*	*(0.13)*	*(0.92)*	*(0.38)*
Marjoram	8.79	7.25	8.67	8.68	9.06	10.98	4.19	3.61
	(0.07)	*(0.53)*	*(0.22)*	*(0.22)*	*(0.08)*	*(0.45)*	*(0.18)*	*(0.39)*
Oregano	8.83	7.50	8.21	8.27	9.13	11.36	3.77	3.57
	(0.41)	*(0.40)*	*(0.04)*	*(0.66)*	*(0.24)*	*(0.12)*	*(0.44)*	*(0.34)*
Rosemary	8.62	7.24	8.51	8.15	9.21	11.12	2.41	2.80
	(0.37)	*(0.12)*	*(0.15)*	*(0.49)*	*(0.24)*	*(0.14)*	*(0.72)*	*(0.10)*
Yarrow	8.60	8.40	8.76	8.63	8.62	10.99	2.57	2.00
	(0.18)	*(0.44)*	*(0.07)*	*(0.36)*	*(0.32)*	*(0.13)*	*(0.68)*	*(1.16)*
Thyme	8.32	7.82	8.74	8.05	9.21	11.15	3.68	2.27
	(0.81)	*(0.81)*	*(0.25)*	*(0.53)*	*(0.24)*	*(0.17)*	*(0.62)*	*(0.77)*
s.e.d.	0.70	0.78	0.49	0.69	0.35	0.33	0.76	0.75
	NS	NS	NS	NS	NS	NS	NS	NS

Data comparisons are done within each column. NS (P>0.05)
Data are means (SEM) in colony forming units per gram sample of the logarithms of 3 treatment replicates.

The proportions of LAB to coliforms for each of the samples were calculated by dividing the log_{10} of the bacterial counts for LAB by those for the coliforms (**Table 3.17**). There were no significant differences between the treatments in the ratios of LAB to coliforms. The healthiest faecal lactic acid: coliform ratios were observed for birds fed diets containing yarrow and thyme herbs, where the LAB outnumbered the coliforms. However, the birds fed on the yarrow and thyme supplemented treatments had the lowest lactic acid: coliform ratios in the caecal samples. Perhaps a more balanced ratio of lactic acid: coliform bacteria should represent a better measure of gut health, as opposed to larger proportions of LAB.

Table 3.17 *Effect of dietary supplementation with plant herbs on the ratios of lactic acid bacteria (LAB): coliforms in the intestine of broilers at 28 days of age*

	LAB: coliform ratios	
Supplement	*Faecal*	*Caecal*
Control	1.000 *(0.019)*	1.217 *(0.094)*
Marjoram	0.987 *(0.032)*	1.206 *(0.066)*
Oregano	0.933 *(0.038)*	1.117 *(0.138)*
Rosemary	0.992 *(0.053)*	1.124 *(0.054)*
Thyme	1.077 *(0.137)*	1.061 *(0.157)*
Yarrow	1.020 *(0.014)*	1.028 *(1.010)*
s.e.d.	0.095	0.150
	NS	NS

Significance comparisons are done within each column. NS (P>0.05)
Data are proportions (SEM) of 3 replicate sample means for each count measured in $\log_{10}$ c.f.u. g^{-1}.

Different bacterial species could perhaps be looked at as a ratio of the total population for faecal and caecal samples by expressing them as a proportion of total anaerobes (**Table 3.18**). This was calculated by dividing the $\log_{10}$ data for the various bacterial counts by that of anaerobes as a baseline. No significant treatment differences were observed between the various bacterial proportions. Numerically, faecal samples from birds fed the diets with thyme and yarrow had highest proportions of LAB in relation to total anaerobes, with oregano and rosemary the lowest. In general, very low numbers of *C. perfringens* were isolated, in some cases <50, indicating that this bacterium did not appear to be a problem in these birds. The birds fed diets with marjoram and oregano supplements had the highest faecal and caecal *C. perfringens* proportions. In general, lower bacterial proportions were present in caecal rather than faecal samples for all species except *C. perfringens*, which was expected due to the higher number of anaerobes in the caeca, which constitutes the main microbial fermentation organ.

The frequency of occurrence of potentially pathogenic bacteria in the faecal and caecal samples for the sampled birds was shown (**Table 3.19**). One replicate in each case tested positive for haemolytic *E. coli* and for *C. jejuni* from different birds fed diets with the thyme and yarrow supplements. The catalase tests on all bacterial isolates produced negative results, indicating that all bacteria isolated may well be LAB. It may be the case that the numbers of pathogenic bacteria were reduced, given that the birds were housed in the metabolism cages, thus providing no substrate for bacterial growth.

Table 3.18 *Effect of dietary supplementation with plant herbs on the proportions of various bacterial species to total anaerobes in broilers at 28 days of age*

	Proportions of intestinal microflora to total anaerobes in broilers					
Treatment	*Coliforms: Anaerobes*		*LAB: Anaerobes*		*C. perfringens: Anaerobes*	
	Faecal	*Caecal*	*Faecal*	*Caecal*	*Faecal*	*Caecal*
Control	0.947	0.618	0.947	0.742	0.373	0.350
	(0.028)	*(0.060)*	*(0.029)*	*(0.036)*	*(0.090)*	*(0.032)*
Marjoram	0.970	0.661	0.957	0.794	0.462	0.332
	(0.003)	*(0.047)*	*(0.031)*	*(0.046)*	*(0.016)*	*(0.044)*
Oregano	0.967	0.661	0.901	0.727	0.412	0.314
	(0.020)	*(0.042)*	*(0.019)*	*(0.051)*	*(0.039)*	*(0.028)*
Rosemary	0.936	0.651	0.925	0.734	0.263	0.252
	(0.037)	*(0.012)*	*(0.021)*	*(0.048)*	*(0.080)*	*(0.008)*
Thyme	0.901	0.700	0.949	0.722	0.403	0.203
	(0.077)	*(0.065)*	*(0.034)*	*(0.050)*	*(0.070)*	*(0.070)*
Yarrow	1.001	0.765	1.020	0.785	0.296	0.185
	(0.053)	*(0.040)*	*(0.042)*	*(0.033)*	*(0.070)*	*(0.110)*
s.e.d.	0.059	0.068	0.044	0.069	0.081	0.068
	NS	NS	NS	NS	NS	NS

Significance comparisons are done within each column. NS (P>0.05)
LAB – Lactic acid bacteria
Data are proportions (SEM) of 3 replicate sample means for each count measured in $\log_{10}$ c.f.u. g^{-1}

Table 3.19 *Effect of dietary supplementation with plant herbs on the occurrence of potentially pathogenic bacteria in intestinal samples from broilers aged 28 days*

	Confirmatory testing of bacterial samples from cultured isolates		
Treatment	*Campylobacter jejuni*	*Haemolytic E. coli*	*Catalase test*
Control	-	-	All negative
Marjoram	-	-	All negative
Oregano	-	-	All negative
Rosemary	-	-	All negative
Yarrow	1 replicate (faecal)	1 replicate (faecal)	All negative
Thyme	1 replicate (faecal)	1 replicate (caecal)	All negative

The catalase test was performed on the presumptive LAB to distinguish these from yeasts, where LAB are catalase negative.

3.3.7 Effect of herbs or oils included in diets on nutrient digestibility in broilers

The effects of inclusion of herbs or their EO in the diets on the coefficients of apparent DM digestibility (ADMD), OM digestibility (DOMD) and the apparent metabolisability of nitrogen (AMN) were measured and calculated. The contents of apparent metabolisable energy (AME) and also apparent metabolisable energy corrected to zero nitrogen retention (AMEn) were measured in broilers at 25 days of age. The digestible content of OM and also the metabolisable nitrogen content in the treatment diets were then calculated, along with the metabolisability of the dietary ration (AME:GE). This section presents the results of these analyses on the excreta samples as a measure of total tract digestibility.

There were no effects of treatment on the AME in the birds, either with or without a correction for nitrogen equilibrium (**Table 3.20**), nor was there an effect of treatment on the AME:GE or AMEn:GE. As a whole, the dietary AME values were rather low in these birds compared to those fed on commercial diets. This was not entirely unexpected as the diets were formulated to be marginal in energy and protein in order to enhance the effects of the plant treatments. The determined AME values in the birds fall about 3 MJ short of the calculated ME values from the diet formulation, so the wheat may have been of a lower quality than expected. No enzymes were included in these diets, and the birds may not have been able to access some of the energy stored within the cereal grains. The diets were also formulated to have a low concentration of vitamin E, and all these factors will reduce their feeding value.

Table 3.20 *Effect of herb and essential oil supplementation on the apparent metabolisable energy (AME) and energy metabolisability (AME:GE) in broilers at 28 days, both with and without a nitrogen correction (AMEn)*

Apparent metabolisable energy content and coefficient of energy metabolisability				
Supplement	*AME*	*AMEn*	*AME:GE*	*AMEn:GE*
	(MJ kg^{-1} DM)			
Control	10.46 *(0.73)*	9.96 *(0.67)*	0.567 *(0.040)*	0.579 *(0.027)*
Marjoram herb	10.39 *(0.70)*	9.89 *(0.62)*	0.568 *(0.039)*	0.583 *(0.048)*
Oregano herb	10.59 *(0.90)*	10.14 *(0.78)*	0.572 *(0.048)*	0.592 *(0.049)*
Rosemary herb	10.11 *(0.48)*	9.76 *(0.44)*	0.550 *(0.026)*	0.574 *(0.032)*
Yarrow herb	10.78 *(0.48)*	10.26 *(0.43)*	0.582 *(0.026)*	0.608 *(0.033)*
Thyme herb	10.34 *(0.83)*	9.94 *(0.74)*	0.558 *(0.045)*	0.588 *(0.033)*
Marjoram oil	10.05 *(0.52)*	9.56 *(0.45)*	0.556 *(0.028)*	0.570 *(0.044)*
Oregano oil	10.65 *(0.76)*	10.21 *(0.64)*	0.576 *(0.040)*	0.607 *(0.034)*
Rosemary oil	10.59 *(0.55)*	10.12 *(0.49)*	0.576 *(0.030)*	0.607 *(0.057)*
Yarrow oil	10.31 *(0.89)*	9.96 *(0.80)*	0.553 *(0.048)*	0.569 *(0.034)*
Thyme oil	10.63 *(0.55)*	10.15 *(0.48)*	0.572 *(0.030)*	0.593 *(0.051)*
Con*Herb_oil	NS	NS	NS	NS
Con*Plant	NS	NS	NS	NS
Con*Herb_oil*plant	NS	NS	NS	NS
s.e.d.	0.553	0.498	0.030	0.036

Data are the means (SEM) of 5 replicates for each dietary treatment.
Significance is indicated in each column by the presence of superscripts. NS (P>0.05)

No treatment differences were observed in either the apparent coefficients of ADMD or DOMD in relation to the dietary treatment (**Table 3.21**). The coefficients of ADMD for these diets were low compared to commercial values of about 0.75-0.80, but these values are in agreement with the low AME values determined in the birds. There were no differences due to treatment in relation to the digestible OM content in the diet. This data was analysed at the interaction level, where Genstat compares the gradient for the digestibility values from the birds fed the diets with EO and herbs of the various plant species against each other and

against the control treatment. No interactions were present between the EO and herb samples from any treatment in comparison with each other.

Table 3.21 *Effect of inclusion of oils or herbs on the coefficients of apparent digestibility of dry and organic matter (ADMD and DOMD) of diets fed to broilers at 28 days, with the content of digestible OM in the diets*

DM and OM digestibility, along with the content of utilised OM from the diets			
Treatment	*ADMD*	*DOMD*	*Digestible OM content in diet (g kg^{-1} DM)*
Control	0.534 *(0.034)*	0.574 *(0.036)*	531 *(33)*
Marjoram herb	0.539 *(0.037)*	0.578 *(0.037)*	536 *(34)*
Oregano herb	0.538 *(0.051)*	0.580 *(0.036)*	540 *(34)*
Rosemary herb	0.515 *(0.026)*	0.562 *(0.052)*	526 *(48)*
Yarrow herb	0.544 *(0.024)*	0.589 *(0.026)*	547 *(24)*
Thyme herb	0.516 *(0.040)*	0.558 *(0.026)*	520 *(24)*
Marjoram oil	0.526 *(0.028)*	0.568 *(0.042)*	527 *(39)*
Oregano oil	0.536 *(0.046)*	0.573 *(0.029)*	534 *(27)*
Rosemary oil	0.539 *(0.032)*	0.576 *(0.045)*	533 *(42)*
Yarrow oil	0.523 *(0.045)*	0.561 *(0.032)*	525 *(30)*
Thyme oil	0.539 *(0.035)*	0.579 *(0.046)*	540 *(43)*
Con*Herb_oil	NS	NS	NS
Con*Plant spp.	NS	NS	NS
Con*herb_oil*type	NS	NS	NS
s.e.d.	0.029	0.029	27.09

Significant differences are indicated in each column by the presence of superscripts. NS (P>0.05)
Data are the mean (SEM) of 5 replicates of each treatment.

The coefficient of AMN and also the metabolisable N content in the diet after the removal of 1 replicate (pen 46 from the yarrow EO treatment) is presented (**Table 3.22**). There was a tendency (P=0.07) for the birds fed diets with rosemary herb to utilise nitrogen less effectively from the diet than the remaining treatments. The data from pen 46 was observed to have a large standard error of the residuals in Genstat (3.65), which made it an abnormal value. The bird from the pen in question was measured with a low concentration of titanium dioxide, from which a negative value was calculated for both the coefficient of AMN and for the metabolisable N content in the diet. As these were growing birds, it is impossible that they should be catabolising N from the body, so this pen was removed.

3.3.8 Effect of herb and oil supplementation in diets on the endogenous cell loss in broilers as measured by the concentration of sialic acid in the excreta

The inclusion of herbs and EO in diets fed to broilers decreased the concentration of sialic acid in the excreta by 6.6% and 3.5% respectively when compared to the control treatment, but this difference was not significant (**Table 3.23**).

Table 3.22 *Effect of dietary oil or herb inclusion on the coefficients of apparent metabolisable nitrogen (AMN) for diets fed to broilers at 28 days, with the content of metabolisable N in the diets*

N metabolisability and the contents of utilised N from the diets		
Supplement	*AMN*	*Metabolisable N content of the diet (g kg^{-1} DM)*
Control	0.405 *(0.063)*	13.61 *(2.12)*
Marjoram herb	0.399 *(0.052)*	13.64 *(1.77)*
Oregano herb	0.371 *(0.091)*	12.45 *(3.07)*
Rosemary herb	0.307 *(0.043)*	9.72 *(1.37)*
Yarrow herb	0.421 *(0.054)*	14.29 *(1.82)*
Thyme herb	0.331 *(0.060)*	10.89 *(1.99)*
Marjoram oil	0.382 *(0.071)*	13.25 *(2.45)*
Oregano oil	0.359 *(0.043)*	11.96 *(1.42)*
Rosemary oil	0.377 *(0.074)*	12.89 *(2.54)*
Yarrow oil	0.365 *(0.064)*	11.49 *(2.01)*
Thyme oil	0.386 *(0.071)*	13.12 *(2.41)*
Con* herb_oil	NS	NS
Con*plant spp.	NS	NS
Con*herb_oil*plant spp.	NS	P=0.07
s.e.d.1	0.045	1.556
s.e.d.2	0.048	1.651

Significant differences are indicated in each column by the presence of superscripts. NS (P>0.05)
Data are the mean (SEM) for 5 replicates of each treatment.
For most of the treatment comparisons, s.e.d. 1 is used. However, when comparing yarrow oil to the rest of the treatments, s.e.d. 2 should be used.

Table 3.23 *Effect of dietary herb or essential oil inclusion on the concentration of sialic acid in broiler excreta*

Sialic acid as a proportion of total feed intake (mg g^{-1} intake)	
Control	88.4 *(7.70)*
Plant herbs	82.5 *(2.74)*
Essential oils	85.3 *(2.76)*
s.e.d.	3.39
	NS

NS (P>0.05)
Data are means (SEM), pooled over the herbs and oils from each of the plant species

However, when looking at the different plant species, there was a tendency (P=0.087) for sialic acid concentration to be reduced in the excreta when oregano was fed in the diet (**Table 3.24**). The birds fed diets with oregano excreted 13.7% less sialic acid, when compared to the birds fed the control treatment.

The birds fed with thyme EO in the diet excreted more sialic acid, compared to those fed with yarrow, marjoram and oregano herbs as well as with oregano and rosemary EOs (P=0.019; **Table 3.25**). When compared to birds fed the control diet, sialic acid concentration was increased by 10.9% in the excreta of birds fed diets with thyme EO, but was not significant.

The lowest concentrations of sialic acid were excreted by birds fed diets with oregano herb and rosemary EO, when compared to those in birds fed diets with marjoram EO, rosemary herb and also the control diets (P=0.019).

Table 3.24 *Effect of the inclusion of various plant species in diets on the sialic acid concentration in broiler excreta*

	Sialic acid as a proportion of total feed intake	
	Concentration(mg SA g^{-1} intake)	*% change relative to control treatment*
Control	88.4 *(7.70)*	0
Marjoram	86.6 *(2.90)*	-2.0
Oregano	76.3 *(4.65)*	-13.7
Rosemary	82.1 *(6.21)*	-7.1
Yarrow	82.9 *(2.16)*	-6.2
Thyme	91.4 *(3.82)*	+3.3
s.e.d.	5.36	
	P=0.087	

Significant differences are shown in columns by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM), pooled for each plant species over the various oil and herb treatments

Table 3.25 *Effect of supplementation with plant herbs and their essential oils on the concentration of sialic acid in broiler excreta*

	Sialic acid concentration expressed as a proportion of feed intake	
	Concentration (mg SA g^{-1} intake)	*% change in relation to control treatment*
Control	88.4^{a} *(7.70)*	0
Marjoram herb	80.2^{bc} *(3.28)*	-9.28
Oregano herb	72.9^{c} *(7.34)*	-17.53
Rosemary herb	92.4^{a} *(7.47)*	+4.52
Yarrow herb	82.2^{bc} *(3.66)*	-7.01
Thyme herb	84.9^{abc} *(6.45)*	-3.96
Marjoram oil	93.1^{a} *(2.50)*	+5.32
Oregano oil	79.7^{bc} *(6.13)*	-9.84
Rosemary oil	71.9^{c} *(8.07)*	-18.67
Yarrow oil	83.6^{abc} *(2.72)*	-5.43
Thyme oil	98.0^{a} *(1.69)*	+10.86
s.e.d.	5.36	
	P=0.019	

Significant differences are expressed by the presence of non-identical superscripts within a column.
Data was compared at P<0.05, but the largest treatment differences were significant to the level shown.
Data are means (SEM) for 5 replicates of each treatment.

3.3.9 Effect of herb and essential oil supplements on the digestibility of amino acids

There were no differences due to treatment on the apparent digestibility coefficients of any AA in broilers at 25 days of age (**Table 3.26**).

Table 3.26 *Effect of inclusion of herbs and their essential oils in diets on the apparent digestibility coefficients of amino acids in broilers at 25 days*

	Apparent coefficients of digestibility of amino acids in broilers												
	Cont	Marj herb	Oreg herb	Rose herb	Yarr herb	Thyme herb	Marj oil	Oreg oil	Rose oil	Yarr oil	Thyme oil	*s.e.d.*	
Ala	0.628	0.615	0.646	0.679	0.645	0.605	0.629	0.635	0.659	0.648	0.635	0.027	NS
	(0.045)	*(0.023)*	*(0.040)*	*(0.033)*	*(0.027)*	*(0.034)*	*(0.042)*	*(0.028)*	*(0.036)*	*(0.043)*	*(0.059)*		
Asp	0.720	0.716	0.723	0.680	0.728	0.721	0.714	0.734	0.744	0.720	0.714	0.025	NS
	(0.036)	*(0.018)*	*(0.042)*	*(0.021)*	*(0.019)*	*(0.024)*	*(0.035)*	*(0.018)*	*(0.031)*	*(0.036)*	*(0.044)*		
Glu	0.835	0.844	0.846	0.830	0.848	0.846	0.839	0.839	0.848	0.840	0.838	0.015	NS
	(0.026)	*(0.010)*	*(0.022)*	*(0.011)*	*(0.013)*	*(0.015)*	*(0.021)*	*(0.015)*	*(0.018)*	*(0.025)*	*(0.029)*		
Ser	0.730	0.718	0.732	0.668	0.711	0.717	0.711	0.727	0.733	0.706	0.718	0.024	NS
	(0.036)	*(0.022)*	*(0.039)*	*(0.021)*	*(0.023)*	*(0.028)*	*(0.033)*	*(0.021)*	*(0.032)*	*(0.040)*	*(0.047)*		
Tyr	0.743	0.769	0.777	0.700	0.752	0.741	0.758	0.747	0.748	0.744	0.744	0.024	NS
	(0.039)	*(0.019)*	*(0.037)*	*(0.023)*	*(0.019)*	*(0.027)*	*(0.034)*	*(0.023)*	*(0.035)*	*(0.033)*	*(0.045)*		
Σ Disp	**0.772**	**0.775**	**0.782**	**0.746**	**0.780**	**0.774**	**0.771**	**0.777**	**0.786**	**0.773**	**0.772**	**0.020**	**NS**
	(0.032)	***(0.015)***	***(0.031)***	***(0.017)***	***(0.017)***	***(0.021)***	***(0.028)***	***(0.018)***	***(0.025)***	***(0.031)***	***(0.038)***		
Arg	0.798	0.793	0.813	0.772	0.804	0.786	0.810	0.801	0.816	0.782	0.807	0.020	NS
	(0.032)	*(0.011)*	*(0.023)*	*(0.012)*	*(0.018)*	*(0.027)*	*(0.025)*	*(0.025)*	*(0.022)*	*(0.035)*	*(0.030)*		
His	0.752	0.807	0.769	0.742	0.773	0.794	0.791	0.804	0.801	0.796	0.785	0.029	NS
	(0.046)	*(0.011)*	*(0.043)*	*(0.019)*	*(0.027)*	*(0.019)*	*(0.026)*	*(0.013)*	*(0.034)*	*(0.039)*	*(0.050)*		
Iso	0.730	0.756	0.764	0.711	0.760	0.729	0.748	0.760	0.736	0.752	0.738	0.027	NS
	(0.040)	*(0.014)*	*(0.042)*	*(0.021)*	*(0.017)*	*(0.020)*	*(0.030)*	*(0.018)*	*(0.035)*	*(0.033)*	*(0.047)*		
Leu	0.753	0.758	0.758	0.716	0.762	0.740	0.745	0.754	0.764	0.756	0.751	0.022	NS
	(0.034)	*(0.016)*	*(0.039)*	*(0.018)*	*(0.017)*	*(0.022)*	*(0.030)*	*(0.018)*	*(0.029)*	*(0.032)*	*(0.043)*		
Lys	0.784	0.796	0.774	0.746	0.788	0.779	0.785	0.782	0.794	0.776	0.778	0.021	NS
	(0.033)	*(0.018)*	*(0.039)*	*(0.020)*	*(0.019)*	*(0.022)*	*(0.029)*	*(0.020)*	*(0.027)*	*(0.034)*	*(0.043)*		
Phe	0.776	0.784	0.793	0.741	0.784	0.769	0.776	0.787	0.782	0.776	0.767	0.020	NS
	(0.031)	*(0.015)*	*(0.035)*	*(0.017)*	*(0.015)*	*(0.021)*	*(0.028)*	*(0.016)*	*(0.028)*	*(0.031)*	*(0.040)*		
Thr	0.672	0.674	0.636	0.565	0.634	0.620	0.632	0.614	0.642	0.634	0.627	0.032	NS
	(0.041)	*(0.025)*	*(0.049)*	*(0.033)*	*(0.028)*	*(0.032)*	*(0.045)*	*(0.024)*	*(0.049)*	*(0.044)*	*(0.064)*		
Val	0.611	0.674	0.666	0.576	0.658	0.629	0.659	0.646	0.634	0.659	0.637	0.039	NS
	(0.048)	*(0.017)*	*(0.052)*	*(0.025)*	*(0.025)*	*(0.033)*	*(0.047)*	*(0.025)*	*(0.043)*	*(0.052)*	*(0.062)*		
Σ Indisp	**0.741**	**0.757**	**0.753**	**0.703**	**0.752**	**0.735**	**0.748**	**0.749**	**0.753**	**0.745**	**0.742**	**0.023**	**NS**
	(0.037)	***(0.015)***	***(0.038)***	***(0.018)***	***(0.019)***	***(0.024)***	***(0.032)***	***(0.019)***	***(0.031)***	***(0.036)***	***(0.045)***		
Σ Tot AA	**0.758**	**0.766**	**0.769**	**0.726**	**0.767**	**0.756**	**0.760**	**0.764**	**0.771**	**0.760**	**0.758**	**0.021**	**NS**
	(0.034)	***(0.015)***	***(0.034)***	***(0.017)***	***(0.018)***	***(0.022)***	***(0.030)***	***(0.018)***	***(0.028)***	***(0.034)***	***(0.041)***		

Data are means (SEM) of 5 treatment replicates. Significant differences are expressed in rows for each amino acid and identified by the presence of superscripts.

3.3.10 Effect of plant herbs and essential oils on ileal morphology in broilers at 28 days

Unfortunately, a preliminary examination of the prepared slides revealed disruptions in the villus architecture, which prevented the measurement of a large number of the sections. In these sections, the top or a substantial amount of the villus was severed, making it impossible to measure villus height and other intestinal measurements with respect to treatment. The ileal tissues were re-sectioned at SAC Veterinary diagnostic labs in Edinburgh, but unfortunately no better quality sections could be obtained. Thus, at this stage it is impossible to report any effects on the histology of the ileum in these birds.

3.3.11 Principal Components Analysis

Principal component analysis (PCA) is a multivariate statistical method for the analysis of many highly correlated variables. Whilst there may be considerable variation between experimental units/observations for each variable, a strong correlation between several variables means that it may be possible to describe the variation between samples in a small number, either 2 or 3, linear combinations of the response variables. The variation between the samples can then be displayed by plotting the 2 principal components against each other. Univariate ANOVA can then be carried out on the principal components in the same way as for any other variable. Essentially, PCA is a data reduction tool, which aims to provide a more concise representation of the salient features of the data.

Each principal component is a linear combination of the response variables. The weightings given to each of the variables are known as "loadings". As the intention is to use as few principal components as possible to reproduce the variation in the original variables, the loadings for the first component are estimated in such a way that the variation in the first component is maximised. Subsequent components maximise the remaining variation, which has not been represented by the earlier components.

For each component in turn, an examination of the loadings gives some indication of which response variables are dominating the respective component's scores and are therefore important in discriminating between the experimental units. Either very high or very low loading values when compared to those of the other variables potentially indicates a difference for that variable. Where ANOVA of the principal component shows a significant treatment

effect, a corresponding ANOVA of the individual dominating variables is likely to give supporting results.

3.3.12 Dietary nutrient digestibility

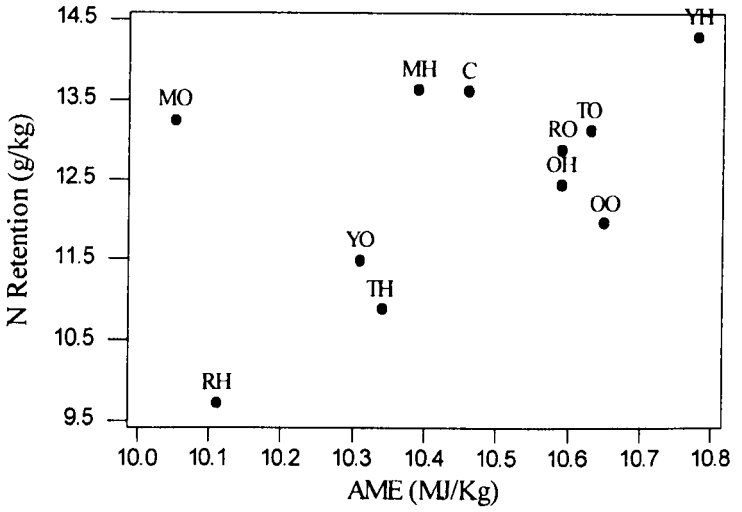
When the digestibility variables were grouped together, 98.3% of the variation in the dataset could be accounted for in the calculation of the principal components PC1 and PC2 (**Table 3.27**). These components were then inserted into a multidimensional graphical matrix to explain the dataset variation. Analysis of variance revealed no differences due to treatment on the PC1 component ($P>0.05$), thus confirming that the bulk of the information was not significantly different (data not shown). However, the remaining 3% of the dataset variation was made up of variations between the type of plant supplements in the relationship between the dietary utilisation of energy and protein, in the calculation of the component PC2. The calculation of the component PC2 refers to maximisation of the amount of variation contained in the treatments, and a plot of the parameter of nitrogen retention *vs* AME is shown (**Figure 3.1**). This graph suggests that the best treatment overall for energy and protein utilisation was the yarrow herb supplement, followed by thyme EO and rosemary EO. The birds fed the control diets and those with marjoram supplements were biased towards N retention rather than AME, based on the information provided by the contrast in the PC2 component.

Table 3.27 *Loadings calculated for insertion into the correlation matrix after grouping the variables associated with dietary nutrient digestibility in the calculation of principal components for broilers fed diets with herbs and essential oils over 21 days*

Calculated loadings for PCA grouped over the nutrient digestibility variables		
Variable	*PC1*	*PC2*
AME (MJ kg^{-1} DM)	-0.339	-0.187
AMEn (MJ kg^{-1} DM)	-0.337	-0.291
AME:GE	-0.340	-0.151
AMEn:GE	-0.324	-0.504
Apparent Metabolisability of N	-0.327	0.528
Metabolisable N content of diet (g kg^{-1})	-0.324	0.562
App DM digestibility	-0.338	0.090
Coefficient of OM digestibility (DOMD)	-0.338	0.013
Digestibility OM in Diet (g kg^{-1})	-0.333	-0.041
% of variation explained	**95.3**	**3.1**
Cumulative variation explained	**95.3%**	**98.4%**

PC1 ANOVA ($p>0.05$); PC2 ANOVA (Variation in plants, $p<0.05$).

Figure 3.1 *Plot of nitrogen retention against apparent metabolizable energy (AME) to show the variation in energy and protein utilisation with terpene supplementation*



3.3.13 Amino acid digestibility

Over 95% of the total variation in the digestibility coefficients of the AA could be explained by calculation of the first 2 principal components, after the high correlation of variables was confirmed. An example of the regression analysis used to correlate several variables can be found in **Appendix 8**. The loadings calculated for each AA are shown (**Table 3.28**). Analysis of variance on each principal component revealed no differences due to treatment ($P>0.05$; data not shown), thus confirming the results of the individual analysis of variance testing on the coefficients of digestibility of AA.

3.3.14 Bacteriology and performance data

The bacteriological counts were also regressed to show the extent of correlation, but the R^2 values were not highly correlated enough for the calculation of principal components. It was not possible to carry out this technique for the performance data, due to the complication of repeated measurements over time on the same variables.

Table 3.28 *Loadings calculated for insertion into the covariance matrix after grouping the variables associated with amino acid digestibility in the calculation of principal components in broilers fed diets supplemented with herbs and essential oils over 21 days*

Calculated loadings for PCA grouped over the AA variables		
Variable	*PC1*	*PC2*
Histidine	-0.248	-0.376
Threonine	-0.327	0.362
Arginine	-0.186	0.207
Valine	-0.327	-0.639
Phenylalanine	-0.213	-0.006
Isoleucine	-0.243	-0.264
Leucine	-0.229	0.031
Lysine	-0.229	0.097
Tyrosine	-0.254	-0.040
Alanine	-0.305	0.276
Glutamic acid	-0.258	0.291
Serine	-0.152	-0.005
Aspartic acid	-0.245	0.125
Total dispensible AA's	-0.217	0.106
Total indispensible AA's	-0.262	-0.084
Total AA's	-0.241	0.005
% of variation explained	**94.1**	**2.0**
Cumulative variation explained	**94.1%**	**96.1%**

ANOVA on PC1 & PC2 ($p>0.05$; not shown)

3.4 Discussion

This discussion is composed of several parts, dealing firstly with the chemical composition of each plant supplement in turn and its current use in industry trials. This is followed by a general description to consider a number of factors involved in this work in relation to the taste perceptions of the terpenes by the birds and their interaction with other substrates, their effects on nutrient digestibility, intestinal bacteriology and ileal morphology measurements. The effects of the housing environment and of the form of terpenes presented in the diet are also included. These findings are placed into context with those from the industry trials, and with previous work detailing the effect of antibiotics on birds. The discussion section concludes with a paragraph to summarise the main findings.

3.4.1 Oregano

Oregano EO is a potent antimicrobial (Sivropoulou *et al.*, 1996) and antioxidant (Botsoglou *et al.*, 2002; Papageorgiou *et al.*, 2003), due to its content of phenolic terpenes, but had negative effects in this experiment and was the poorest treatment. Although the EO exhibited some antimicrobial activity *in vitro*, its application in the diets was associated with restricted feed intakes and weight gain in the birds. There may have been an astringent taste with this

supplement in relation to either its tannin or terpene content, which later reduced feed intake and consequently average daily gain. The oregano herb had the highest concentration of condensed tannins (CT) of the plant samples analysed at approximately 16g kg^{-1}. Chickens have been shown to limit their feed intake in response to the presence of unpleasant flavours in the diets (Balog & Millar, 1989). Despite the inclusion of the herb at only 10g kg^{-1} in the diet, the birds still consumed a calculated quantity of 0.16g CT kg^{-1} diet from the oregano, resulting in a decreased average daily gain over the experimental period of between 10.4%-13.2%. This level of reduction is consistent with that observed after feeding tannic acid and sorghum together in chick diets at this concentration (Dale *et al*, 1980). It is well known that CT bind to other dietary components, but as neither AME nor the ratio of ME:GE was reduced in the birds with oregano herb or EO inclusion in the diets, it is considered that no problems existed with the binding of CT's to starch. However, the reduction in performance with both oregano treatments could imply that tannins or terpenes may be binding to some dietary components and rendering them unavailable. It is possible that other unidentified secondary plant compounds may be present in these plants, such as hydrolysable tannins, which were not measured, but the main differences are considered in terms of the terpene composition.

The chemotype of oregano EO is genetically determined and is apparent through differences in the concentrations of the main phenolic components. Environmental conditions can influence the chemotype, leading to the dominance of a certain terpene in the EO, either thymol, carvacrol or a relatively equal proportion of both these terpenes. Carvacrol can be present in oregano EO at concentrations between 0.12-56.6%, thymol between 7.91-52.62%, and the precursors of these two components, *p*-cymene and γ-terpinene, can make up between 72-92.8% of its total composition (Sivropoulou *et al.*, 1996; Russo *et al.*, 1998). Thymol and carvacrol exhibit up to 96% of the total antimicrobial activity of oregano EO and have been suggested to have additive effects (Sivropoulou *et al.*, 1996; Adam *et al.*, 1998; Daferera *et al*, 2000; Lambert *et al.*, 2001). The activity of the phenolic precursors *p*-cymene and γ-terpinene are questionable and are not established conclusively (Sivropoulou *et al.*, 1996; Adam *et al.*, 1998). However, they are generally considered to be inactive, perhaps due to the absence of a phenolic hydroxyl group on the aromatic molecule (Dorman & Deans, 2000). Thymol has been reported to have a higher antimicrobial activity compared to carvacrol, even in assessing the activity against several bacterial strains within a species (Sivropoulou *et al.*, 1996; Russo *et al.*, 1998; Daferera *et al.*, 2003; Olasupo *et al.*, 2003). Some studies report the opposite

effect, however, with carvacrol having the higher antimicrobial activity when compared to thymol (Nostro *et al.*, 2003), so the extent of antimicrobial activity with each component is unclear. It has been suggested that these main phenolic components in oregano act by penetrating the bacterial cell membrane and increasing its permeability, resulting in a dissipation of the pH gradient and subsequent leakage of ions such as potassium and phosphate out of the cell (Lambert *et al.*, 2001). Thus, it is probable that these phenolic components are responsible for the bulk of any bioactive or antimicrobial activity observed in the supplement.

A typical Greek oregano EO contains high concentrations of phenols, with 45.2% thymol, 33.1% carvacrol, 5.54% γ-terpinene and 7.35% *p*-cymene as the major components (Adam *et al.*, 1998). The oregano EO used in our study had an atypical composition, containing no thymol or carvacrol, with contents of *p*-cymene and γ-terpinene at only 1.3% and 17-19% respectively. Additionally, the EO used in our experiment was tested twice by GC analysis, and experienced slight changes in its composition during storage. Oregano EOs are generally of a very stable nature, so this provides further evidence that the EO used in our study was of a dubious chemical quality for antibacterial or medicinal purposes and may have been better suited for use in fragrances. The main constituent in our EO was 4-terpineol at between 35-40% of total composition, with 4.9-5.4% α–terpineol. These terpenes were present only in trace amounts in the range of 24 EOs measured by Russo *et al.*, (1998). These authors measured one anomalous oregano EO sample with a similar composition to that in our EO, which was collected after the flowering period of growth, and Russo *et al.* (1998) suggested that this EO may have been derived from senescent tissues after phenolic decomposition. Herbal material is extremely variable in quality. The composition of an EO sample can reflect a specific botanical source, a different geographical origin, different environmental influences and a given time of collection. It is therefore possible that the *in vitro* antimicrobial activity observed in the oregano EO used in the present study was due to synergism in some other chemical components, such as 1,8-cineole and α–terpineol, as reported by Sivropoulou *et al.*, (1996). The antimicrobial activity may well have been much greater when using oregano EO chosen for a higher content of either thymol or carvacrol. Complete bactericidal activity has been observed for oregano EO *in vitro* at dilutions as low as 1 in 10,000, with high activity at

dilutions of 1 in 50,000 over an 8 hour period (Sivropoulou *et al.*, 1996), thus oregano is normally a strong antimicrobial.

One of the main commercially available products, namely Orego-Stim (Ecopharm Hellas, SA, Kilkis, Greece), marketed by Meridien Animal Health, Luton, UK, has been associated with positive responses in FCR and weight gain in pigs (Gill, 1999), at smaller doses than in this study. Orego-Stim contains 5% EO of *O. vulgare* ssp. *hirtum* in the form of a powder, along with 95% natural feed grade inert carrier. Although work in pigs resulted in improved productivity, work on a similar theme carried out in chickens reduced the incidence of coccidiosis without affecting the productive responses of the birds (Gill, 1999). The level of EO inclusion in our experiment was around ten times that used in studies with pigs using Orego-stim. No effects were observed in supplementing Orego-Stim from the day of hatch on broiler performance in floor pens (Botsoglou *et al.*, 2002), or alternatively on the performance of turkeys measured at 12 or 16 weeks of age (Papageorgiou *et al.*, 2003). Both Botsoglou *et al.* (2002) and Papageorgiou *et al.* (2003) used lower inclusion levels than in this study and also found the oregano to have an antioxidant effect. Other studies in broiler chicks also reported no effects on performance with the dietary inclusion of plant extracts of oregano (Hertrampf, 2001; Lewis *et al.*, 2003; Demir *et al.*, 2003). These results were surprising, given the highly antimicrobial nature of the EO, but there is nonetheless an indication that oregano supplements may not exert a growth-promoting effect in broilers, and the effect may even be negative if the quality of the EO is less than optimal. However, oregano may be associated with a health-promoting effect, as it has also been observed to stop diarrhoea in broilers associated with coccidiosis and increase subsequent growth and FCR (Hertrampf, 2001), although both Hertrampf (2001) and Gill (1999) contain information from non-refereed sources. Supplementation of Orego-Stim in organic broiler flocks did increase feed intake and BM, without affecting the FCR (Waldenstedt, 2004). However, this study was carried out in conjunction with a vaccination programme against coccidiosis, where the vaccination programme produced the bulk of the effects (Waldenstedt, 2004). It may be the case that the increased rates of intestinal transit in chickens do not allow for the same response as that observed in pigs, but further studies would be required to test oregano more fully.

3.4.2 Thyme

In this experiment, thyme EO was observed to have the greatest effect in terms of its antimicrobial activity *in vitro,* and also for its positive influences on BM and average weight gain in the birds, without an associated rise in feed consumption. Blended commercial supplements of EO, known as APEX Poultry, have also resulted in increased weight gains in industry trials (Tucker, 2002), but the chemical composition of these EOs were not similar to those used in this experiment. It would therefore appear that several types of EO can result in positive benefits on growth in poultry. The causes for the increased weight gains in chicks associated with thyme EO supplementation are unclear, and will be discussed further later on in this section. Thyme EO is well known for its potent antimicrobial activity *in vitro* (Deans & Ritchie, 1987; Piccaglia *et al.*, 1993; Dorman & Deans, 2000), even at dilutions of 1 in 10 in ethanol (Deans & Ritchie, 1987), but the antimicrobial effect was not shown *in vivo* in this experiment. Although the composition of *Thymus* spp. is complex and very variable, most of its antimicrobial activity has also been attributed to the thymol and carvacrol monoterpene hydrocarbon fractions. The activity of both components may be additive, as previously mentioned for oregano, but in this EO the concentrations of both these phenolics were intermediate. Different terpene components in *Thymus* spp. contribute to the antimicrobial activity in each case, such as linalol, 1,8-cineole and a mixture of both, suggesting the presence of a synergistic effect between a number of EO components (Daferera *et al.*, 2000 & 2003; Faleiro *et al.*, 2003). It is unclear which terpenes may be responsible for such a synergistic effect. There is a lack of information within the literature on either industry or research experiments using thyme as a bioactive supplement, as most studies carried out to date in poultry report the effects of oregano in diets. Additionally, the composition of the thyme EO used in the present study was not consistent with those normally selected in the literature for their good antimicrobial quality. The thyme EO used here contained α-terpineol at 29.69% as its most abundant major component, followed by 10.21% thymol, 7.32 % carvacrol, then by 9.93% of an unidentified terpene and 8.35% β-pinene, 5.18% γ-terpinene, 4.30% α-pinene and 3.98% linalol. Nonetheless, the EO exerted considerable antimicrobial activity *in vitro*, although it may be possible that a higher proportion of either thymol or carvacrol may have increased this still further or changed the properties of this EO. Further studies should look at thyme more fully, but it may be the case that the mixture of both terpenes increased the palatability of this thyme in the birds. Thymol is an astringent compound, whereas carvacrol has been described as having a sweet taste (Svoboda, 2002,

Pers. comm). However, poultry have fewer taste buds than pigs and are thus affected to a lesser extent than pigs by taste.

The composition of thyme EO has also been reported to be greatly dependent on the method of EO extraction; for instance, the terpene thymol was obtained by simultaneous distillation-solvent extraction at 2-48%, compared to 2.5-4.8% by headspace analysis from the same plant (Venskutonis, 1997). The EO composition will depend on the method of its extraction and on the drying conditions employed in the plant. Venskutonis (1997) suggested that the drying temperature may change the biological structure of the glandular trichomes in the plant, thus modifying or reducing some of the terpene compounds, after reporting a 43% loss in thymol when the plant material was oven-dried at 60°C. In the present study, all the plant herbs were obtained commercially, and there was no information available on the conditions under which they were dried.

3.4.3 Yarrow

Medicinal compounds for broilers should be chosen to be easily incorporated into the diet and have sufficient strength to promote a bioactive effect in the birds. The EO had a light consistency and watery nature, which caused concern and may be responsible for its lack of activity *in vitro*. The plant herb may be a much more suitable form for feeding this compound, as the bioactive sesquiterpene (15C) chamazulene component of the plant is not present in the EO. From the PCA on nutrient digestibility and the analysis of variance tests, the yarrow herb resulted in the highest retention of dietary N and the greatest values for AME in the birds. In this study, the yarrow herb outperformed its EO in all the parameters studied, and this may indicate the presence of another unidentified compound in the herb material. These components have not been identified, but may be related to the inactive precursor of chamazulene, matricin, which is only present in the herb material. The yarrow included in these diets was especially imported from Slovakia for its high chamazulene content in the EO, but the plant had only been introduced into the field one year previously. It is known that the yarrow plant may take 3-4 years in field conditions to establish a stable chemical nature (Svoboda, 2002, pers. comm). This compound has not been widely used in broiler diets, and so literature on its performance is very limited. Positive benefits have been reported for FCE in broilers with dietary inclusion of yarrow from 17-27 days of age, using a lower inclusion level at 1.8 g kg^{-1} (Lewis *et al.*, 2003). However, Lewis *et al.* (2003) provide no information

on the chemical composition of the EO apart from the presence of the bioactive component 1,8-cineole. The yarrow used in our experiment contained no identifiable 1,8-cineole, but a substantial proportion of the monoterpenes β-pinene, α-terpineol and linalol and some chamazulene sesquiterpene. This plant is also known for its variable chemical composition. The yarrow used in this experiment was of a similar terpene composition when compared to the EO analysed by Rohloff *et al.* (2000), but had a much higher composition of linalol and α-terpineol at 10-17% and 6.29%. These two compounds have antimicrobial activity (Kim *et al*, 1995; Faleiro *et al.*, 2003), proving that the bioactive nature of this EO is complex. This yarrow was harvested while flowering, when the EO content is at its highest concentration in the plant, so the lack of *in vitro* antimicrobial activity in the feeding trial was disappointing. However, Candan *et al.* (2003) have established the antimicrobial nature of yarrow EO *in vitro*.

3.4.4 Marjoram

The EO of marjoram is known to contain a large number of compounds. Daferera *et al.* (2000) stated that the EO of marjoram has two clearly defined types in the literature, the phenolic thymol/carvacrol chemotype and the *cis*-sabinene hydrate/terpinen-4-ol chemotypes. The variability of terpene composition in marjoram has been reported (Lawrence, 1976-1994; Appendix 1). The EO of marjoram used in this experiment clearly falls into the phenolic chemotype, being mainly composed of carvacrol at 54-56%. The additional presence of the inactive terpene precursor compounds for carvacrol suggests that the EO used was of a reasonable quality and chemical composition. Again, this plant has not previously been supplemented into poultry diets, so it is impossible to compare this data with results from other experiments. The antimicrobial activity of this EO *in vitro* was quite disappointing, although it did numerically reduce the numbers of *C. perfringens*.

3.4.5 Rosemary

The composition of rosemary EO in this experiment appeared to be stable, and agreed well with previous compositional analyses from the literature. Rosemary EO is characterised by the presence of a high component of 1,8-cineole, which may form 78-89% of the EO composition (Daferera *et al.*, 2000). The antimicrobial activity of 1,8-cineole has been demonstrated *in vitro* (Cox *et al.*, 2001). The rosemary EO used in our experiment contained 46% 1,8-cineole as the main component, which was lower than in the literature, but would

appear to suggest that this EO was of a reasonably good antimicrobial quality. Camphor, present in our EO at a concentration of 16-17.7%, is also known for its antimicrobial activity. The concentration of camphor agrees well with that present within the rosemary EO used by Piccaglia *et al.*, (1993). Rosemary EO has been reported in the literature as having antimicrobial activity against both Gram-positive and Gram-negative organisms (Narasimha Rao & Nigam, 1970), but very little antimicrobial activity was observed *in vitro* in this EO, except against *C. perfringens* and *S. enteritidis* from the bacterial species tested.

3.4.6 Taste perception of flavour compounds and their interaction with other substrates

Fruit-eating birds may respond more positively to "sweeter" diets than insect eating or graminivorous birds such as chicken. Chickens possess only 24 taste buds at the base of the tongue, but have a moderately well developed sense of taste (Kare & Rogers, 1976), suggesting that their responses to dietary flavours cannot reliably be predicted. The taste perception of chickens has not been widely reported in the literature, but should be considered when dealing with aroma compounds. Birds may or may not be able to differentiate between flavours in the food. Taste-testing work in broiler chickens has demonstrated that sweet-tasting diets were preferred more readily than diets including saccharin, salt and the astringent quinine (Balog & Millar, 1989). However, Balog & Millar (1989) reported inconclusive results as they failed to check these dietary flavour preferences with physiological measurements of electrical brain impulses, therefore failing to confirm a biochemical perception of these compounds. Increases in the β-wave production within rat and water vole brain tissue have been reported when using stimuli from various known anti-feedant aromatic compounds, including the terpenes camphor and carvacrol (Vanderwolf *et al.*, 2002). A biochemical basis has been demonstrated for the initiation of olfactory activity in the domestic chicken (Koch *et al.*, 1991). Koch *et al.* (1991) removed olfactory tissue containing the enzyme NaK-ATPase, which is involved in sweetness taste perception, demonstrating an alteration in its activity in converting NADH to NAD^+ when exposed to various aromatic aldehydes and ketones. There is therefore a possibility that the basis of the positive response in weight gain with the thyme EO in this experiment was due to its palatable terpene composition. There may also be some importance of the smell of the aromatic compound in relation to its stimulatory effect, but this cannot be quantified here.

Aroma or volatile compounds are known to interact with starch, other carbohydrates and protein molecules, and possibly also lipids, which may affect their digestibility. Higher molecular weight aroma compounds will associate more strongly and with increased stability to carbohydrates, depending on the nature of their functional groups, ie. acid binding<aldehydes<esters≤ketones≤alcohols, whereas aroma compounds that show increasing polarity and volatility are less likely to associate with carbohydrates (Goubet *et al.*, 1998). The presence of hydrophobic non-covalent and hydrogen-bonding interactions have been reported to result in strong associations between low molecular weight hydrocarbons and the hydroxyl groups of corn starch polysaccharides in an aqueous environment (Golovnya *et al.*, 1998). These effects ranged from 80-100% for 8 compounds, and 50-70% association for the remaining 4 of 12 hydrocarbons isolated from *Rosmarinus officinalis* L. (Golovnya *et al.*, 1998). Whether these associations are beneficial or detrimental remain to be determined, but it is clear that free terpenes will associate in the intestinal environment with feed particles or the digesta. This may have some impact on the AME of the dietary ration, or may cause the terpenes to be absorbed and metabolised through the gut walls at the brush border membrane.

Changes in the rheological properties of starch molecules present in low concentrations have been described *in vitro* due to the presence of the flavour compounds (-)fenchone and decanal, leading to increased gelation (Nuessli *et al.*, 1995). The presence of aroma compounds may therefore affect the digestibility of starch and other nutrients, by causing changes in the intestinal viscosity. The degree to which such a situation may be influential would require careful study, as feeding terpenes as supplements in broiler chick diets should not reduce the potential digestibility of the dietary ration. However, the type and nature of the intestinal carbohydrate is important. A lower retention of aroma compounds was observed *in vitro* on lower molecular weight starch hydrolysates and sugars, with an increased rate of diffusion (Goubet *et al.* 1998). In low viscosity solutions, the lower retention of aroma compounds by carbohydrate should enable them to diffuse more fully around the intestine and thus lead them to have greater potential to exert their effects, when compared to complete starches and higher molecular weight compounds. Goubet *et al.* (1998) also observed a greater release of volatile compounds from crystalline rather than amorphous materials. This would suggest that the use of enzymes may facilitate the action of terpenes and other aroma compounds, by reducing the digesta viscosity and enabling a more complete breakdown of dietary substrates through intestinal mixing. When they added the simple sugars glucose, sucrose and maltose to their

template matrix of starch *in vitro*, Golovnya *et al.* (1998) also observed an increased tendency for associations between these compounds and aroma compounds, in agreement with Goubet *et al.*, (1998). A more complete breakdown of starch may therefore imply that terpenes would associate with simple sugar molecules, then be absorbed and metabolised as digestion products with these simple sugars, but this is unclear. However, Williams & Losa (2001) suggest that EOs and their component terpenes themselves also act to reduce intestinal viscosity. Weurding *et al.* (2001) observed starch digestion in diets without enzymes at 88.2 and 89.8% in wheat and barley, with only a further 4.7 and 5.2% digestion for each in the terminal ileum and no bacterial fermentation. In the present study, the low nutrient digestibility coefficients would have resulted in a high proportion of intestinal starch, thus the terpenes may associate with intestinal bacteria rather than feed or digesta components. This could alter or disable bacterial activity, either selectively or non-selectively, lead to the terpenes being excreted with bacteria, or alternatively cause the release of terpenes in the distal gastrointestinal tract close to the caeca as bacteria themselves undergo metabolism reactions. In the present experiment, there were only numerical decreases in bacterial number, thus the action of these terpenes on intestinal bacteria are unclear and will require further work. Alternatively, some bacteria may be themselves involved in the harvesting of energy in the lower gut through volatile fatty acid (VFA) production, and it may not be beneficial to deprive them of potential substrates.

The site of terpene action in the intestine has not been described, and these results have not suggested this action to be antimicrobial. It may be more beneficial for terpenes to bind to dietary components rather than to disable bacteria, then to be released as these components are absorbed, thus decreasing the intestinal pH towards the distal end of the gastrointestinal tract where pathogenic bacteria may exist. Unfortunately, the variability in observation of lower tract microbiological populations of *C. perfringens* in this study, and the lack of a clear antimicrobial effect *in vivo*, did not substantiate any of the mechanisms involved. A reduction in intestinal pH should prevent the growth of pathogenic bacteria in the lower tract by making the growth conditions unfavourable and preventing the regeneration of bacterial spores, thus being indirectly responsible for the bioactive effect of the compounds. EO blends have been reported to reduce the intestinal colonisation of *C. perfringens* in industry trials (Losa & Köhler, 2001; Williams & Losa, 2001; Mitsch *et al.*, 2002; Sims *et al.*, 2004), so further studies are required. Commercial EO blends have also selectively reduced *Escherichia coli*

and increased *Lactobacillus* spp. populations in a positive way, suggesting a different mode of action to that of antibiotics (Tucker, 2002). It is possible that the effects are visible only on a much larger scale of experimentation. Only very limited information is available concerning the composition of the EO blends used in trials.

The polarity of molecules will also be extremely important, as polar terpene compounds may potentially associate with water molecules and thus may be absorbed or removed from the intestine. The effect of water intake could therefore serve to alter the balance of active terpenes within the diet by dilution, if the intestinal matrix acts as a semi-permeable membrane. Non-polar compounds would therefore remain attached to undigested feed particles in the intestinal matrix, which may have a balance on their bioactivity. It has been reported that the presence of 6% of mainly proteinaceous OM, such as milk and bovine serum albumin, considerably compromised the *in vitro* antimicrobial activity of *Ocimum* var *thyrsiflorum* EO, although this activity was unaffected by the presence of saliva (Narasimha Rao & Nigam, 1970). Clearly, further studies are required to assess the effects of digesta and excreta components on the efficacy of the terpenes within the intestine and also the presence of bacteria. This is important in terms of the desired site of action of the terpene constituents, within the proximal or distal regions of the intestine or for a general effect throughout. It has been suggested that the phenolic compounds in oregano are metabolised, but no experimental techniques have been developed to identify or quantify any of these phenols in biological tissues (Botsoglou *et al.*, 2002; Papageorgiou *et al.*, 2003), so their bioavailability cannot currently be demonstrated.

3.4.7 Effects of the terpenes on nutrient digestibility

The analytical results would suggest that the dietary AME values in this experiment were extremely low in comparison with the literature. Wheat has an extremely variable feeding quality, ranging from around 9.5-16.5 MJ kg^{-1} DM. An acceptable value for AME in good quality wheat should be around 14.5-15 MJ kg^{-1} DM (Choct *et al.*, 1995). The calculations of apparent digestibility in this study revealed dietary AME values in the region of 10.5 MJ kg^{-1} DM, although formulated to be 13.43 MJ kg^{-1} DM, so the wheat quality was clearly sub-optimal. Although these diets were designed to be marginal, this should at least partly explain the very low digestibility and slow growth of these birds. In order to incorporate the plant material supplements, the birds were also fed on mash, normally associated with a reduced

AME in comparison with pelleted diets, which are denser in energy with less bulk. In addition, around 7-25% lower AME values (average 10%) were consistently observed with dietary wheat inclusion at 70% and above, suggesting that wheat may contain toxic substances or inhibitors (Payne *et al.*, 1977). Poorer quality wheat is commonly associated with indigestible arabinoxylan anti-nutrient fractions, which are components of non-starch polysaccharides (NSP), and may also contain lectins, which will reduce the utilisation of the wheat in the diet by the bird. In this experiment, wheat was included in high quantities at just under 68%. These diets were fed to the birds with no antimicrobials or anticoccidials, and additionally without any enzymes, so the EO and herb supplements in the diet were the only substances differentially affecting bird performance.

Dietary fibre has a very limited digestibility in the birds, consisting of cellulose, hemicellulose, pectin and lignin (Mc Donald *et al.* 1995). It is considered that the presence of a carbohydrase would be beneficial in the utilisation of NSP contained within the fibre components and specifically arabinoxylans, which are known to be a problem in poorer quality wheat diets for poultry. Enzymes are frequently supplemented in the diet for this reason. In this experiment, enzymes were not included, as it is possible at least some may have an antimicrobial action of their own (Fuglsang *et al.*, 1995). However, the access of the bird to the cereal starch endosperm may then be restricted due to its inability to digest the fibrous components in the cereal cell walls, thus also negatively affecting the digestibility of starches, protein and fat. It has been reported previously that birds fed on a high fibre diet weighed less than birds on low fibre, ate more and had a larger gut size as a portion of body weight, suggesting that more energy was used for nutrient partitioning (Savory & Gentle, 1976a). The size of the gut responds to changes in the dietary fibre content, indicating that the digestive system is "elastic" (Savory & Gentle, 1976b). Additionally, higher levels of dietary fibre are observed to affect the rate of absorption of different intestinal sugars (Savory, 1992). It is possible that terpenes affect nutrient partitioning in some similar manner, as this study shows slight differences in protein utilisation with respect to the various terpenes, even in diets with a constant level of dietary fibre. The proportion of the broiler intestine in relation to BM changes as a direct result of dietary composition and the presence of stressors, which suggests that higher weight gains and feed intakes may be due to a larger intestinal size (Bedford, 1996). This increased size may occur *via* increases in the allometric growth or production of endogenous enzymes in the bird (Bedford, 1996). If terpenes further increase the viscosity in

connection with starch gelation, this may also indirectly account for increased intestinal weight.

The rate of feed passage will influence the efficiency of digestion and absorption of dietary nutrients, and a large presence of undigested food in the small intestine may reduce food intake as a result. Herbs and spices are traditionally thought to enhance the properties of digestion in a food by stimulating the production of endogenous enzymes in the small intestinal mucosa, along with the pancreas and liver. Spices such as ginger, ajowan, cumin, piperine, coriander and capsaicin have been reported to increase the intestinal transit time in experimental rats by 31, 28, 26, 25 and 19%, associated with increased endogenous enzyme, bile acid and pancreatic juice stimulation (Platel & Srinivasan, 1996, 2000 & 2001). However, even if the herbs in the present study acted in this manner, the presence of the large indigestible dietary fibre component would provide only a limited supply of nutrients due to restricted access. The initial viewing of histological sections revealed undigested cereal components with partially unbroken cell walls in these birds, which would seem to confirm this. Platel & Srinivasan (2001) viewed no changes to the excreta components reflecting poorer absorption of digested nutrients, suggesting the faster rate of transit took place after nutrient absorption was complete. Protein within the cereal grain may be bound or linked to the fibre components, thus a reduction in ability to utilise fibre components may also determine protein digestion and availability (Grala *et al.*, 1999). Exogenous xylanase enzymes in wheat diets have improved bird performance by reducing arabinoxylans, improving AME and nutrient digestibility (especially starch), reducing variation between birds and increasing the rate of passage by an associated reduction in the digesta viscosity (Choct *et al.*, 1995; Crouch *et al.*, 1997). Enzymes also reduce the size and water holding capacity of NSP, decreasing the intestinal viscosity (Choct *et al.*, 1995). In future studies, feeding a low ME wheat sample in conjunction with a dietary enzyme should increase nutrient utilisation and improve bird performance, which may be advisable when considering the poor responses in the birds on the present experiment. It is also possible that the endogenous digestive enzymes of the young broiler may be limited or insufficiently produced. Comparative studies have reported that broilers and other higher weight line birds produced similar amounts of digestive enzymes as layers and also low weight line birds, but had to cope with a larger digesta volume as the birds ate to meet their requirements (Nir *et al.*, 1993; Dunnington & Siegel, 1995). The positive benefits of enzyme supplementation are more evident in chicks

than in mature birds, increasing intakes, rates of passage and nutrient digestibility, thus there is an age-dependent effect of enzyme inclusion (Almirall & Esteve-Garcia, 1994). Enzyme supplementation in wheat diets containing high levels of NSP improved the digestibility of both crude protein and energy, and also the performance of chickens to a greater extent than wheat of good feeding quality (Crouch *et al.*, 1997). This was also observed in pigs, but to a lesser extent, by 3-5% when compared to 1-2% for good quality diets (Yin *et al.*, 2000). The ileal digestion varies considerably between different feedstuffs due to changes in the starch digestion in the anterior portion of the digestive tract (Weurding *et al.*, 2001). There is also a link between starch digestibility and AME, when cereals are fed as the main components in diets. Svihus & Hetland (2001) observed a positive correlation between the insoluble dietary fibre content, or a large presence of wheat starch in the intestinal chyme, which then resulted in poor starch availability. Protein digestibility has also been reduced in diets with low AME, but this has not been improved with the presence of an enzyme (Choct *et al.*, 1995), and it may be possible that the level of endogenous enzyme production or protein utilisation may be involved. As shown in the present experiment, different herbs may also affect the digestibility of protein within this experiment. It is possible for diets without exogenous enzymes to be utilised efficiently, but this may be dependent on the use of wheat with a good feeding quality, which was not the case in this study. The digestion of starch was measured in diets without exogenous enzymes for both wheat and barley at 88.2 and 89.8% respectively, with only a further 4.7 and 7.5% digestion for each in the terminal ileum (Weurding *et al.*, 2001). In their birds, Weurding *et al.* (2001) observed no small intestinal bacterial fermentation to be taking place. However, feeding whole wheat diets are suggested to be subjected to an increased grinding action of the gizzard to regulate nutrient release with no ill effects on digestion (Svihus *et al.*, 2002), suggesting that hormonal control is involved and the site of starch digestion may be relatively unimportant. In future studies, any work on phytochemicals will be carried out in the presence of a dietary carbohydrase, as these are commonly found in commercial poultry diets.

In this study, the excreta samples taken allowed the measurement of total tract AA digestibility values, which will be artificially raised or lowered due to the contribution of the relevant gut microflora. The availability of these AA cannot be determined, due to the inability to distinguish between endogenous and dietary N losses, which are nutritionally very expensive to the bird. However, the rosemary herb treatment may have contained some

compound resulting in reduced digestibility coefficients of AA. The depression in dietary protein digestibility and AA absorption from the ileum with increased levels of dietary NSP has been clearly linked to the increased endogenous secretion of ileal N (Yin *et al.*, 2000). The protein retention from the diet in the birds fed with rosemary herb tended to be quite low in the birds of the present study, while the sialic acid production in the excreta with this treatment was numerically higher than that in the control birds. Endogenous secretions in the bird, including saliva, pancreatic secretions, bile, enzymes, sloughed-off epithelial cells, serum albumin and mucin, are likely to be increased where there is an elevated synthesis of protein and may be underestimated in the measurement of apparent AA digestibility (Nyachoti *et al.*, 1997). This will also cause an associated loss of energy. Any undigested components of starch along with NSP will be available for bacterial fermentation in the lower gut (Choct *et al.*, 1996). Thus, diets with high fibre levels, such as those in the present study, will encourage such an energy loss to take place. The tissue deposition of energy and protein may be reduced by as much as 10%, due to the increased gut protein turnover by the microflora and the lower energy deposition efficiency of VFA production in the caeca (Muramutsu *et al.*, 1994). Additionally, metabolism of the prokaryotic intestinal microflora will produce polyamines and other harmful bacterial metabolites, especially in the presence of blockages or slow intestinal movement, degrading dietary AA such as lysine and arginine in the absence of antibiotics by the action of ornithine decarboxylase (Osborne & Seidel, 1989). It is generally considered that the action of intestinal bacteria in the proximal intestine should be discouraged. Therefore, in experiments with a high fibre level, the action of intestinal microflora will reduce the utilisation of dietary nutrients.

Muramutsu *et al.* (1994) observed a high correlation between the fat and protein energy deposition in their study, by varying the dietary ME level. When feeding diets with varying protein but constant sulphur AA and lysine concentrations, Sklan & Plavnik (2002) reported that for a given energy concentration, the feed intake was influenced by the dietary protein content, and suggested that one or several AA concentrations regulated feed intake. Thus, if the terpenes were in some way to influence AA digestibility or availability from the diet, they could potentially alter the energy: protein ratio, and thus the efficiency of dietary utilisation. Both the digestibility coefficients and PCA analyses from this experiment suggested that the terpene supplements in the plants appeared to vary the relationship between AME and protein deposition slightly, but further studies should be carried out. Numerically increased

endogenous losses associated with thyme EO, marjoram EO and rosemary herb may be helpful in enhancing enzyme production with regards to nutrient digestibility, but this will be very expensive to the birds in terms of energy requirements. Larsen *et al.* (1993) observed that the loss of endogenous N and AA at the terminal ileum increased with certain types and levels of dietary fibre, thus producing increased concentrations of sialic acid and resulted in an increased digesta viscosity. It may be possible that endogenous enzyme production is related to the release of sialic acid from gastrointestinal mucin. The removal of sialic acid from duodenal mucin has been shown to restrict its ability to perform viscous fingering, or the ability for HCl to travel over the intestinal surface, by reducing its viscosity (Fujita *et al.*, 2000). This may be important in the development of lesions, as it may damage intestinal integrity (Fujita *et al.*, 2000).

This experiment observed that most terpene-supplemented diets reduced the concentrations of sialic acid in the excreta of the birds, when compared to the non-supplemented controls. High viscosity wheat diets are associated with a decreased rate of digestion, by increasing both the intestinal mass and size as the bird compensates by secreting more endogenous N (Bedford, 1996). Different types of dietary fibre have also been suggested to enhance endogenous N loss, but the mechanism by which they do this is poorly understood (Nyachoti *et al.*, 1997). It is possible that the numerically increased sialic acid production with thyme and marjoram EO and with rosemary herb when compared to the birds on the control treatment diets may be related to the potential for increased endogenous enzyme production, perhaps stimulated by the presence of carvacrol or thymol. Herbs and spices have traditionally been used to stimulate digestion in the literature, and this should also be the case with concentrations used for therapeutic purposes. Certain plant spices stimulate proteolysis, especially in conjunction with the supplementation of organic acids, by lowering the pH to the optimal level for pepsin activity (Kamel, 2001). The inclusion of spices in rat diets at 5 times the average supplementation concentrations in the diet increased the endogenous pancreatic lipase, trypsin, amylase and chymotrypsin secretion; ginger increasing amylase by 184%, with curcumin, ginger, capsaicin and piperine increasing trypsin by 120-165% and chymotrypsin by 24-73% (Platel & Srinivasan, 2000). Platel & Srinivasan (2000) observed some spices to reduce endogenous secretions, along with similar food intakes for all treatments, but failed to see the same effects with a large single oral dose of the spices. An increased amylase activity in the intestinal chyme was observed in birds fed Crina® Poultry at 21 days of age, but this effect

decreased with increasing age (Lee *et al.*, 2003). Clearly, there is a trade-off between the energy expense of the process with any potential benefits received, and the choice of terpene supplement may have an individually positive or negative effect on dietary protein and energy utilisation with respect to its chemical composition. This means that there is considerable scope for the testing of herbal materials, as effective secondary plant supplements may also be able to increase the digestibility coefficients for energy, protein and AA's.

In the present experiment, there was no effect on the digestibility coefficients of the various AA's. However, the diets supplemented with rosemary herb had a tendency to reduce the utilisation of protein in the birds. The effect of EO may be at the level of free amino acids in the intestine, rather than acting against proteins or peptides. A blend of thymol, eugenol, vanillin and limonene fed to ruminants, known as Crina HC, did not break down either proteins or peptides, but had a selective effect against some ammonia-producing bacteria, therefore decreasing the rate of free AA deamination (McIntosh *et al.*, 2003). If this is the case, EO's may enhance or inhibit endogenous enzyme production by influencing the processes of transamination, thus affecting the availability of dietary AA. Some terpenes clearly favour digestibility of certain AA over others. Diets supplemented with plant extracts have been observed to increase the availability of the AA's lysine, asparagine, phenylalanine, histidine, serine and threonine (Kamel, 2001; Jamroz *et al.*, 2003). McIntosh *et al.* (2003) suggest that the EO-induced toxicity against the proteolytic and amylolytic bacterium *Ruminobacter amylophilus* may mean that a dietary EO presence may slow the breakdown of starch or protein, which may therefore restrict the breakdown of fibre in ruminants. In poultry, which have only a limited ability to digest fibre, this may be significant as it may inhibit the formation of uric acid or restrict AA absorption by the birds.

The levels of vitamin E were restricted in this diet formulation. In addition, wheat has been observed to contain less vitamin E than other major feed cereals such as corn (Leeson & Summers, 2001). This reduced content of vitamin E may have been one other reason for the reduced performance of these birds. It must be considered that any benefits of feeding thyme EO, marjoram EO or marjoram herb supplements in animal diets may be related to their activity as antioxidants, due to their substantial contents of thymol and carvacrol (Deighton *et al.*, 1993), rather than or as well as their antimicrobial action. Botsoglou *et al.* (2002) and Papageorgiou *et al.* (2003) reported an antioxidant effect in oregano EO, using ten times less

oil as that in our study. The marjoram used in the present study had a substantial carvacrol component at 56%, and the thyme contained both carvacrol and thymol. Supplementation of terpene antioxidants in EO may be beneficial in diets with reduced vitamin E levels. In the present study, the birds fed these 3 plant supplements were observed to be equivalent or greater in performance than the birds fed on the control treatment diets.

3.4.8 Form of terpene presentation in the diets

The form of supplementation of bioactive compounds in the diets may additionally have some relevance in determining their activity. The productivity responses in this present study illustrated interesting differences between the species of plants supplemented and whether they were included as EO or herbs. The activity of marjoram was consistent when fed as either an EO or as a plant herb, and the composition of both may be similar as a result. Yarrow had a positive effect, and was much better fed as an herb than as the EO. The reduced quality of the EO used in the present study may have resulted in an overly negative perception of the use of yarrow as an EO supplement. Dorman & Deans (2000) have suggested that the active terpenes in the whole plant may be trapped within secretory gland structures, which may favour the antimicrobial activity of EO rather than herbs. In contrast, Shelef *et al.* (1984) reported a greater inhibitory effect of sage herb than the EO from this plant against bacteria when incubated in meat broth. Thus, bioactivity within the plant herb cannot be excluded. It may be the case that milling of the herb fraction may ameliorate these effects, releasing the active terpenes in the gut during digestion and absorption. In this study, the herbs were fed as they were purchased, apart from the yarrow, which was milled on arrival, and in reflection it may be more beneficial to feed these herbs after milling. If these plants were milled before use, it was considered that the volatile components may be at least partially lost through vaporisation, and the leaves used in the present study were well chopped before they were dried. A higher activity of terpinene-4-ol was observed separately *in vitro* than when present as part of the EO fraction (Cox *et al.*, 2001). The use of an EO supplement will depend on a demonstrable uniformity of its antimicrobial characteristics, its price in the dietary ration and its acceptability to the birds as a feed additive. A significant amount of antioxidant activity in an EO has been suggested to depend on synergism between minor glycosidically bound components, such as thymoquinone aglycone at a content of 8 mg kg^{-1} with carvacrol and thymol (Milos *et al.*, 2000). This would further suggest that isolation of the active terpene

constituents is not the right approach in the identification of a suitable dietary supplement, but that the whole EO extract or herb should be used.

More research is therefore required to determine the best form of supplementation of bioactive phytochemicals and their constituents. The dried plant herbs used in this experiment, although perhaps easier to incorporate into diets, were not composed of pure terpenes and will contain other compounds that may affect bioactivity. The drying process may also have affected the concentration of terpenes and phytochemicals within the plant material, so that less bioactivity may prevail. The drying conditions in these plants were unknown, although the quality of the dried herb appeared good visually. The EO's in the present study were relatively easy to incorporate, mixing well with the vegetable oil fraction. However, the EO fraction may well be lost during the pelleting process in commercial poultry feeds. These fractions would probably have to be fed after some form of encapsulation in pelleted feeds, to protect them from being lost to the atmosphere. Venskutonis (1997) reported a 43% loss in thymol when EO-containing plants were oven-dried at 60°C, rather than the normal 40°C. The use of biotechnology and cell culture may help to overcome the widespread variations in the medicinal quality of an EO to make it cheaper for use in animal feeds.

It may be possible that the faster intestinal transit and smaller volumes of digesta increase the variability of chickens in a study situation when compared with pigs, or the response of herbal supplementation is innately more variable than that of conventional antimicrobials. Thyme and oregano EO's are both strongly phenolic compounds which are well known for their antimicrobial activity *in vitro*, although the dose rate and degree of replication in this study may have been insufficient to demonstrate significant effects *in vivo*, and therefore further studies are required. The use of more replicates in future feeding trials may reduce the high variation observed between bacterial counts, and therefore increase the likelihood of observing significant effects due to treatment. Several trends were observed within this experiment that could potentially be significant under a different experimental methodology. However, information within the literature on previous studies in this area was limited and caused complications at the design stage of this work.

3.4.9 Effect of the herb and essential oil supplements on intestinal bacteriology

At around seven days after hatch, bacterial populations in the chick intestinal tract stabilise, with the small intestine predominantly containing LAB, which are normally *Streptococcus* and *Lactobacillus* spp. at 65-80% along with *Escherichia coli* (Salanitro *et al.*, 1978). The caeca of broiler chicks contains mainly anaerobes such as *Eubacterium* and *Bacteroides* spp. (Salanitro *et al.*, 1978). It was our intention in the present study to focus on the main populations of interest, whether harmful or as main components of the intestinal tract. The gut microflora utilise at least 10% or 1 MJ of dietary AME in their metabolism and turnover (Muramutsu *et al.*, 1994), and an increased level of intestinal viscosity will decrease the rate of digestion and permit more dietary nutrients to be utilised by the microflora themselves. Selective inhibition of the growth of the hyper-ammonia-producing bacterial species *Clostridium sticklandii* and *Peptostreptococcus anaerobius* has been demonstrated in ruminants fed with a blend of terpenes, consisting of thymol, eugenol, vanillin and limonene (McIntosh *et al.*, 2003). A selective inhibition of growth has also been demonstrated in broilers when using blends of EO in industry trials for bacterial species such as *E. coli* and *C. perfringens* (Losa & Köhler, 2001; Tucker, 2002). When using an encapsulated product known as XTRACT in broiler diets, which contained the terpenes capsaicin, carvacrol and cinnamaldehyde, a reduction in *E. coli* and *C. perfringens* concentrations in rectal contents were reported, to the same efficiency as avilamycin antibiotic supplement (Jamroz *et al.*, 2003). Thus, it is possible for EO to selectively inhibit bacterial growth. In reflection, the results in the present study were disappointing, and no clear effects were established when using the plant herbs on the intestinal microflora populations. In future studies, it may be beneficial to test the EO against the growth of bacteria *in vitro*. These components are not restricted by structural barriers against exerting an antimicrobial effect, as suggested by Dorman & Deans, (2000). Alternatively, the concentration of active herbal material may have been diluted extensively when feeding the herb plant as a supplement. The high variation present between treatment replicates may have caused problems in the present experiment, and thus an increased replication may be beneficial in future work. When testing a range of bacteria, McIntosh *et al.* (2003) reported that *Peptostreptococcus anaerobius* and *Clostridium sticklandii* were the bacteria most likely to inhibit N retention in ruminants. In the present experiment, the inclusion of thyme, yarrow and rosemary in the diets numerically decreased the populations of *C. perfringens* in the caeca by more than 1 log, so it is possible that the high sample variation masked some treatment effects. However, the attachment of anaerobic

bacteria in the small intestine may act to control the rates of bacterial metabolism or fermentation (Salanitro *et al.*, 1978), and thus may be associated with the development of disease. The inclusion of a blend of EO marketed as CRINA poultry has been shown to reduce the intestinal colonisation of *C. perfringens*, and thus may help to prevent bacterial disease problems such as necrotic enteritis (Mitsch *et al.*, 2002; Sims *et al.*, 2004). The mucin layers at the brush border appear to be associated with bacterial adherence, and both mucin and bacterial composition may be diet dependent (Salanitro *et al.*, 1978). If this is the case, and the bacterial composition is stabilised at day 7 in the birds, then the birds should perhaps be fed with the test diets from the day of hatch to influence the bacterial composition.

When measuring bacterial growth *in vitro*, it was observed that thymol prolonged the 'lag growth phase' of both *E. coli* and *P. aeruginosa*, even when included at a sub-inhibitory concentration, suggesting that phenolic agents may interact with primary non-vital target sites within the bacteria (Walsh *et al.*, 2003). In the 'stationary phase' of growth, bacterial cell walls are more tolerant against disruption (Cox *et al.*, 1998), which is suggested to be due to the changed composition of fatty acids and in the production of lipopolysaccharide by the cell. Olasupo *et al.* (2003) observed that increasing concentrations of the antimicrobial compound diacetyl caused a prolonged 'lag phase' of growth in Gram negative bacteria. Increasing concentrations of diacetyl led to both a reduced growth rate and final cell density of the Gram-negative bacteria (Olasupo *et al.*, 2003). This may be important when adding EO supplements at low concentrations into diets, as it indicates that even below concentrations where a natural compound is exerting an antimicrobial effect, there may be some beneficial suppression of bacterial growth. However, this may also predispose these compounds to develop antimicrobial resistance through selective pressures, so they should be used wisely and in rotation.

In this study, birds fed the diets containing all herbal supplements displayed increased caecal coliform populations compared with controls, suggesting that the dietary presence of these herbs may select more against Gram positive rather than Gram negative bacteria. Bacterial isolates counted in various studies suggest that the majority of organisms contained in the tract are Gram positive at 65-80%, and most are facultatively anaerobic, but strict anaerobes are present in the caeca (Salanitro *et al.*, 1978). Conventional antimicrobials have been known to preferentially target Gram-positive bacteria in broiler production, primarily *C. perfringens* in

the control of necrotic enteritis (Engberg & Petersen, 2001). If this is the case, conditions suitable for the presence of Campylobacter or Salmonella may exist in birds fed diets supplemented with herbs. From the results obtained for the oregano herb supplement, it is also impossible to quantify the effect of the presence of CT in the diets on the bacterial populations in this experiment. Synergistic effects of the various components of EO have been shown for basil *in vitro* (Lachowicz *et al.*, 1998) and for several constituents of tea tree EO *in vitro* (Cox *et al.*, 2001), which may even be of greater bioactivity than the complete EO. One herb contains an extensive variety of phytochemicals, all with variable bacteriostatic or bacteriocidal activity, and use of herbal combinations may well prove to be the best course of action in poultry when effective compounds have been identified. The survival rates of bacteria within the two sample types should have been comparable in this experiment, yet differences existed between the caecal and faecal microflora. It may be the case that a decreased exposure time to air would have resulted in the survival of a more stable and less sub-lethally injured bacterial population. Unfortunately, the samples had to be transported before analysis, which will have influenced the population composition, although all received similar treatment.

3.4.10 Effects of environment and housing

In this experiment, the facilities used included two different cage types at different locations within the room facility, as a result of last minute changes in the experimental design. Although every precaution was taken to ensure uniformity within an experimental block, the location of the cages in blocks 4 and 5 provided numerically lower responses for some of the experimental variables. These 2 blocks were placed in the experimental room with a much closer proximity to the single gas brooder. In the experiment, there were 2 mortalities at the end of the first week, one each from treatment pens 34 & 36 in block 4. It is possible that the close proximity of these cages to the gas brooder may have caused a slight dehydrating effect in the neighbouring cages during the early stages of the experiment. The design of the experimental room is such that these cages received less light, as they were located at the bottom of a tier of cages. They may also have experienced a small increase in temperature than the birds in blocks 1-3, which were located at a much further point from the brooder.

It is considered that the responses to plant antimicrobials may be greater in a more challenging environment, in agreement with the conclusions of Lee *et al.*, (2003). This initial experiment

was mainly for screening purposes, and it focussed on using herbs as natural antimicrobials in growing chickens in a caged experimental situation. With hindsight, there may have been an insufficient pathogenic challenge against these birds for beneficial effects to become apparent, despite the poorer nutritional quality of the diet. Previously, it has been shown that cage environments are the cleanest option in broiler chicken production in terms of helminth populations (Permin *et al.*, 1999) and red mite infestation (Höglund *et al.*, 1995). In future, it may prove more beneficial to raise birds under a different environmental situation, more representative of the commercial environment. Litter provides a substrate for pathogenic bacterial growth in conditions close to a neutral pH level (Pope & Cherry, 2000).

3.4.11 Histological analysis of the ileal samples

The failure to measure the histological samples in this study was disappointing, and was thought to be due to insufficient volumes of buffered formal saline in the containers used for sample preservation, causing a lysis of the tissue material (Pennycott, 2001, Pers. comm). The intestinal samples were sent to diagnostic labs and re-sectioned, but there was still no measurable appearance of intestinal villi. However, it is possible that the quality of this diet may have had some destructive effect on the intestinal surface, through the production of harmful bacterial metabolites. Garlic preparations at pharmacological levels administered directly onto the intestinal mucosa have been observed to cause loss of epithelial cells from the top of the crypts, reddening and also severe mucosal damage (Hoshino *et al.*, 2001). However, the supplements in our study were fed in much lower concentrations and in a normal dietary ration, which should have ameliorated any potential negative effects. Alternatively, the presence of an intestinal disease cannot be ruled out, which might disrupt intestinal integrity, although the cultured populations of *C. perfringens* were relatively low in number in the microbiological analysis.

In summary, marked differences were found between the plant species and also in their form of supplementation in the diets on the bird performance characteristics. Several of the EO's used had an atypical composition to those recommended for their antimicrobial efficacy in the literature, and it is recommended that more emphasis be put on the use of EO with a suitable concentration of the active terpenes for antimicrobial purposes. Despite this, both thyme and oregano EO demonstrated good antimicrobial activity *in vitro* against 9 bacterial species, including both Gram-positive and Gram-negative bacteria. Generally, thyme EO and yarrow

herb supplements in diets appeared to result in the best bird performance, while oregano herb and yarrow EO were the worst for inclusion in diets. By 21 days of age, the birds fed diets with thyme EO were heavier, when compared to those fed diets with both rosemary and yarrow EO as well as those with oregano EO and herb ($P<0.01$). On day 28 of the study, birds fed diets with thyme EO had an improved BM compared to those fed diets with thyme herb, oregano EO and herb, rosemary EO and yarrow EO supplements ($P<0.01$). The birds fed diets with yarrow herb achieved a greater BM than those fed diets with oregano herb and rosemary EO ($P<0.01$, days 21 & 28) and also yarrow EO ($P<0.01$, day 28). The lightest birds were those fed on the diets with yarrow EO, weighing 11.8% less than those on the control diet at day 28 ($P>0.05$). When compared to the controls, the positive increases with thyme EO supplements on BM remained relatively constant over time, whereas those with yarrow herb diminished slightly with age. As the birds got older, the decreased BM with the dietary inclusion of oregano herb and yarrow EO supplements became gradually worse. Over the study period, the weight gains in birds fed diets with thyme EO were greater than those fed the non-supplemented control diets ($P=0.001$). The birds fed diets supplemented with thyme EO and also yarrow herb had improved weight gains compared to those fed diets with thyme and oregano herbs, and oregano and rosemary EOs ($P<0.001$). Poorest weight gains over the study were observed in birds fed diets with yarrow EO, compared to those fed diets with thyme EO, marjoram EO and herb, rosemary herb and also yarrow herb ($P=0.001$). Over the study period, the diets with thyme EO were not consumed in greater quantities than the control diets, but were more readily consumed than those with yarrow EO and oregano EO ($P<0.05$). However, the diets with oregano herb and rosemary EO supplements were consumed less than the control diets over the study period ($P<0.05$). The FCR values in this study were lowest for birds fed on diets with rosemary EO, compared to those with rosemary and thyme herbs ($P<0.05$). The diets with yarrow EO had the highest FCR values, when compared to those with rosemary and thyme EOs ($P<0.05$). There were no treatment effects on the populations of the main microfloral species in faecal or caecal samples, the ratios of lactic acid: coliform bacteria for any treatment, or the proportions of each bacterial species in relation to total anaerobes. When the nutrient digestibility was measured, there were no significant effects of treatment on the AME, AMEn, AME:GE, AMEn:GE ADMD, AMN or DOMD. Overall, the values for AME were quite low in these diets. Dietary supplementation with rosemary herb resulted in a tendency ($P=0.07$) for birds to retain less N from the diet, compared to those fed the control diets. The birds fed thyme EO in the diet excreted a higher concentration of sialic

acid, compared to those fed diets with yarrow, marjoram and oregano herbs, and the EOs of oregano and rosemary (P=0.019). The lowest concentrations of sialic acid were excreted by the birds fed diets with oregano herb and rosemary EO, compared to those fed diets with marjoram EO, rosemary herb and the control diets (P=0.019). There was no effect of any treatment diet on the apparent digestibility coefficients of AA's in the present experiment. Due to methodology problems in the assessment of the ileal morphology in these birds, the samples could not be measured.

3.5 Conclusions

This study has demonstrated that various terpene supplements have a differential effect on broiler performance, when fed as supplements in the diet. These differences are primarily related to their chemical composition, and there does not always appear to be a similarity in the results of supplementing an herb and its extracted EO in broilers. As the chemical composition of plant material is variable, it will therefore be imperative to obtain a measurement of the compositional quality of plant materials before their use in diets for animals. The differential effects of plant compounds and their extracts in broilers may relate to their influence on the digestion or absorption of dietary substrates, or may be related to how they are perceived by the birds in terms of taste within the diets. There may be an effect of dietary terpene supplements on the intestinal microflora in broilers, but this was not demonstrated clearly in the present experiment and will require further assessment. In future experiments, the effects of plant compounds may be more suitably evaluated in an environment more closely related to that faced by birds grown under commercial conditions. It would be beneficial to study these plant compounds in combination with enzymes, in order to appreciate fully the mode of action of both supplements together in the birds, and the possibility for any interactions between them. This may be particularly true in situations where the diet is reduced in its nutritional value. Herbs or EO in broiler diets appear to influence the production of endogenous secretions in the birds, but it is not clear whether these changes are positive or negative. These EO or herb supplements may increase or decrease the digestibility of the dietary ration, in terms of the availability of protein, and may influence its relationship with energy utilisation, resulting in an overall effect on the bird performance.

CHAPTER 4

4. ASSESSMENT OF THE INCLUSION OF VARIOUS CONCENTRATIONS OF THYME OIL IN BROILER DIETS, IN THE PRESENCE OR ABSENCE OF A COMMERCIAL CARBOHYDRASE

4.1 Introduction

Essential oils (EO) are rich in bioactive secondary plant products, predominantly mono- and sesqui-terpene compounds. Many studies have shown antimicrobial effects for these oils *in vitro* both against Gram-negative and Gram-positive bacteria (Helander *et al.*, 1998; Hammer *et al.*, 1999, Dorman & Deans, 2000; Faleiro *et al.*, 2003). However, few reports exist in the literature describing an antimicrobial effect *in vivo,* when using EO as feed supplements for animals. If such an effect could be established for bioactive plants, this should increase their acceptance within the poultry industry as alternatives to synthetic antimicrobials. EO's are aromatic compounds used in flavouring, and are known to form associations with or bind to organic dietary components such as carbohydrates and proteins (Narasimha Rao & Nigam, 1970; Goubet *et al.*, 1998; Golovnya *et al.*, 1998). There may also be an interaction of EO with the digesta, which may alter their bioactive activity as supplements.

The previous growth trial investigated a range of plants and their various forms of supplementation. This trial focussed on the use of a good compositional quality *Thymus vulgaris* L. oil with a high thymol content, and assessed various concentrations of this oil in order to determine an optimum dietary inclusion level for broilers. The phenolic monoterpenes thymol and carvacrol are known for their ability to attack bacterial cell walls (Helander *et al.*, 1998), are present in thyme EO and are two of the strongest natural phenolic compounds available. These phenolic compounds penetrate the cell membranes of Gram-negative bacteria, increasing its permeability, thus dissipating the pH gradient and causing the leakage of intracellular phosphate and potassium ions from the cell (Lambert *et al.*, 2001). Very few terpenes have been shown to have the ability to penetrate Gram-negative bacterial cell walls and their exact mechanism of action has not yet been established. However, the presence of other terpenes may still alter the bioactivity of EO. Several terpenes isolated from tea tree EO have shown a greater antimicrobial effect *in vitro* than the complete EO (Cox *et al.*, 2001). The effect of variations in the content of other components on the bioactivity or antimicrobial activity of an EO remains unknown.

Enzyme supplementation is a common feature in most broiler diets in the UK, especially those based on wheat, which has a variable quality in relation to its content of non-starch polysaccharides (NSP). As the use of exogenous enzymes in poultry diets is ubiquitous, the use of an additional supplement such as thyme EO could not be recommended without first determining the existence of either synergistic or antagonistic effects between these two supplements. Carbohydrase inclusion may improve broiler performance in the first 3 weeks by breaking down the indigestible fibre component of the cereal cell wall, thus increasing the availability of nutrients to the bird and reducing the adverse effects of NSP and other antinutritional factors (Bedford 1996). This has a positive impact on AME, reduces the viscosity of digesta and modifies the ileal and caecal microbial fermentation profiles, by providing more nutrients to the bird in the proximal intestine (Bedford, 1996, Choct *et al.*, 1999). If more nutrients are available to the bird directly, then fewer nutrients will be available for bacterial fermentation in the distal gut. Thus, thyme EO and an exogenous carbohydrase may act in tandem, by improving nutrient availability from the diet and also by reducing the bacterial challenge faced by the birds. This experiment was designed to investigate the effects of both supplements together on broiler performance.

The previous experiment was conducted in cage housing, which may have presented an insufficient environmental challenge to fully appreciate the effects of EO as dietary supplements for broilers. A growing environment based on a cage system has been shown to be the cleanest available in terms of helminths and red mite prevention (Höglund *et al.*, 1995; Permin *et al.*, 1999). In contrast, the use of sawdust litter provides a substrate for the growth of pathogenic bacteria (Pope & Cherry, 2000), and is widely used in commercial broiler housing. Therefore, this study was set up to look at the growth of broilers housed on sawdust litter, to provide them with a greater environmental challenge than in the previous experiment.

4.2 Aims of the experiment and Methodology

This experiment was designed to assess the effects of inclusion of various concentrations of thyme EO (*Thymus vulgaris* L.) in diets, with or without a dietary carbohydrase, on the growth performance and other characteristics in broilers. The experiment therefore sought to establish whether an interaction was present between the thyme EO and carbohydrase supplements. An additional aim was to establish whether or not the enzyme itself had an antimicrobial or bioactive effect in birds.

4.2.1 Sources of supplements for the feeding trial

A commercial carbohydrase (Avizyme 1210; xylanase [EC3.2.1.8]=2776 U kg^{-1} & β-glucanase [EC3.2.1.6]=177 U kg^{-1}), was provided by Danisco Animal Nutrition, Marlborough, UK for use in this experiment. The EO of *Thymus vulgaris* L. was purchased from Essentially Oils Ltd., Oxfordshire, UK.

4.2.2 Housing and Environment

The pens used in the study were constructed to allow the recommended commercial stocking density of 34 kg m^{-2} to be achieved. Each pen was equipped with a single bell drinker and feed trough. Heating was provided by means of electrical heaters on the ceiling of each room, set at the start of the experiment to give a temperature of 32°C, and then gradually decreased to give a final temperature of 21°C at 21 days of age. Supplementary heating was provided as required by mobile butane gas heaters in each room. The birds were provided with a standard lighting pattern, with 1 hour of darkness following a standard period of 23 hours light, from the start to the finish of the study.

4.2.3 Experimental Design

The feeding trial was carried out between March-April 2002 in floor pens using wood shavings as bedding. In total, 480 female Ross 308 broilers were purchased at day old (Grampian Country Chickens, Whitburn, Midlothian) and reared from day of hatch to 42 days. A 4 X 2 factorial balanced incomplete block design was used in the experiment, with 12 pens set up in each room of the poultry house. The pens were assigned randomly to treatment, using six replicates of eight treatments, to give a total of 48 pens in the study. On arrival at the day of hatch, ten birds were randomly assigned into each pen by body weight. The birds were fed on a starter ration from days 0-22, followed by a finisher ration from 23-42 days (**Table 4.1**). All birds were weighed individually on arrival, grouped into categories by weight and introduced into the treatment pens at day 0. The birds were then weighed again on study days 7, 14, 22, 35 and 42. The feed requirements for each pen were also weighed in advance before allocation to the birds. At the end of each dietary period, the weight of uneaten feed in each pen was determined. This allowed the calculation of the feed intake and feed conversion ratio (FCR) for each pen within each dietary period. Samples of the starter and finisher diets were retained for laboratory analysis. At study days 7, 19 and 42, one bird in each pen was

euthanased by administering sodium pentobarbitone (euthatal) at 1 ml kg^{-1} body mass, before ileal and caecal digesta and ileal tissue samples were collected as described in Section 2.4.3.

4.2.4 Diet formulation and nutritional composition

Barley and wheat were both included in the basal ration to provide a substantial source of NSP's in the diet (**Table 4.1**). The diet was prepared as a basal control ration, balanced for its provision of energy and protein and fed to satisfy the requirements for growth of broilers of the age and genotype used in the study. This basal ration was then split into eight, and the treatment-specific supplements were added. The dietary treatments were supplemented with thyme EO at 0, 1, 3 and 5 g kg^{-1}, and fed with or without carbohydrase at 0.5 g kg^{-1} (**Table 4.2**). No synthetic antimicrobials or anticoccidials were added to any treatment diets. The diet was supplied in the form of a mash for the duration of the study.

Table 4.1 *Diet formulation and calculated chemical composition of each ration*

Feed Ingredient	*Amount in diet (g kg^{-1})*		*Calculated composition*	*(g kg^{-1})*	
	Starter	Finisher		Starter	Finisher
Wheat	406.0	477.5	ME (MJ kg^{-1})	12.2	12.9
Barley	161.5	155.0	Crude Protein	222.2	190.6
Soya bean meal (Hi-pro)	143.5	45.0	Ether Extract/Fat	66.8	83.9
Soya bean meal (Full fat)	232.5	256.0	Crude Fibre	40.3	39.5
Soya oil	15.0	28.0	Calcium	9.5	8.8
Mono di-calcium phosphate	12.5	10.0	Phosphorus	7.2	6.5
Limestone	15.0	15.0	Lysine	13.7	11.6
Sodium chloride	3.0	3.0	Methionine + Cysteine	9.9	8.6
Lysine	2.0	2.0			
Methionine	4.0	3.5			
Vit/ Min premix[1]	5.0	5.0			

[1]Supplied per kg diet: Vitamin A 12,000 IU, Vitamin D3 5000 IU, Vitamin E (as α-tocopherol) 50 mg, Vitamin K 3 mg, Folic acid 1mg, Nicotinic Acid 50 mg, Vitamin B1 (Thiamine) 2 mg, Vitamin B2 (Riboflavin) 7 mg, Vitamin B6 (Pyridoxine) 5 mg, Vitamin B12 15 µg, Biotin 200 µg, Calcium pantothenate 15 mg, Iodine 1mg, Molybdenum 0.5 mg, Selenium 200 µg, Cobalt 0.5 mg, Copper 10 mg, Iron 80 mg, Manganese 100 mg, Zinc 80 mg, Limestone 4.18 g

4.2.5 Statistical analysis

The experimental data were analysed in Genstat Release 5.2, using analysis of variance. For any main treatment effects, Fisher's least significant difference (L.S.D.) test was used to separate the treatment means. Comparisons were done for the responses to supplementation with thyme EO using data pooled and averaged over the treatments with carbohydrase, and for the responses to carbohydrase inclusion by averaging over the treatments with thyme EO. Interactions between carbohydrase and thyme EO in the data variables were determined by entering the term enz*herb in the ANOVA model.

Table 4.2 *Details of study treatments and inclusion levels of compounds*

Treatment	*Thyme oil inclusion levels (g kg^{-1})*	*Carbohydrase inclusion (g kg^{-1})*
1	Control (no inclusion)	0
2	1	0
3	3	0
4	5	0
5	Control (no inclusion)	0.5
6	1	0.5
7	3	0.5
8	5	0.5

4.3 Results

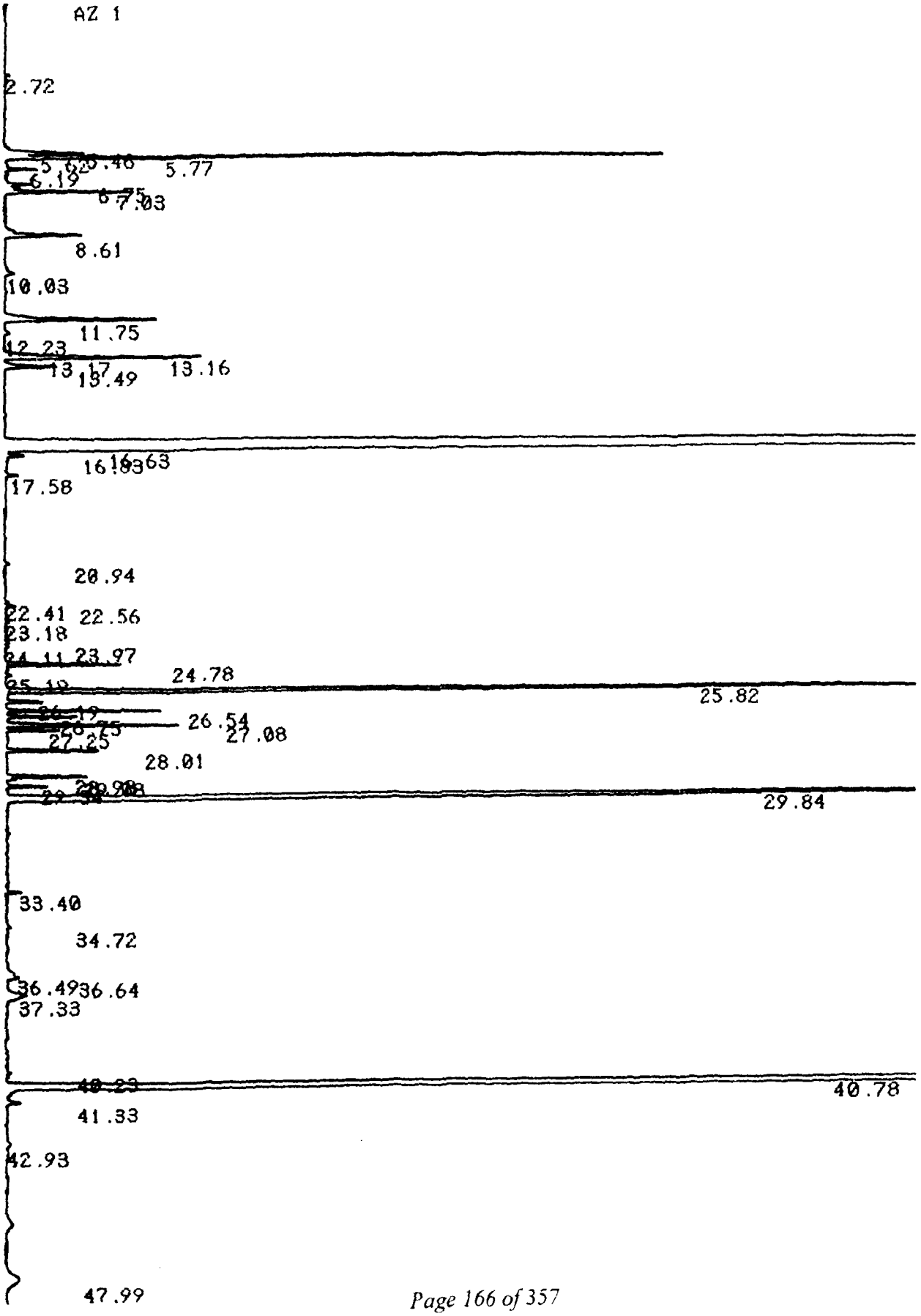
In this section, the composition of the thyme EO and analysis of the experimental diets are presented. This is followed by the bird performance data and then by the remaining experimental measurements, which were analysed at the end of each dietary phase (19 and 42 days). These measurements included the intestinal bacteriology, nutrient and amino acid (AA) digestibilities, sialic acid concentration and caecal VFA concentrations. The ileal morphology was measured in the birds at 7 and 42 days. Finally, due to the large numbers of experimental variables, the statistical technique of principal component analysis (PCA) was used to confirm the results of the individual analyses of variance.

The birds in the study were exposed to a *colisepticaemia* infection in the first week, which was unrelated to treatment. The total mortality for this experiment was 7.4% over the first 11 days, after which no more deaths occurred. *Colisepticaemia* is normally a secondary bacterial infection in chickens. It occurs following the proliferation and subsequent overgrowth of *Escherichia coli* within the intestine. The source of the infection could not be determined.

4.3.1 Composition of the essential oil of Thymus vulgaris L.

Analysis of the thyme EO by gas chromatography revealed 5 major terpene components (**Figure 4.1**). These main components included thymol (44.08%), *p*-cymene (32.04%), α-terpineol (9.59%), linalol (4.62%) and α-pinene (2.11%). The total percentage of the EO identified was 92.44%. The remaining 7.56% was composed of another 37 components, but each contributed less than 1% to the EO. After incorporation of the EO into the rations, samples were taken and the EO re-distilled from the diets, to assess the amount incorporated into the mash. About 60% recovery was achieved, suggesting a volatilisation of around 40%.

Figure 4.1 *Terpene composition within the essential oil of Thymus vulgaris L.*



4.3.2 Diet Compositional Analysis

The gross energy (GE) content of the basal starter/grower diet was determined as 18.94 MJ kg^{-1} DM and the GE content of thyme EO as 39 MJ kg^{-1}. The GE within each supplemented treatment was not determined for the starter rations, as these were not included within the nutrient digestibility analyses and thyme EO was not included at a high enough concentration to change the dietary nutrient composition. However, the finisher diets were included in the assessment of nutrient digestibility and the GE contents of each treatment diet were measured. Calculated dietary compositional values are presented (**Table 4.1**). The determined composition of crude protein was similar between each starter ration at 235-240 g kg^{-1} DM, and between the finisher rations at 200-205 g kg^{-1} DM (**Table 4.3**), which are comparable to the calculated values. The apparent metabolisable energy (AME) values of the diet are presented later in this chapter.

Table 4.3 *The determined chemical composition of dietary rations supplemented with or without thyme oil and enzyme (Enz), as fed to broilers over the study period (0-42 days)*

	Determined chemical composition of dietary treatments								
Treatment	*DM (g kg^{-1})*		*CP (g kg^{-1} DM)*		*Ash (g kg^{-1} DM)*		*OM (g kg^{-1} DM)*		*GE (MJ kg^{-1} DM)*
	S	F	S	F	S	F	S	F	F
Control	887	898	243	202	73	58	927	942	19.85
Thyme (1)	889	898	242	200	67	58	933	942	20.04
Thyme (3)	888	898	236	202	63	59	937	941	19.97
Thyme (5)	886	899	233	203	65	58	935	942	19.98
Cont + Enz	888	900	235	201	67	60	933	940	20.07
Thy (1) + Enz	891	898	238	206	68	58	932	942	20.20
Thy (3) + Enz	890	898	240	212	71	59	929	941	20.20
Thy (5) + Enz	889	895	232	207	70	58	930	942	20.36

N=2 samples for each measurement. S and F refer to starter (0-22 days) and finisher (23-42 days) rations respectively.

The amino acid (AA) concentrations supplied in each starter/grower ration up to 22 days were determined (**Table 4.4**). However, the contents of the sulphur AA, proline and hydroxyproline were not determined, so the values fall short of the determined crude protein concentration. The concentrations of the dietary AA supplied by the finisher rations to 42 days were also determined (**Table 4.5**), excluding the sulphur AA, proline and hydroxyproline.

Table 4.4 *The content of amino acids (g kg^{-1} DM) determined within the treatment diets as fed to the birds between 0-22 days, with and without thyme oil and enzyme inclusion*

	Control diets		*Diets including Thyme oil (g kg^{-1})*			*Diets including Thyme oil (g kg^{-1}) & 0.5 g kg^{-1} enzyme*		
(in g kg^{-1} DM)	C	C + Enz	1	3	5	1	3	5
Alanine	8.89	8.27	8.60	8.39	8.40	8.80	9.22	9.34
Aspartic acid	20.33	19.94	20.10	19.98	20.27	20.21	21.09	21.78
Glutamic acid	45.90	45.47	46.38	45.86	46.40	45.26	47.24	48.86
Serine	10.13	9.56	9.78	9.67	9.87	9.83	10.47	10.96
Tyrosine	5.26	5.32	5.51	5.15	5.37	5.34	5.76	5.52
Σ Dispensible	**90.51**	**88.55**	**90.36**	**89.05**	**90.30**	**89.43**	**93.77**	**96.45**
Arginine	12.27	12.33	12.70	12.18	12.72	12.55	13.24	13.30
Glycine	9.16	7.78	8.29	7.99	8.19	8.31	9.38	10.11
Histidine	5.02	4.69	4.93	4.82	4.90	4.93	5.28	5.51
Isoleucine	8.87	9.23	9.23	9.01	9.25	9.22	9.43	9.54
Leucine	15.29	15.46	15.74	15.30	15.57	15.95	16.58	16.91
Lysine	13.26	12.49	12.63	12.13	12.14	13.72	14.95	15.47
Phenylalanine	10.05	10.21	10.31	10.08	10.21	10.57	11.05	11.28
Threonine	7.61	7.46	7.53	7.46	7.69	7.63	7.95	8.13
Valine	9.95	10.29	10.29	10.05	10.33	10.13	10.45	10.57
Σ Indispensible	**91.47**	**89.95**	**91.63**	**89.01**	**90.99**	**93.01**	**98.31**	**100.80**
Σ Total AA	**181.97**	**178.50**	**182.00**	**178.06**	**181.29**	**182.40**	**192.08**	**197.30**

N=3 for all treatments.

Table 4.5 *The content of amino acids (g kg^{-1} DM) determined within the treatment diets as fed to the birds between 23-42 days, with and without thyme oil and enzyme inclusion*

	Control diets		*Diets including Thyme oil (g kg^{-1})*			*Diets including Thyme oil (g kg^{-1}) & 0.5 g kg^{-1} enzyme*		
(in g kg^{-1} DM)	C	C + Enz	1	3	5	1	3	5
Alanine	7.67	7.05	7.34	6.91	7.27	7.07	6.98	7.25
Aspartic acid	16.75	15.87	16.70	16.15	16.61	15.63	16.15	16.27
Glutamic acid	41.99	39.18	40.97	39.71	40.79	38.68	40.21	40.67
Serine	8.98	8.11	8.72	8.31	8.46	8.17	8.28	8.39
Tyrosine	4.30	4.38	4.38	4.12	4.61	4.14	4.25	4.52
Σ Dispensible	**79.69**	**74.59**	**78.12**	**75.19**	**77.74**	**73.69**	**75.86**	**77.09**
Arginine	10.38	9.99	10.23	10.00	10.51	9.72	10.06	10.26
Glycine	7.83	6.55	7.11	6.68	6.83	6.80	6.60	6.59
Histidine	4.29	3.86	4.06	3.91	4.05	3.89	3.91	3.95
Isoleucine	7.40	7.34	7.45	7.24	7.51	7.05	7.35	7.49
Leucine	13.25	12.83	13.17	12.65	13.07	12.45	12.67	13.06
Lysine	10.92	10.70	10.35	10.47	10.31	10.32	9.89	10.17
Phenylalanine	8.69	8.47	8.58	8.27	8.53	8.20	8.25	8.56
Threonine	6.64	6.23	6.55	6.31	6.48	6.07	6.29	6.39
Valine	8.38	8.32	8.47	8.21	8.55	8.01	8.36	8.51
Σ Indispensible	**69.94**	**67.74**	**68.86**	**67.06**	**69.02**	**65.72**	**66.78**	**68.39**
Σ Total AA	**157.46**	**148.88**	**154.09**	**148.93**	**153.58**	**146.20**	**149.23**	**152.10**

N=3 for all treatments.

4.3.3 The effect of carbohydrase inclusion in the diets on broiler performance

Analysis of the pooled experimental data showed that carbohydrase inclusion in the diets improved body mass (BM) (P<0.001) at each measurement, except at 0 days of age in the birds (**Table 4.6**).

Table 4.6 *Effect of carbohydrase in diets on average broiler body mass from 0-42 days*

	Average body mass (g)					
Treatment	*Day 0*	*Day 7*	*Day 14*	*Day 23*	*Day 35*	*Day 42*
Enz -ve	37.8 *(0.5)*	93^{a} *(2)*	216^{a} *(6)*	619^{a} *(14)*	1344^{a} *(26)*	1746^{a} *(34)*
Enz +ve	37.6 *(0.4)*	117^{b} *(2)*	302^{b} *(6)*	785^{b} *(16)*	1548^{b} *(25)*	1937^{b} *(29)*
s.e.d	0.241	3.17	8.62	21.5	38.5	47.5
	NS	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) of 6 pen replicates pooled across the thyme oil supplemented treatments.

Increases in weight gain of 29.2%, 34.1% and 16.6% respectively were achieved at 0-7, 8-14 and 15-23 days of age, in birds fed diets supplemented with carbohydrase (P<0.001), compared to those without supplement (**Table 4.7**). There was a tendency (P=0.086) for carbohydrase inclusion to cause an increase in weight gain (5.2%) between days 24-35. Carbohydrase inclusion had no effect on the older birds in the study. However, due to the early improvement in body mass, the weight gain increased in the study from 0-42 days by 9.2% with the enzyme inclusion (P<0.001).

Table 4.7 *Effect of dietary carbohydrase inclusion on average weight gain from 0-42 days*

	Average weight gain (g bird^{-1} day^{-1})					
Treatment	*0-7 days*	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*0-42 days*
Enz –ve	8.0^{a} *(0.4)*	17.4^{a} *(0.7)*	44.8^{a} *(1.0)*	55.8 *(1.2)*	67.0 *(2.3)*	39.3^{a} *(0.8)*
Enz +ve	11.3^{b} *(0.2)*	26.4^{b} *(0.7)*	53.7^{b} *(1.1)*	58.7 *(1.0)*	64.8 *(1.8)*	43.3^{b} *(0.7)*
s.e.d	0.4	1.0	1.6	1.6	2.7	1.096
	P<0.001	P<0.001	P<0.001	P=0.086	NS	P<0.001

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled across the thyme oil supplemented treatments.

The inclusion of a dietary carbohydrase increased feed consumption by 25.3 and 16.1% within the first few weeks (days 8-14, P<0.001; days 15-23, P<0.001; **Table 4.8**). The data between days 0-7 was excluded due to excessive feed wastage, which resulted in artificially high values for consumption in birds of that age. After day 23, the carbohydrase had no effect on feed consumption. There was an increase in feed consumption of 7.4% during the study overall (8-42 days) with the inclusion of carbohydrase in the diets (P=0.002).

Table 4.8 *Effect of carbohydrase inclusion on broiler feed consumption over 42 days*

	Average feed consumption (g feed bird^{-1} day^{-1})				
Treatment	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Enz –ve	26.5^{a} *(0.8)*	68.4^{a} *(1.7)*	134.5 *(2.5)*	135.0 *(4.9)*	94.5^{a} *(1.8)*
Enz +ve	35.5^{b} *(0.9)*	81.5^{b} *(1.2)*	138.0 *(2.5)*	139.8 *(4.0)*	102.1^{b} *(1.4)*
s.e.d	1.19	2.15	3.41	5.14	2.32
	P<0.001	P<0.001	NS	NS	P=0.002

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled across the thyme oil supplemented treatments.

The inclusion of carbohydrase in diets reduced the feed conversion ratio (FCR) only between 8-14 days (1.37 *vs* 1.56; P=0.02; **Table 4.9**). There was no effect of enzyme on FCR in broilers after this point in the study. The FCR's were at the poorer end of the spectrum when compared to commercially fed birds.

Table 4.9 *Effect of carbohydrase inclusion on FCR in broilers from 8-42 days of age*

	Feed Conversion Ratio (feed unit gain^{-1})				
Treatment	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Enz –ve	1.56^{a} *(0.05)*	1.54 *(0.03)*	2.24 *(0.05)*	2.03 *(0.06)*	2.01 *(0.04)*
Enz +ve	1.37^{b} *(0.05)*	1.53 *(0.03)*	2.19 *(0.07)*	2.19 *(0.09)*	1.98 *(0.05)*
s.e.d	0.077	0.047	0.083	0.113	0.066
	P=0.02	NS	NS	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled across the thyme oil supplemented treatments.

4.3.4 Effect of thyme oil inclusion in diets on broiler performance

There were no significant effects on BM as a result of thyme EO inclusion in the diets (**Table 4.10**). However, on day 23 of the study, the inclusion of thyme EO at 5 g kg^{-1} caused a decrease of approximately 10.4% in BM (P=0.07). This may indicate that higher dietary concentrations of thyme EO have an adverse effect on broiler BM.

Table 4.10 *Effect of thyme oil inclusion in diets on average body mass (BM) over 42 days*

	Average BM (g)					
Supplement (g kg^{-1})	*Day 0*	*Day 7*	*Day 14*	*Day 23*	*Day 35*	*Day 42*
Control (0)	37.2 *(0.6)*	106 *(5)*	269 *(15)*	733 *(30)*	1474 *(34)*	1874 *(41)*
Thyme (1)	37.8 *(0.7)*	107 *(4)*	262 *(15)*	705 *(33)*	1462 *(56)*	1867 *(69)*
Thyme (3)	37.8 *(0.7)*	106 *(6)*	263 *(17)*	713 *(35)*	1477 *(51)*	1869 *(51)*
Thyme (5)	37.9 *(0.6)*	102 *(4)*	241 *(13)*	657 *(26)*	1371 *(42)*	1756 *(44)*
s.e.d	0.32	4.22	11.5	28.7	51.4	63.4
	NS	NS	NS	P=0.07	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled across Enz-ve and Enz +ve treatments.

Thyme EO had no effect on weight gain during the first week (**Table 4.11**). However, the inclusion of thyme EO from 8-14 days at 5 g kg^{-1} tended to decrease weight gain by 14.6% compared to the controls (P=0.076). There was also a tendency for weight gain to be depressed by 10.7% when thyme EO was added to the diets from 15-23 days at this level (P=0.089). After day 23, there was no effect of thyme EO inclusion on weight gain, suggesting that an initial adaptation period may be necessary for this supplement, or that 5 g kg^{-1} may be too high in terms of dietary supplementation in broilers.

Table 4.11 *Effect of dietary thyme oil inclusion on average weight gain in broilers*

	Average weight gain (g bird^{-1} day^{-1})					
Inclusion (g kg^{-1})	*0-7 days*	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*0-42 days*
Control (0)	9.8 *(0.7)*	23.3 *(1.5)*	51.6 *(1.8)*	57.0 *(1.3)*	66.7 *(3.2)*	42.1 *(0.9)*
Thyme (1)	9.9 *(0.5)*	22.0 *(1.7)*	49.3 *(2.1)*	58.2 *(1.9)*	67.6 *(3.3)*	41.9 *(1.6)*
Thyme (3)	9.7 *(0.8)*	22.4 *(1.8)*	50.0 *(2.2)*	58.8 *(1.7)*	65.4 *(2.4)*	42.0 *(1.1)*
Thyme (5)	9.2 *(0.6)*	19.9 *(1.5)*	46.1 *(1.5)*	55.0 *(1.6)*	64.0 *(2.9)*	39.3 *(1.1)*
s.e.d.	0.59	1.30	2.12	2.19	3.65	1.46
	NS	P=0.076	P=0.089	NS	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled over Enz-ve and Enz +ve treatments.

Average feed consumption tended (P=0.07) to decline linearly with increasing thyme EO concentrations from 8-14 days (**Table 4.12**). This decine was linear with increasing concentration of thyme oil in the diet (P=0.007; s.e.d.=11.14), where diets with an inclusion level of 5 g kg^{-1} of thyme EO reduced feed consumption by 16.2% compared to the birds fed the control ration. After this time, there was no effect of the thyme EO inclusion in diets on feed consumption. There were no significant effects on FCR as a result of the dietary inclusion of thyme EO at any concentration (**Table 4.13**).

Table 4.12 *Effect of thyme oil inclusion on average feed consumption in broilers*

	Average feed consumption (g bird^{-1}day^{-1})				
Supplement (g kg^{-1})	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Control (0)	34^{a} *(2)*	77 *(3)*	135 *(3)*	139 *(7)*	99 *(2)*
Thyme (1)	32^{ab} *(1)*	73 *(3)*	134 *(2)*	136 *(6)*	97 *(2)*
Thyme (3)	30^{ab} *(2)*	76 *(3)*	136 *(4)*	133 *(6)*	98 *(3)*
Thyme (5)	28^{b} *(2)*	73 *(3)*	139 *(4)*	142 *(7)*	99 *(3)*
s.e.d	1.6	2.9	4.5	6.9	3.09
	P=0.007	NS	NS	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled over Enz-ve and Enz +ve treatments.

Table 4.13 *Thyme oil inclusion in diets and its effect on broiler FCR from 8-42 days*

	Feed conversion ratio (feed gain^{-1})				
Treatment (g kg^{-1})	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Control (0)	1.49 *(0.07)*	1.50 *(0.03)*	2.20 *(0.07)*	2.11 *(0.01)*	1.97 *(0.03)*
Thyme (1)	1.50 *(0.09)*	1.49 *(0.03)*	2.15 *(0.07)*	2.03 *(0.06)*	1.95 *(0.04)*
Thyme (3)	1.38 *(0.07)*	1.55 *(0.06)*	2.15 *(0.08)*	2.07 *(0.12)*	1.95 *(0.06)*
Thyme (5)	1.49 *(0.10)*	1.59 *(0.04)*	2.36 *(0.11)*	2.25 *(0.15)*	2.11 *(0.09)*
s.e.d	0.103	0.063	0.110	0.151	0.088
	NS	NS	NS	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM) from 6 replicate pens pooled over Enz-ve and Enz +ve treatments.

4.3.5 Effect of the combined inclusion of thyme oil and enzymes in diets on performance

There were no interactions between the dietary supplements on BM (**Table 4.14**). The greatest BM occurred in birds fed diets containing both thyme EO at 3 g kg^{-1} and the carbohydrase. No interactions were observed between the supplements with respect to weight gain, and the slight increases observed in weight gain were due to the presence of carbohydrase (**Table 4.15**).

Table 4.14 *The effect of thyme oil and enzyme inclusion in diets on average BM*

	Average BM (g)					
Inclusion (g kg^{-1})	*Day 0*	*Day 7*	*Day 14*	*Day 23*	*Day 35*	*Day 42*
Control (0) – Enz	37.6 *(1.1)*	92 *(6)*	222 *(8)*	639 *(13)*	1391 *(31)*	1776 *(51)*
Thyme (1) – Enz	37.9 *(1.1)*	98 *(4)*	224 *(15)*	635 *(38)*	1351 *(69)*	1762 *(103)*
Thyme (3) – Enz	37.7 *(1.1)*	91 *(6)*	209 *(10)*	603 *(26)*	1335 *(47)*	1747 *(61)*
Thyme (5) – Enz	37.9 *(1.1)*	94 *(5)*	210 *(16)*	598 *(35)*	1299 *(61)*	1699 *(58)*
Control (0) + Enz	37.3 *(0.7)*	118 *(3)*	316 *(8)*	827 *(26)*	1553 *(34)*	1972 *(30)*
Thyme (1) + Enz	37.8 *(0.9)*	117 *(4)*	300 *(14)*	776 *(39)*	1572 *(64)*	1972 *(76)*
Thyme (3) + Enz	38.0 *(0.9)*	121 *(4)*	316 *(10)*	822 *(16)*	1619 *(32)*	1992 *(36)*
Thyme (5) + Enz	37.9 *(0.8)*	111 *(4)*	273 *(10)*	715 *(21)*	1444 *(41)*	1812 *(61)*
s.e.d.	0.482	6.07	17.09	41.2	73.8	91.1
Enz*Thyme	NS	NS	NS	NS	NS	NS

NS (P>0.05)
Means (SEM) are displayed, where each mean is based on an average of six replicates.

There were no interactions between the supplements with respect to average feed consumption between days 8-42 in the birds (**Table 4.16**). Any increases in consumption up to day 23 were primarily due to the carbohydrase inclusion.

Table 4.15 *Effect of thyme oil and enzyme inclusion on broiler weight gain*

	Average weight gain (g bird^{-1} day^{-1})					
Inclusion (g kg^{-1})	*0-7 days*	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*0-42 days*
Control (0) – Enz	7.8 *(0.8)*	18.5 *(0.5)*	46.4 *(0.5)*	57.8 *(1.8)*	64.2 *(5.2)*	40.1 *(1.1)*
Thyme (1) – Enz	8.5 *(0.5)*	17.9 *(1.7)*	45.8 *(2.6)*	55.1 *(2.7)*	68.5 *(6.1)*	39.6 *(2.4)*
Thyme (3) – Enz	7.6 *(0.9)*	16.8 *(1.1)*	43.8 *(2.5)*	56.3 *(2.4)*	68.8 *(3.7)*	39.5 *(1.4)*
Thyme (5) – Enz	8.0 *(0.8)*	16.5 *(2.0)*	43.2 *(2.2)*	54.0 *(2.7)*	66.6 *(4.1)*	38.2 *(1.4)*
Control (0) + Enz	11.8 *(0.5)*	28.1 *(1.1)*	56.8 *(2.1)*	56.2 *(2.1)*	69.2 *(3.9)*	44.1 *(0.7)*
Thyme (1) + Enz	11.3 *(0.5)*	26.2 *(1.8)*	52.8 *(3.0)*	61.2 *(2.1)*	66.8 *(3.1)*	44.2 *(1.8)*
Thyme (3) + Enz	11.8 *(0.5)*	28.0 *(0.8)*	56.2 *(1.0)*	61.3 *(1.6)*	62.0 *(2.6)*	44.6 *(0.8)*
Thyme (5) + Enz	10.4 *(0.5)*	23.2 *(1.2)*	49.1 *(1.5)*	56.0 *(1.8)*	61.4 *(4.1)*	40.5 *(1.4)*
s.e.d.	0.85	1.9	3	3.1	5.2	2.10
Enz*Thyme	NS	NS	NS	NS	NS	NS

NS (P>0.05)
Means (SEM) are displayed, where each mean is based on an average of six replicates.

Table 4.16 *Effect of thyme oil and enzyme inclusion on feed consumption*

	Average feed consumption (g bird^{-1}day^{-1})				
Inclusion (g kg^{-1})	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Control (0) – Enz	28.7 *(1.2)*	69.6 *(1.9)*	137.6 *(4.2)*	140.1 *(13.2)*	97.5 *(3.6)*
Thyme (1) – Enz	28.4 *(1.0)*	67.5 *(4.8)*	133.5 *(2.9)*	133.2 *(9.4)*	94.1 *(4.0)*
Thyme (3) – Enz	24.9 *(1.6)*	68.6 *(2.8)*	135.9 *(8.4)*	131.9 *(9.6)*	94.1 *(4.1)*
Thyme (5) – Enz	23.9 *(1.9)*	68.0 *(4.0)*	131.2 *(3.7)*	135.1 *(8.9)*	92.4 *(2.9)*
Control (0) + Enz	39.6 *(2.7)*	84.2 *(1.9)*	132.9 *(4.0)*	137.6 *(5.9)*	101.3 *(1.1)*
Thyme (1) + Enz	34.7 *(1.1)*	79.4 *(3.3)*	134.4 *(3.4)*	139.0 *(9.0)*	100.0 *(2.7)*
Thyme (3) + Enz	34.7 *(1.1)*	84.4 *(1.5)*	136.9 *(3.5)*	134.5 *(6.9)*	101.5 *(2.6)*
Thyme (5) + Enz	33.0 *(1.5)*	78.0 *(1.9)*	147.6 *(6.6)*	148.1 *(10.7)*	105.7 *(4.3)*
s.e.d.	2.28	4.12	6.52	9.84	8.98
Enz*Thyme	NS	NS	NS	NS	NS

NS (P>0.05)
Means (SEM) are displayed, where each mean is based on an average of six replicates.

No interactions were observed between the supplements in relation to the FCR (**Table 4.17**). The FCR's were at the poorer range of the spectrum, in comparison with those of commercial birds.

Table 4.17 *Effect of thyme oil and enzyme inclusion in relation to FCR in broilers*

	Feed conversion ratio (gain feed^{-1})				
Inclusion (g kg^{-1})	*8-14 days*	*15-23 days*	*24-35 days*	*36-42 days*	*8-42 days*
Control (0) – Enz	1.55 *(0.05)*	1.50 *(0.03)*	2.20 *(0.03)*	2.18 *(0.08)*	2.03 *(0.04)*
Thyme (1) – Enz	1.65 *(0.14)*	1.47 *(0.06)*	2.26 *(0.10)*	1.97 *(0.08)*	2.00 *(0.06)*
Thyme (3) – Enz	1.51 *(0.11)*	1.59 *(0.12)*	2.23 *(0.13)*	1.96 *(0.20)*	2.01 *(0.12)*
Thyme (5) – Enz	1.52 *(0.14)*	1.58 *(0.04)*	2.27 *(0.13)*	2.03 *(0.02)*	2.02 *(0.07)*
Control (0) + Enz	1.43 *(0.14)*	1.42 *(0.06)*	2.20 *(0.13)*	2.04 *(0.19)*	1.92 *(0.04)*
Thyme (1) + Enz	1.35 *(0.09)*	1.51 *(0.04)*	2.04 *(0.06)*	2.08 *(0.10)*	1.89 *(0.06)*
Thyme (3) + Enz	1.24 *(0.03)*	1.50 *(0.04)*	2.07 *(0.03)*	2.18 *(0.14)*	1.90 *(0.05)*
Thyme (5) + Enz	1.46 *(0.15)*	1.60 *(0.08)*	2.45 *(0.17)*	2.47 *(0.27)*	2.20 *(0.17)*
s.e.d.	0.148	0.091	0.159	0.216	0.127
Enz*Thyme	NS	NS	NS	NS	NS

NS (P>0.05).
Means (SEM) are displayed, where each mean is based on an average of six replicates.

4.3.6 *Effect of carbohydrase on caecal microflora at 19 days*

Coliform bacteria were increased in caecal contents from birds fed diets supplemented with carbohydrase from 8.76 to 9.56 log_{10} c.f.u. g^{-1} sample ($P<0.05$), suggesting that either the enzyme was not effective against *E. coli*, or it did not act antimicrobially in these birds (**Table 4.18**). The inclusion of the dietary carbohydrase numerically reduced caecal counts of *C. perfringens*, from 4.21 to 2.95 log_{10} c.f.u. g^{-1} material ($p>0.05$).

Table 4.18 *Effect of carbohydrase on caecal microflora at 19 days of age*

	Microfloral populations (log_{10} c.f.u. g^{-1})			
Treatment	*Lactic Acid Bacteria*	*Coliforms*	*Total Anaerobes*	*C. perfringens*
Enz –ve	10.03 *(0.17)*	8.76^{a} *(0.24)*	10.95 *(0.15)*	4.21 *(0.57)*
Enz +ve	9.58 *(0.20)*	9.56^{b} *(0.31)*	10.99 *(0.19)*	2.95 *(0.49)*
s.e.d.	0.264	0.338	0.240	0.797
	NS	P=0.032	NS	NS

Means within a column without a common superscript are significantly different. NS ($P>0.05$)
Data are logarithms of 3 means in colony forming units g^{-1} sample (SEM) pooled over thyme oil treatments.

The proportion of caecal lactic acid bacteria to coliforms in birds was significantly reduced by the addition of a dietary carbohydrase at 19 days ($P<0.001$; **Table 4.19**), due to increases in the coliform populations. There was a tendency ($P=0.055$) for caecal coliform numbers to increase in relation to total anaerobes with the inclusion of carbohydrase, either due to the previous infection or the increased coliform numbers with this supplement.

Table 4.19 *Effect of carbohydrase on the proportions of caecal microflora at 19 days*

	Proportions of caecal microflora in relation to total anaerobes		
Treatment	*LAB: COL*	*LAB: ANR*	*COL: ANR*
Enz–ve	1.152^{a} *(0.029)*	0.918 *(0.022)*	0.801 *(0.024)*
Enz+ve	1.011^{b} *(0.035)*	0.873 *(0.020)*	0.874 *(0.035)*
s.e.d	0.343	0.031	0.035
	P<0.001	NS	P=0.055

Means within a column without a common superscript are significantly different. NS ($P>0.05$)
Data are proportions (SEM) of 3 replicate sample means for each count pooled over thyme oil treatments.
LAB = lactic acid bacteria, COL = coliforms, ANR = total anaerobes.

4.3.7 *Thyme oil inclusion and its effect on the caecal microflora at 19 days*

Caecal coliforms were reduced by 1.5 logs (9.94 to 8.46) by the inclusion of thyme EO in the diets at 1 g kg^{-1}, and by 1 log with 5 g kg^{-1} in the diets ($P=0.028$, **Table 4.20**). This would appear to indicate that thyme EO had an antimicrobial effect in these birds, but not at the inclusion level of 3 g kg^{-1}. Lactic acid bacteria, which are normally considered beneficial in

the gut, were increased by 0.5 log when thyme EO was included at higher concentrations, but there was no effect of the EO on the other bacterial species.

Table 4.20 *Effect of thyme oil inclusion on caecal microflora at 19 days of age*

	Microfloral populations in samples of caecal contents (log_{10} c.f.u. g^{-1})			
Inclusion (g kg^{-1})	*Lactic Acid Bacteria*	*Coliforms*	*Anaerobes*	*C. perfringens*
Control (0)	9.47 *(0.28)*	9.94^{a} *(0.32)*	10.73 *(0.21)*	2.62 *(1.03)*
Thyme (1)	9.58 *(0.28)*	8.46^{b} *(0.44)*	11.32 *(0.20)*	2.23 *(0.75)*
Thyme (3)	10.12 *(0.25)*	9.35^{ab} *(0.19)*	11.21 *(0.26)*	3.81 *(1.04)*
Thyme (5)	10.03 *(0.27)*	8.88^{b} *(0.41)*	10.63 *(0.17)*	2.54 *(0.66)*
s.e.d.	0.352	0.451	0.32	1.062
	NS	P=0.028	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are logarithms of 3 means in colony forming units g^{-1} sample (SEM) pooled over enzyme treatments.

The proportion of lactic acid bacteria to coliforms was altered with the inclusion of thyme EO (P=0.003; **Table 4.21**), but no other bacterial species was affected by the dietary EO inclusion. The proportion of coliforms to caecal anaerobes (COL:ANR) was reduced (P=0.015) by the inclusion of thyme EO at 1 g kg^{-1}, but not at the other inclusion levels.

Table 4.21 *The effect of thyme oil on proportions of caecal microflora at 19 days*

	Proportions of caecal microflora in relation to total anaerobes		
Inclusion (g kg^{-1})	*LAB: COL*	*LAB: ANR*	*COL: ANR*
Control (0)	0.959^{a} *(0.047)*	0.885 *(0.036)*	0.928^{a} *(0.033)*
Thyme (1)	1.145^{b} *(0.056)*	0.850 *(0.038)*	0.751^{b} *(0.049)*
Thyme (3)	1.082^{b} *(0.016)*	0.905 *(0.022)*	0.836^{ab} *(0.023)*
Thyme (5)	1.139^{b} *(0.047)*	0.944 *(0.020)*	0.835^{ab} *(0.036)*
s.e.d.	0.045	0.041	0.046
	P=0.003	NS	P=0.015

Means within a column without a common superscript are significantly different. NS (P>0.05)
Comparisons were done at the p<0.05 level, but the largest treatment differences are as shown.
Data are proportions (SEM) of 3 sample means for each count pooled over enzyme treatments.
LAB = lactic acid bacteria, COL = coliforms, ANR = total anaerobes.

There were no significant interactions between the thyme EO and the carbohydrase with regards to the caecal microbial populations, either when expressed on a log basis (**Table 4.22**), or as ratios of one microbial population to another (**Table 4.23**), with the inclusion of both supplements in the diets.

Table 4.22 *Effects of thyme oil with or without the presence of an enzyme on caecal microflora at 19 days of age*

	Microflora populations in samples of caecal contents (log_{10} c.f.u. g^{-1})			
Inclusion (g kg^{-1})	*Lactic acid bacteria*	*Coliforms*	*Total anaerobes*	*C. perfringens*
Control (0) – Enz	9.80 *(0.39)*	9.43 *(0.22)*	10.94 *(0.34)*	4.60 *(1.16)*
Thyme (1) – Enz	9.99 *(0.49)*	7.96 *(0.26)*	11.21 *(0.29)*	3.62 *(0.57)*
Thyme (3) – Enz	10.16 *(0.14)*	9.37 *(0.26)*	10.95 *(0.29)*	5.20 *(1.31)*
Thyme (5) – Enz	10.24 *(0.36)*	8.27 *(0.45)*	10.69 *(0.33)*	2.83 *(1.49)*
Control (0) – Enz	9.13 *(0.27)*	10.46 *(0.53)*	10.52 *(0.27)*	0.63 *(0.81)*
Thyme (1) – Enz	9.26 *(0.12)*	8.95 *(0.86)*	11.42 *(0.31)*	0.83 *(1.01)*
Thyme (3) – Enz	10.09 *(0.53)*	9.34 *(0.30)*	11.46 *(0.43)*	2.41 *(1.50)*
Thyme (5) – Enz	9.82 *(0.41)*	9.48 *(0.64)*	10.57 *(0.19)*	2.26 *(0.27)*
s.e.d.	0.506	0.648	0.460	1.525
Enz*Thyme	NS	NS	NS	NS

NS (P>0.05)
Data are means (SEM) in colony forming units per gram sample of the logarithms of 3 treatment replicates.

Table 4.23 *Effect of thyme oil either in the presence or absence of an enzyme on the proportions of caecal microflora at 19 days of age*

	Proportions of caecal microflora in relation to total anaerobes		
Inclusion (g kg^{-1})	*LAB: COL*	*LAB: ANR*	*COL: ANR*
Control (0) – Enz	1.041 *(0.026)*	0.900 *(0.062)*	0.865 *(0.045)*
Thyme (1) – Enz	1.241 *(0.023)*	0.887 *(0.066)*	0.712 *(0.043)*
Thyme (3) – Enz	1.086 *(0.016)*	0.928 *(0.021)*	0.856 *(0.022)*
Thyme (5) – Enz	1.240 *(0.038)*	0.958 *(0.007)*	0.773 *(0.028)*
Control (0) + Enz	0.877 *(0.067)*	0.870 *(0.047)*	0.992 *(0.027)*
Thyme (1) + Enz	1.050 *(0.086)*	0.813 *(0.033)*	0.789 *(0.098)*
Thyme (3) + Enz	1.079 *(0.032)*	0.881 *(0.032)*	0.817 *(0.036)*
Thyme (5) + Enz	1.038 *(0.027)*	0.929 *(0.038)*	0.897 *(0.056)*
s.e.d.	0.065	0.060	0.067
Enz*Thyme	NS	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Data are proportions (SEM) of 3 replicate sample means for each count.
LAB = lactic acid bacteria, COL = coliforms, ANR = total anaerobes

4.3.8 Effect of supplementation with thyme oil or enzyme on caecal microflora at 42 days

Samples were analysed from the control diets with or without carbohydrase, and from the diets with 5 g kg^{-1} thyme EO, again with or without the carbohydrase, in order that the main effects of each supplement in the experiment could be observed. This was done to maximise the number of replicates of each sample in the statistical analysis, as it was only possible to analyse 3 sample replicates at 19 days of age. There were no significant effects on the caecal microflora populations with the inclusion of either carbohydrase or thyme EO in the diets (**Table** 4.24), although the thyme EO numerically decreased the caecal enterococci population by 0.5 log, either with or without the presence of the carbohydrase. There was no effect on the proportions of each species within the caeca in relation to total anaerobes (**Table 4.25**).

Table 4.24 *Effect of thyme oil with or without an enzyme on caecal microflora at 42 days*

	Microflora populations in samples of caecal material (log_{10} c.f.u. g^{-1})				
Inclusion (g kg^{-1})	*Lactic acid bacteria*	*Coliforms*	*Total anaerobes*	*Enterococci*	*C. perfringens*
Control (0) - Enz	10.01 *(0.24)*	8.97 *(0.21)*	11.28 *(0.20)*	8.61 *(0.32)*	5.21 *(0.85)*
Control (0) + Enz	10.27 *(0.37)*	8.69 *(0.18)*	11.19 *(0.14)*	8.5 *(0.28)*	5.15 *(0.70)*
Thyme (5) – Enz	9.99 *(0.31)*	8.42 *(0.30)*	10.96 *(0.18)*	8.07 *(0.25)*	4.70 *(0.58)*
Thyme (5) + Enz	10.13 *(0.36)*	8.74 *(0.40)*	11.32 *(0.15)*	8.07 *(0.34)*	4.20 *(0.69)*
s.e.d.	0.457	0.435	0.232	0.417	0.841
Variation source					
Enz	NS	NS	NS	NS	NS
Thyme	NS	NS	NS	NS	NS
Enz*Thyme	NS	NS	NS	NS	NS

Significant differences are shown in each column by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 6 treatment replicates, expressed as log colony forming units g^{-1} sample.

Table 4.25 *Effect of thyme oil with or without an enzyme on bacterial proportions within the caeca at 42 days*

	Proportions of caecal microflora in relation to total anaerobes				
Inclusion (g kg^{-1})	*LAB: COL*	*LAB: ANR*	*COL: ANR*	*ENT: ANR*	*C.PERF: ANR*
Control (0) – Enz	1.120 *(0.046)*	0.887 *(0.015)*	0.797 *(0.028)*	0.764 *(0.026)*	0.467 *(0.08)*
Control (0) + Enz	1.186 *(0.052)*	0.918 *(0.030)*	0.777 *(0.021)*	0.760 *(0.026)*	0.459 *(0.06)*
Thyme (5) + Enz	1.192 *(0.049)*	0.914 *(0.038)*	0.770 *(0.034)*	0.738 *(0.031)*	0.430 *(0.054)*
Thyme (5) + Enz	1.175 *(0.078)*	0.897 *(0.038)*	0.772 *(0.034)*	0.714 *(0.029)*	0.372 *(0.06)*
s.e.d.	0.082	0.045	0.044	0.038	0.074
Variation source					
Enz	NS	NS	NS	NS	NS
Thyme	NS	NS	NS	NS	NS
Enz*Thyme	NS	NS	NS	NS	NS

Significances within columns are indicated by superscripts. NS (P>0.05)
Data are proportions (SEM) based on the means of 6 treatment replicates for each count.
LAB=lactic acid bacteria, COL=coliforms, ANR=Anaerobes, C.PERF=*C. perfringens*, ENT=Enterococci

Confirmatory testing on all bacterial isolates showed negative results for catalase tests, indicating that all the bacteria cultured should be lactic acid bacteria. No bacterial isolates tested positive for either *C. jejuni* or haemolytic *E. coli* in these analyses.

4.3.9 Effects of thyme oil and carbohydrase on dietary nutrient digestibility at 19 days

The effects of thyme EO and carbohydrase supplementation in the diets were measured at the end of each experimental dietary phase (19 and 42 days). The coefficients of apparent DM digestibility (ADMD), apparent OM digestibility (DOMD), apparent digestibility of nitrogen (AND), apparent metabolisability of nitrogen (AMN), apparent metabolisable energy (AME) and also apparent metabolisable energy corrected to zero nitrogen retention (AMEn) were calculated for the birds. The coefficient of metabolisability of energy within the dietary ration was also calculated (AME:GE and AMEn:GE), as well as the contents of digestible OM and N

within the treatment diets. This section presents the results of measurements taken on the ileal samples (ileal digestibility) at 19 days and the excreta samples (total gastrointestinal tract digestibility) at 42 days. Due to the small volume of sample material obtained, it was not possible to measure the GE content of the ileal samples at 19 days, and as a result neither the apparent digestible energy (ADE) nor the ratio of digestible: gross energy (ADE:GE) could be calculated. No significant differences were observed in the digestibility of nutrients at 19 days with the inclusion of a dietary enzyme, the various concentrations of thyme EO or with both supplements together (**Table 4.26**). However, only 3 treatment replicates were analysed and significant differences may have occurred with an increased level of replication.

Table 4.26 *Effect of thyme oil and carbohydrase in diets on the coefficients of apparent ileal digestibility of dry and organic matter (ADMD & DOMD) and also nitrogen (AND), presented with the dietary contents of digestible OM and nitrogen, measured in the ileum of broilers aged 19 days*

DM, OM and N digestibility, and the contents of utilised OM and N from the diets					
Supplement	*ADMD*	*DOMD*	*Digestible OM content in the diet (g kg^{-1} DM)*	*AND*	*Dietary content of digestible N (g kg^{-1} DM)*
Control (0) – Enz	0.557 *(0.044)*	0.551 *(0.045)*	511 *(42)*	0.734 *(0.049)*	28.5 *(1.9)*
Thyme (1) – Enz	0.568 *(0.065)*	0.565 *(0.072)*	527 *(67)*	0.781 *(0.020)*	30.3 *(0.8)*
Thyme (3) – Enz	0.574 *(0.047)*	0.573 *(0.047)*	537 *(44)*	0.736 *(0.043)*	27.8 *(1.6)*
Thyme (5) – Enz	0.579 *(0.064)*	0.572 *(0.066)*	535 *(62)*	0.733 *(0.034)*	27.3 *(1.3)*
Control (0) + Enz	0.540 *(0.055)*	0.541 *(0.057)*	504 *(53)*	0.762 *(0.026)*	28.7 *(1.0)*
Thyme (1) + Enz	0.542 *(0.027)*	0.545 *(0.025)*	508 *(24)*	0.767 *(0.006)*	29.2 *(0.2)*
Thyme (3) + Enz	0.562 *(0.004)*	0.561 *(0.006)*	521 *(5)*	0.759 *(0.016)*	29.2 *(0.6)*
Thyme (5) + Enz	0.633 *(0.023)*	0.636 *(0.024)*	592 *(22)*	0.758 *(0.011)*	28.1 *(0.4)*
Source of variation					
Enz inclusion	NS	NS	NS	NS	NS
Thyme inclusion	NS	NS	NS	NS	NS
Enz*Thyme	NS	NS	NS	NS	NS
s.e.d. 1	0.067	0.070	65.5	0.044	1.667
s.e.d. 2				0.032	1.216
s.e.d. 3				0.033	1.270

Significances are expressed in each column by the presence of superscripts.
Data are the means (SEM) of 3 treatment replicates, and have been corrected for missing values.
Treatment comparisons: s.e.d. 1 refers to treatment comparisons for all diets except those with 1 & 3 g kg^{-1} thyme oil and 5 g kg^{-1} thyme oil + enzyme. s.e.d. 2 refers to the comparison of these named diets with the remaining treatments, and s.e.d. 3 to the comparison of these named diets with each other.

4.3.10 Effects of thyme oil and carbohydrase on dietary nutrient digestibility at 42 days

Carbohydrase inclusion within the diet increased both the AME (P<0.001) and energy metabolisability (AME:GE) (P=0.004) of the diets in broilers at 42 days of age, both with and without a correction to nitrogen equilibrium (**Table 4.27**). The carbohydrase also increased the ADMD (P=0.003) and DOMD (P=0.004), along with the digestible OM content (P=0.004)

of the diet (**Table 4.28**). Both the AMN (P<0.05) and the dietary content of metabolisable N (P=0.007) were increased with carbohydrase.

Table 4.27 *Effect of a dietary carbohydrase on the apparent metabolisable energy (AME) and energy metabolisability (AME:GE) at 42 days in broilers, both with and without a nitrogen correction (AMEn)*

	Apparent metabolisable energy content and coefficient of energy metabolisability			
Supplement	*AME (MJ kg^{-1} DM)*	*AMEn (MJ kg^{-1} DM)*	*AME:GE*	*AMEn:GE*
No Enzyme	13.78^{a} *(0.20)*	13.17^{a} *(0.19)*	0.690^{a} *(0.010)*	0.660^{a} *(0.009)*
Enzyme	14.74^{b} *(0.23)*	14.08^{b} *(0.22)*	0.729^{b} *(0.011)*	0.697^{b} *(0.011)*
s.e.d.	0.258	0.242	0.013	0.012
	P<0.001	P<0.001	P=0.004	P=0.004

Significances within each column are indicated by the presence of different superscripts.
Data are the means (SEM), pooled across the treatments containing thyme oil.

Table 4.28 *Effect of carbohydrase inclusion on the coefficients of apparent digestibility of dry and organic matter (ADMD & DOMD) and the coefficient of metabolisable nitrogen (AMN) of diets fed to broilers at 42 days, with the contents of digestible OM and metabolisable N*

	DM and OM digestibility, N metabolisability and contents of utilised nutrients from the diets				
Supplement	*ADMD*	*DOMD*	*Digestible OM content (g kg^{-1} DM)*	*AMN*	*Metabolisable N content (g kg^{-1} DM)*
Enz -ve	0.649^{a} *(0.009)*	0.671^{a} *(0.009)*	632^{a} *(8)*	0.515^{a} *(0.012)*	16.6^{a} *(0.4)*
Enz +ve	0.684^{b} *(0.011)*	0.706^{b} *(0.011)*	664^{b} *(10)*	0.548^{b} *(0.014)*	18.1^{b} *(0.5)*
s.e.d.	0.011	0.011	10.44	0.0156	0.515
	P=0.003	P=0.004	P=0.004	P<0.05	P=0.007

Significances within each column are indicated by the presence of different superscripts.
Data are the means (SEM), pooled across the treatments containing thyme EO.

Thyme EO inclusion in the diets at 5 g kg^{-1} increased the AME by 0.74 MJ kg^{-1} DM (P=0.055), and the AMEn by 0.68 MJ kg^{-1} DM (P<0.05) (**Table 4.29**). There was a tendency (P=0.095) for the inclusion of thyme EO in diets to increase the metabolisability of energy (AMEn:GE) in broilers fed these diets at 42 days.

Table 4.29 *Effect of thyme oil inclusion on the apparent metabolisable energy (AME) and energy metabolisability (AME:GE) of diets at 42 days, with a nitrogen correction (AMEn)*

	Apparent metabolisable energy content and coefficient of energy metabolisability			
Inclusion (g kg^{-1})	*AME (MJ kg^{-1} DM)*	*AMEn (MJ kg^{-1} DM)*	*AME:GE*	*AMEn:GE*
Control (0)	13.95 *(0.39)*	13.35^{b} *(0.37)*	0.699 *(0.019)*	0.669 *(0.018)*
Thyme (1)	14.52 *(0.37)*	13.87^{ab} *(0.34)*	0.721 *(0.018)*	0.689 *(0.016)*
Thyme (3)	13.87 *(0.33)*	13.24^{b} *(0.31)*	0.691 *(0.017)*	0.659 *(0.016)*
Thyme (5)	14.69 *(0.20)*	14.03^{a} *(0.19)*	0.728 *(0.010)*	0.696 *(0.009)*
s.e.d.	0.344	0.322	0.017	0.016
	P=0.055	P<0.05	NS	P=0.095

Significances within each column are indicated by the presence of different superscripts. NS (P>0.05)
Data are the means (SEM), pooled across the treatments containing enzyme.

There was a trend (P=0.08) for thyme EO inclusion at concentrations of 3 and 5 g kg^{-1} to increase the ADMD of the diet at 42 days (**Table 4.30**). There was no effect of thyme EO on the coefficients of DOMD or AMN, and therefore also no effect on the digestible OM and metabolisable N contents of the diets.

Table 4.30 *Effect of thyme oil on the coefficients of apparent dry and organic matter digestibility (ADMD & DOMD) and apparent metabolisability of nitrogen (AMN) in diets at 42 days, presented with the digestible OM and metabolisable N contents in the diet*

	DM and OM digestibility, N metabolisability and contents of utilised nutrients from the diets				
Inclusion (g kg^{-1})	*ADMD*	*DOMD*	*Digestible OM content of diet (g kg^{-1} DM)*	*AMN*	*Metabolisable N in diet (g kg^{-1} DM)*
Control(0)	0.657 *(0.016)*	0.680 *(0.016)*	640 *(15)*	0.514 *(0.019)*	16.6 *(0.6)*
Thyme (1)	0.678 *(0.017)*	0.700 *(0.018)*	659 *(17)*	0.542 *(0.024)*	17.6 *(0.8)*
Thyme (3)	0.649 *(0.015)*	0.671 *(0.015)*	632 *(15)*	0.524 *(0.020)*	17.3 *(0.7)*
Thyme (5)	0.682 *(0.009)*	0.702 *(0.009)*	662 *(9)*	0.544 *(0.012)*	17.9 *(0.4)*
s.e.d.	0.014	0.015	13.93	0.021	0.686
	P=0.082	NS	NS	NS	NS

Significances within each column are indicated by the presence of different superscripts. NS (P>0.05)
Data are the means (SEM), pooled across the treatments containing enzyme.

When both supplements were included together in diets, the energy availability was improved, with the enzyme providing the bulk of the contribution in increasing both AME and AME:GE with and without a nitrogen correction (**Table 4.31**). In each case, the best result was obtained with the inclusion of 1 g kg^{-1} thyme EO + enzyme, and the AME and AMEn of the diet with 3 g kg^{-1} thyme EO and enzyme was inconsistent with the other data.

Table 4.31 *Effect of the inclusion of thyme oil with or without a carbohydrase on the apparent metabolisable energy (AME) and energy metabolisability (AME:GE) of diets fed to broilers at 42 days, with a correction to nitrogen equilibrium (AMEn)*

	Apparent metabolisable energy content and coefficient of energy metabolisability			
Supplement (g kg^{-1})	*AME (MJ kg^{-1} DM)*	*AMEn (MJ kg^{-1} DM)*	*AME:GE*	*AMEn:GE*
Control (0) – Enz	13.02^{d} *(0.53)*	12.44^{d} *(0.49)*	0.656^{d} *(0.027)*	0.627^{d} *(0.025)*
Thyme (1) – Enz	13.51^{cd} *(0.37)*	12.95^{cd} *(0.35)*	0.674^{cd} *(0.019)*	0.646^{cd} *(0.017)*
Thyme (3) – Enz	14.10^{bc} *(0.14)*	13.46^{bc} *(0.13)*	0.706^{bcd} *(0.007)*	0.674^{bc} *(0.007)*
Thyme (5) – Enz	14.48^{bc} *(0.25)*	13.83^{bc} *(0.24)*	0.725^{abc} *(0.012)*	0.692^{abc} *(0.012)*
Control (0) + Enz	14.89^{ab} *(0.18)*	14.25^{ab} *(0.16)*	0.742^{ab} *(0.009)*	0.710^{ab} *(0.008)*
Thyme (1) + Enz	15.52^{a} *(0.24)*	14.79^{a} *(0.23)*	0.768^{a} *(0.012)*	0.732^{a} *(0.011)*
Thyme (3) + Enz	13.65^{cd} *(0.66)*	13.02^{cd} *(0.62)*	0.676^{cd} *(0.033)*	0.645^{cd} *(0.030)*
Thyme (5) + Enz	14.89^{ab} *(0.32)*	14.24^{ab} *(0.30)*	0.731^{ab} *(0.016)*	0.699^{ab} *(0.015)*
s.e.d.	0.494	0.463	0.025	0.023
Enz*Thyme	P=0.002	P=0.002	P≤0.001	P≤0.001

Significances within each column are indicated by the presence of different superscripts.
Data are the means (SEM) of 6 replicates for each treatment.

When there was no carbohydrase present, both the coefficients of apparent DM and OM digestibility were improved at 5 g kg^{-1} thyme EO inclusion. Both of these parameters also increased significantly for each treatment when carbohydrase was included in the diets, compared to those treatments without enzyme (**Table 4.32**). The greatest increase in digestibility was observed with the 1 g kg^{-1} thyme EO + enzyme treatment ($p<0.001$), supporting an interaction between both supplements, with the enzyme producing the bulk of the effects.

Table 4.32 *Effect of thyme oil inclusion either with or without a carbohydrase on the coefficients of apparent digestibility of dry and organic matter (ADMD & DOMD) in diets fed to broilers at 42 days, presented with the digestible OM content in the diets*

	Coefficients of DM and OM digestibility, with the dietary contents of digestible OM		
Inclusion (g kg^{-1})	*ADMD*	*DOMD*	*Digestible OM content of diet (g kg^{-1} DM)*
Control (0) – Enz	0.621^{c} *(0.022)*	0.644^{d} *(0.022)*	606^{c} *(21)*
Thyme (1) – Enz	0.631^{c} *(0.017)*	0.652^{d} *(0.017)*	615^{c} *(16)*
Thyme (3) – Enz	0.664^{bc} *(0.009)*	0.687^{bcd} *(0.009)*	646^{bc} *(8)*
Thyme (5) – Enz	0.682^{b} *(0.010)*	0.702^{abc} *(0.010)*	662^{b} *(10)*
Control (0) + Enz	0.694^{ab} *(0.008)*	0.718^{ab} *(0.008)*	675^{ab} *(8)*
Thyme (1) + Enz	0.725^{a} *(0.010)*	0.747^{a} *(0.010)*	704^{a} *(10)*
Thyme (3) + Enz	0.634^{c} *(0.030)*	0.656^{cd} *(0.030)*	617^{c} *(28)*
Thyme (5) + Enz	0.682^{b} *(0.017)*	0.702^{bc} *(0.016)*	662^{b} *(15)*
s.e.d.	0.021	0.021	20.00
Enz* Thyme	P<0.001	P<0.001	P<0.001

Significance was tested at the $p<0.05$ level, but the largest differences are as shown, and is expressed in each column by non-identical superscripts.
Data are the means (SEM) of 6 treatment replicates.

The diet with thyme EO at 1 g kg^{-1} and carbohydrase was observed to have the highest coefficient of AMN (P=0.003), and the highest metabolisable dietary N content (P=0.004), suggesting the presence of an interaction between both supplements (**Table 4.33**). Nitrogen utilisation appeared to be greatest at a higher concentration of dietary thyme EO, or a low concentration of thyme EO with carbohydrase.

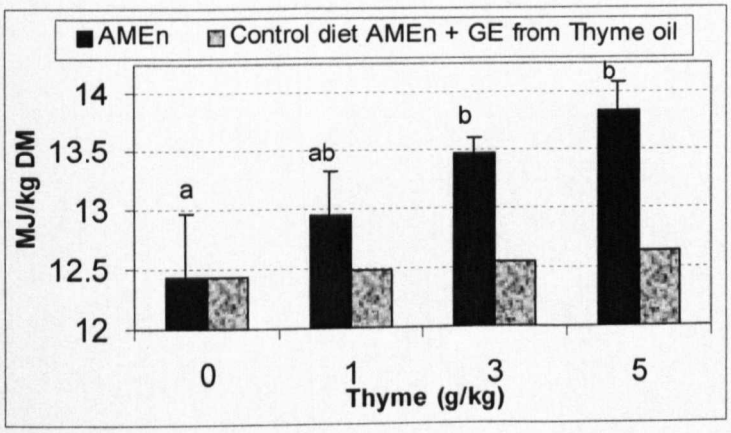
The gross energy of thyme EO was determined to be 39.139 MJ kg^{-1}. At each increasing level of dietary supplementation, thyme EO increased the AMEn by around 0.5 MJ kg^{-1} DM. This increase in AMEn was significant with the inclusion of 3 and 5 g kg^{-1} thyme EO, compared to the control diet ($P<0.05$) (**Figure 4.2**). The increase in AMEn seen with thyme EO inclusion in diets was much greater than the contribution of the thyme EO itself to the GE content of the diet.

Table 4.33 *Effect of thyme oil and carbohydrase inclusion on the coefficient of apparent metabolisable nitrogen (AMN) and the metabolisable N content of the diets at 42 days*

	Coefficient of N metabolisability and dietary metabolisable N content	
Inclusion (g kg^{-1})	*AMN*	*Metabolisable N content of diet (g kg^{-1})*
Control (0) – Enz	0.490^{bc} *(0.033)*	15.8^{cd} *(1.0)*
Thyme (1) – Enz	0.480^{c} *(0.024)*	15.3^{d} *(0.8)*
Thyme (3) – Enz	0.541^{bc} *(0.011)*	17.5^{bc} *(0.4)*
Thyme (5) – Enz	0.550^{ab} *(0.013)*	17.9^{b} *(0.4)*
Control (0) + Enz	0.538^{bc} *(0.017)*	17.4^{bc} *(0.6)*
Thyme (1) + Enz	0.605^{a} *(0.017)*	19.9^{a} *(0.5)*
Thyme (3) + Enz	0.508^{bc} *(0.040)*	17.2^{bcd} *(1.3)*
Thyme (5) + Enz	0.539^{bc} *(0.020)*	17.9^{b} *(0.7)*
s.e.d.	0.03	0.985
Enz*Thyme	P=0.003	P=0.004

Significance was tested at the $p<0.05$ level but the largest differences are as shown, and expressed in each column by the presence of different superscripts. Data are means (SEM) of 6 treatment replicates.

Figure 4.2 *Apparent metabolisable energy (AMEn) as affected by an increasing dietary thyme oil concentration and contribution of the thyme oil to the dietary energy content*



4.3.11 Effect of thyme oil and carbohydrase on the endogenous losses in broilers measured by concentrations of sialic acid in the ileal digesta or excreta

The dietary carbohydrase increased the concentration of sialic acid in relation to the feed intake at 19 days (P=0.012), but not at 42 days in broilers (**Table 4.34**). Thus, the carbohydrase caused a greater endogenous cell loss within the younger birds and may have increased energy expenditure to replace lost tissue.

Thyme EO inclusion in the diets had no effect on the sialic acid concentration, at either 19 or 42 days (**Table 4.35**). There was a tendency (P=0.065) towards an interaction effect between the supplements at 42 days on the concentration of sialic acid, but no effect at 19 days (**Table**

4.36). However, this effect occurred mainly within both treatments including 3 g kg^{-1} thyme EO, and was not clear enough to be explained in terms of effects on the other treatments.

Table 4.34 *Effect of a dietary carbohydrase on the sialic acid concentration as a measure of endogenous cell loss in broilers*

	Sialic acid concentration as a proportion of total feed intake (mg SA g^{-1} intake)	
	19 days (Ileal digesta)	*42 days (Excreta)*
Enz -ve	74^{a} *(3)*	157 *(5)*
Enz +ve	90^{b} *(4)*	150 *(5)*
s.e.d.	5.55	5.82
	P=0.012	NS

Significances are expressed in each column by the use of different superscripts. NS (P>0.05)
Data are the mean (SEM), pooled across the treatments containing thyme EO.

Table 4.35 *Effect of thyme oil inclusion in diets on the sialic acid concentration as a measure of endogenous cell loss in broilers*

	Sialic acid concentration in relation to total feed intake (mg SA g^{-1} intake)	
Inclusion (g kg^{-1})	*19 days (Ileal digesta)*	*42 days (Excreta)*
Control (0)	83 *(5)*	148 *(6)*
Thyme (1)	87 *(8)*	154 *(7)*
Thyme (3)	80 *(3)*	157 *(8)*
Thyme (5)	77 *(8)*	156 *(5)*
s.e.d.	7.40	7.76
	NS	NS

Significances are expressed in each column by the use of different superscripts. NS (P>0.05)
Data are the mean (SEM), pooled across the treatments containing carbohydrase.

Table 4.36 *Effect of dietary thyme oil and carbohydrase inclusion on the sialic acid concentration as a measure of endogenous cell loss in broilers*

	Sialic acid concentration in relation to feed intake (mg SA g^{-1} intake)	
Treatment (g kg^{-1})	*19 days (Ileal digesta)*	*42 days (Excreta)*
Control (0) – Enz	76.2 *(5.7)*	145 *(9)*
Thyme (1) –Enz	73.8 *(11.9)*	150 *(11)*
Thyme (3) – Enz	78.9 *(2.7)*	172 *(11)*
Thyme (5) – Enz	66.8 *(6.4)*	163 *(6)*
Control (0) + Enz	89.0 *(4.8)*	151 *(9)*
Thyme (1) + Enz	100.5 *(4.2)*	158 *(11)*
Thyme (3) + Enz	82.0 *(6.5)*	143 *(10)*
Thyme (5) + Enz	87.4 *(11.1)*	149 *(8)*
s.e.d.	10.46	11.14
Enz	P=0.012	NS
Thy	NS	NS
Enz*Thy	NS	P=0.065

Significances were displayed in each column by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 3 treatment replicates for ileal digesta and 6 treatment replicates for excreta.

4.3.12 Amino acid digestibility in broilers fed diets with or without thyme oil and enzymes

There was no effect of treatment on the apparent coefficients of AA digestibility in birds aged 19 days (**Table 4.37**), although the analysis of more treatment replicates may have elucidated some treatment effects. However, the apparent digestibility coefficient of threonine was increased by 11.71% in birds fed the control diet with carbohydrase, and by 21.33% in birds fed diets containing 5 g kg^{-1} thyme EO and carbohydrase, compared to the non-supplemented control treatment at 19 days. Birds fed diets with 5 g kg^{-1} thyme EO and carbohydrase showed an increase in the coefficient of digestibility of aspartic acid (12.4%) and tyrosine (10.8%), when compared to birds fed the control diet.

Table 4.37 *Effect of dietary inclusion of thyme oil and carbohydrase on the coefficients of apparent ileal amino acid digestibility measured in broilers at 19 days of age*

Apparent digestibility of amino acids in broilers at 19 days									
	Diets without enzyme				*Diets with enzyme @ 0.5 g kg^{-1}*				*s.e.d.*
Thyme (g kg^{-1})	Con (0)	1	3	5	Con (0)	1	3	5	
Alanine	0.737	0.740	0.730	0.745	0.760	0.751	0.730	0.791	0.042
	(0.055)	*(0.038)*	*(0.022)*	*(0.054)*	*(0.014)*	*(0.020)*	*(0.018)*	*(0.004)*	NS
Aspartic acid	0.720	0.755	0.741	0.774	0.777	0.764	0.741	0.809	0.042
	(0.065)	*(0.031)*	*(0.032)*	*(0.036)*	*(0.010)*	*(0.011)*	*(0.012)*	*(0.009)*	NS
Glutamic acid	0.841	0.866	0.861	0.881	0.881	0.873	0.856	0.894	0.025
	(0.041)	*(0.019)*	*(0.019)*	*(0.011)*	*(0.008)*	*(0.005)*	*(0.008)*	*(0.005)*	NS
Serine	0.740	0.753	0.747	0.762	0.772	0.759	0.745	0.813	0.038
	(0.052)	*(0.026)*	*(0.022)*	*(0.047)*	*(0.011)*	*(0.014)*	*(0.011)*	*(0.007)*	NS
Tyrosine	0.722	0.762	0.738	0.761	0.783	0.755	0.744	0.800	0.046
	(0.059)	*(0.039)*	*(0.025)*	*(0.048)*	*(0.019)*	*(0.010)*	*(0.009)*	*(0.008)*	NS
Σ Disp AA	**0.786**	**0.811**	**0.802**	**0.825**	**0.829**	**0.816**	**0.798**	**0.851**	**0.032**
	(0.050)	***(0.025)***	***(0.023)***	***(0.026)***	***(0.009)***	***(0.008)***	***(0.010)***	***(0.006)***	**NS**
Arginine	0.808	0.832	0.832	0.842	0.848	0.833	0.815	0.862	0.029
	(0.044)	*(0.029)*	*(0.016)*	*(0.018)*	*(0.016)*	*(0.007)*	*(0.006)*	*(0.006)*	NS
Histidine	0.795	0.822	0.805	0.818	0.837	0.821	0.803	0.864	0.033
	(0.048)	*(0.027)*	*(0.022)*	*(0.035)*	*(0.008)*	*(0.019)*	*(0.019)*	*(0.007)*	NS
Isoleucine	0.765	0.792	0.770	0.794	0.805	0.791	0.766	0.821	0.038
	(0.048)	*(0.036)*	*(0.026)*	*(0.043)*	*(0.014)*	*(0.011)*	*(0.012)*	*(0.005)*	NS
Leucine	0.788	0.805	0.787	0.805	0.809	0.797	0.783	0.831	0.034
	(0.042)	*(0.028)*	*(0.019)*	*(0.041)*	*(0.014)*	*(0.013)*	*(0.012)*	*(0.002)*	NS
Lysine	0.838	0.846	0.838	0.839	0.848	0.845	0.842	0.880	0.027
	(0.036)	*(0.016)*	*(0.016)*	*(0.035)*	*(0.008)*	*(0.016)*	*(0.011)*	*(0.003)*	NS
Phenylalanine	0.806	0.826	0.813	0.821	0.827	0.820	0.802	0.849	0.031
	(0.041)	*(0.023)*	*(0.017)*	*(0.038)*	*(0.012)*	*(0.012)*	*(0.011)*	*(0.001)*	NS
Threonine	0.572	0.600	0.586	0.621	0.639	0.619	0.589	0.694	0.067
	(0.067)	*(0.047)*	*(0.032)*	*(0.086)*	*(0.020)*	*(0.022)*	*(0.018)*	*(0.028)*	NS
Valine	0.728	0.759	0.730	0.762	0.777	0.752	0.728	0.795	0.044
	(0.055)	*(0.041)*	*(0.030)*	*(0.047)*	*(0.019)*	*(0.016)*	*(0.014)*	*(0.007)*	NS
Σ Indisp AA	**0.764**	**0.784**	**0.770**	**0.787**	**0.796**	**0.782**	**0.768**	**0.825**	**0.036**
	(0.049)	***(0.029)***	***(0.024)***	***(0.043)***	***(0.011)***	***(0.015)***	***(0.011)***	***(0.003)***	**NS**
Σ Total AA	**0.780**	**0.802**	**0.790**	**0.810**	**0.816**	**0.803**	**0.788**	**0.841**	**0.033**
	(0.048)	***(0.027)***	***(0.024)***	***(0.034)***	***(0.009)***	***(0.012)***	***(0.010)***	***(0.004)***	**NS**

Significant differences in each row are indicated by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 3 treatment replicates.

In the analysis of the data at 42 days, one pen was removed, due to a large residual standard error (1 g kg^{-1} thyme EO + enzyme, pen 48). The experimental design complicated the description of a clear treatment effect on the digestibility of AA in the birds. With unequal numbers of treatment replicates for diets with carbohydrase in each room of the facility, the information on variation within the dataset was split into two levels, 89% and 11%, and most of the information is taken from the 89% level.

At the 11% level of variation, the inclusion of carbohydrase in diets increased glutamic acid, leucine, lysine, phenylalanine and also total AA digestibilities at 42 days (all $P<0.05$; **Table 4.38**). The digestibility of alanine (11% level; $P=0.07$) was also increased with carbohydrase inclusion. These observed differences at the 11% level were due to its extremely low residual mean square (r.m.s.), whereas at the 89% level, the larger r.m.s. described most of the experimental variation. The inclusion of carbohydrase resulted in a tendency for increased threonine ($P=0.055$), glutamic acid ($P=0.07$), tyrosine ($P=0.085$) and valine ($P=0.094$) digestibilities in the birds at the 89% level. Unfortunately, these two levels cannot be combined in Genstat, and are thus not overall effects of treatment. There were no effects of thyme EO inclusion in diets on the digestibility coefficients of AA's in broilers at 42 days, and no interactions between the factors.

Table 4.38 *Effect of thyme oil and carbohydrase in diets on the apparent digestibility coefficients of amino acids, measured in broiler excreta at 42 days*

	Apparent digestbility of amino acids in broilers at 42 days of age													
Thyme (gkg^{-1})	Cont (0)	Thy (1)	Thy (3)	Thy 5	Cont (0)+E	Thy (1)+E	Thy (3)+E	Thy (5)+E	*Enz (11%)*	*Enz (89%)*	*Thy*	*T*E*	*s.e.d.*	*s.e.d. 2*
Alanine	0.759	0.760	0.746	0.768	0.784	0.794	0.749	0.778	P=0.07	NS	NS	NS	0.025	0.026
	(0.016)	*(0.028)*	*(0.014)*	*(0.011)*	*(0.012)*	*(0.030)*	*(0.017)*	*(0.011)*						
Aspartic acid	0.804	0.810	0.810	0.816	0.833	0.805	0.814	0.831	NS	NS	NS	NS	0.023	0.024
	(0.012)	*(0.021)*	*(0.014)*	*(0.007)*	*(0.008)*	*(0.021)*	*(0.011)*	*(0.010)*						
Glutamic acid	0.895	0.898	0.896	0.902	0.917	0.919	0.908	0.916	P=0.011	P=0.07	NS	NS	0.010	0.010
	(0.006)	*(0.010)*	*(0.007)*	*(0.004)*	*(0.004)*	*(0.010)*	*(0.006)*	*(0.006)*						
Serine	0.817	0.820	0.817	0.814	0.837	0.848	0.821	0.829	NS	NS	NS	NS	0.017	0.018
	(0.010)	*(0.019)*	*(0.011)*	*(0.008)*	*(0.008)*	*(0.017)*	*(0.012)*	*(0.009)*						
Tyrosine	0.790	0.800	0.791	0.814	0.831	0.823	0.801	0.827	NS	P=0.085	NS	NS	0.018	0.019
	(0.014)	*(0.017)*	*(0.014)*	*(0.009)*	*(0.008)*	*(0.020)*	*(0.011)*	*(0.010)*						
Σ Disp	**0.851**	**0.855**	**0.851**	**0.859**	**0.871**	**0.852**	**0.855**	**0.868**	**NS**	NS	NS	NS	0.018	0.019
	(0.009)	***(0.015)***	***(0.010)***	***(0.006)***	***(0.006)***	***(0.016)***	***(0.009)***	***(0.008)***						
Arginine	0.878	0.880	0.884	0.888	0.898	0.903	0.885	0.889	NS	NS	NS	NS	0.011	0.012
	(0.001)	*(0.011)*	*(0.008)*	*(0.006)*	*(0.005)*	*(0.010)*	*(0.008)*	*(0.009)*						
Histidine	0.874	0.864	0.869	0.871	0.882	0.893	0.871	0.872	NS	NS	NS	NS	0.013	0.014
	(0.009)	*(0.010)*	*(0.010)*	*(0.008)*	*(0.008)*	*(0.012)*	*(0.009)*	*(0.012)*						
Isoleucine	0.809	0.809	0.814	0.827	0.838	0.839	0.823	0.837	NS	NS	NS	NS	0.018	0.020
	(0.014)	*(0.017)*	*(0.014)*	*(0.009)*	*(0.008)*	*(0.018)*	*(0.012)*	*(0.012)*						
Leucine	0.815	0.818	0.816	0.829	0.852	0.852	0.830	0.848	P<0.05	NS	NS	NS	0.018	0.019
	(0.011)	*(0.019)*	*(0.012)*	*(0.007)*	*(0.006)*	*(0.018)*	*(0.011)*	*(0.009)*						
Lysine	0.867	0.865	0.868	0.867	0.888	0.892	0.855	0.871	P<0.05	NS	NS	NS	0.016	0.017
	(0.007)	*(0.019)*	*(0.008)*	*(0.007)*	*(0.007)*	*(0.019)*	*(0.012)*	*(0.007)*						
Phenylalanine	0.841	0.843	0.842	0.855	0.874	0.875	0.852	0.868	P<0.05	NS	NS	NS	0.015	0.016
	(0.009)	*(0.017)*	*(0.010)*	*(0.006)*	*(0.005)*	*(0.016)*	*(0.011)*	*(0.007)*						
Threonine	0.653	0.673	0.654	0.658	0.712	0.712	0.667	0.697	NS	P=0.055	NS	NS	0.030	0.031
	(0.019)	*(0.035)*	*(0.020)*	*(0.015)*	*(0.013)*	*(0.034)*	*(0.014)*	*(0.011)*						
Valine	0.772	0.776	0.776	0.793	0.810	0.806	0.791	0.807	NS	P=0.094	NS	NS	0.021	0.022
	(0.017)	*(0.020)*	*(0.016)*	*(0.010)*	*(0.009)*	*(0.021)*	*(0.013)*	*(0.013)*						
Σ Indisp	**0.820**	**0.823**	**0.822**	**0.831**	**0.847**	**0.824**	**0.824**	**0.839**	**NS**	NS	NS	NS	0.021	0.022
	(0.011)	***(0.018)***	***(0.012)***	***(0.007)***	***(0.007)***	***(0.018)***	***(0.011)***	***(0.009)***						
Σ Total AA	**0.833**	**0.836**	**0.834**	**0.842**	**0.863**	**0.865**	**0.844**	**0.858**	**P<0.05**	NS	NS	NS	0.016	0.016
	(0.010)	***(0.017)***	***(0.011)***	***(0.006)***	***(0.006)***	***(0.017)***	***(0.010)***	***(0.008)***						

Data are means (SEM) of the digestibility coefficients for each amino acid, based on 6 treatment replicates, and significance in each row is indicated by the presence of superscripts.
s.e.d. values were corrected after the removal of 1 replicate from the Thy (1) + Enzyme treatment, thus s.e.d. 2 should be used only in the comparison of means on this treatment with the others.

4.3.13 Effect of inclusion of thyme oil and carbohydrase on caecal fermentation profiles

When a carbohydrase was included in the diet, the caecal concentration of acetic acid in broilers was reduced by 27.4% (P=0.021), lactic acid by 57.9% (P=0.015) and *n*-butyric acid by 46% (P=0.037) respectively (**Table 4.39**). The caecal concentration of propionic acid was also reduced by 20.6% when a carbohydrase was added to the diet. This resulted in an overall reduction of 37.6% in total caecal volatile fatty acids (VFA) (P=0.009).

Table 4.39 *Effect of carbohydrase in diets on volatile fatty acid concentrations (VFA) in the caeca of broilers at 19 days of age*

	Concentrations of caecal VFA (g kg^{-1})				
	Acetic Acid	*Lactic Acid*	*n-Butyric Acid*	*Propionic Acid*	*TOTAL VFA*
Enz –ve	3.53^{a} *(0.28)*	1.07^{a} *(0.24)*	2.00^{a} *(0.27)*	0.36 *(0.04)*	7.10^{a} *(0.63)*
Enz +ve	2.56^{b} *(0.24)*	0.45^{b} *(0.06)*	1.08^{b} *(0.24)*	0.29 *(0.04)*	4.43^{b} *(0.50)*
s.e.d.	0.375	0.226	0.400	0.064	0.882
	P=0.021	P=0.015	P=0.037	NS	P=0.009

Significances are expressed in each column by the use of different superscripts. NS (P>0.05)
Data are the mean (SEM), pooled across the treatments containing thyme oil.

Carbohydrase inclusion in the diets increased the percentage of acetic acid by 8.4% (P<0.05) in relation to the total VFA. However, the addition of carbohydrase had no effect on the levels of lactic, n-butyric and propionic acid and actually tended (P=0.094) to decrease the proportions of the minor VFA (isovaleric, valeric and isobutyric acids) (**Table 4.40**). The carbohydrase appears to have decreased caecal microbial activity, as acetic acid represented the most abundant caecal fermentation product.

Table 4.40 *Effect of a dietary carbohydrase on individual caecal VFA concentrations in broilers at 19 days of age, where each is presented as a percentage of the total*

	Proportions (%) of individual VFA in relation to total VFA concentration				
	Acetic Acid	*Lactic Acid*	*n-Butyric Acid*	*Propionic Acid*	*Other*
Enz –ve	51.2^{a} *(2.9)*	14.3 *(2.6)*	27.4 *(2.1)*	5.19 *(0.5)*	1.84 *(0.4)*
Enz +ve	59.6^{b} *(2.2)*	12.0 *(2.3)*	20.7 *(3.5)*	6.76 *(0.8)*	0.85 *(0.3)*
s.e.d.	3.84	3.18	4.39	0.914	0.55
	P<0.05	NS	NS	NS	P=0.094

Significances are expressed in each column by the use of different superscripts. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing thyme oil.

Thyme EO inclusion in the diet had no significant effect on the caecal VFA concentrations at 19 days (**Table 4.41**). However, the lactic acid concentration was doubled in the caeca of broilers fed diets with thyme EO at 3 g kg^{-1} compared to the other treatments. There was an

increase of 14% in the total concentration of caecal VFA in birds fed diets with 3 g kg^{-1} thyme EO, when compared to those birds fed the control treatment.

Table 4.41 *Effect of thyme oil inclusion in the diet on VFA concentrations in the caeca of broilers at 19 days of age*

	Concentrations of caecal VFA (g kg^{-1})				
Inclusion (g kg^{-1})	*Acetic Acid*	*Lactic Acid*	*n-Butyric Acid*	*Propionic Acid*	*TOTAL VFA*
Control (0)	3.15 *(0.67)*	0.69 *(0.17)*	1.52 *(0.58)*	0.30 *(0.07)*	5.73 *(1.47)*
Thyme (1)	2.90 *(0.43)*	0.63 *(0.15)*	1.34 *(0.50)*	0.32 *(0.04)*	5.25 *(1.00)*
Thyme (3)	3.07 *(0.16)*	1.16 *(0.51)*	1.79 *(0.11)*	0.34 *(0.04)*	6.53 *(0.56)*
Thyme (5)	3.05 *(0.24)*	0.55 *(0.09)*	1.51 *(0.30)*	0.35 *(0.07)*	5.54 *(0.53)*
s.e.d.	0.500	0.302	0.533	0.085	1.177
	NS	NS	NS	NS	NS

Significance is shown in each column by the presence of different superscripts. NS (P>0.05)
Data are the mean (SEM), pooled across the treatments containing carbohydrase.

There were no significant effects due to the inclusion of thyme EO in the diets at 19 days on the proportions of caecal VFA (**Table 4.42**). The inclusion of thyme EO in diets at 3 g kg^{-1} decreased the percentage of acetic acid in the caeca of broilers by 11.1%, but increased the percentages of lactic acid, n-butyric acid and minor VFA (other) by 3, 6.7 and 1.5% respectively, compared to birds fed the control treatment, indicating a selective effect of thyme EO on fermentation.

Table 4.42 *Effect of thyme oil in diets on the individual caecal VFA concentrations in broilers at 19 days of age, where each is presented as a percentage of the total*

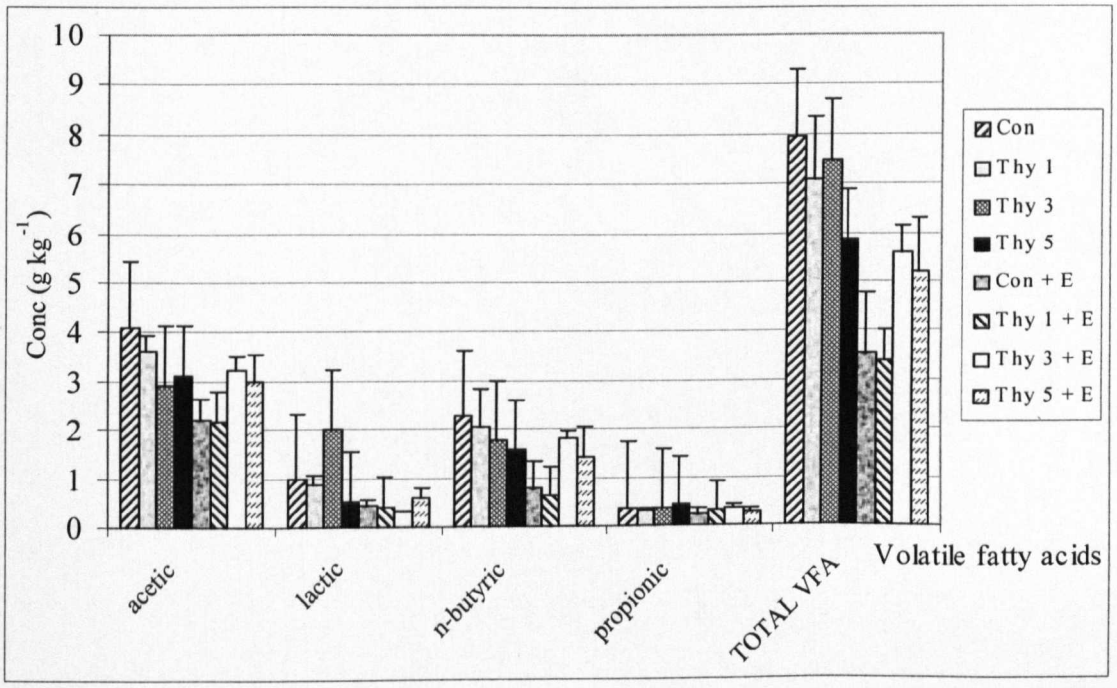
	Proportions (%) of individual VFA in relation to total VFA concentration				
	Acetic Acid	*Lactic Acid*	*n-Butyric acid*	*Propionic acid*	*Other*
Control (0)	59.8 *(5.0)*	12.7 *(1.9)*	21.3 *(5.0)*	5.3 *(0.6)*	0.8 *(0.4)*
Thyme (1)	57.4 *(3.5)*	12.8 *(2.5)*	21.4 *(5.1)*	7.3 *(1.6)*	1.1 *(0.4)*
Thyme (3)	48.7 *(4.4)*	15.7 *(5.7)*	28.0 *(2.0)*	5.2 *(0.6)*	2.3 *(0.8)*
Thyme (5)	55.8 *(1.7)*	11.3 *(3.4)*	25.6 *(4.1)*	6.1 *(0.9)*	1.2 *(0.4)*
s.e.d.	5.13	4.24	5.86	1.22	0.74
	NS	NS	NS	NS	NS

Significances are expressed in each column by the presence of different superscripts. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing carbohydrase.

The presence or absence of the dietary enzyme had a substantial effect on the caecal lactic acid concentration. There was a tendency (P=0.055) for caecal lactic acid concentrations to be increased in birds fed the diet with 3 g kg^{-1} thyme EO at 19 days, compared to all other treatments (**Figure 4.3**). There appeared to be a generally higher caecal VFA concentration in birds when the diets were fed without carbohydrase at the lower inclusion levels of thyme EO.

However, this was not the case at higher inclusion levels of thyme EO. The high variation within the data due to the small number of replicates may have accounted for some of the effects observed, but the lack of interaction between the dietary supplements suggests that each acted independently.

Figure 4.3 *Effect of thyme oil and carbohydrase inclusion in diets on VFA concentrations in the caeca of broiler chicks at 19 days of age*



When the diets containing 3 g kg^{-1} thyme EO were fed with or without carbohydrase, the large difference in the caecal lactic acid concentration was confusing, but the data indicated an interaction between the dietary supplements in this treatment at 19 days ($P<0.05$; **Table 4.43**). However, in both treatments showing significant differences, namely those birds fed with 5 g kg^{-1} thyme EO and 3 g kg^{-1} thyme EO with carbohydrase in their diets, the variation around the mean was small compared to the other treatments. Carbohydrase inclusion in the diet doubled the percentage of propionic acid in the caeca, in birds fed diets with 1 g kg^{-1} thyme EO ($P=0.073$), but not in any other treatment. There were no other effects due to the dietary treatments, and the use of only 3 replicates in this analysis may have masked some effects, due to the higher level of variation in the data.

Table 4.43 *Effect of thyme oil and carbohydrase in diets on caecal VFA concentrations in broilers at 19 days of age, where each is presented as a percentage of the total*

	Proportions (%) of individual VFA in relation to total VFA concentration				
Treatment	*Acetic acid*	*Lactic acid*	*n-Butyric acid*	*Propionic acid*	*Other*
Cont – Enz	54.1 *(5.1)*	12.1^{ab} *(2.1)*	28.0 *(4.1)*	4.6 *(0.3)*	1.2 *(0.5)*
Thy (1) – Enz	54.6 *(7.1)*	11.5^{ab} *(1.4)*	28.1 *(8.0)*	4.6 *(0.6)*	1.1 *(0.3)*
Thy (3) – Enz	41.3 *(6.1)*	25.3^{a} *(7.3)*	25.5 *(3.0)*	4.5 *(0.5)*	3.3 *(1.4)*
Thy (5) – Enz	54.8 *(3.1)*	8.2^{b} *(0.9)*	28.2 *(1.9)*	7.0 *(1.6)*	1.7 *(0.4)*
Cont + Enz	65.6 *(8.0)*	13.3^{ab} *(3.6)*	14.6 *(8.4)*	6.0 *(1.0)*	0.4 *(0.6)*
Thy (1) + Enz	60.2 *(2.0)*	14.0^{ab} *(5.4)*	14.7 *(5.6)*	9.9 *(2.5)*	1.1 *(0.8)*
Thy (3) + Enz	56.1 *(1.2)*	6.2^{b} *(1.0)*	30.6 *(0.5)*	5.9 *(1.0)*	1.2 *(0.8)*
Thy (5) + Enz	56.8 *(2.1)*	14.4^{ab} *(7.3)*	23.0 *(8.8)*	5.2 *(0.2)*	0.6 *(0.8)*
s.e.d.	7.36	6.09	8.41	1.751	1.056
Enz*Thy	NS	P<0.05	NS	P=0.073	NS

Significances between treatments are shown in each column by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM) of 3 treatment replicates.

The carbohydrase tended to increase the acetic (P=0.061) and valeric (P=0.071) acid concentrations by 14.0% and 15.2% respectively at 42 days (**Table 4.44**). The total caecal VFA concentration tended to increase by 8.2% from 10.75 to 11.63 g kg^{-1} (P=0.089). Acetic acid was increased in the caeca by 2.3% (P<0.05), and lactic acid tended to decrease by 1.9% (P=0.069) at the 11% level of variation with carbohydrase inclusion (**Table 4.45**).

Table 4.44 *Effect of a dietary carbohydrase on caecal VFA concentrations at 42 days*

	Concentrations of caecal VFA (g kg^{-1})			
	Enz -ve	*Enz +ve*	*s.e.d.*	
Acetic acid	3.32 *(0.16)*	3.86 *(0.18)*	0.268	P=0.061
Lactic acid	2.05 *(0.19)*	2.01 *(0.24)*	0.282	NS
n-Butyric acid	4.02 *(0.21)*	4.26 *(0.21)*	0.341	NS
Propionic acid	0.71 *(0.08)*	0.73 *(0.06)*	0.102	NS
Valeric acid	0.38 *(0.02)*	0.45 *(0.03)*	0.036	P=0.071
Isobutyric acid	0.09 *(0.01)*	0.12 *(0.02)*	0.018	NS
Isovaleric acid	0.18 *(0.02)*	0.20 *(0.02)*	0.024	NS
TOTAL VFA	10.75 *(0.28)*	11.63 *(0.35)*	0.489	P=0.089

Significances are expressed in each row by the presence of non-identical superscripts. NS (P>0.05)
Data are the means (SEM), pooled across the treatments containing thyme oil.

The caecal lactic acid concentration was selectively increased at 42 days (P<0.05), but there was a tendency (P=0.092) for the concentration of propionic acid to be reduced, when thyme EO was included at 5 g kg^{-1} in the diet of broilers (**Table 4.46**). Lactic acid as a proportion of the total VFA was increased by 4.9% (P<0.05) with the inclusion of thyme EO in the diet, while both propionic and isobutyric acid proportions tended to be decreased by 1.88% (P=0.09) and 0.3% (P=0.066) respectively (**Table 4.47**).

Table 4.45 *Effect of a dietary carbohydrase on individual caecal VFA concentrations in broilers at 42 days of age, where each is presented as a percentage of the total*

Proportions (%) of individual VFA in relation to total VFA concentration					
	Enz –ve	*Enz +ve*	*s.e.d.*	*Sig @ 11%*	*Sig @ 89%*
Acetic Acid	30.9 *(1.2)*	33.2 *(1.1)*	1.805	P<0.05	NS
Lactic Acid	19.1 *(1.7)*	17.2 *(1.8)*	2.360	P=0.069	NS
n-Butyric Acid	37.2 *(1.5)*	36.4 *(0.9)*	1.953	NS	NS
Propionic Acid	6.7 *(0.9)*	6.4 *(0.6)*	1.111	NS	NS
Valeric Acid	3.6 *(0.2)*	3.9 *(0.3)*	0.305	NS	NS
Isobutyric Acid	0.8 *(0.1)*	1.1 *(0.2)*	0.162	NS	NS
Isovaleric Acid	1.7 *(0.2)*	1.8 *(0.2)*	0.245	NS	NS

Significances in each row are indicated by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing thyme oil.
Overall significance in this dataset is at the 89% level of variation, but effects were also shown at the 11% level.

Table 4.46 *Effect of thyme oil in diets on the caecal VFA concentrations at 42 days*

Concentration of caecal VFA (g kg^{-1})				
	Thyme -ve	*Thyme +ve (5)*	*s.e.d.*	
Acetic Acid	3.64 *(0.21)*	3.54 *(0.18)*	0.252	NS
Lactic Acid	1.70^{a} *(0.06)*	2.36^{b} *(0.27)*	0.266	P=0.023
n-Butyric Acid	4.04 *(0.26)*	4.24 *(0.17)*	0.321	NS
Propionic Acid	0.80 *(0.05)*	0.63 *(0.08)*	0.096	P=0.092
Valeric Acid	0.43 *(0.02)*	0.41 *(0.03)*	0.034	NS
Isobutyric Acid	0.12 *(0.01)*	0.09 *(0.02)*	0.017	NS
Isovaleric Acid	0.20 *(0.02)*	0.18 *(0.02)*	0.023	NS
TOTAL VFA	10.93 *(0.41)*	11.45 *(0.25)*	0.46	NS

Significances are expressed in each row by the presence of different superscripts. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing carbohydrase.

Table 4.47 *Effect of thyme oil inclusion in diets on individual caecal VFA concentrations in broilers at 42 days of age, where each is presented as a percentage of the total*

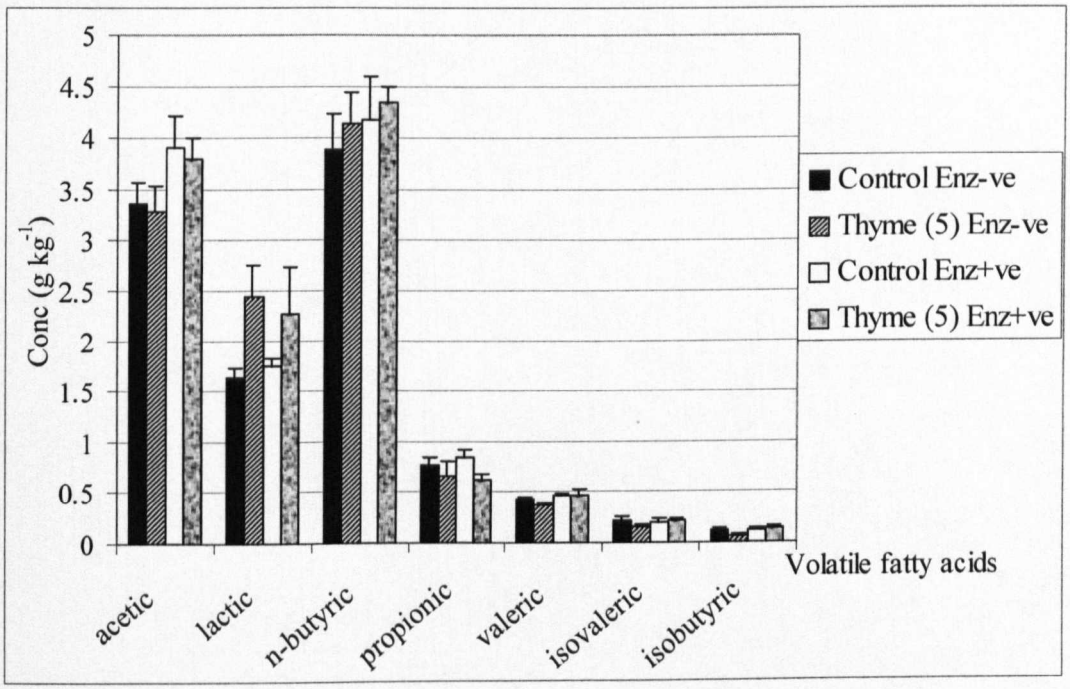
Proportions (%) of individual VFA in relation to total VFA concentration				
	Thyme -ve	*Thyme +ve (5)*	*s.e.d.*	
Acetic Acid	33.1 *(0.9)*	30.9 *(1.5)*	1.70	NS
Lactic Acid	15.7^{a} *(0.7)*	20.6^{b} *(2.2)*	2.23	P<0.05
n-Butyric Acid	36.7 *(1.3)*	37.0 *(1.2)*	1.84	NS
Propionic Acid	7.5 *(0.7)*	5.6 *(0.8)*	1.05	P=0.09
Valeric Acid	4.0 *(0.2)*	3.5 *(0.3)*	0.28	NS
Isobutyric Acid	1.1 *(0.1)*	0.8 *(0.1)*	0.15	P=0.066
Isovaleric Acid	1.9 *(0.2)*	1.5 *(0.2)*	0.23	NS

Significances are expressed in each row by the use of different superscripts. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing carbohydrase.

There were no interactions between the supplements with regards to the concentrations of caecal lactic, n-butyric, propionic or valeric acids in the caeca at 42 days (**Figure 4.4**). Thyme EO inclusion decreased the concentration of isobutyric acid, but the carbohydrase increased it, so the birds fed with 5 g kg^{-1} thyme EO had the lowest caecal isobutyric acid concentration

(P≤0.05). Isovaleric acid concentrations tended (P=0.076) to be increased by carbohydrase inclusion, but decreased by thyme EO inclusion in diets. There was no effect of any supplement on the total VFA concentrations in the caeca of broilers fed any supplemented diet at 42 days (not shown).

Figure 4.4 *Effect of thyme oil in the presence or absence of carbohydrase on VFA concentrations in the caeca of broilers at 42 days of age*



The percentage of isobutyric acid in the caeca tended (P=0.054) to be reduced with the inclusion of thyme EO at 5 g kg^{-1} in diets fed to broilers at 42 days (**Table 4.48**). Both the thyme EO dietary treatments resulted in a numerically increased percentage of lactic acid in the caeca, but there was no interaction between the supplements. The presence of the carbohydrase had no effect on the caecal VFA percentages.

As each pen containing several birds represented the experimental replicate, it was possible to include time as a factor within the comparisons. Transformation of the data to log_{10} was carried out to compensate for the 10-fold differences in VFA concentrations between 19 and 42 days of age. Only four VFA's were compared between 19 and 42 days in birds fed each supplemented diet, as some of the minor VFA's were not detectable at 19 days, ie. isovaleric, valeric and isobutyric acids. Analysis of variance was carried out in Genstat on the

transformed data by REML, incorporating Wald's test for treatment effects by Chi-squared analysis. The summary of the main treatment effects shows a distinct effect of time on the caecal VFA concentrations (**Table 4.49**).

Table 4.48 *Effect of dietary thyme oil and carbohydrase inclusion on the concentrations of caecal VFA in broilers at 42 days, where each is presented as a percentage of the total*

Proportions (%) of individual VFA in relation to total VFA concentration						
	Control Enz –ve	*Thyme (5 g kg^{-1}) Enz –ve*	*Control Enz +ve*	*Thyme (5 g kg^{-1}) Enz +ve*	*s.e.d.*	
Acetic Acid	32.2 *(1.4)*	29.6 *(2.0)*	34.0 *(0.9)*	32.3 *(2.1)*	2.48	NS
Lactic Acid	15.9 *(0.8)*	22.2 *(2.7)*	15.5 *(1.1)*	18.9 *(3.5)*	3.24	NS
n-Butyric Acid	37.2 *(2.0)*	37.2 *(2.3)*	36.1 *(1.7)*	36.7 *(1.0)*	2.68	NS
Propionic Acid	7.4 *(0.9)*	5.9 *(1.6)*	7.5 *(1.0)*	5.3 *(0.5)*	1.53	NS
Valeric Acid	4.0 *(0.2)*	3.2 *(0.2)*	4.0 *(0.3)*	3.9 *(0.5)*	0.42	NS
Isobutyric Acid	1.1 *(0.1)*	0.5 *(0.1)*	1.1 *(0.3)*	1.1 *(0.2)*	0.22	P=0.054
Isovaleric Acid	2.1 *(0.3)*	1.3 *(0.3)*	1.8 *(0.3)*	1.7 *(0.3)*	0.34	NS

Significance is shown in each row by the presence of different superscripts. NS (P>0.05)
Data are means (SEM) of 6 treatment replicates.

Table 4.49 *Summary of the main effects when thyme oil and a carbohydrase were included in diets on the caecal VFA concentrations in broilers measured at 19 and 42 days*

Transformed concentrations of caecal VFA (Log_{10} g kg^{-1})					
Supplement	*Time*	*Lactic Acid*	*Acetic Acid*	*Propionic Acid*	*n-Butyric Acid*
Control (none)	19d	-0.12 *(0.29)*	1.35 *(0.25)*	-1.10 *(0.26)*	0.64 *(0.45)*
Control (none)	42d	0.53 *(0.06)*	1.19 *(0.06)*	-0.29 *(0.11)*	1.33 *(0.08)*
Control + Thyme	19d	-0.79 *(0.09)*	1.11 *(0.02)*	-0.96 *(0.29)*	0.44 *(0.14)*
Control + Thyme	42d	0.88 *(0.13)*	1.16 *(0.08)*	-0.57 *(0.23)*	1.39 *(0.09)*
Control + Enzyme	19d	-0.95 *(0.29)*	0.75 *(0.19)*	-1.66 *(0.49)*	-1.15 *(1.07)*
Control + Enzyme	42d	0.52 *(0.05)*	1.36 *(0.08)*	-0.20 *(0.09)*	1.42 *(0.10)*
Control + Thyme + Enzyme	19d	-0.72 *(0.33)*	1.07 *(0.18)*	-1.34 *(0.25)*	-0.04 *(0.75)*
Control + Thyme + Enzyme	42d	0.70 *(0.19)*	1.34 *(0.06)*	-0.51 *(0.10)*	1.48 *(0.04)*
Source of variation					
Age		P<0.001	P=0.005	P<0.001	P<0.001
Enzyme		NS	NS	NS	NS
Thyme		NS	NS	NS	NS
Enzyme*Thyme		NS	NS	NS	NS
Age*Enzyme		P=0.067	P<0.001	P=0.074	P<0.01
Age*Thyme		P=0.002	NS	P=0.082	NS
Age*Thyme*Enzyme		P<0.001	P<0.05	NS	NS
s.e.d. 1		0.241	0.175	0.349	0.542
s.e.d. 2		0.192	0.126	0.247	0.383
s.e.d. 3		0.156	0.136	0.136	0.469
s.e.d. 4		0.218	0.152	0.152	0.469

At 19 days, n=3 and at 42 days, n=6. Values displayed in parentheses are the S.E.M.
s.e.d. comparisons are as follows: s.e.d. 1 refers to means compared within the same level of enzyme and between levels of thyme at 19 days; s.e.d. 2 refers to comparisons within the same level of enzyme and between levels of thyme at 42 days; s.e.d. 3 refers to means with the same combination of enzyme and herb between 19 and 42 days; s.e.d. 4 refers to means compared with different levels of enzyme and thyme between 19 and 42 days.

4.3.14 The effect of dietary thyme EO and enzyme inclusion on ileal tissue dimensions

The measurements presented in this section are based on the dimensions of intestinal sections cut transversely from the ileum in broilers of 7 and 42 days of age after they were fed diets supplemented with thyme EO and carbohydrase over the period of growth. The perimeter was measured for each transverse ileal section (section perimeter) and the diameter of each transverse section between the outer edges (section diameter). Other measurements included the average diameter of the intestinal lumen between the top of the villi across the centre of the ileum (lumen diameter) and the average height of villi within the ileum (villus height). The average depth of the crypts of Lebarkuhn at the base of the villi (crypt depth) and also the average diameter of intestinal walls in the ileum (wall diameter) were also measured.

Carbohydrase inclusion in the diets reduced (P<0.05) the ileal crypt depth by 21.4% and tended (P=0.082) to reduce villus height by 17.4% at 7 days (**Table 4.50**). When the data for crypt depth were adjusted to show BM at 7 days as a covariate, the same effect was observed (0.135 mm without carbohydrase *vs* 0.098 mm with carbohydrase inclusion; P=0.05, s.e.d. = 0.017 with 13 degrees of freedom, not shown). The ileal wall diameter was reduced by 13.5% by the inclusion of carbohydrase, but this was not significant.

Table 4.50 *Effect of dietary carbohydrase inclusion on ileal morphology at 7 days*

Dimensional measurements within the ileum of broilers at 7 days (mm)				
	Enz -ve	*Enz +ve*	*s.e.d.*	
Section diameter	4.53 *(0.15)*	4.47 *(0.15)*	0.217	NS
Villus height	0.70 *(0.05)*	0.58 *(0.03)*	0.070	P=0.082
Crypt depth	0.13^{a} *(0.01)*	0.10^{b} *(0.01)*	0.011	P=0.022
Wall diameter	0.32 *(0.03)*	0.28 *(0.01)*	0.334	NS
Section perimeter	14.12 *(0.43)*	14.16 *(0.63)*	0.765	NS
Lumen diameter	3.41 *(0.17)*	3.50 *(0.18)*	0.248	NS

Means within a row without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing thyme oil and are based on an average of 5 measurements taken on each pen.
s.e.d.'s are adjusted to correct for missing data points.

Thyme EO inclusion in the diets at 5 g kg^{-1} reduced the ileal section diameter (P<0.05) by 9.1% and the section perimeter (P<0.05) by 9.5% (**Table 4.51**). In addition, the diameter of the lumen tended (P=0.055) to be reduced by 12% with thyme EO inclusion in the diets. No interactions were present in this data between thyme EO and carbohydrase with regards to the ileal morphology (**Table 4.52**).

Table 4.51 *Effect of thyme oil inclusion in diets on ileal morphology in broilers at 7 days*

Dimensional measurements in the ileum of broilers at 7 days (mm)				
	No Thyme	*Thyme (5 g kg^{-1})*	*s.e.d.*	
Section diameter	4.71^{a} *(0.16)*	4.28^{b} *(0.10)*	0.201	P<0.05
Villus height	0.65 *(0.06)*	0.64 *(0.04)*	0.066	NS
Crypt depth	0.12 *(0.01)*	0.11 *(0.01)*	0.011	NS
Wall diameter	0.31 *(0.03)*	0.28 *(0.02)*	0.038	NS
Section perimeter	14.85^{a} *(0.42)*	13.43^{b} *(0.45)*	0.721	P<0.05
Lumen diameter	3.67 *(0.17)*	3.23 *(0.15)*	0.230	P=0.055

Means within a row without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing carbohydrase and are based on an average of 5 measurements taken on each pen.
s.e.d.'s are adjusted to correct for missing data points.

Table 4.52 *Effect of thyme oil and carbohydrase in diets on ileal morphology at 7 days*

Dimensional measurements in the ileum of broilers at 7 days (mm)						
	Control Enz -ve	*Thyme (5 g kg^{-1}) Enz -ve*	*Control Enz +ve*	*Thyme (5 g kg^{-1}) Enz +ve*	*s.e.d.*	*Enz* Thyme*
Section diameter	4.71 *(0.27)*	4.34 *(0.13)*	4.72 *(0.24)*	4.23 *(0.14)*	0.267	NS
Villus height	0.75 *(0.08)*	0.66 *(0.052)*	0.55 *(0.05)*	0.62 *(0.04)*	0.089	NS
Crypt depth	0.13 *(0.02)*	0.13 *(0.01)*	0.11 *(0.01)*	0.10 *(0.01)*	0.015	NS
Wall diameter	0.34 *(0.05)*	0.30 *(0.03)*	0.28 *(0.01)*	0.27 *(0.02)*	0.052	NS
Section perimeter	14.74 *(0.49)*	13.50 *(0.58)*	14.95 *(1.03)*	13.36 *(0.63)*	0.795	NS
Lumen diameter	3.55 *(0.27)*	3.26 *(0.20)*	3.80 *(0.21)*	3.21 *(0.17)*	0.305	NS

Significance is shown in each row by the presence of superscripts. NS (P>0.05)
Data are mean (SEM), based on 5 measurements taken from each pen, and 6 pen replicates of each treatment.

As several sections were incomplete, measurements of the section perimeters were not included due to the numbers of missing data values. The diameter of the ileum was reduced (P=0.024) with carbohydrase inclusion at 42 days (**Table 4.53**). However, the lumen diameter was only numerically reduced when carbohydrase was included, and it is possible that this may be due to a reduction in dietary particle size. No effects were observed on ileal morphology after thyme EO inclusion at 42 days of age (**Table 4.54**).

Table 4.53 *Effect of carbohydrase in diets on the ileal morphology in broilers at 42 days*

Dimensional measurements in the ileum of broilers at 42 days (mm)				
	Enz –ve	*Enz +ve*	*s.e.d.*	*Sig*
Section diameter	11.50^{a} *(0.80)*	9.63^{b} *(0.39)*	0.756	P=0.024
Villus height	2.24 *(0.09)*	2.00 *(0.14)*	0.184	NS
Crypt depth	0.20 *(0.01)*	0.18 *(0.01)*	0.018	NS
Wall diameter	0.50 *(0.02)*	0.44 *(0.02)*	0.035	NS
Lumen diameter	8.30 *(0.94)*	6.81 *(0.46)*	0.942	NS

Means within a row without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing thyme EO and are based on an average of 5 measurements taken on each pen.
s.e.d.'s are adjusted to correct for missing data points.

Table 4.54 *Ileal tissue dimensions at 42 days as affected by dietary thyme oil inclusion*

Dimensional measurements within the ileum of broilers at 42 days (mm)				
	No Thyme	*Thyme (5 g kg^{-1})*	*s.e.d.*	*Sig*
Section diameter	11.03 *(0.71)*	10.11 *(0.58)*	0.712	NS
Villus height	2.03 *(0.11)*	2.21 *(0.13)*	0.173	NS
Crypt depth	0.19 *(0.01)*	0.19 *(0.01)*	0.016	NS
Wall diameter	0.48 *(0.03)*	0.46 *(0.02)*	0.033	NS
Lumen diameter	8.00 *(0.81)*	7.11 *(0.67)*	0.889	NS

Means within a row without a common superscript are significantly different. NS (P>0.05)
Data are means (SEM), pooled across the treatments containing carbohydrase and are based on an average of 5 measurements taken on each pen.
s.e.d.'s are adjusted to correct for missing data points.

No interactions were observed between the supplements on any of the ileal morphology measurements in broilers at 42 days, when both were included in diets (**Table 4.55**).

Table 4.55 *Effect of dietary thyme oil and carbohydrase inclusion on ileal morphology in broilers at 42 days*

	Dimensional measurements in the ileum of broilers at 42 days (mm)				
Inclusion (g kg^{-1})	*Section diam*	*Villus height*	*Crypt depth*	*Wall diameter*	*Lumen diam*
Control (0) Enz –ve	11.92 *(1.33)*	2.23 *(0.10)*	0.196 *(0.025)*	0.51 *(0.03)*	8.51 *(1.55)*
Thyme (5) Enz –ve	11.09 *(1.00)*	2.24 *(0.17)*	0.194 *(0.015)*	0.48 *(0.03)*	8.08 *(1.19)*
Control (0) Enz +ve	10.14 *(0.60)*	1.82 *(0.18)*	0.178 *(0.011)*	0.44 *(0.04)*	7.48 *(0.65)*
Thyme (5) Enz +ve	9.12 *(0.50)*	2.17 *(0.20)*	0.186 *(0.018)*	0.44 *(0.02)*	6.13 *(0.59)*
s.e.d.	1.038	0.253	0.024	0.048	1.295
Enz*Thyme	NS	NS	NS	NS	NS

Significance is shown in each column by the presence of superscripts. NS (P>0.05)
Data are mean (SEM), based on 5 measurements taken from each pen, using 6 treatment replicates.

4.3.15 Principal Components Analysis (PCA)

As previously described in Chapter 3, PCA was included in this experiment to compare the results of the separate analyses of variance with a different statistical methodology, due to the large numbers of experimental variables involved in this experiment. The aim was to reduce the numbers of variables by grouping (correlating) related variables together to explain the sources of variation within the dataset by regression.

4.3.15.1 The correlation of related data variables associated with nutrient digestibility in broilers as affected by the inclusion of thyme EO and carbohydrase in diets

At 19 days of age, the variables associated with nutrient digestibility were not sufficiently highly correlated to be considered in an analysis by PCA. Insufficient correlation (R<0.900)

makes for a larger variation when grouping several data variables together, which can only then be explained using a larger number of principal components to describe the dataset variation. As described previously in Chapter 3, the objective of using PCA is to reduce the numbers of variables as far as possible. The loadings used in the calculation of the first 3 principal components explained 99% of the total variation in the dataset associated with dietary nutrient digestibility at 42 days of age (**Table 4.56**). Analysis of variance on the PC1 component showed that carbohydrase affected nutrient digestibility (P=0.003), but thyme EO did not (P=0.156), although there was a significant interaction between both factors (P<0.001) at 42 days (**Table 4.57**). In the calculation of PC2, there was no effect of carbohydrase inclusion on nutrient digestibility (P=0.298), but thyme EO tended (P=0.075) to have an effect, and a significant interaction was present between both factors (P=0.004). When PC3 was calculated, there was no effect of either supplement included alone in the diets, but an interaction between both factors (P=0.005) when supplemented together, although the PC3 component represented only a tiny part of the variation. Generally, these results are in agreement with those of the individual ANOVA tests for the data from these experimental birds at 42 days, describing the effects of both thyme EO and carbohydrase in the diet on the coefficients of ADMD, DOMD and AMN, as well as the AME content and metabolisability (AME:GE) of the diets.

Table 4.56 *The loadings calculated for insertion into the correlation matrix in the calculation of principal components for variables relating to nutrient digestibility at 42 days, as affected by the dietary inclusion of thyme oil or carbohydrase*

Loadings used in Principal Component Analysis for nutrient digestibility at 42 days			
Variable	*PC1*	*PC2*	*PC3*
AME (MJ kg^{-1} DM)	-0.34	-0.21	-0.31
AME: GE	-0.34	-0.20	-0.23
Coefficient AMN	-0.32	0.59	-0.11
AMEn (MJ kg^{-1} DM)	-0.34	-0.27	-0.32
AMEn: GE	-0.34	-0.26	-0.24
Metabolisable N in diet (g)	-0.32	0.64	-0.14
App DM digestibility	-0.34	-0.07	0.28
Coefficient of OM digestibility (DOMD)	-0.34	-0.10	0.45
Digestibility of OM in diet (g kg^{-1} DM)	-0.33	-0.07	0.62
% of variation explained	94.9	3.4	1.2
Cumulative variation explained	94.9%	98.3%	99.5%

An example of a correlation analysis used in the initial stages of PCA is included (**Appendix 8**).

Table 4.57 *Effect of thyme oil and carbohydrase in diets on analysis of variance on the calculated principal components of the correlation matrix of nutrient digestibility variables in broilers at 42 days*

Nutrient digestibility as analysed by Principal Component Analysis			
Treatment	*PC1*	*PC2*	*PC3*
Control (0) – Enz	2.71^{a} *(1.36)*	0.36^{a} *(0.17)*	0.07^{ab} *(0.10)*
Thyme (1) – Enz	2.12^{ab} *(0.97)*	-0.29^{bc} *(0.18)*	-0.09^{bc} *(0.20)*
Thyme (3) – Enz	0.14^{bc} *(0.40)*	-0.23^{ab} *(0.15)*	0.03^{ab} *(0.00)*
Thyme (5) – Enz	-0.85^{cd} *(0.60)*	0.01^{ab} *(0.0)*	0.25^{a} *(0.06)*
Control (0) + Enz	-1.40^{cd} *(0.50)*	-0.61^{a} *(0.23)*	0.17^{ab} *(0.09)*
Thyme (1) + Enz	-3.20^{d} *(0.61)*	0.17^{ab} *(0.20)*	-0.09^{bc} *(0.10)*
Thyme (3) + Enz	1.40^{abc} *(1.70)*	0.41^{a} *(0.25)*	0.09^{ab} *(0.20)*
Thyme (5) + Enz	-0.92^{cd} *(0.86)*	-0.26^{bc} *(0.07)*	-0.41^{c} *(0.06)*
s.e.d.	1.250	0.275	0.162
Enz	P=0.003	NS	NS
Thyme	NS	P=0.075	NS
Enz*Thyme	P<0.001	P<0.004	P=0.005

Significance was tested at the $p<0.05$ level, but the greatest differences between means are as shown.
Significance is illustrated in each column by the presence of non-identical superscripts.
Data are means (SEM) of 6 treatment replicates used in the calculation of each principal component.

4.3.15.2 Amino acid digestibility

At 19 days of age in these broilers, over 95% of the variation between the coefficients of ileal digestibility of AA could be explained in the calculation of the first 2 principal components, and the loadings used in their calculation are presented (**Table 4.58**). There were no effects of treatment on these principal components (not shown). There were no significant effects of inclusion of either thyme EO and/or carbohydrase in diets in the ANOVA tests carried out on each individual coefficient of AA digestibility at 19 days, thus the two sets of statistical analyses were in agreement.

The principal components were calculated for the coefficients of the digestibility of AA at 42 days after the removal of experimental pen 48 (treatment 6), due to its high residual standard error. Over 96% of the dataset variation could be explained in the first 2 principal components, and the loadings used in their calculation are presented (**Table 4.59**). At the 11% level of variation in the dataset, carbohydrase affected AA digestibility ($P<0.05$) within PC1, but there was no effect at the 89% level (**Table 4.60**). There was an effect of thyme EO inclusion on AA digestibility for PC2 ($P<0.05$), which was not demonstrated in the individual ANOVA results. However, this may be indicative of a minor effect, perhaps in the numerical percentage changes observed between the digestibility coefficients of threonine and histidine.

Table 4.58 *The loadings calculated for insertion into the covariance matrix in the calculation of principal components for variables relating to the coefficients of amino acid digestibility at 19 days in broilers, as affected by thyme EO and carbohydrase inclusion in diets*

Loadings used in Principal Component Analysis for amino acid digestibility in broilers at 19 days		
Variable	*PC1*	*PC2*
Aspartic acid	-0.27	0.03
Glutamic acid	-0.15	0.03
Serine	-0.24	0.05
Histidine	-0.23	-0.10
Glycine	-0.36	-0.83
Threonine	-0.37	0.45
Arginine	-0.17	0.09
Alanine	-0.26	-0.03
Tyrosine	-0.26	0.25
Valine	-0.27	0.07
Phenylalanine	-0.18	0.05
Isoleucine	-0.23	0.09
Leucine	-0.20	0.07
Lysine	-0.17	-0.09
Total dispensible AA	-0.20	0.01
Total indispensible AA	-0.23	0.05
Total AA	-0.21	-0.01
% of variation explained	**92.3**	**3.6**
Cumulative variation explained	**92.3%**	**95.9%**

ANOVA on PC1 and PC2 (P>0.05)

Table 4.59 *The loadings calculated for insertion into the covariance matrix in the calculation of principal components for variables associated with the coefficients of amino acid digestibility at 42 days, when broilers were fed diets including thyme oil and carbohydrase*

Loadings used in Principal Component Analysis for amino acid digestibility in broilers at 42 days of age		
Variable	*PC1*	*PC2*
Aspartic acid	-0.262	0.008
Glutamic acid	-0.133	0.001
Serine	-0.230	-0.012
Histidine	-0.171	0.369
Threonine	-0.413	-0.770
Arginine	-0.156	0.192
Alanine	-0.351	0.031
Tyrosine	-0.266	0.188
Valine	-0.294	0.293
Phenylalanine	-0.211	0.038
Isoleucine	-0.254	0.284
Leucine	-0.240	0.060
Lysine	-0.211	-0.156
Total dispensible AA	-0.199	0.015
Total indispensible AA	-0.240	0.037
Total AA	-0.218	0.025
% of variation explained	**93.13**	**2.94**
Cumulative variation explained	**93.13%**	**96.07%**

Table 4.60 *Effects of thyme oil and carbohydrase in diets on analysis of variance of the calculated principal components of the covariance matrix, based on the coefficients of amino acid digestibility at 42 days in broilers*

Amino acid digestibility in broilers at 42 days as analysed by PCA		
Inclusion (g kg^{-1})	*PC1*	*PC2*
Control (0) – Enz	0.063 *(0.046)*	0.001 *(0.009)*
Thyme (1) – Enz	0.046 *(0.076)*	-0.014 *(0.001)*
Thyme (3) – Enz	0.061 *(0.049)*	0.001 *(0.007)*
Thyme (5) – Enz	0.023 *(0.028)*	0.016 *(0.013)*
Control (0) + Enz	-0.077 *(0.030)*	-0.007 *(0.005)*
Control (1) + Enz	-0.085 *(0.077)*	-0.006 *(0.008)*
Control (3) + Enz	0.015 *(0.043)*	0.006 *(0.008)*
Control (5) + Enz	-0.052 *(0.036)*	-0.001 *(0.011)*
s.e.d. 1	0.0713	0.0079
s.e.d. 2	0.0750	0.0083
Enz @ 11% level	P<0.05	NS
Enz @ 89% level	P=0.142	NS
Thyme	NS	P<0.05
Enz*Thyme	NS	P=0.100

s.e.d.'s were calculated for the combined treatment means, where s.e.d. 2 refers to comparisons between Control (1) + Enz treatment and all other treatments, and s.e.d. 1 for comparisons between all treatments except Control (1) + Enz.
Data are means (SEM) for 6 replicates of each treatment, except for Control (1) + Enz, where n=5.

4.3.15.3 Other data variable groups

The bacteriological, histological and caecal VFA measurements were regressed to show the extent of correlation between data variables, but the R^2 values were not sufficiently high enough to justify the use of multivariate analysis. It was not possible to carry out PCA on the performance data, due to the complication of repeated measurements over time on the same experimental variables.

4.4 Discussion

4.4.1 Chemical composition of thyme EO

The composition of thyme EO is variable, due to environmental influences on the plant during its growth period and also the complex genetic morphology of the plant. The content of thymol and its inactive precursor *para*-cymene in this EO would suggest it has a thymol chemotype and subsequently a highly antibacterial nature. Recent studies have established that thyme EO and its components are bioactive antimicrobially. Both thyme EO and thymol have shown antimicrobial properties against both Gram-positive and Gram-negative bacteria (Deans & Ritchie, 1987; Sivropoulou *et al.*, 1996; Dorman & Deans, 2000; Walsh *et al.*, 2003; Sagdic, 2003). Apart from thymol, other monoterpenes from *Thymus* spp. plants have shown strong antimicrobial properties, such as linalol, 1,8-cineole (Faleiro *et al.*, 2003) and also α-

terpineol (Cox *et al.*, 1998), but these monoterpenes may not be present in all thyme EO samples. The antimicrobial activity of thyme EO is dependent on more than one chemical component, and these effects may be either synergistic or antagonistic (Daferera *et al.*, 2000 & 2003). There may therefore be some contribution from α-pinene, linalol, and α-terpineol in this thyme EO towards its bioactivity, although the bioactivity and high content of thymol in the EO would suggest that this compound is responsible for most of the bioactive effects. Walsh *et al.* (2003) reported that nerolidol synergistically improved the activity of thymol and eugenol against *E. coli* and *S. aureus*, and suggested that both thymol and eugenol had a similar mode of action and target site. Faleiro *et al.* (2003) reported that *E. coli* was susceptible to linalol, but not to a mixture of linalol and 1,8-cineole. Oregano EO had a larger minimum inhibitory concentration (MIC) against *S. aureus* than the MIC of its components carvacrol and thymol, suggesting that this was due to the lower concentration (38%) of these 2 components within the EO (Nostro *et al.*, 2003). Although the bioactivity of terpenes may sometimes be greater when used in isolation than when present in the complete EO, the terpene composition in an EO sample will determine its bioactivity.

The variability of composition in an EO, even in one species of plant, means that it is important to source products carefully and to perform a chemical characterisation of the material to check its quality before use (Faleiro *et al.*, 2003). For this reason, we measured the chemical composition of the thyme EO by GC before using it in the experiments. Within animal nutrition, most industry trials carried out to date have used thymol or carvacrol, either within oregano EO, which is another strongly phenolic EO, or alternatively as blended compounds with one or other of these two terpenes as primary components. There have been no previous experiments in poultry that have used thyme EO as a feed additive and very few studies have used additives based predominantly on thymol. Reports from studies done using EO or their components as dietary additives within animal nutrition usually contain very little information on the chemical composition of the materials used in these supplements.

4.4.2 Experimental Design

The statistical analysis for the factor of enzyme was based mainly on the variation within (89%), as opposed to between (11%) the rooms, but the presence of a significant effect at the 11% level for the enzyme factor should not be overlooked. At the planning stage of this experiment, it was considered that the carbohydrase may potentially have a very large effect in

these diets, which had high fibre content, so a loss of 11% should not greatly compromise the robustness of the experiment. A different experimental design may have highlighted significant overall increases in the digestibility of AA at 42 days when the diets were fed with the carbohydrase, but cannot be determined outright using this data.

Throughout the present experiment, both the pooled data and the interaction level between the two treatment factors indicated an anomaly in the data when thyme EO was included in diets at 3 g kg^{-1}. The sampling of one bird per pen should have been sufficient, as the pen represented the treatment replicate, rather than the individual bird. Although every effort was made to randomise the sampling of birds from each pen, it would appear that 2 of the birds sampled from the treatment with 3 g kg^{-1} thyme EO + carbohydrase were smaller than the others. Unfortunately, these 2 pens decreased the treatment mean and increased the variability of the treatment, although all pens represented valid experimental replicates, and as such could not be removed from the analysis. This affected the pooled data when each factor was considered in isolation, numerically lowering the treatment mean.

4.4.3 Effects of thyme oil and carbohydrase on broiler performance

There was an initial reduction in feed intake in this study from days 8 to 14, which may have been due to an astringent taste of the thyme EO, caused by its terpene composition. In these diets, thyme EO was fed at relatively high concentrations, around 10 times higher than in other studies using thymol. In this study, the early reduction in feed intake associated with the inclusion of thyme EO, which led to a numerical reduction in average weight gain, suggested that concentrations of 5 g kg^{-1} may be too high in the diets of birds up to 15 days of age. Although the birds appeared to adapt to the thyme EO after 15 days of age, this initial reduction in bird performance suggests that the inclusion of thymol at 1 g kg^{-1} may be more suitable to minimise this effect. In previous studies, the inclusion of either thymol (at 99% purity) or CRINA poultry (containing 29% active compounds including thymol) had no effect on broiler performance (Lee *et al.*, 2003). However, the product concentrations used by Lee *et al.* (2003) were ten times more dilute than in our experiment. In the same way, when feeding oregano EO with a high thymol composition as a supplement to chickens and turkeys, no effects of supplementation were observed by either Botsoglou *et al.*, (2002) or Papageorgiou *et al.*, (2003). In mature rats, dietary thyme EO supplementation had no effect on the BM, except at age 22 months, where rats fed the control diets had a significantly higher BM than

those fed diets with thyme EO (Youdim & Deans, 1999a). It is possible that the negative effect on feed intake may have been due to the presence of one of the other terpene components within the EO, but the evidence in the literature suggests that the presence of thymol in diets does not promote a gain in weight. No detailed analysis of the composition of the supplements used was available from these authors, so their composition cannot be compared. These authors, apart from Youdim & Deans (1999a), also used a powdered dietary supplement, which may have been retained in diets more effectively than the liquid thyme EO used in the present study. It may be useful to check for astringency with choice testing, using varying concentrations of thyme EO offered to broilers in the feed, and assessing the preferences of the birds.

In general, the antimicrobials previously fed in poultry diets primarily improved productivity and health by suppression of the activity of microfloral populations, thus reducing the competition for dietary substrates and the incidence of sub-clinical infections. In small-scale experiments such as the present study, the facilities utilised will not necessarily represent commercial housing conditions. Lee *et al.* (2003) considered that good housing conditions and a highly digestible diet may have reduced the effects of terpene supplementation in broilers. Experimental housing conditions with a high level of hygiene were also suggested to have prevented the widely used antimicrobial avoparcin from improving broiler performance (Vukic Vranjes & Wenk, 1995). In the design of this experiment, attempts were made to maximise the effects of treatment by including barley at 15% to increase the fibre content and limiting the dietary provision of vitamin E to 50 mg kg^{-1} to encourage intestinal microfloral growth.

The experiment described in Chapter 3 used thyme EO with an intermediate thymol and carvacrol composition and a high content of α-terpineol, reporting positive effects on body mass and weight gain between days 21 and 28 without an associated rise in feed consumption. However, the initial feed intake reduction observed in the birds from 8-14 days of age in this experiment with increasing dietary concentrations of this thyme EO suggests a definite influence of the chemotype of the EO and chemical composition of its terpenes on feed intake and overall bird performance. A highly antimicrobial thyme EO such as this one may be more suitable if fed within a blend of bioactive compounds, especially in young broilers, which have a relatively immature gastrointestinal tract. This supply strategy may also help to reduce the

cost of its inclusion as a supplement. There is evidence to support the perception and preference of sweet tastes compared to astringent ones in broilers, using various aromatic terpenes, aldehydes and ketones (Balog & Millar, 1989; Koch *et al.*, 1991), but the extent of sensory perception is not well understood. Despite having only 24 taste buds each (Kare & Rogers, 1976), broiler chickens are considered to have a moderately well developed sense of taste. More research will elucidate the extent and influence of taste perception in chickens and its possible manipulation. It is likely that various aromatic compounds supplemented within chicken diets will have different influences on the taste and palatability of these diets, and the nature of their functional groups may determine this effect.

Although the mortalities in birds caused by the *colisepticaemia* were not confined to any particular treatment, the infection will have reduced both feed intake and weight gain generally within the first ten days, as sick or unhealthy birds will not eat to meet their requirements. It is unclear whether the reduced feed intake effects in the first few weeks of the present study were due to the *colisepticaemia* outbreak in the birds or the chemical composition of the thyme EO supplement, or both of these factors in combination. Repeating this experimental design using birds free from infection should help to clarify this. It may be possible that the inclusion of thyme EO in the diets of healthy chicks may not have such a marked effect in reducing early performance. A measurement of water intake in broilers fed a diet containing thyme EO may also be useful in assessing the potential astringency of thyme EO. The consumption of water was reduced by 10% in broilers when diets including cinnamaldehyde were fed, and there was an increase in the DM content of the excreta, without an associated effect on bird performance (Lee *et al.*, 2003). This may imply a beneficial effect of cinnamaldehyde and perhaps also other terpenes in reducing sticky droppings and thus improving litter quality.

When considered in isolation, thyme EO was observed to have a negligible or slightly negative effect on bird performance, whereas the inclusion of this carbohydrase in diets markedly increased the body mass, weight gains, feed intakes and the associated FCR in these birds. This was not entirely unexpected, as the diets were formulated to be of a poor quality and contained a high proportion of fibre, which should act as a substrate for the enzyme. Xylanases have been fed in broiler diets to reduce the adverse effects of cereal NSP. Xylanases have reduced the variation in growth between birds and also improved bird

performance characteristics, as well as the digestibility of dietary nutrients and AME in the birds (Choct *et al.*, 1995; Crouch *et al.*, 1997; Hew *et al.*, 1998; Choct *et al.*, 1999; Jamroz *et al.*, 2002). Enzymes reduce the viscosity of digesta, increasing the amount of monosaccharides and other nutrients released from dietary cereals, both in studies carried out *in vitro* and within the intestinal tract of broilers (Malathi & Devegowda, 2001; Silva & Smithard, 2002). At commercial levels of inclusion in the diet, xylanase still retained 15-20% of its original activity after travelling through the broiler gut, significantly decreasing the viscosity of small intestinal fluid, indicating that it can survive acid digestion to act throughout the intestinal tract (Silva & Smithard, 2002). Exogenous enzymes have an age-dependent effect, with benefits in increasing the rates of passage of digesta and the nutrient digestibility more evident in chicks than in mature birds (Almirall & Esteve Garcia, 1994). An increased rate of digesta passage will increase the amount of feed intake, and may therefore improve feed utilisation. A greater improvement in broiler performance between 0 and 21 days of age was reported when carbohydrase was included in diets based on wheat rather than on barley (Jamroz *et al.*, 2002). However, the opposite effect occurred between 22 and 49 days of age, where carbohydrase in barley-based diets improved bird performance more than in wheat-based diets, and it was suggested that the response of the bird to dietary NSP may change with increasing age (Jamroz *et al.*, 2002). The carbohydrase in the present study significantly improved the body mass, weight gains and feed intakes in broilers fed wheat-based diets up to 23 days of age but not after this time point. However, these response variables were sufficiently increased that the birds fed diets including the carbohydrase showed a significant improvement overall through the period from 8 to 42 days of age.

When an enzyme complex and avoparcin were included in broiler diets from 7 to 21 days of age, both supplements significantly improved broiler food conversion efficiency (FCE) when added in isolation, and also additively at a level approaching significance (P=0.053) during this period (Vukic Vranjes & Wenk, 1995). However, although the dietary enzyme complex improved broiler FCE after 21 days of age, there were no effects due to avoparcin inclusion, or any interaction between the supplements on broiler FCE (Vukic Vranjes & Wenk, 1995). The thyme EO and enzyme in the present experiment did not appear to interact in their mode of action for any characteristic of broiler performance. However, the inclusion of avilamycin and an enzyme in diets additively improved broiler performance, increasing body mass by 7.7% and feed conversion by 2.8% (Jamroz *et al.*, 1995). There were increases in broiler weight

gain of 4.7% and 5.1% when diets were supplemented with xylanase and zinc bacitracin, and feed conversion in the birds was improved by 6.5% and 3% respectively (Hock *et al.*, 1997). However, neither the 6.8% improvement in body mass, nor the 7.6% improvement in feed conversion were significant when both additives were included together in diets (Hock *et al.*, 1997). In addition, a blend of the terpenes capsaicin, cinnamaldehyde and carvacrol added in diets improved broiler performance, increasing body mass by 5.4% (150 ppm) or 8.1% (300 ppm), and improving the FCR by 3.1% (150 ppm) or 7.1% (300 ppm) (Jamroz *et al.*, 2003). The carbohydrase and thyme EO used in the present study did not reduce the performance of the birds when they were administered together, which suggests that potentially negative effects of one supplement on one area of the digestive tract may be counteracted by a positive effect of the other. The combined effects of dietary supplementation will depend on individual circumstances, but these studies show that both enzymes and an antimicrobial supplement can potentially improve broiler performance, either alone or synergistically by different mechanisms.

4.4.4 Effects of thyme oil and carbohydrase on intestinal microflora populations

Thymol, carvacrol and eugenol disintegrate the hydrophilic outer membrane of *E. coli, S. typhimurium, S. aureus* and other Gram-negative bacteria (Lambert *et al.*, 2001; Walsh *et al.*, 2003). Their action leads to a dissipation of the pH gradient, increased cellular ATP turnover, and a gradually increasing membrane permeability leading to the release of potassium and phosphate ions into the environment (Helander *et al.*, 1998; Lambert *et al.* 2001; Cox *et al.*, 2000; Walsh *et al.*, 2003). The phenolic terpenes thymol and carvacrol acted to destabilise the outer cell membrane in Gram-negative bacteria and cause the release of intracellular ATP, which did not happen with cinnamaldehyde, and yet cinnamaldehyde inhibited enterobacterial growth and prevented bioluminesce in *Photobacterium leiognathi* (Helander *et al.*, 1998). The presence of the phenolic component in terpene molecules is suggested to be very important, if not essential, in disintegrating the outer cell membrane and thus in the paralysis of the cell (Helander *et al.*, 1998; Evans *et al.*, 1999). Tea tree EO also inhibited glucose-dependent respiration *in vitro*, and exerted a bacteriostatic or bactericidal effect on *E. coli* (Cox *et al.*, 1998), but the susceptibility to tea tree EO differed between *E. coli, Candida albicans* and *S. aureus* (Cox *et al.*, 2000). As tea tree EO is a broad-spectrum antimicrobial, Cox *et al.* (2000) proposed that this variation in susceptibility was due to different rates of diffusion of the active terpene components across the cell membranes. Walsh *et al.* (2003) showed *in vitro*

that EDTA facilitated the antimicrobial action of thymol against *E. coli*, *P. aeruginosa* and *S. aureus*, by removing metal cations from the lipopolysaccharide surface of the Gram negative cell wall, enabling easier access to the target sites of the cell wall. Therefore, there may be effects of other compounds in conjunction with the phenolic EO, which could enhance or contribute to their activity. Nostro *et al.* (2003) have demonstrated the equal effectiveness of carvacrol and thymol *in vitro* against both methicillin-resistant and -susceptible strains of *Staphylococcus aureus* and *S. epidermidis*, suggesting that these EO components also have potential as a topical agent against antibiotic-resistant bacteria. The work of Cox *et al.* (1998 & 2000), Walsh *et al.* (2003) and Nostro *et al.* (2003) provide evidence for the strong activity of low molecular weight, hydrophobic phenolic compounds, such as thymol, for their antimicrobial activity *in vitro*. If thymol and carvacrol paralyse bacteria by inducing cell lysis and the subsequent leakage of cell contents, this suggests that bacteria are less likely to develop resistance mechanisms to these compounds as they destroy the structural integrity of the bacterial cell. This may then support their use as natural antimicrobial feed additives.

Although the *in vitro* antimicrobial properties of thyme EO and thymol are well established (Piccaglia *et al.*, 1993; Sivropoulou *et al.*, 1996; Russo *et al.*, 1998; Dorman & Deans, 2000; Daferera *et al.*, 2003; Olasupo *et al.*, 2003), they were not demonstrated *in vivo* prior to the start of this project. As the diets in the present study contained no anticoccidials or other antimicrobials, the thyme EO and carbohydrase supplements provided the only potential means of self-medication for these birds. In this study, thymol was shown to decrease the *E. coli* population *in vivo*, even with low numbers of treatment replicates, which should have helped in combatting the colisepticaemia infection in these birds. However, the mechanism by which this occurred is uncertain. Hammer *et al.* (1999) reported that the presence of OM in their *in vitro* study reduced the antimicrobial efficacy of tea tree EO, but this was dependent on the type of microorganism tested. It was suggested that each of the 100 or so constituent terpenes in an EO could have some antimicrobial efficacy against specific microorganisms (Hammer *et al.*, 1999). It is possible that thyme EO may not exert antimicrobial properties in the proximal intestine of the chicken, but these properties may become more apparent further down the tract as OM is removed from the lumen. The fate of thyme EO within the intestinal tract is unknown, and it may be excreted, modified intestinally or metabolised. Thyme EO was supplied continuously in the diets of birds in the present study, and this inclusion decreased the numbers of caecal coliforms by 1 log even 10 days after the infection peak, so

the protective effect of thymol against coliforms outlasted the period of the infection in these birds. During the infection, the reduction in bacterial number with dietary thyme EO inclusion may initially have been more extensive in the birds. Further studies are necessary to elucidate the intestinal fate of thyme EO, whether or not it is metabolised or excreted, perhaps by radioactively labelling the terpenes or feeding them as a single dose.

Gram-negative bacteria, such as coliforms, have a relatively hydrophilic surface with small hydrophobic areas, due to their lipopolysaccharide content and especially Lipid A (Nikaido & Vaara, 1985), and represent the bacterial type most resistant to antimicrobial agents. These hydrophobic areas are known as porin channels, which may allow the transport of small hydrophobic molecules such as thymol, but prevent the cellular entry of most antibiotic drugs and larger molecules. The mode of action of these porin channels has not yet been confirmed. EO's have an effect on both Gram-negative and Gram-positive bacteria. Various EO blends incorporating thymol have reduced both the numbers and proliferation rate of *C. perfringens* in the broiler intestine (Losa, 2001; Mitsch *et al.*, 2002; Mitsch *et al.*, 2004). In broilers infected with *Mycoplasma gallisepticum*, there were prebiotic effects when polysaccharide extracts of mushrooms and herbs (*Lentinus edodes*, *Tremella fuciformis* and *Astragalus membranaceus* extracts) were supplemented in the diets, when compared to the antimicrobial apramycin (Guo *et al.*, 2004). These plant extracts stimulated *Bifidobacteria* and *Lactobacilli*, but reduced the numbers of *Bacteroides* spp. and *E. coli* in the caeca (Guo *et al.*, 2004). 'CRINA for piglets', a commercially blended terpene product, was also reported to reduce haemolytic *E. coli* numbers as a proportion of total *E. coli* excreted by piglets (Losa, 2001). This would seem to suggest a definite potential for the action of bioactive plant extracts in diseased animals to aid in the recovery from infection by modifications to the intestinal microflora.

The presence of barley kernels in diets increased the endogenous losses of nitrogen in pigs, which was attributed, in the main, to the insoluble fibre fraction in the kernel as opposed to the β–glucan fraction (Leterme *et al.*, 2000). The diet in the present study was formulated with 15% barley in order to increase the fibre fraction provided to the birds, which may have slowed the rate of digesta passage. This was done with the aim of maximising any differences associated with the inclusion of this thyme EO or carbohydrase, which was mainly composed of xylanase. When present as the only source of fermentable carbon, β–glucan did not support

the growth of any of the lactobacilli, bifidobacteria, enterococci or *E. coli* strains tested *in vitro* (Crittenden *et al.*, 2002). However, in the same study, β–glucan was utilised as the only fermentable substrate by many *Bacteroides* spp. and by *C. beijerinckii* (Crittenden *et al.*, 2002). If the rate of digesta passage was slower or the environment was less anaerobic in nature, is possible that an enhanced fermentation of the β–glucan fraction of the barley could impact on the nitrogen-utilising bacteria, thus promoting the growth of species such as *C. perfringens*. By including barley in these diets at this level, we provided a substrate for potential growth of this bacterium. Supplementation of the diets with both thyme EO and carbohydrase produced only a numerical decrease in numbers of *C. perfringens*, but there was a greater variability around the means for each treatment with this bacterium than with the others tested. The analysis of more replicates from each pen may have diminished this variability. The carbohydrate oligomers produced after the bacterial fermentation of β–glucans from barley may represent a secondary energy source for the birds. However, Vukic Vranjes *et al.* (1994) report that it is possible to replace 20% of wheat in broiler diets with barley, when an enzyme is added, without reducing bird performance, so our inclusion rate of barley may have been too low to sufficiently raise the *C. perfringens* concentration.

In the present experiment, thyme EO reduced coliform concentrations in broilers, but there was no effect on caecal *C. perfringens* concentrations. The carbohydrase only numerically decreased the concentration of *C. perfringens*. It would also appear that the concentration of the thyme EO was not as important as its presence, since there was a similar effect on coliforms at both the inclusion levels of 1 and 5 g kg^{-1} thyme EO. The caecal *C. perfringens* concentration in the present experiment was generally lower when compared to intestinal concentrations of 10_6 c.f.u. $gram^{-1}$ sample in the non-supplemented control treatment of Mitsch *et al.*, (2004). After 12 field trials, Mitsch *et al.* (2004) reported that EO blends included in broiler diets reduced intestinal *C. perfringens* concentrations in the broiler intestine from 10_6 to about 10_2 c.f.u. $gram^{-1}$ sample. Bacterial concentrations were not measured in the ileum or elsewhere in the intestinal tract in the present experiment, but there did not appear to be a problem with *C. perfringens* in the birds. In broilers fed barley-based diets, avoparcin reduced *C. perfringens* but increased the total coliform concentrations (Hofshagen & Kaldusdahl, 1992), which is similar to the effect of the carbohydrase in the present experiment. Diets supplemented with EO reduced the number and diversity of ammonia-producing bacteria in the rumen of sheep (Mc Ewan *et al.*, 2002b), which should

correspond to populations of *Clostridium* spp. in chickens. The work of Mitsch *et al.* (2004) and McEwan *et al.* (2002b) would also suggest that EO's are efficacious in reducing clostridial populations in chickens. When broilers were fed diets including a blended extract of carvacrol, cinnamaldehyde and capsaicin, the numbers of *E. coli* and *C. perfringens* decreased in the rectal digesta from 4.65 X 10^6 to 1.56 X 10^5 and from 3.91 X 10^3 to 1.86 X 10^2 c.f.u. g^{-1} respectively (Jamroz *et al.*, 2003).

Addition of the antimicrobial diacetyl to Gram-negative bacteria at concentrations below the MIC value prolonged the 'lag phase' of bacterial growth, reducing both the growth rate and final cell density with increasing concentration (Olasupo *et al.*, 2003). Thus, there is an indication that even where a natural compound is not killing microorganisms antimicrobially, there may still be some beneficial suppression of bacterial growth. However, this may still predispose the development of bacterial resistance to natural compounds, and therefore these compounds should be used in a treatment rotation or in a blended product. There may be a difference between EO blends and antibiotics in their action against various bacterial populations. It may be possible that EO have a buffering effect on microbial populations, rather than a bactericidal effect, where no one bacterial species is allowed to grow unchecked. The quality of the environmental housing conditions will obviously have an important effect on the response to dietary EO or terpene supplementation in broilers. Likewise, variations in the quality of the dietary substrate in terms of its NSP content and bacterial load will affect intestinal bacterial concentrations. Clearly, at this stage of the research on EO or terpenes, each diet or environmental situation must be taken individually in assessing the effectiveness of the supplement and interactions between EO blends and other supplements such as enzymes.

The activity of the intestinal microflora in chickens will have an indirect effect on the associated nutrient digestibility of the diet. Microbial populations in both the ileal and caecal tissues can reach as high as 10^9 and 10^{11} c.f.u. g^{-1} respectively, and it is estimated that around 10-20% of the dietary energy of the bird is required to sustain them (Apajalahti & Bedford, 2000). In this experiment, the increased numbers of coliforms seen in birds fed diets with carbohydrase may be due to the increased availability of substrate for these bacteria, through the intestinal action of carbohydrase on dietary NSP's. Thus, a separate mode of action appears to exist for the thyme oil and carbohydrase supplements. In broilers fed diets based

on wheat, xylanases numerically increased the caecal populations of *Peptostreptococcus*, *Eubacterium*, *Bifidobacterium*, *Propionibacterium* and *Bacteroides*, and decreased caecal *Clostridium*, *Enterobacteriaceae* and *Campylobacter* populations (Apajalahti & Bedford, 2000). Dietary supplementation with enzymes and zinc bacitracin significantly altered bacterial populations in the small intestine of broilers, but the only effects on the caecal microfloral populations were due to the zinc bacitracin (Hock *et al.*, 1997). Thus, there are conflicting studies on the mode of action of enzymes. As the carbohydrase in the present experiment had no effect on caecal coliforms or the other bacterial populations, this does not support an antimicrobial action. Exogenous carbohydrases act by depolymerising cereal arabinoxylans, thus reducing the viscosity of digesta, improving the rate of passage of the digesta and reducing fermentation in the ileum, while allowing more fermentation of smaller carbohydrates in the caeca (Choct *et al.*, 1999; Bedford, 2000; Apajalahti & Bedford, 2000). The simultaneous supplementation of carbohydrase and thyme EO as used in the present experiment has the potential to improve dietary usage synergistically, both by reduction of the arabinoxylan fraction and the contribution of an antimicrobial effect in the large intestine. This experiment does not support an antimicrobial action for the carbohydrase.

4.4.5 Effect of thyme oil and carbohydrase on dietary nutrient digestibility and the gut environment

Supplementation of carbohydrases and avoparcin in broiler diets positively influenced both the energy metabolisability and also the utilisation of dietary fat, with enzymes having a higher efficacy than antibiotics when each supplement was included separately (Vukic Vranjes & Wenk, 1995). The present experiment suggests that thyme EO inclusion in diets at concentrations around 5 g kg^{-1} may enhance the digestibility of nutrients in broilers, but the inclusion level of thyme EO could be reduced along with the cost of supplementation if an exogenous carbohydrase is also included. In this experiment, the optimal nutrient digestibility occurred when diets were supplemented with carbohydrase and thyme EO at 1 g kg^{-1}, and it seems reasonable to suggest that both synthetic and natural antimicrobials act in the same manner in the improvement of nutrient digestibility. An improved digestibility of dry matter (4.1%), higher crude fat digestibility at 21 days and a tendency towards increased energy digestibility at 32 days was observed in chicks fed diets including 300 ppm of the commercial compound XTRACT, which is based on capsaicin, carvacrol and cinnamaldehyde (Jamroz *et al.*, 2003). NSP-degrading enzymes act to decrease the digesta viscosity, which is caused by

polysaccharides leaching from the bran and endosperm cells of wheat grains, and simultaneously release other nutrients previously trapped inside the cell walls of the grain (Chesson, 2000). However, the mode of action of terpenes in the diet is less clear. The data from the present experiment supports the data in Chapter 3, in that terpenes in diets may exert their effect by modifying the digestibility and utilisation of energy and protein by the birds, which may be mediated through the action of terpenes on the gut microflora. The microflora produce polyamines and other harmful bacterial metabolites as a result of their metabolic activities. The absorption of glucose and some B-complex vitamins, namely nicotinic acid, pantothenic acid and biotin, was increased in the guts of germ-free compared to conventional animals (Ford & Coates, 1971). Therefore, the presence of the gut microflora can decrease the intestinal absorption of simple sugars and other nutrients.

Dietary avoparcin inclusion suppressed intestinal cell turnover, both in the duodenal epithelium and in the liver hepatocytes of broilers, and also the development of reactive lymphoid tissue within the bursa, suggesting that it decreased microflora-related intestinal activity (Krinke & Jamroz, 1996). Any positive action of antimicrobials may therefore be at least partly related to a reduction in energy expenditure associated with an immune response when certain bacteria are present. Studies in broiler chicks showed that *S. faecium* thickened the wall of the small intestine and depressed the growth of broiler chicks through a possible allergic reaction, but showed no effects on the uptake of intestinal glucose by the birds (Coates *et al.*, 1981). However, Coates *et al.* (1981) suggested that this bacterium could negatively affect the utilisation of other nutrients. If thymol or other terpenes preferentially bind to bacteria such as *S. faecium* rather than the bacterium binding to the intestinal wall, the available energy for the birds may increase. The composition of the bacteria in the lumen of the intestine is unknown, whereas the bacterial composition at the brush border membrane has been characterised. Low molecular weight terpene hydrocarbons have an ability to form hydrophobic bonds by absorption and adsorption to starch compounds in the digesta (Golovnya *et al*, 1998; Goubet *et al.*, 1998). Thus, this may indicate that terpenes do not bind to the brush border membrane, but may instead have some effect on the bacteria associated with the digesta within the intestinal lumen. However, it is possible that the effects of terpenes may be modulated through changes in brush border membrane cells, as is the case with other secondary plant compounds. Piperine, an alkaloid, increases the efficiency of nutrient absorption in rats, by increasing the brush border membrane fluidity and the absorptive area in

the small intestine, through altering the composition and kinetics of intestinal enzymes and membrane lipids and by lengthening the intestinal microvilli (Khajuria *et al.*, 2002). Khajuria *et al.* (2002) suggested that this may be due to its apolar nature, and that the turnover of membrane proteins or cytoskeletal components may be involved in increasing the efficiency of permeation through the intestinal barrier. The daily precision feeding of tannic acid directly into the crop of adult chickens at 200 and 300 mg $bird^{-1}$ reduced feed consumption in these birds, but increased glucose absorption and also the activities of trypsin, amylase, alkaline phosphatase and lipase (Majumdar & Moudgal, 1994). Majumdar & Moudgal (1994) suggested that the tannic acid created conditions suitable for the increased digestibility and absorptive efficiency of fat, protein and carbohydrates in these birds, mediated by the association of the increased alkaline phosphatase with $Na^{+}K^{+}$dependent ATPase at the brush border membrane. There was no effect on feed consumption or the activity of any enzymes when tannic acid at 100 mg $bird^{-1}day^{-1}$ was precision fed (Majumdar & Moudgal, 1994).

It is known that low molecular weight aromatic compounds such as terpenes will interact with starch, carbohydrate and protein molecules (Narasimho & Nigam, 1970; Golovnya *et al.*, 1998; Hammer *et al.*, 1999), which may affect their activity *in vivo* either positively or negatively. The presence of organic matter has been reported to affect the antimicrobial activity of thyme EO *in vitro* (Narasimho & Nigam, 1970; Hammer *et al.*, 1999). The improvement in AME and AMEn in the present study with increasing levels of thyme EO supplementation suggests that there may be an effect of terpenes on starch digestion. However, Lee *et al.* (2003) reported that there was no effect of the dietary inclusion of terpenes, including thymol, on ileal starch digestibility in broilers over a growth period of 40 days. However, the presence of the compounds –fenchone and decanal have been associated with changes in the rheological properties of starch molecules *in vitro* (Nuessli *et al.*, 1995), which may lead to changes in the digesta viscosity *in vivo*. Changes in the viscosity of digesta have been reported to be one of the modes of action of terpenes found in EO (Williams & Losa, 2001). In this experiment, the GE provided directly from the concentrations of thyme EO added to the diets of broilers was much less than the increased AME in these birds, when increasing concentrations of thyme EO were added in the diet. Thus, some other mechanism must be improving AME other than the energy provided directly by the thyme EO, and this may be a reduction in the digesta viscosity, perhaps through the action of the terpenes on the intestinal wall enabling an increased nutrient utilisation. Increased viscosity of the digesta

decreases intestinal mixing, which will make the gut environment more aerobic, and increase the potential for invasion of the ileum by obligate anaerobes, where they can compete for resources and may proliferate to cause disease (Apajalahti & Bedford, 2000). The viscosity of digesta in broilers was reduced when CRINA POULTRY, a commercial blend of EO, was included in diets based on wheat and barley (Francesch *et al.*, 1999; Losa, 2001).

The antimicrobial efficacy of thymol and thyme EO against *S. typhimurium* has been increased *in vitro* in highly anaerobic conditions (Juven *et al.*, 1994). However, the type and nature of available carbohydrate is influential in determining whether or not the terpenes attach to the dietary substrate. In the intestine, thymol and the other terpenes may not be bound to the dietary fraction but instead to the gut wall or to bacteria, and may therefore aid the binding of enzymes to starch and other dietary substrates. Aroma molecules containing oxygen atoms are twice less likely to be adsorbed or absorbed by a synthetic corn-based starch polysaccharide matrix than terpene hydrocarbons (Golovnya *et al.*, 1998). The phenolic terpenes thymol and carvacrol contain hydroxyl groups, and are generally associated with a higher bioactivity when compared to terpene hydrocarbons. Golovnya *et al.* (1998) suggested that polar compounds containing hydroxyl groups were more likely to associate with water molecules through hydrogen bonding, which would allow them to reach more areas in the intestinal tract. Thyme EO and other EO's have been observed to stimulate and influence different contractural properties of both smooth muscle (guinea pig ileum) and skeletal muscle (rat diaphragm), either when applied directly or through nerve endings, with a greater variety of effects on skeletal muscle (Lis Balchin & Hart, 1997). Different EO's and different samples of the 'same EO' resulted in different effects on muscle contraction, which was suggested to be due to different enantiomeric ratios of terpenes or other compounds within the EO (Lis-Balchin & Hart, 1997). Lis Balchin & Hart (1997) observed that thyme EO caused only a contraction effect in the smooth muscle of the guinea pig ileum. If thyme EO acts to induce intestinal contractions, this may speed up the flow of digesta through the intestine, which may increase the rate of passage and in turn may increase nutrient availability, but this must be studied within broilers.

After observing that *C. perfringens* and its toxins suppressed growth in broilers, it was suggested that broilers gradually improve their ability to digest cereal diets containing NSP by means of an increased endogenous enzyme production with age (Hofshagen & Kaldusdahl,

1992). If thyme and other EO's act by stimulating the production of endogenous enzymes, this may imply that such supplements will have a beneficial effect in early bird life. Endogenous enzyme production may even be limiting in young broiler chicks selected for rapid muscle tissue growth, where the intestinal nutrient supply system has not fully developed and must work harder to fulfill the demands of the muscle tissue. Sklan & Noy (2003) observed dramatic increases in the secretion of pancreatic amylase from 100-900 U day^{-1}, trypsin from 75-520 U day^{-1} and lipase from 40-150 U day^{-1}, between 5 and 19 days of age in broiler chicks. Nir *et al.* (1993) have suggested that endogenous enzymes are limiting in broiler chicks from 0-14 days of age, due to the slower rate of small intestinal and pancreatic development compared with demand tissues like muscle, and that these birds would benefit from exogenous enzyme supplementation in early life. Comparative studies have indicated that broilers produce similar amounts of digestive enzymes as do layers and other slower growing chickens, but have a much greater volume of digesta to act upon (Nir *et al.*, 1993; Dunnington & Siegel, 1995). The effect of various herbs and spices in increasing the endogenous enzyme, bile acid and pancreatic juice production has already been demonstrated (Platel & Srinivasan, 1996, 2000 & 2001). This has resulted in a shorter time for the transit of digesta passage in experimental rats (Platel & Srinivasan, 2001). These increased endogenous secretions will undoubtedly affect the digestion and absorption of the various dietary substrates, and in doing so may alter the size of the associated internal organs. Nutrient digestibility and retention is poor in monogastrics fed cereals with a high antinutrient content, such as rye with pentosans. When broiler chicks were fed a rye-based diet, the mass of the gall bladder as a proportion of liver mass increased, which was suggested to indicate that the production of bile had also increased (Campbell *et al.*, 1983). Lee *et al.* (2003) observed that thymol increased the mass of the avian liver by 13% ($p<0.05$) and the pancreas by 8% ($p>0.05$) at 21 days of age, compared to control birds, but this effect disappeared by 40 days of age. Hypertrophy of the liver and pancreas might suggest a high level of endogenous secretion in these birds, which may be energetically expensive, although the size of the organ may not directly affect the production of its main secretions. Unfortunately, we did not measure organ weights in the present experiment, but instead correlated endogenous losses to sialic acid concentration. However, the sialic acid concentration was only increased by inclusion of carbohydrase in the diet at 19 days and not with thyme EO inclusion. When the terpene blend CRINA poultry was added to broiler diets, amylase activity was increased by 30% within the intestinal chyme at 21 days of age compared to control birds (Lee *et al.*, 2003).

Lee *et al.* (2003) also observed that birds fed thymol in their diet had the highest trypsin activity at 40 days, compared to birds fed diets with cinnamaldehyde or CRINA poultry. It is apparent that the sensitivity of the endogenous enzymes to terpene additives in the diet changes with increasing bird age, but it is necessary to establish whether or not this effect is either negative or positive. It may be necessary to alter the composition of a dietary supplement over the lifetime of the bird. Achieving optimum gains when feeding EO supplements may also depend on individual environmental conditions in a flock. By feeding a blended product of phytochemical supplements, this may alleviate such concerns. There may also be some biotransformation and degradation of terpenes within the anaerobic environment of the intestinal tract. Novel enzymes have already been isolated for the 7α-dehydroxylation of primary bile acids (Hylemon & Harder, 1999). Muhlbauer *et al.* (2003) suggested that inactive terpenes may be metabolised to active ones within the intestinal environment. The digestion of dietary substrates may therefore be affected by the intestinal metabolism of terpenes within broilers, by novel metabolic pathways.

More research clearly needs to be undertaken in terms of the effects of terpenes in combination with other dietary supplements, and the interaction of both these types of supplement in the diets. Other types of exogenous enzymes should be considered for their effects in broilers in conjunction with the inclusion of thyme EO and other EO's in the diet. EO's are used extensively in food preservation environments, for their antibacterial properties in preventing food spoilage (Banks *et al.*, 1986; Dillon & Board, 1994). They are used to extend the shelf life of meats (Aureli *et al.*, 1981; Skandamis & Nychas, 2001), bread (Nielsen & Rios, 2000; Guynot *et al.*, 2003), seafood (Mejlholm & Dalgaard, 2002) and pre-prepared fruit (Roller & Seedhar, 2002), among other foods. The combination of nisin, a bacteriocin produced by *Lactococcus lactis,* with organic terpenes failed to enhance the antimicrobial activity of the terpenes *in vitro* (Olasupo *et al.*, 2003). In combination with diacetyl, nisin acted antagonistically in relation to controlling populations of *S. typhimurium* but not *E. coli* (Olasupo *et al.*, 2003). However, other components may have a different effect. Using a range of concentrations between 5 and 10%, the terpenes cinnamaldehyde, perillaldehyde, cuminaldehyde, citronellol, citral, geraniol, perillalcohol and eugenol were observed to have a strong synergy with a 7% solution of NaCl *in vitro* in inhibiting the growth of various fungi (Kurita & Koike, 1982). However, only a very weak synergistic anti-fungal effect was observed between this NaCl and the terpenes D-carvone, vanillin, linalol, citronellal or D-

limonene (Kurita & Koike, 1982). Thus, synergism may occur between some terpenes with components of the diet, but not others. Under anaerobic intestinal conditions, a synergistic effect may be observed between thyme EO and lower concentrations of either NaCl or some other dietary component. If not directly antimicrobial in action, the combined effect may delay or modify the growth of intestinal bacteria.

After the diets used in the present study were mixed, the EO was re-distilled from the diet and approximately 60% of the thyme EO recovered, indicating a loss of nearly 40% of the EO. This loss was relatively consistent across the treatments, and indicates that thyme EO mixed into the diets may need to be protected in some way, in order to prevent its volatilisation before the diets are ingested. It is possible that some EO may not have been recovered in the distillation process and may have become incorporated into some component of the feed material. The volatilised aromatic terpenes may still have shown some benefits when these diets were exposed to air, but these benefits are likely to be reduced.

4.4.6 Effects of thyme oil and carbohydrase on amino acid digestibility

In the present study, a clear effect of carbohydrase was shown in increasing the apparent coefficients of digestibility of AA, but there did not appear to be much of an effect of thyme EO in isolation at 19 or 42 days. When corrected to nitrogen equilibrium, this experiment showed that each addition of thyme EO in the diet up to 5g kg^{-1} increasingly improved the AMEn. The dietary inclusion of the commercial blend XTRACT, containing capsaicin, cinnamaldehyde and carvacrol, improved the apparent ileal digestibility of nitrogen, and also improved digestibilities of threonine, serine, asparagine, phenylalanine, histidine and lysine in broilers at 21 and 32 days of age (Jamroz *et al.*, 2003). Kamel (2000) also mentions separate studies where supplementation of XTRACT has increased the ileal availability of lysine and threonine to broilers, when it was fed as a dietary supplement. Thus, there may be some effect of EO on AA and N utilisation in broilers, but this is not supported by the present experiment apart from the slightly higher digestible N content of the diet when 5 g kg^{-1} thyme EO was fed in isolation. In Chapter 5, when broilers were fed diets supplemented with this same thyme EO, it had no effect on the apparent digestibility of AA's and only a numerical decrease in the digestible N content of the diet, when compared to birds on the control treatment. However, in the present experiment, a significantly higher coefficient of AMN and a higher dietary content of metabolisable N was observed in birds when the diets were supplemented with both

carbohydrase and thyme EO at 1 g kg^{-1}. It must be noted that the data in the present experiment used excreta samples at 42 days in a measure of total tract AA digestibility, whereas the analysis in Chapter 5 excluded any action of the microflora by the analysis of ileal samples. As such, it is suggested that ileal samples are used in future analyses, as used by Jamroz *et al.* (2003), in order to counteract the effect of the intestinal microflora and define a clear effect of the thyme EO in birds. The effect of this thyme EO on nitrogen utilisation from the diet is unclear, and changing the contents of dietary protein and/or energy in future studies may allow a more detailed analysis and help to clarify the situation.

By measuring true protein digestibility *in vivo*, it is assumed that ileal endogenous nitrogen losses are constant. However, these losses vary according to the presence of antinutritional factors or fibre content of the diet (Leterme *et al.*, 2000). Broilers fed wheat-based diets supplemented with antinutrient pentosans from rye were reported to have an increased variation in growth, and a general depression in the digestibilities of starch, protein and fat, by as much as 14.6, 18.7 and 25.8% respectively (Choct & Annison, 1992). As a foreign substance in diets fed to poultry, thyme EO may be regarded as an antinutrient, especially as it reduced feed intake in birds in the present experiment between 8-14 days of age. As such, thyme EO may require a period of adaptation in the diet. In heifers fed diets supplemented with the commercial product 'CRINA for ruminants', which contained EO's, no effects were observed due to the inclusion of this supplement after 10 days, but there was an increased *in situ* degradation of protein after 28 days supplementation (Molero *et al.*, 2004). Molero *et al.* (2004) suggested that a longer period of feeding the supplement would be required, in order to increase the protein supply to the small intestine and thus improve heifer performance. In sheep, Mc Ewan *et al.* (2002a) observed that EO's reduced the attachment of rumen bacteria to peas and rapeseed meal protein substrates, which decreased the breakdown of these substrates in the rumen. Therefore, this suggests that dietary EO may also influence protein utilisation or the utilisation of other nutrients in poultry. This may account in part for the increases in AME and AMEn observed in the present study, as more protein may then become directly available to the birds and there may be a saving in energy through the reduction in protein turnover.

The intestinal microflora of broilers may contribute positively towards the utilisation of dietary protein by releasing AA from peptides, but conversely may also act negatively by

deaminating AA, or incorporating AA into microbial protein (Salter *et al.*, 1974). These microbial proteins may then be excreted, resulting in an unchanged overall dietary protein utilisation. Alternatively, the action of the gut flora may be through a buffering effect. In a comparison of germ-free and conventional broilers fed a range of protein sources and also nitrogen-free diets, there were no direct effects observed due to the action of intestinal microflora on the utilisation of dietary proteins (Salter *et al.*, 1974; Salter & Fulford, 1974). However, after several changes were observed in the composition of the excreta, it was suggested that the microflora serve an important role in endogenous protein degradation, as well as nitrogen recycling and the route of nitrogen excretion from the gut of broilers (Salter & Fulford, 1974; Salter *et al.*, 1974). Thyme EO supplementation in the diets could therefore potentially alter protein and AA digestibility directly by reducing bacterial attachment to dietary substrates, or indirectly by modifying the populations of intestinal bacteria utilising this substrate in favour of the birds. This does not seem to be the case from these experimental results. However, dietary supplementation with EO may affect proteolysis. A higher level of crude protein degradation was observed in the rumen of heifers fed diets with low rather than high levels of protein concentrate (Molero *et al.*, 2004), but these authors considered this effect to be both diet and substrate dependent.

In the present experiment, xylanase supplementation in wheat-based diets improved the digestibility coefficients of AA in the total tract, which agrees with previous reports (Bedford, 1995; Hew *et al.*, 1998). Xylanase may increase AA digestibility in the birds by liberating protein-based compounds from cell walls structurally, or from their association with fibre in the intestinal matrix. Hew *et al.* (1998) reported particularly large effects of xylanase in improving the digestibility of threonine, aspartic acid, lysine, serine, glutamic acid and alanine in the excreta. The results of the present experiment indicated tendencies for the increased digestibility of threonine, tyrosine and valine in the total tract of broilers at 42 days with carbohydrase, so responses to xylanase inclusion in diets will differ according to each experimental situation. However, both the present study and that of Hew *et al.* (1998) observed an increased threonine digestibility. As we used excreta samples in the analysis, the action of the microflora will have affected the digestibility coefficients. A large proportion of the total AA pool is contained within the caecal flora, but the microflora also acts to convert some AA into others on a regular basis (Apajalahti & Bedford, 2000). Improvements in the

AA digestibility coefficients with dietary xylanase inclusion may also be related to a reduction in the losses of endogenous AA, through the reduced effects of antinutritive wheat NSP (Larsen *et al.*, 1993; Hew *et al.*, 1998). Salter & Fulford (1974) reported that broiler intestinal microflora preferentially utilise undigested N from endogenous proteins, therefore the inclusion of enzymes in diets may act to modify or limit microbial metabolism in the hindgut to prevent this action. This may also be true of various EO's. Inclusion of mint EO was found to significantly alter the AA production of both *Salmonella enteritidis* and *Staphylococcus aureus* when incubated in nutrient agar (Tassou *et al.*, 2000). However, the present study did not show any effects of thyme EO inclusion on AA digestibility. With the ability to exclude other environmental factors, results *in vitro* may be more defined than the results of dietary supplementation *in vivo*. However, using the intestinal contents of broilers and an *in vitro* fermentation technique, Shanmugavelu *et al.* (2004) reported that thyme EO suppressed microbial fermentation throughout the intestinal tract. This may indicate that thyme EO does not affect dietary utilisation of AA, but instead acts directly on the microflora.

4.4.7 Effects of thyme oil and carbohydrase on ileal morphology

In the present experiment, dietary carbohydrase inclusion decreased the depth of intestinal crypts in the ileum in broilers at 7 days of age, and tended to reduce the ileal villus height, which may indicate a decreased production of endogenous secretions or cell turnover in these birds. It would also suggest a reduced surface area for absorption and less tissue turnover. Overall, the growth of the birds fed carbohydrase was much greater, and the addition of body mass (BM) as a covariate did not change the observation of this significant effect. However, the concentration of sialic acid was increased at 19 days with carbohydrase inclusion, which would suggest a greater endogenous loss from the gut. These results are contradictory, and are difficult to explain. A numerically reduced crypt cell proliferation rate was observed in birds fed diets based on rye, supplemented with xylanase at commercial inclusion levels at 24 days of age (Silva & Smithard, 2002). This reduction in crypt cell proliferation rate was significant when the birds were fed diets including 10 times the commercial inclusion level of xylanase (Silva & Smithard, 2002). Mathlouthi *et al.* (2002) observed an increased ileal villus height and an increased villus height to crypt depth ratio at 22 days of age, when xylanase and β-glucanase were included to reduce the effects of a rye-based diet with tallow in broilers. However, ileal morphology was similar in the birds fed rye diets with carbohydrase, when compared to those fed the soya-based control diet (Mathlouthi *et al.*, 2002). This increased

age makes it difficult to compare the birds in these 2 studies with birds in the present study. As mentioned earlier in this chapter, the production of endogenous enzymes and other secretions are lower in young broilers up to 14-19 days (Nir *et al.*, 1993; Sklan & Noy, 2003). At 42 days of age, carbohydrase decreased the diameter of the ileum in the study birds. This may suggest a smaller gut tissue mass in the birds fed with carbohydrase in the diet, perhaps due to the greater efficiency in nutrient utilisation in these birds, which prevented the need for increased intestinal growth to cope with dietary processing. However, there was a larger variation around this treatment mean, and the delicate nature of these samples may have been disrupted slightly during tissue processing, despite careful handling. Not all of the ileal samples were of a circular nature, due to either the presence or absence of digesta, and not all contained digesta, which presented problems during section handling. This was the reason for taking several measurements on each section. It is possible that the use of more replicates of each treatment would increase the accuracy of the analysis, as the sampled sections represented only a very small portion of the ileum.

The size of the intestinal lumen (diameter and perimeter) was reduced in birds fed diets containing thyme EO supplements at 7 days of age in the present study, but there were no effects at 42 days with this supplement. Entering BM as a covariate in the analysis did not change these results. The reduced lumen diameter may have been a reflection of a slightly reduced feed intake by the birds, or of the presence of food within the gut at 7 days when fed diets with thyme EO. Thyme EO inclusion had no appreciable effect on the ileal morphology in these broilers, but the variability within the samples may have diminished some treatment effects. Some monoterpenes are known to have a protective effect on intestinal tissue. There was an anti-inflammatory effect of 1,8-cineole on induced intestinal colitis in rats, with an increased wet weight of intestine and reduction in intestinal lesions in pre-treated animals (Santos *et al.*, 2004), suggesting 1,8-cineole could be used in the prevention of gastrointestinal inflammation or ulceration.

4.4.8 Effect of thyme oil and carbohydrase on products of caecal bacterial fermentation

Enzyme inclusion within broiler diets in the present study decreased the concentrations of ileal VFA but increased the caecal VFA concentrations (Choct *et al.*, 1999), suggesting that enzymes decreased the activity of the gut microflora, probably by reducing the level of potential fermentable substrate through breakdown of arabinoxylans. Improvements in feed

utilisation and a significant reduction in the number of starch degrading bacteria in the caeca were also observed at 12 weeks of age, when turkey hens were fed wheat-based diets supplemented with enzyme (Persia *et al.*, 2002). This suggested that the effect of NSP in this wheat was reduced at an earlier stage in the digestive tract, and that the portion of the diet degraded by the microflora was decreased and could be manipulated. The concentration of OM available for caecal bacterial fermentation may be of considerable relevance in the action of other dietary supplements. When using thyme EO with swine and cattle waste *in vitro*, a higher OM concentration was considered to have increased the concentration of the EO required to control bacterial fermentation (Varel & Miller, 2001).

Dietary avoparcin supplementation reduced the concentration of some VFA in the caeca (acetate, propionate, butyrate and total VFA), but xylanase inclusion increased propionate and total caecal VFA concentration (Apajalahti & Bedford, 2000). The carbohydrase used in the present study had a selective action on the microflora, reducing lactic, n-butyric and overall caecal VFA, but increasing acetic acid concentrations in broilers at 19 days. In contrast, the avoparcin used by Apajalahti & Bedford (2000) appeared to be non-selective in its action. The inclusion of xylanase in wheat-based diets increased the occurrence of xylo-oligomers in the small intestine available for caecal fermentation, which increased the propionic acid concentration by promoting growth conditions suitable for *Propionibacter* and *Bifidobacteria* spp. instead of *Clostridium* spp. (Apajalahti & Bedford, 2000). Bifidobacteria may be able to grow by utilising these xylo-oligosaccharides, so slowly fermentable larger polysaccharides could actually be used as prebiotics (Crittenden *et al.*, 2002). Arabinoxylan was fermented slowly but efficiently by *Bifidobacterium longum*, but not by other species of *Bifidobacterium*, *lactobacilli*, *enterococci*, *E. coli*, *C. perfringens* and *C. difficile* when present as the sole carbon substrate (Crittenden *et al.*, 2002). This suggests that arabinoxylan itself may not be the reason for the overgrowth of potentially damaging bacteria, but this may be caused instead by the presence of other substrates. Alternatively, reflux in the caeca may introduce bacteria to an area with available substrate. However, it is generally considered that bacterial action in the small intestine should be minimised. The type or quantity of dietary starch has also been shown to manipulate the bacterial composition of the large intestine. Feeding amylomaize starch at 30% in mice diets increased bifidobacteria and lactobacilli, while elevating the faecal butyrate concentration, suggesting a prebiotic effect of this starch type (Wang *et al.*, 2002). If

terpenes change the *in vitro* rheological properties of starch, as suggested by Nuessli *et al.* (1995), further studies should focus on the variety of starch types used in diets.

The proportion of certain VFA in the caeca at 42 days in birds fed wheat/soya bean meal diets varied between 50-53% acetate, 22-25% propionate and 16-21% butyrate, either with or without an enzyme (Jamroz *et al.*, 2002). In the experiment of Jamroz *et al.* (2002), the birds were fed diets with a similar composition to those in the present study. However, there was a substantial fermentation shift in the present experiment, independent of the action of carbohydrase, where the caecal VFA composition ranged from 29-34% acetate, 15-22% lactate, 36-37% n-butyrate, 5-7% propionate 3-4% valeric acid, 0.5-1% isobutyric acid and 1-2% isovaleric acid at 42 days. The incubation of thymol and carvacrol with cattle and swine waste resulted in a complete inhibition of volatile fatty acids and lactate fermentation over 23 days *in vitro* (Varel & Miller, 2001). However, mint EO has been observed to reduce growth in *S. enteritidis,* by preventing its ability to take up glucose and therefore metabolise lactate and acetate *in vitro* (Tassou *et al.* 2000). However, this same action was not observed against *S. aureus* with the mint EO, thus there was a selective effect of the EO against some bacteria only (Tassou *et al.*, 2000). Changes in bacterial fermentation have been observed under neutral pH conditions *in vitro* using carvacrol, thymol and also mint EO, suggesting that the antimicrobial effect is not dependent on a decrease in pH (Tassou *et al.*, 2000; Varel & Miller, 2001). However, changes or reductions in the pH will increase the volatility of the VFA as they become deionised.

Fermentation studies on the same thyme EO used in the present experiment have also been carried out using broiler caecal contents *in vitro.* These studies indicated that thyme EO completely inhibited gas production and thus bacterial metabolism *in vitro* (Shanmugavelu *et al.*, 2004). A complete inhibition of bacterial fermentation may be undesirable, as fermentation provides an additional potential supply of dietary energy and protein to the bird. The impact of the microflora presence and activity is unclear with regards to nutrient utilisation in chickens. The utilisation of energy and protein from the diet is estimated to be reduced by about 10% as a result of the microflora presence, due to the increased protein turnover by the activity of the microflora and the lower energy efficiency of VFA production (Muramutsu *et al.*, 1994). However, Muramutsu *et al.* (1994) also suggest the microflora may be of benefit to the birds by preserving energy losses from the body when there is no available

dietary supply, and as such may act in a buffering capacity. Jamroz *et al.* (2002) also describes the potential energy value to the birds as a result of bacterial metabolism. It is perhaps more prudent to balance the action of the microflora, by choosing the dietary substrate carefully in an attempt to manipulate the predominant species fermented. In the absence of dietary substrates and the gut environment, the effects *in vitro* may be overly simplified, as the results of this present growth trial were not in agreement with those of Shanmugavelu *et al.* (2004). However, Shanmugavelu *et al.* (2004) did not attempt to identify the main bacteria present in the gut contents they used. Thyme EO inclusion in the present experiment was shown to enhance lactic acid concentrations, and it also tended to enhance caecal propionic acid concentrations, so thyme EO would appear to have a selective effect on microbial fermentation. Testing of the fermentation occurring within anaerobically stored minced meat, to which oregano EO had been added at 0.5 and 1%, showed that EO increased the concentration of lactic acid on the meat surface (Skandamis & Nychas, 2001). Lactic acid, which is used in the food industry to reduce microbial contamination of meat and other products, has antimicrobial properties, which may be partly due to its effect in lowering the pH (Shelef, 1994).

The present experiment showed clear differences in concentrations for all VFA in the caeca between 19 and 42 days. Increasing acetate and lactate concentrations have been reported between 3 to 15 days in commercially housed broilers, while propionate and butyrate were first detected from 12-15 days of age (van der Wielen *et al.*, 2000). After this time, lactate was undetectable, but the concentrations of the 3 other VFA remained stable (van der Wielen *et al.*, 2000), which would indicate that after the initial establishment in the caeca, they remained relatively stable. The difference in VFA concentration between 19 and 42 days in this experiment may be related to changes over time in the composition and utilisation of the diet, or they may have changed once the birds fully recovered from the infection. The exact reason is unclear. The antimicrobial properties of VFA are well known, especially at an acid pH, where the VFA exist in their lipophilic, undissociated form and can penetrate the microbial cell and disrupt its homeostasis. Formic acid and propionic acid have been reported to disrupt the synthesis of DNA, RNA, protein, lipid and cell walls in *E. coli* when added into an *in vitro* growth medium, but the mechanism for this effect is unknown (Cherrington *et al.*, 1990). In the presence of an infection, the caecal VFA concentrations in broilers have been reported to change substantially over several days, before reverting to normal levels once

more, suggesting that an infection will significantly alter microbial fermentation (Apajalahti & Bedford, 2000b). Diets fed to broilers containing NSP from rye or pectin resulted in a proliferation of bacteria and an increased production of n-butyric acid within the ileum of the birds (Wagner & Thomas, 1978), which was suggested to be associated with intestinal *Clostridium* spp. populations. Wagner & Thomas (1978) observed that penicillin treatment significantly decreased the production of n-butyric acid. In this experiment, the thyme EO had no effect on n-butyric acid, but the carbohydrase decreased its production by 7% at 19 days. As this effect was not observed at 42 days, this may mean that any adverse effects of NSP decreased over time or that the increased coliforms at 19 days were also associated with a higher activity of *Clostridium* spp., which was less important at 42 days. In piggery effluent fermented *in vitro*, VFA were equally effective against both shiga-toxigenic and resident *E. coli* strains over a 3 hour period, but at pH 4.3 the shiga-toxigenic strains were killed twice as quickly than resident strains after a 1 hour period (Harris *et al.*, 2001). When these strains were tested at pH 6.8, the VFA did not act antimicrobially towards either strain (Harris *et al.*, 2001). In the present study, there was an increased caecal lactic acid concentration at 19 days with inclusion of 3g kg^{-1} thyme EO. At 42 days, the concentration of lactic acid increased with thyme EO, and the proportion of acetic acid increased with the inclusion of carbohydrase, which may indicate a shift in metabolism towards more probiotic bacterial species with these supplements. There may be a synergistic antimicrobial action between lactic and acetic acids, especially under conditions of a decreased pH. Apart from the direct antimicrobial control of populations of *C. perfringens* and *E. coli* within the intestinal tract, Jamroz *et al.* (2003) also suggested that the terpene blend XTRACT may stimulate *Lactobacillus* spp., to produce lactic acid, which may act on bacteria through an alternative mechanism of competitive exclusion. However, in the present study, the increased concentration of lactic acid with thyme EO inclusion occurred towards the end of the growth period, when the birds had a mature gut flora. At this time, there was no infection detected, and it may have been beneficial to assess the caecal concentrations more frequently during growth to report any changing effects over time.

4.4.9 Other effects of thyme oil

The effects of terpenes should be considered holistically, rather than directly on the gut in terms of potential use as dietary supplements. There may be an application for the use of thyme EO in laying hen diets, in the protection of bone health. In mature rats, osteoclast

activity in bone resorption was inhibited by both the leaves and EO's of several plants, including sage, rosemary and thyme, and also pine, dwarf pine, juniper, and eucalyptus (Muhlbauer *et al.*, 2003). Terpene compounds, including thymol, borneol and camphor, directly inhibited the number of resorption pits in each rat osteoclast when studied *in vitro*, which were restored after the removal of these substances from the feed (Muhlbauer *et al.*, 2003). The antioxidative properties of thyme EO have also been described *in vitro* (Piccaglia *et al.*, 1993), and in older rats (Youdim & Deans, 1999a). Thyme EO, and particularly its content of thymol, has been associated with the maintenance of higher tissue polyunsaturated fatty acid concentrations in older rats (Youdim & Deans, 1999b). Botsoglou *et al.* (2002) and Papageorgiou *et al.* (2003) showed that oregano supplementation in chickens and turkeys increased the antioxidative properties of the meat.

In summary, the data suggests that both carbohydrase and thyme EO could be included together in diets to improve the growth of broilers. There were significant benefits in broiler performance characteristics with the inclusion of the enzyme but not the thyme EO. The thyme EO reduced feed intake slightly from days 8-14, but after this period there was no effect due to its inclusion on bird performance. Optimal digestibilities of wheat-based diets with a low to average quality by broilers may be achieved with a combination of both thyme EO and carbohydrase supplements. In this study, the combination was most effective when including a dietary carbohydrase at commercial levels of inclusion (0.5 g kg^{-1}) along with 1 g kg^{-1} thyme EO, but this should be validated in conjunction with other exogenous and endogenous enzymes. At 42 days of age in the birds, this treatment had the highest values for the coefficients of ADMD, DOMD and AMN, as well as the highest dietary contents of digestible OM and metabolisable N. The treatment with 1 gkg^{-1} thyme EO and carbohydrase also had the largest values for AME and AME:GE, both with and without a correction for nitrogen equilibrium. The benefits in these measurements were mainly due to the inclusion of carbohydrase in the diet, but generally indicated an additive effect of the inclusion of both supplements together on nutrient digestibility. Thyme EO inclusion in the diets in isolation had no effect on the dietary N or AA digestibility, but it may have benefits in reducing the viscosity of digesta. Inclusion of the carbohydrase had a positive effect on the digestibility of some AA, which probably occurred through liberation of nutrients within the cell wall components as part of a reduction in the effects of wheat NSP. Carbohydrase inclusion increased the concentration of sialic acid in the ileal digesta at 19 days, which may be

reflective of increased endogenous losses in these birds at this age. There was no synergistic effect between the supplements on the intestinal microflora populations. However, each supplement exerted a separate effect on the microflora, with thyme EO reducing the populations of caecal coliforms, and the carbohydrase numerically decreasing caecal *C. perfringens*. The carbohydrase reduced the depth of intestinal crypts and tended to reduce the height of villi in the ileum, an effect that was unchanged when the BM of the birds at 7 days was added as a covariate. The acetic, lactic and butyric acid concentrations in the caeca were increased with carbohydrase at 7 days, but at 42 days this supplement only tended to increase acetic, valeric and total caecal VFA concentrations, thus the effect of carbohydrase diminished with time. Thyme EO inclusion at 19 days numerically increased the concentration of lactic acid, but at 42 days, this increase in lactic acid was significant, and there was a tendency for this supplement to increase propionic acid and reduce isobutyric acid concentrations in the caeca. These effects may be synergistic, as several interactions were noted in VFA, between the age of the birds and with the inclusion of each supplement. The above effects should be balanced with the fact that the birds in this study were affected by *colisepticaemia* at an early age, and the impact of this infection is uncertain in the results of the analysis of the study material.

4.5 Conclusions

Thyme EO is a strong antimicrobial compound and its phenolic terpene content may give the diets a taste that is initially disagreeable to the birds. However, thyme EO may have a protective effect in the intestine, by a selective antimicrobial or inhibitory action against coliform populations in naturally infected birds. After the initial adaptation to the presence of thyme EO in the diet, the birds appeared to recover and showed a compensated growth. Thyme EO may be better fed when the birds have a more developed gut, or at a low concentration initially within a phytochemical blend. The beneficial effects of thyme EO in the diet of broilers were much less evident than those of exogenous enzymes, when both were included together. Carbohydrase inclusion in the diet had a positive effect on performance and also nutrient digestibility in the dietary ration. It also reduced the availability of substrate for intestinal microflora, but stimulated caecal fermentation. There is some potential for thyme EO to enhance the effects of a carbohydrase within the dietary ration for broilers, in terms of the availability of energy for the birds. However, both supplements appear to have a different mode of action in the intestine. The thyme EO may influence the viscosity of

intestinal contents. It appears to have a positive effect on the bacterial production of lactic acid, and may decrease the requirement for substrate by the microflora in the distal gut. The supplementation of thyme EO at 1 g kg^{-1} together with a carbohydrase in broiler diets is likely to synergistically improve the digestibility of an average quality ration and may have beneficial effects on the microflora within the intestine.

CHAPTER 5

5. AN ASSESSMENT OF THE INCLUSION OF VARIOUS PHYTOCHEMICALS OBTAINED FROM HERBS, GARLIC AND CONDENSED TANNINS, IN THE DIETS OF BROILERS GROWN FROM 0-42 DAYS OF AGE, WHEN ALL DIETS WERE FED WITH A CARBOHYDRASE

5.1 Introduction

Previous experiments presented in this thesis have suggested that some phytochemicals are suitable for inclusion in poultry diets as potentially beneficial bioactive compounds. The experiment described in Chapter 4 reports the effects of thyme essential oil (EO) in the presence of a carbohydrase, on various measurements associated with gut health in broilers, showing an interaction between both supplements in several parameters. The experiment reported in this chapter includes thyme EO as a dietary supplement, to assess its effects in birds free from *colisepticaemia*. However, this experiment was also designed to assess the effects of other bioactive plant supplements in broilers, notably the secondary plant compounds from garlic and tannins, when supplemented in broiler diets. To follow commercial trends as closely as possible, an exogenous carbohydrase was included in all treatment diets fed to the birds in an attempt to reduce variation caused by components such as NSPs. An emulsifier was added to the treatment rations at low concentrations to provide a source of supplementary choline in the diet, which is required by the body to help in the digestion of fat-soluble vitamins.

Garlic is noted for its wide range of bioactive properties, which include antimicrobial (Naganawa *et al.*, 1996; Benkeblia, 2004), anti-fungal (Pai & Platt, 1995), and antioxidant (Yin & Cheng, 1998b) effects. The antimicrobial properties of garlic have been noted against Vancomycin-resistant enterococci (VRE) (Jonkers *et al.*, 1999). Tannins have previously been associated with negative effects when included in the diets of broiler chicks (Dale *et al.*, 1980; Jansman, 1993), due to their strong binding associations with protein and fibre compounds. However, this reduction in the quality of the diet is not always consistent when tannins are used. The presence of a mixture of polyphenolic and simple phenolic compounds may be responsible for these adverse effects, as they are different structurally, which may influence the formation of hydrogen bonds or hydrophobic associations between the tannin and other dietary components. No depression in growth was associated with the feeding of sorghum tannins to ducks (Elkin & Rogler, 1990). Sorghum tannins are condensed tannins (CT), which

are known antimicrobials and antioxidants, so these CT may have beneficial effects in poultry. As a result, the present experiment includes 3 commercial sources of purified CT, namely grapeseed, mimosa and also cranberry. The results of the previous feeding trial (Chapter 3) indicated that yarrow herb had the potential to affect the gut microflora in birds, while maintaining the nutritional quality of the diet. Information on yarrow (*Achillea millefolium* L.) is not well reported in scientific literature, but its antimicrobial properties (Candan *et al.*, 2003) and other bioactive properties are described (Chandler *et al.*, 1982a & b). The plant may take a period of 2-3 years to become established as a crop, and for its chemical composition to stabilise accordingly (Svoboda, 2003, personal communication). This present experiment also used supplementary rosemary herb in the diets, with a different chemical composition to the rosemary used in the experiment reported in Chapter 3.

The experiment described in Chapter 3 reported the effects of diets supplemented with several plant herbs and their associated EO in broilers grown in a cage environment, which is atypical of the environmental situation faced by broilers grown commercially. A growing environment based on a cage system has been shown to be the cleanest available in terms of helminth and red mite prevention (Höglund *et al.*, 1995; Permin *et al.*, 1999). In contrast, the use of sawdust litter provides a substrate for the growth of pathogenic bacteria (Pope & Cherry, 2000), and is widely used in commercial broiler housing. The current experiment was therefore designed to maximise the effects of treatment by increasing the potential of the disease challenge within the growing environment.

5.2 Aims of the Experiment and Methodology

This experiment aimed to test the effect of various secondary plant compounds in broilers, including several herbs, thyme EO, and sources of CT's on bird performance and associated parameters. These effects could then be compared for each type of supplement. Additionally, a subsidiary experiment aimed to test the effects of some of the plant treatments on the organoleptic properties of the meat, using a panel of trained assessors.

5.2.1 Provision of dietary supplements for the feeding trial

A commercial carbohydrase (Avizyme 1210; xylanase [EC3.2.1.8]= 2776 U kg^{-1} & β-glucanase [EC3.2.1.6] = 177 U kg^{-1}) was provided by Danisco Animal Nutrition, Marlborough, UK for use in this experiment. The EO of *Thymus vulgaris* L. (thyme) was

purchased from Essentially Oils Ltd., Oxfordshire, UK, and was identical to the EO used in the experiment reported in Chapter 4. Oven-dried *Rosmarinus officinalis* L. (rosemary) was provided as chopped herb material, and *Allium sativum* L. (garlic) in the form of a spray-dried powder by Devenish Nutrition, Belfast, Co. Antrim, UK. Both of these materials were sourced by Devenish Nutrition from Park Tonks Ltd., Great Abington, Cambridge, UK. *Achillea millefolium* L. (yarrow) was grown on site at SAC Ayr, and provided by Katja Svoboda, Department of Plant Biology, SAC. Powdered extracts of *Vaccinium macrocarpon* Ait (cranberry) and *Vitis vinifera* L. (grapeseed) CT's were provided by Braes Feed Ingredients Ltd., Chester, UK. Extracts of CT from *Acacia mollissima* (previously known as *Acacia mearnsii),* or mimosa CT, was provided as a dry powder by Roy Wilson Dickson Ltd., Flintshire, UK. Lysoforte™ Booster Dry emulsifier containing lecithin (E322) was purchased by Devenish Nutrition and sourced from Kemin Europa NV (Kemin UK Ltd., Castlethorpe, Brigg, North Lincolnshire, UK).

5.2.2 Housing and Environment

The birds in this experiment were housed in open-span poultry housing, in floor pens with a litter substrate of wood-shavings, in pens of a size that allowed the birds to be stocked as closely as possible to a commercial stocking density of 34 kg m^{-2}. A small quantity of used litter (about 1kg/30 kg of wood-shavings) was sprinkled into the pens to change their environmental quality. This was intended to present a microbial challenge in the developing gut of the birds in order to maximise any treatment effects. Each pen was equipped with a single tube-feeder, and a nipple drinker line was suspended throughout the house. Heating was provided by means of a gas burner situated at one end of the house to maintain the house temperature. The temperature was set at the start of the experiment to 32°C, and then gradually decreased to achieve a final temp of 21°C at 21 days of age. The birds were provided with a standard lighting pattern for broilers, with 1 hour of darkness following a standard period of 23 hours light, from the start to the finish of the study.

5.2.3 Experimental Design

The study was conducted between November 2002 and January 2003. Using a simple randomised experimental design, the pens in the trial were randomly arranged as 6 replicates of 8 dietary treatments, to give a total of 48 pens in the study. Day-old broiler chickens (n=960) were purchased (Grampian Country Chickens, Whitburn, Midlothian) and reared

from day of hatch to 42 days. The birds were bulk-weighed, in groups of 20 on their arrival, and assigned randomly to each pen. All birds were fed a basal wheat/ soya bean meal ration, which was also supplemented with carbohydrase at 0.5 g kg^{-1}. The diets in the study were fed to the birds as starter rations from study days 0-7, grower rations from study days 8-21 and finally as finisher rations from study days 22-42 (**Table 5.1**). In addition to being weighed on day 0 of the study, the birds were also weighed again in bulk on study days 7, 21 and 42. Additionally, the birds were weighed individually on day 17, in order to assess the variation in body mass (BM) within each pen. The feed requirement for each pen was weighed in advance before allocation at the start of each feeding period, and the weight of uneaten feed at the end allowed for calculation of the feed intake and the feed conversion ratio (FCR) during that period. Samples of the grower and finisher diets were retained for analysis of their chemical composition in the laboratory. On study days 7, 21 and 42, one bird from each pen was euthanased by administration of sodium pentobarbitone (euthatal) at 1 ml kg^{-1} body mass, and samples of ileal and caecal digesta were collected as described in section 2.4.3. At the end of the trial (day 42), all birds from the control, yarrow, garlic, thyme EO and rosemary treatments were taken for use in the subsidiary study, killed and processed as whole birds within the carcass evaluation unit at SAC Auchincruive. The birds were frozen to a temperature of -20°C and transported to CHARIS Innovative Food Services (Hannah Research Institute, Ayr). Meat samples from these whole birds were retained for organoleptic analysis.

5.2.4 Diet and Nutrition

Barley and wheat were both included in the basal ration to provide a source of NSP in the diet (**Table 5.1**). The diets were formulated at SAC Ayr and were prepared as a basal control ration, balanced in their provision of energy and protein and fed to satisfy the requirements for growth of broilers of the age and genotype used in the study. The diets used were mixed at Target Feeds, (Whitchurch, Shropshire, UK) and included commercial levels of a carbohydrase at 0.5 g kg^{-1} and an emulsifier at 0.5 g kg^{-1}. This basal ration was then split into 8 treatment diets, and the treatment-specific supplements were added at SAC Ayr. Details of the inclusion levels for each plant supplement are shown (**Table 5.2**). No additional antimicrobials or anticoccidials were added to diets. The diet was supplied in the form of a mash for the duration of the study.

Table 5.1 *Diet formulation and calculated chemical composition of the basal rations*

Feed Ingredient	*Amount in diet (g kg^{-1})*		
	Starter	Grower	Finisher
Wheat	489.4	478.3	530.3
Barley	120.0	120.0	120.0
Soya bean meal (hi pro)	302.5	242.2	187.0
Soya bean meal (full fat)	0.0	57.9	64.1
Soya oil	45.0	60.0	60.0
Mono dicalcium phosphate	12.6	11.1	12.6
Limestone	15.5	15.5	12.5
Sodium chloride	3.0	3.0	3.0
Lysine	3.0	3.0	2.0
Methionine	4.0	4.0	3.5
Vit/ Min premix[1]	5.0	5.0	5.0
Calculated chemical composition (g kg^{-1})			
	Starter	Grower	Finisher
ME (MJ kg^{-1})	12.3	12.9	13.1
Crude Protein (CP)	223.5	213.5	194.0
Ether Extract/Fat	56.8	81.4	82.7
Crude Fibre	33.7	34.2	33.9
Calcium	9.5	9.2	8.3
Phosphorus	7.1	6.7	6.9
Lysine	14.5	13.9	11.6
Methionine + Cysteine	9.8	9.5	8.6

[1]Supplied per kg diet: Vitamin A 12,000 IU, Vitamin D3 5000 IU, Vitamin E (as α-tocopherol) 50 mg, Vitamin K 3 mg, Folic acid 1mg, Nicotinic Acid 50 mg, Vitamin B1 (Thiamine) 2 mg, Vitamin B2 (Riboflavin) 7 mg, Vitamin B6 (Pyridoxine) 5 mg, Vitamin B12 15 µg, Biotin 200 µg, Calcium pantothenate 15 mg, Iodine 1mg, Molybdenum 0.5 mg, Selenium 200 µg, Cobalt 0.5 mg, Copper 10 mg, Iron 80 mg, Manganese 100 mg, Zinc 80 mg, Limestone 4.18 g

Table 5.2 *Details of study treatments and inclusion levels of compounds*

Treatment	*Plant supplement*	*Inclusion level (g kg^{-1})*
1	Control (no supplement)	0
2	Yarrow herb	10
3	Thyme EO	1
4	Rosemary herb	10
5	Garlic powder	10
6	Mimosa CT powder	1
7	Grapeseed CT powder	1
8	Cranberry CT powder	1

5.2.5 Statistical analysis

The experimental data were analysed in Genstat Release 5.2, using analysis of variance. Where there was a main treatment effect, Fisher's least significant difference test (L.S.D.) was used to separate means.

5.3 Results

In this section, the composition of the supplements and experimental diets are presented. This is followed by the bird performance data, and then by the remaining experimental measurements, which were analysed at the end of each dietary phase (21 and 42 days). These measurements included the nutrient and amino acid (AA) digestibilities, sialic acid concentration and the concentration of volatile fatty acids (VFA) in the caeca. Due to the large number of experimental variables, the statistical technique of principal component analysis (PCA) was used to confirm the results of the individual analyses of variance.

All the birds remained free from disease in the study, with a mean BM at 42 days on each treatment around 200g lighter when compared to Ross 308 performance objectives. However, variability in the birds was higher in the CT treatments, and 15 birds were culled because they were not thriving or failed to keep up with the raising of the drinker line. Of the 15 birds culled throughout the trial, 26% (6 birds) were from the treatment with grapeseed CT and 42% (8 birds) were from the mimosa CT treatment. Only 1 bird was culled from those fed with cranberry CT.

5.3.1 *Experimental analysis of the plant samples and extracts used as supplements*

The garlic used in these experiments was compared against a refrigerated reference sample and was measured to have an activity of 69.9% against *Candida albicans*. For each of the herb and tannin extracts used in the experiment, the CT concentration was determined by the butanol/HCl method (**Table 5.3**). As expected, the CT concentration was greatest in the purified extracts of grapeseed and mimosa CT at approximately 373 and 258 g kg^{-1} DM respectively, but the cranberry CT content was considerably lower at 24 g kg^{-1} DM.

Table 5.3 *Condensed tannin contents in the plant supplements used in this feeding trial*

	Measurement of condensed tannins (CT)	
Supplement	*(g kg^{-1})*	*(g kg^{-1} DM)*
Yarrow	0.2	---
Rosemary	0.5	---
Mimosa CT	239.8	258.1
Grapeseed CT	343.9	372.6
Cranberry CT	22.2	24.0

Each sample is expressed as an average of 2 measurements
Dry matter corrections were not possible for rosemary and yarrow due to the content of volatile substances in the plant material

The EO's were distilled from the herb material, as described (**Appendix 2**). The thyme, yarrow and rosemary EO's were analysed by gas chromatography (GC) analysis, and the composition of the terpenes can be found (**Appendix 3**).

5.3.2 Diet Compositional Analysis

The calculated dietary compositional values are presented (**Table 5.4**). The determined composition of crude protein was similar between each grower ration at 231-239 g kg^{-1} DM, and between the finisher rations at 204-212 g kg^{-1} DM, which are comparable to the calculated values. The determined values for organic matter (OM) and gross energy (GE) were similar between the dietary treatments. The apparent metabolisable energy (AME) values are presented later in this chapter.

Table 5.4 *Composition of the dietary rations fed to broilers over the study as measured by proximate analysis when supplemented with secondary plant compounds*

	Determined chemical composition of the dietary treatments									
Treatment	*DM* ($g\ kg^{-1}$)		*CP* ($g\ kg^{-1}$ *DM*)		*Ash* ($g\ kg^{-1}$ *DM*)		*OM* ($g\ kg^{-1}$ *DM*)		*GE* ($MJ\ kg^{-1}$ *DM*)	
	G	F	G	F	G	F	G	F	G	F
Control	892	887	239	209	55	54	945	946	19.88	19.65
Yarrow herb	891	889	234	205	55	56	945	945	20.04	19.68
Thyme EO	892	888	234	210	56	54	944	946	20.01	19.74
Rosemary herb	893	887	234	204	57	55	943	945	20.37	19.74
Garlic powder	892	889	235	205	56	53	944	947	19.72	19.64
Mimosa powder	891	889	231	211	57	54	943	945	20.09	19.90
Grapeseed CT	893	888	235	210	57	53	943	947	20.45	19.72
Cranberry CT	892	894	237	212	58	55	942	945	21.19	19.78

G and F refer to grower (8-21) and finisher (22-42 days) rations. Each value is based on an average of 2 measurements.

The amino acid (AA) concentrations supplied in each grower ration, as fed to the birds from 8-21 days of age, are presented (**Table 5.5**). The acid hydrolysis procedure in the AA analysis partially destroyed the content of methionine, and the other sulphur AA and proline and hydroxyproline were not determined, thus the values obtained fall short of the determined CP concentrations. Each supplemented diet was relatively similar to the control treatment ration in the content of its AA supplied.

The concentrations of AA supplied in the ration for the finisher dietary treatments were determined (**Table 5.6**), excluding methionine and the other sulphur AA, as well as proline and hydroxyproline. Each treatment was similar in composition to the control ration in composition as fed to the birds, assuming errors during the analysis to be ±10%.

Table 5.5 *The concentrations of amino acids as fed to the birds in each dietary treatment, which included the various secondary plant compounds, between 8-21 days of age*

Amino acid composition of the grower treatment diets as fed between 8-21 days (g kg^{-1} DM)								
Treatment	*Cont*	*Yarrow*	*Thyme*	*Rosemary*	*Garlic*	*Mimosa*	*Grapeseed*	*Cranberry*
Alanine	7.92	8.09	8.47	8.54	8.60	8.32	8.08	8.01
Aspartic acid	20.59	19.89	20.55	20.75	20.76	20.42	20.22	19.85
Glutamic acid	45.01	44.99	45.76	45.99	45.54	45.36	44.65	43.69
Serine	10.53	10.69	10.76	10.89	10.86	10.38	10.02	9.87
Tyrosine	5.03	5.06	5.45	5.60	5.30	5.53	5.55	5.34
Σ Disp	**89.08**	**88.71**	**90.99**	**91.76**	**91.06**	**90.01**	**88.52**	**86.77**
Arginine	11.86	12.18	12.81	12.97	13.24	12.88	12.85	12.50
Glycine	7.57	7.78	8.00	7.92	8.46	7.89	7.15	7.05
Histidine	4.48	4.53	4.73	4.79	4.93	4.75	4.53	4.42
Isoleucine	8.94	8.90	9.25	9.23	9.08	9.02	9.11	9.02
Leucine	15.42	15.56	15.96	16.12	16.02	15.71	15.33	15.00
Lysine	12.43	12.68	12.91	13.47	13.82	13.78	12.70	12.43
Phenylalanine	10.24	10.42	10.85	10.95	10.94	10.66	10.34	10.09
Threonine	7.47	7.52	7.76	7.76	7.95	7.72	7.44	7.36
Valine	10.15	10.00	10.30	10.26	10.09	9.94	10.08	10.05
Σ Indisp	**88.55**	**89.58**	**92.56**	**93.48**	**94.53**	**92.37**	**89.53**	**87.92**
Σ Tot AA	**177.63**	**178.29**	**183.56**	**185.24**	**185.59**	**182.38**	**178.05**	**174.69**

N=3 for all treatments

Table 5.6 *The concentration of amino acids as fed to the birds in each dietary treatment, which included the various secondary plant compounds, between 22-42 days of age*

Amino acid composition of the finisher treatment diets as fed between 22-42 days (g kg^{-1} DM)								
	Control	*Yarrow*	*Thyme*	*Rosemary*	*Garlic*	*Mimosa*	*Grapeseed*	*Cranberry*
Alanine	7.40	7.35	7.03	7.30	7.34	7.05	6.88	7.05
Aspartic acid	17.64	17.30	16.41	17.65	17.11	16.84	16.53	17.18
Glutamic acid	43.84	41.99	41.35	41.94	42.29	41.32	40.58	41.95
Serine	9.09	9.03	8.79	9.09	9.15	8.96	8.71	9.03
Tyrosine	5.05	5.00	4.92	4.65	4.63	4.56	4.61	4.70
Σ Disp	**83.01**	**80.67**	**78.50**	**80.64**	**80.52**	**78.73**	**77.32**	**79.92**
Arginine	11.35	11.00	10.75	10.58	10.94	10.43	10.27	10.41
Glycine	6.83	6.71	6.55	6.59	6.83	6.21	5.98	6.28
Histidine	4.13	4.03	3.94	4.02	4.13	3.88	3.75	3.89
Isoleucine	8.27	8.02	7.70	7.89	7.57	7.42	7.23	7.47
Leucine	14.21	13.72	13.32	13.66	13.45	13.03	12.70	13.15
Lysine	10.81	10.61	10.41	10.66	10.91	10.16	9.78	9.69
Phenylalanine	9.65	9.30	9.08	9.03	9.14	8.80	8.55	8.81
Threonine	6.70	6.57	6.35	6.53	6.52	6.39	6.18	6.33
Valine	9.42	9.16	8.74	9.00	8.63	8.43	8.23	8.49
Σ Indisp	**81.37**	**79.12**	**76.84**	**77.95**	**78.12**	**74.75**	**72.67**	**74.52**
Σ Total AA	**164.38**	**159.79**	**155.34**	**158.59**	**158.64**	**153.48**	**149.99**	**154.44**

N=3 for all treatments

5.3.3 Effect of the inclusion of phytochemicals in diets on broiler performance

The inclusion of garlic in the diet improved body mass (BM) in the birds at 7 days of age (P<0.001), when compared to all other treatments, but there was no effect of this supplement on BM after 7 days (**Table 5.7**). The treatments containing thyme EO and mimosa CT had the lowest values for BM throughout the study period, but were not significantly lower than any treatment at any time other than at 7 days.

Table 5.7 *Effect of the dietary inclusion of phytochemicals on average body mass (BM) in broilers from 0-42 days of age*

	Average BM (g)			
Treatment	*Day 0*	*Day 7*	*Day 21*	*Day 42*
Control	36.2 *(0.6)*	113^{b} *(3)*	593 *(9)*	1944 *(20)*
Yarrow	37.1 *(0.7)*	111^{b} *(3)*	583 *(10)*	1931 *(46)*
Thyme	36.0 *(0.5)*	107^{b} *(3)*	576 *(8)*	1880 *(44)*
Rosemary	36.6 *(0.5)*	111^{b} *(1)*	564 *(13)*	1958 *(84)*
Garlic	36.4 *(0.8)*	120^{a} *(2)*	601 *(11)*	1960 *(44)*
Mimosa	37.3 *(0.7)*	107^{b} *(3)*	571 *(11)*	1900 *(53)*
Grapeseed	36.8 *(0.5)*	112^{b} *(2)*	573 *(21)*	1923 *(45)*
Cranberry	36.3 *(0.6)*	113^{b} *(2)*	587 *(7)*	1952 *(17)*
s.e.d	0.539	2.58	16.35	67.2
	NS	P<0.001	NS	NS

Means within a column without a common superscript are significantly different. NS (P>0.05)
Significant differences were compared at the P<0.05 level, but the greatest differences are as shown
Data are means (SEM) of 6 pen replicates

From study days 0-7, there was a higher average weight gain in the birds fed diets with garlic, when compared to those birds fed diets with yarrow and rosemary herbs, thyme EO and also mimosa, grapeseed and cranberry CT's (P<0.001; **Table 5.8**). However, those birds fed diets with garlic did not have a higher average gain, when compared to those birds fed the control diet. The birds fed diets including mimosa CT and thyme EO were associated with the lowest average gains between 0-7 days, when compared to yarrow herb and garlic, as well as grapeseed and cranberry CT's and the control diets without supplement (P<0.001). There was no difference due to treatment in average weight gain in these birds after 7 days of age.

In the first week of the study, the diets with mimosa CT were consumed to a lesser extent than all other diets except those with grapeseed CT and yarrow herb (P<0.05; **Table 5.9**). Average feed consumption was highest in birds given the diets with garlic between 0-7 days, when compared to those diets with mimosa and grapeseed CT's and also yarrow (P=0.008). The data for the second and third weeks of the study showed that the diets with garlic were again

favoured above the others, especially when compared to thyme oil and mimosa CT supplemented diets (P<0.05). During days 8-21, the control diet without supplement was only consumed in greater quantities than the diets with mimosa CT (P<0.05), and there were no differences in feed consumption between birds fed the control diet and those on the remaining dietary treatments. No treatment effects were observed in average feed consumption in either the finisher rations fed between 22-42 days, or over the study period as a whole (0-42 days).

Table 5.8 *Effect of the inclusion of dietary phytochemicals on average weight gain from 0-42 days of age in broilers*

	Average weight gain (g bird^{-1} day^{-1})			
Treatment	*0-7 days*	*8-21 days*	*22-42 days*	*0-42 days*
Control	11.2^{ab} *(0.4)*	34.6 *(0.4)*	64.1 *(0.9)*	45.4 *(0.5)*
Yarrow	10.5^{bc} *(0.3)*	34.2 *(0.7)*	64.2 *(1.9)*	45.2 *(1.2)*
Thyme	10.2^{d} *(0.4)*	33.5 *(0.4)*	62.1 *(2.0)*	43.9 *(1.0)*
Rosemary	10.7^{bcd} *(0.1)*	32.4 *(0.8)*	66.4 *(3.7)*	45.8 *(2.0)*
Garlic	11.9^{a} *(0.2)*	34.4 *(0.7)*	64.7 *(1.7)*	45.8 *(1.0)*
Mimosa	10.0^{d} *(0.4)*	33.1 *(0.6)*	64.2 *(1.8)*	44.8 *(1.0)*
Grapeseed	10.8^{bc} *(0.3)*	33.7 *(1.3)*	64.0 *(1.2)*	45.0 *(1.0)*
Cranberry	10.9^{bc} *(0.3)*	33.9 *(0.4)*	65.0 *(0.6)*	45.6 *(0.4)*
s.e.d	0.36	1.02	2.67	1.53
	P<0.001	NS	NS	NS

Significant differences are illustrated in each column by the presence of non-identical superscripts
Significance was compared at P<0.05 level, but the largest differences are as shown. NS (P>0.05)
Data are means (SEM) of 6 treatment replicates

Table 5.9 *Effect of the inclusion of dietary phytochemicals on average feed consumption over 42 days in broilers*

	Average feed consumption (g bird^{-1} day^{-1})			
Treatment	*0-7 days*	*8-21 days*	*22-42 days*	*0-42 days*
Control	17.3^{abc} *(0.6)*	50.2^{ab} *(0.4)*	119 *(3)*	77 *(1)*
Yarrow	16.7^{bcd} *(0.6)*	50.0^{ab} *(0.7)*	118 *(3)*	77 *(1)*
Thyme	17.4^{abc} *(0.7)*	47.9^{bc} *(0.7)*	115 *(2)*	74 *(1)*
Rosemary	17.8^{ab} *(0.5)*	48.4^{abc} *(0.9)*	121 *(4)*	78 *(2)*
Garlic	18.0^{a} *(0.2)*	50.3^{a} *(0.9)*	119 *(2)*	78 *(1)*
Mimosa	15.8^{d} *(0.4)*	46.7^{c} *(1.2)*	117 *(2)*	74 *(2)*
Grapeseed	16.7^{bcd} *(0.4)*	48.0^{abc} *(1.2)*	117 *(3)*	75 *(1)*
Cranberry	17.4^{abc} *(0.4)*	49.1^{ab} *(0.4)*	119 *(1)*	77 *(1)*
s.e.d	0.55	1.20	3.55	1.99
	P=0.008	P<0.05	NS	NS

Significance was compared at P<0.05 level, but the largest differences are as shown. NS (P>0.05)
Means within a column without a common superscript are significantly different.
Data are means (SEM) of 6 treatment replicates

Dietary garlic inclusion resulted in birds with the best feed conversion ratio (FCR) between 0 and 7 days of age, when compared to those birds fed diets with thyme EO and rosemary herb (P<0.05; **Table 5.10**). The birds fed diets with rosemary herb had the highest FCR's between

study days 8-21, compared to those fed diets including thyme EO, and those with mimosa, grapeseed and cranberry CT's (P=0.013). After this time, there was no effect of any diet between study days 22 and 42, or throughout the trial period overall on broiler FCR.

Table 5.10 *Effect of the inclusion of dietary phytochemicals on feed conversion ratio (FCR) in broilers over 42 days*

	Feed conversion ratio per pen (FCR) (feed unit gain^{-1})			
Treatment	*0-7 days*	*8-21 days*	*22-42 days*	*0-42 days*
Control	1.56^{ab} *(0.04)*	1.45^{ab} *(0.01)*	1.85 *(0.03)*	1.70 *(0.02)*
Yarrow	1.59^{ab} *(0.04)*	1.47^{ab} *(0.02)*	1.85 *(0.02)*	1.70 *(0.02)*
Thyme	1.72^{b} *(0.06)*	1.43^{b} *(0.01)*	1.85 *(0.03)*	1.69 *(0.02)*
Rosemary	1.67^{b} *(0.04)*	1.50^{a} *(0.02)*	1.85 *(0.12)*	1.71 *(0.09)*
Garlic	1.52^{a} *(0.03)*	1.46^{ab} *(0.01)*	1.85 *(0.02)*	1.70 *(0.02)*
Mimosa	1.60^{ab} *(0.04)*	1.41^{b} *(0.02)*	1.83 *(0.03)*	1.66 *(0.02)*
Grapeseed	1.56^{ab} *(0.05)*	1.43^{b} *(0.02)*	1.83 *(0.02)*	1.66 *(0.02)*
Cranberry	1.60^{ab} *(0.06)*	1.45^{b} *(0.02)*	1.82 *(0.01)*	1.68 *(0.01)*
s.e.d	0.059	0.022	0.068	0.049
	P<0.05	P=0.013	NS	NS

Means within a column without a common superscript are significantly different at P<0.05. NS (P>0.05)
Data are means (SEM) of 6 treatment replicates

The inclusion of any herb or EO in the diets of broilers over the growth period of 42 days had no effect on the eviscerated (EV) carcass weights (**Table 5.11**). The birds fed diets with CT supplements were not included in this analysis, as they were not sent for organoleptic analysis.

Table 5.11 *Effect of the inclusion of phytochemicals in diets over a 42 day growth period on the eviscerated (EV) carcass weights of broilers processed as whole birds*

Treatment	*EV weight at 42 days (g)*
Control	1295 *(0.016)*
Yarrow herb	1266 *(0.017)*
Thyme oil	1246 *(0.016)*
Rosemary herb	1228 *(0.018)*
Garlic powder	1294 *(0.018)*
s.e.d	0.036
	NS

Comparisons were done at the P<0.05 level. NS (P>0.05)
Data are means (SEM) of 6 replicates of each treatment

5.3.4 Effect of phytochemical inclusion on dietary nutrient digestibility

The effect of inclusion of phytochemicals in broiler diets on the measurement of ileal digestibility in the birds was assessed at the end of each of the grower and finisher dietary phases (21 and 42 days). The coefficients of apparent DM digestibility (ADMD), digestibility of OM (DOMD), apparent digestibility of nitrogen (AND), apparent digestibility of energy

(ADE) and also the apparent digestibility of energy corrected to nitrogen equilibrium (ADEn) were calculated for the birds. The coefficient of the digestibility of energy within the ration was also calculated (DE:GE), along with the digestible OM and N contents of the treatment diets. This section presents the results of these measurements of ileal digestibility at 21 and 42 days of age in the birds.

The gross energy (GE) of thyme EO was determined to be 39.14 MJ kg^{-1}, and that of garlic powder to be 16.51 MJ kg^{-1}. The difference between values for ADE in the birds fed the diet with garlic and those fed the non-supplemented control was calculated as 0.67 MJ kg^{-1} DM at 21 days of age. The energy supplied directly by the garlic to the birds in the ration was 0.165 MJ at this level of dietary inclusion, thus garlic supplementation in the ration improved ADE by about 0.5 MJ more than the energy it supplied within the diet.

There was no significant difference of any dietary treatment on ADE in the broilers, either with or without a nitrogen correction at 21 days (**Table 5.12**). However, there was a tendency (P=0.067) for the DE:GE ratio to be lower in the birds fed with cranberry CT, when compared to those fed with garlic. Several samples were not measurable in this analysis (data from pens 2, 5, 11, 17, 25, 31 and 32) and 1 sample was removed (pen 43) due to the low volume of sample available for analysis. As the material available for measurement at 21 days from the ileal contents was limited, and there were several missing data values, the s.e.d.'s were corrected manually for treatment comparisons as shown in the table.

Both the coefficients of ADMD (P=0.018) and DOMD (P<0.05) were reduced in the birds fed diets with cranberry when compared to all the other treatment diets except that supplemented with yarrow herb at 21 days (**Table 5.13**). Likewise, the content of digestible OM in the diet was reduced in the birds fed diets supplemented with cranberry CT, when compared to those fed all other diets except for yarrow herb (P=0.019).

Table 5.12 *Effect of dietary inclusion of phytochemicals on the digestibility of energy, both with and without a correction for nitrogen equilibrium (ADE and ADEn), along with the ratio of DE:GE within the dietary ration in broilers at 21 days*

	Dietary energy utilisation at 21 days		
Treatment	*ADE*	*ADEn*	*DE:GE*
	(MJ kg^{-1} DM)		
(1) Control	11.66 *(0.71)*	10.92 *(0.68)*	0.587 *(0.036)*
(2) Yarrow	11.47 *(0.21)*	10.78 *(0.21)*	0.572 *(0.011)*
(3) Thyme	11.74 *(0.17)*	11.08 *(0.21)*	0.587 *(0.009)*
(4) Rosemary	11.56 *(0.42)*	10.99 *(0.37)*	0.567 *(0.021)*
(5) Garlic	12.20 *(0.17)*	11.48 *(0.16)*	0.619 *(0.009)*
(6) Mimosa	11.43 *(0.77)*	10.88 *(0.71)*	0.569 *(0.038)*
(7) Grapeseed	11.89 *(0.99)*	11.24 *(0.91)*	0.581 *(0.048)*
(8) Cranberry	10.57 *(0.46)*	10.08 *(0.42)*	0.498 *(0.021)*
s.e.d. 1	0.667	0.619	0.033
s.e.d. 2	0.700	0.649	0.034
s.e.d. 3	0.746	0.692	0.037
s.e.d. 4	0.775	0.719	0.038
s.e.d. 5	0.731	0.678	0.036
s.e.d. 6	0.817	0.758	0.040
	NS	NS	P=0.067

Significant differences are illustrated in each column by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates of each treatment, unless the data is corrected for missing values.
Treatment comparisons are as follows: s.e.d. 1 refers to treatments 1, 4 & 7 in comparison with each other, s.e.d. 2 refers to treatments 1, 4 & 7 in comparison with treatments 2 & 5, s.e.d. 3 describes treatments 1, 4 & 7 in comparison with treatments 3, 6 & 8, s.e.d. 4 refers to treatments 3, 6 & 8 in comparison with 2 & 5, s.e.d. 5 refers to the comparison between treatments 2 & 5 and s.e.d. 6 refers to treatments 3, 6, & 8 in comparison with each other.

Table 5.13 *Effect of the inclusion of dietary phytochemicals on the apparent coefficients of dry and organic matter digestibility (ADMD and DOMD), along with the digestible OM content in the diet as fed to broiler chicks at 21 days of age*

	Coefficients of DM and OM digestibility, with the dietary contents of digestible OM		
Treatment	*ADMD*	*DOMD*	*Dig OM content of the diet (g kg^{-1} DM)*
Control	0.580^{a} *(0.035)*	0.591^{a} *(0.035)*	559^{ab} *(33)*
Yarrow	0.543^{ab} *(0.059)*	0.553^{ab} *(0.059)*	522^{bc} *(56)*
Thyme	0.594^{a} *(0.025)*	0.609^{a} *(0.024)*	575^{ab} *(23)*
Rosemary	0.556^{a} *(0.019)*	0.569^{a} *(0.019)*	537^{ab} *(18)*
Garlic	0.625^{a} *(0.011)*	0.636^{a} *(0.011)*	600^{a} *(10)*
Mimosa	0.590^{a} *(0.023)*	0.605^{a} *(0.024)*	570^{ab} *(22)*
Grapeseed	0.584^{a} *(0.044)*	0.596^{a} *(0.045)*	562^{ab} *(42)*
Cranberry	0.465^{b} *(0.036)*	0.478^{b} *(0.037)*	450^{c} *(35)*
s.e.d.	0.040	0.041	38.4
	P=0.018	P<0.05	P=0.019

Significant differences within each column are indicated by the presence of non-identical superscripts.
Data are means (SEM) of 6 replicates of each treatment.

The birds fed diets with garlic had the highest coefficients of AND when compared to those birds fed diets with rosemary, as well as mimosa and cranberry CT's (P<0.05; **Table 5.14**). The birds fed the diets with cranberry CT had a lower coefficient of AND than those birds fed

diets with rosemary, garlic and mimosa CT respectively (P<0.05). The content of N digested from the diet was highest for those birds fed with garlic, compared to those fed diets with rosemary herb, and mimosa and cranberry CT's (P=0.021). Birds fed cranberry CT in their diets had the lowest digestible N content from their diet, compared to those birds fed diets with yarrow herb, thyme EO, garlic and grapeseed CT (P=0.021).

Table 5.14 *Effect of including phytochemicals on apparent coefficients of nitrogen digestibility (AND) and content of digestible N in diets as fed to broilers at 21 days*

	N digestibility and utilisation from the diet	
Treatment	*AND*	*Digestible N content of diet (g kg^{-1} DM)*
Control	0.508^{ab} *(0.033)*	19.39^{ab} *(1.25)*
Yarrow	0.477^{abc} *(0.059)*	17.86^{abc} *(2.20)*
Thyme	0.453^{abc} *(0.043)*	16.94^{abc} *(1.59)*
Rosemary	0.414^{bc} *(0.044)*	15.52^{bcd} *(1.66)*
Garlic	0.528^{a} *(0.027)*	19.86^{a} *(1.01)*
Mimosa	0.405^{bc} *(0.038)*	14.94^{cd} *(1.38)*
Grapeseed	0.475^{abc} *(0.064)*	17.87^{abc} *(2.40)*
Cranberry	0.325^{c} *(0.027)*	12.32^{d} *(2.24)*
s.e.d.	0.056	2.114
	P<0.05	P=0.021

Significant differences in each column are indicated by the presence of different superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates for each treatment.

There were no effects of treatment on ADE or ADEn in the birds at 42 days of age with respect to treatment (**Table 5.15**), nor were there any effects on the digestibility of energy within the dietary ration (DE:GE). There was an increase in ADE of around 0.8 MJ for the birds fed the diet with garlic when compared to those birds fed the control ration. However, the energy supplied by the garlic within the diet at this inclusion level was 0.165 MJ, indicating that garlic increased the ADE slightly more than was supplied nutritionally.

There were no differences due to treatment at 42 days of age in broilers on the coefficients of ADMD or DOMD, nor was there any difference in the digestible OM content of the diet (**Table 5.16**). No treatment differences were observed on either the coefficient of AND or the digestible N content within the diet as fed at 42 days (**Table 5.17**).

Table 5.15 *Effect of the inclusion of dietary phytochemicals on the digestibility of energy in the ration, both with and without a correction for nitrogen equilibrium (ADE and ADEn) and the ratio of DE:GE in the diet as fed to broilers at 42 days of age*

Energy utilisation in the diets of broilers at 42 days			
Treatment	*ADE*	*ADEn*	*DE:GE*
	(MJ kg^{-1} DM)		
(1) Control	11.24 *(0.56)*	10.84 *(0.52)*	0.572 *(0.029)*
(2) Yarrow	10.10 *(0.39)*	9.65 *(0.41)*	0.513 *(0.020)*
(3) Thyme	10.14 *(1.04)*	9.77 *(0.91)*	0.513 *(0.053)*
(4) Rosemary	10.67 *(1.32)*	10.18 *(1.34)*	0.541 *(0.067)*
(5) Garlic	12.04 *(0.31)*	11.50 *(0.30)*	0.613 *(0.016)*
(6) Mimosa	9.77 *(0.81)*	9.39 *(0.75)*	0.491 *(0.041)*
(7) Grapeseed	11.26 *(1.01)*	10.73 *(0.92)*	0.571 *(0.051)*
(8) Cranberry	10.64 *(0.73)*	10.21 *(0.64)*	0.538 *(0.037)*
s.e.d. 1	1.173	1.092	0.059
s.e.d. 2	1.231	1.145	0.062
s.e.d. 3	1.286	1.196	0.065
	NS	NS	NS

Significant differences in each column are indicated by the presence of different superscripts. NS (P>0.05)
Data are the means (SEM) of 6 replicates of each treatment, unless corrected for missing values.
Treatment comparisons are as follows: s.e.d. 1 refers to treatments 3, 5, 7 & 8 in comparison with each other, s.e.d. 2 refers to treatments 1, 2, 4, & 6 in comparison with treatments 3, 5, 7, & 8 and s.e.d. 3 to treatments 1, 2, 4, & 6 in comparison with each other.

Table 5.16 *Effect of dietary inclusion of phytochemicals on the apparent coefficients of DM and OM digestibility (ADMD and DOMD), along with the content of digestible OM in the diets as fed to broiler chicks at 42 days of age*

Coefficients of DM and OM digestibility, with the dietary contents of digestible OM			
Treatment	*ADMD*	*DOMD*	*Digestible OM content of the diet (g kg^{-1} DM)*
Control	0.556 *(0.029)*	0.575 *(0.030)*	534 *(28)*
Yarrow	0.507 *(0.025)*	0.526 *(0.026)*	497 *(24)*
Thyme	0.519 *(0.052)*	0.540 *(0.052)*	511 *(49)*
Rosemary	0.529 *(0.062)*	0.542 *(0.066)*	513 *(62)*
Garlic	0.594 *(0.018)*	0.615 *(0.019)*	582 *(18)*
Mimosa	0.479 *(0.030)*	0.498 *(0.031)*	471 *(29)*
Grapeseed	0.566 *(0.052)*	0.586 *(0.054)*	555 *(51)*
Cranberry	0.534 *(0.034)*	0.555 *(0.034)*	525 *(32)*
s.e.d.	0.058	0.061	58.0
	NS	NS	NS

Significant differences in each column are indicated by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates of each treatment.

Table 5.17 *Effect of dietary phytochemical inclusion on apparent coefficient of nitrogen digestibility (AND) and the content of digestible N in the diet in broilers at 42 days*

N digestibility and utilisation from the diet at 42 days		
Treatment	*AND*	*Digestible N content of diet (g kg^{-1} DM)*
Control	0.326 *(0.038)*	10.87 *(1.28)*
Yarrow	0.339 *(0.061)*	11.11 *(1.98)*
Thyme	0.294 *(0.108)*	9.91 *(3.65)*
Rosemary	0.398 *(0.030)*	13.00 *(0.99)*
Garlic	0.450 *(0.020)*	14.78 *(0.65)*
Mimosa	0.237 *(0.072)*	8.00 *(2.43)*
Grapeseed	0.428 *(0.077)*	14.36 *(2.59)*
Cranberry	0.349 *(0.086)*	11.80 *(2.90)*
s.e.d.	0.097	3.268
	NS	NS

Significant differences in each column are indicated by the presence of superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates of each treatment.

5.3.5 Effect of dietary phytochemical inclusion on the concentration of sialic acid in ileal digesta as a measure of endogenous loss in broilers

There were no differences in relation to the concentration of sialic acid in the ileal digesta with the dietary inclusion of phytochemicals, at either 21 or 42 days of age (**Table 5.18**).

Table 5.18 *Effect of the inclusion of dietary phytochemicals on the concentration of sialic acid in the ileum of broilers at 21 and 42 days of age, as a measure of endogenous losses when expressed relative to feed intake*

Sialic acid as a proportion of total feed intake (mg g^{-1} intake)		
Supplement	*21 days*	*42 days*
Control	103 *(8.13)*	153 *(12.53)*
Yarrow	90 *(4.87)*	151 *(10.52)*
Thyme	102 *(7.87)*	145 *(15.46)*
Rosemary	97 *(5.73)*	161 *(4.10)*
Garlic	106 *(6.60)*	162 *(3.81)*
Mimosa	106 *(12.63)*	176 *(13.52)*
Grapeseed	101 *(7.23)*	139 *(17.66)*
Cranberry	97 *(7.94)*	164 *(14.20)*
s.e.d. 1	9.53	14.72
s.e.d. 2	9.99	15.44
s.e.d. 3	10.44	
	NS	NS

Significant differences are expressed in each column by the presence of superscripts. NS (P>0.05).
Data are means (SEM) of 6 replicates per treatment, but the s.e.d.'s are corrected for missing data points.
At 21 days, treatment comparisons are as follows: s.e.d. 1 refers to comparisons between all diets except yarrow and thyme, s.e.d. 2 refers to comparisons between yarrow or thyme and the rest, and s.e.d. 3 to yarrow *vs* thyme.
At 42 days, s.e.d. 1 refers to comparisons between all treatments except for the control group, and s.e.d. 2 refers to comparisons between the control group with any other treatment.

5.3.6 Effect of dietary phytochemical supplementation on amino acid digestibility

The calculation of the apparent AA digestibility coefficients at both 21 and 42 days of age in these birds, by comparing the determined concentrations of each AA in the ileal contents and the treatment diets, was complicated by high concentrations of each AA in the ileal digesta. The concentrations of amino acids in the dietary treatments were as expected. This higher concentration of ileal AA nitrogen was confirmed, by repeating several samples in a second analysis. The higher concentrations of AA in the ileal contents in these samples correspond well with the determined concentrations of N in the Dumas method, which was used to calculate the coefficient of apparent nitrogen digestibility using these same samples of ileal digesta. For this reason, the digestibility coefficients of the amino acids were much lower than normally observed, and this will be examined in more detail in the discussion section.

There were no treatment differences in relation to the digestibility coefficients of arginine in the birds (**Table 5.19**). Generally, for each AA, the birds fed those diets with supplementary garlic had the highest digestibility coefficients, when compared to those birds fed the diets with cranberry and mimosa CT's and also rosemary herb. The birds fed the diets with cranberry CT had significantly lower digestibility coefficients for all AA except arginine, when compared to those fed the control diets, and those with thyme EO, rosemary, yarrow and garlic herbs in general. In some specific cases, there were slight differences in the coefficients of digestibility of AA between the treatments from those general observations stated above. The birds fed the control treatments were not different from those fed diets with grapeseed CT in the coefficients of AA digestibility. There was an increased total AA digestibility in the birds fed diets with garlic, compared to those fed diets with rosemary and mimosa CT ($P=0.012$). The birds fed diets with cranberry CT had the poorest total AA digestibility, compared to those fed diets with yarrow and garlic, thyme EO, grapeseed CT and the non-supplemented controls ($P=0.012$). There were no treatment differences in the digestibility of any AA at 42 days (**Table 5.20**).

Table 5.19 *Effect of dietary inclusion of phytochemicals on the apparent coefficients of digestibility of amino acids at 21 days*

	Apparent digestibility coefficients of amino acids in broilers at 21 days of age									
Treat	*Control*	*Yarrow*	*Thyme*	*Rosemary*	*Garlic*	*Mimosa*	*Grapeseed*	*Cranberry*	*s.e.d*	
Ala	0.347^{ab} *(0.051)*	0.313^{ab} *(0.082)*	0.335^{ab} *(0.048)*	0.330^{ab} *(0.101)*	0.439^{a} *(0.038)*	0.267^{bc} *(0.054)*	0.335^{ab} *(0.077)*	0.139^{c} *(0.049)*	0.078	P<0.05
Asp	0.526^{a} *(0.042)*	0.482^{a} *(0.052)*	0.483^{a} *(0.035)*	0.473^{a} *(0.068)*	0.556^{a} *(0.024)*	0.445^{ab} *(0.043)*	0.511^{a} *(0.050)*	0.359^{b} *(0.035)*	0.055	P<0.05
Glu	0.698^{a} *(0.026)*	0.672^{ab} *(0.038)*	0.661^{ab} *(0.026)*	0.625^{bc} *(0.028)*	0.708^{a} *(0.017)*	0.624^{bc} *(0.025)*	0.675^{ab} *(0.026)*	0.564^{c} *(0.024)*	0.033	P=0.003
Ser	0.504^{ab} *(0.039)*	0.479^{ab} *(0.054)*	0.467^{ab} *(0.032)*	0.424^{b} *(0.061)*	0.547^{a} *(0.027)*	0.412^{bc} *(0.045)*	0.466^{ab} *(0.051)*	0.300^{c} *(0.047)*	0.057	P=0.007
Tyr	0.459^{ab} *(0.041)*	0.413^{abc} *(0.070)*	0.428^{abc} *(0.044)*	0.370^{bc} *(0.049)*	0.507^{a} *(0.032)*	0.390^{abc} *(0.058)*	0.497^{a} *(0.045)*	0.307^{c} *(0.038)*	0.061	P<0.05
Σ Disp AA	**0.591^{ab} *(0.034)***	**0.559^{abc} *(0.049)***	**0.554^{abc} *(0.031)***	**0.524^{bc} *(0.045)***	**0.617^{a} *(0.023)***	**0.512^{cd} *(0.035)***	**0.572^{abc} *(0.040)***	**0.432^{d} *(0.031)***	**0.045**	**P=0.009**
Arg	0.602 *(0.034)*	0.565 *(0.052)*	0.563 *(0.032)*	0.567 *(0.063)*	0.640 *(0.024)*	0.536 *(0.050)*	0.625 *(0.039)*	0.473 *(0.031)*	0.055	NS
Gly	0.309^{a} *(0.069)*	0.304^{a} *(0.098)*	0.309^{a} *(0.053)*	0.268^{a} *(0.134)*	0.447^{a} *(0.044)*	0.271^{a} *(0.061)*	0.286^{a} *(0.074)*	0.030^{b} *(0.054)*	0.094	P=0.014
His	0.548^{ab} *(0.045)*	0.507^{ab} *(0.045)*	0.507^{ab} *(0.063)*	0.465^{bc} *(0.048)*	0.601^{a} *(0.029)*	0.478^{bc} *(0.042)*	0.534^{ab} *(0.048)*	0.366^{c} *(0.035)*	0.052	P=0.006
Iso	0.516^{a} *(0.036)*	0.477^{ab} *(0.059)*	0.475^{ab} *(0.035)*	0.436^{abc} *(0.055)*	0.544^{a} *(0.027)*	0.396^{bc} *(0.048)*	0.494^{ab} *(0.054)*	0.350^{c} *(0.044)*	0.056	P<0.05
Leu	0.523^{ab} *(0.035)*	0.487^{ab} *(0.063)*	0.479^{ab} *(0.036)*	0.438^{bc} *(0.051)*	0.558^{a} *(0.029)*	0.411^{bc} *(0.047)*	0.487^{ab} *(0.054)*	0.332^{c} *(0.046)*	0.056	P=0.01
Lys	0.512^{ab} *(0.032)*	0.477^{ab} *(0.075)*	0.456^{b} *(0.041)*	0.431^{b} *(0.048)*	0.584^{a} *(0.032)*	0.448^{b} *(0.041)*	0.490^{ab} *(0.057)*	0.312^{c} *(0.042)*	0.057	P=0.004
Phe	0.603^{ab} *(0.030)*	0.575^{ab} *(0.053)*	0.575^{ab} *(0.028)*	0.538^{bc} *(0.043)*	0.643^{a} *(0.023)*	0.515^{bc} *(0.037)*	0.576^{ab} *(0.043)*	0.451^{c} *(0.035)*	0.045	P=0.008
Thr	0.215^{ab} *(0.064)*	0.181^{ab} *(0.089)*	0.182^{ab} *(0.052)*	0.152^{ab} *(0.113)*	0.306^{a} *(0.032)*	0.106^{bc} *(0.106)*	0.251^{ab} *(0.072)*	-0.068^{c} *(0.080)*	0.093	P=0.017
Val	0.478^{a} *(0.039)*	0.432^{ab} *(0.066)*	0.428^{ab} *(0.040)*	0.383^{abc} *(0.053)*	0.501^{a} *(0.030)*	0.340^{bc} *(0.050)*	0.445^{ab} *(0.061)*	0.292^{c} *(0.049)*	0.060	P<0.05
Σ Ind AA	**0.492^{ab} *(0.039)***	**0.459^{ab} *(0.067)***	**0.454^{ab} *(0.037)***	**0.424^{b} *(0.062)***	**0.547^{a} *(0.029)***	**0.403^{bc} *(0.047)***	**0.478^{ab} *(0.054)***	**0.303^{c} *(0.043)***	**0.059**	**P=0.015**
Σ Tot AA	**0.542^{ab} *(0.036)***	**0.509^{ab} *(0.058)***	**0.504^{ab} *(0.034)***	**0.473^{bc} *(0.053)***	**0.581^{a} *(0.026)***	**0.457^{bc} *(0.041)***	**0.525^{ab} *(0.047)***	**0.367^{c} *(0.037)***	**0.052**	**P=0.012**

In each row, non-identical superscripts are used to show treatments that are significantly different from each other.
Data was tested at P<0.05, but the largest differences are significant to the level shown.
Data are the means (SEM) of 6 replicates on each treatment.

Table 5.20 *Effect of dietary inclusion of phytochemicals on the apparent coefficients of digestibility of amino acids at 42 days of age in broilers*

	Apparent digestibility coefficients of amino acids in broilers at 42 days of age									
	Cont	*Yarrow*	*Thyme*	*Rosemary*	*Garlic*	*Mimosa*	*Grapeseed*	*Cranberry*	*s.e.d.*	
Alanine	0.125 *(0.058)*	0.175 *(0.067)*	0.067 *(0.139)*	0.218 *(0.056)*	0.331 *(0.019)*	0.019 *(0.091)*	0.238 *(0.116)*	0.107 *(0.138)*	0.135	NS
Aspartic Acid	0.347 *(0.049)*	0.373 *(0.053)*	0.258 *(0.116)*	0.400 *(0.033)*	0.453 *(0.016)*	0.253 *(0.070)*	0.422 *(0.086)*	0.334 *(0.090)*	0.102	NS
Glutamic Acid	0.581 *(0.023)*	0.617 *(0.028)*	0.554 *(0.074)*	0.639 *(0.021)*	0.673 *(0.011)*	0.525 *(0.043)*	0.636 *(0.049)*	0.572 *(0.061)*	0.063	NS
Serine	0.311 *(0.058)*	0.359 *(0.050)*	0.278 *(0.114)*	0.362 *(0.063)*	0.486 *(0.022)*	0.240 *(0.076)*	0.422 *(0.103)*	0.351 *(0.104)*	0.113	NS
Tyrosine	0.295 *(0.050)*	0.368 *(0.064)*	0.274 *(0.120)*	0.344 *(0.052)*	0.445 *(0.011)*	0.136 *(0.092)*	0.412 *(0.089)*	0.257 *(0.113)*	0.117	NS
Σ Disp	**0.444** ***(0.036)***	**0.480** ***(0.041)***	**0.400** ***(0.095)***	**0.500** ***(0.031)***	**0.559** ***(0.014)***	**0.367** ***(0.059)***	**0.517** ***(0.071)***	**0.436** ***(0.081)***	**0.085**	NS
Arginine	0.426 *(0.048)*	0.517 *(0.055)*	0.418 *(0.108)*	0.491 *(0.042)*	0.581 *(0.022)*	0.361 *(0.073)*	0.568 *(0.073)*	0.384 *(0.111)*	0.104	NS
Glycine	0.063 *(0.067)*	0.148 *(0.061)*	0.014 *(0.146)*	0.143 *(0.062)*	0.280 *(0.024)*	-0.121 *(0.099)*	0.148 *(0.135)*	-0.001 *(0.160)*	0.149	NS
Histidine	0.365 *(0.043)*	0.445 *(0.051)*	0.331 *(0.114)*	0.416 *(0.048)*	0.536 *(0.016)*	0.283 *(0.066)*	0.457 *(0.091)*	0.343 *(0.111)*	0.107	NS
Isoleucine	0.332 *(0.042)*	0.369 *(0.054)*	0.274 *(0.113)*	0.383 *(0.038)*	0.454 *(0.016)*	0.200 *(0.077)*	0.388 *(0.079)*	0.287 *(0.107)*	0.104	NS
Leucine	0.336 *(0.044)*	0.372 *(0.054)*	0.281 *(0.114)*	0.396 *(0.036)*	0.474 *(0.015)*	0.230 *(0.068)*	0.414 *(0.078)*	0.299 *(0.111)*	0.103	NS
Lysine	0.270 *(0.056)*	0.329 *(0.064)*	0.247 *(0.118)*	0.380 *(0.045)*	0.452 *(0.018)*	0.235 *(0.061)*	0.379 *(0.090)*	0.195 *(0.131)*	0.114	NS
Phenylalanine	0.452 *(0.036)*	0.493 *(0.044)*	0.416 *(0.094)*	0.498 *(0.030)*	0.580 *(0.013)*	0.368 *(0.057)*	0.506 *(0.061)*	0.423 *(0.090)*	0.084	NS
Threonine	-0.081 *(0.082)*	-0.058 *(0.097)*	-0.164 *(0.141)*	0.026 *(0.052)*	0.107 *(0.022)*	-0.278 *(0.127)*	0.040 *(0.138)*	-0.148 *(0.159)*	0.158	NS
Valine	0.285 *(0.045)*	0.316 *(0.062)*	0.222 *(0.117)*	0.336 *(0.038)*	0.407 *(0.015)*	0.131 *(0.079)*	0.342 *(0.087)*	0.228 *(0.112)*	0.110	NS
Σ Indisp	**0.291** ***(0.049)***	**0.343** ***(0.059)***	**0.247** ***(0.116)***	**0.359** ***(0.041)***	**0.443** ***(0.017)***	**0.181** ***(0.075)***	**0.380** ***(0.088)***	**0.242** ***(0.119)***	**0.111**	NS
Σ Tot AA	**0.368** ***(0.042)***	**0.412** ***(0.050)***	**0.324** ***(0.106)***	**0.431** ***(0.036)***	**0.502** ***(0.015)***	**0.276** ***(0.067)***	**0.451** ***(0.079)***	**0.343** ***(0.099)***	**0.098**	NS

Significant differences are shown in the table by the presence of non-identical superscripts in each row. NS (P>0.05)

Data are the means (SEM) of 6 replicates of each treatment

5.3.7 Effect of the inclusion of dietary phytochemicals on the caecal VFA concentration

The concentrations of lactic acid (P=0.002) and n-butyric acid (P<0.001) were decreased in the caeca between 21 and 42 days of age, but the caecal concentrations of propionic (P<0.001) and valeric (P<0.001) acids increased with age in the birds (**Table 5.21**).

Table 5.21 *Effect of age on individual caecal VFA concentrations in broilers fed dietary phytochemical supplements during growth*

	Concentrations of caecal VFA (g kg^{-1})				
	Acetic	*Lactic*	*Propionic*	*Valeric*	*n-Butyric*
21 days	4.59 *(0.12)*	1.28^{a} *(0.05)*	0.81^{a} *(0.09)*	0.48^{a} *(0.06)*	4.13^{a} *(0.15)*
42 days	4.37 *(0.12)*	1.03^{b} *(0.06)*	1.42^{b} *(0.08)*	0.80^{b} *(0.04)*	3.28^{b} *(0.13)*
s.e.d.	0.190	0.075	0.092	0.065	0.175
	NS	P=0.002	P<0.001	P<0.001	P<0.001

Significant differences are shown in each column by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates for each treatment.

The effect of dietary treatment on the caecal VFA concentrations in broilers is presented at 21 and 42 days of age respectively (**Figure 5.1**). There were no effects of treatment with dietary phytochemicals at either 21 or 42 days of age in the birds on the concentrations of caecal VFA. No significant interactions were observed between treatment and time on the caecal VFA concentrations, but n-butyric acid tended (P=0.089) towards an interaction between treatment and the age in these birds.

There were no effects of dietary treatment on the proportions of acetic, lactic, propionic, n-butyric or valeric acids in relation to the total VFA concentration in the caeca (**Table 5.22**). However, there was a tendency (P=0.073) for an increase in the total VFA concentrations in the birds fed diets with yarrow, compared to those fed diets with garlic, indicating that the fermentative activity of the microflora in the birds fed the treatment with garlic may have been suppressed. However, the total VFA in the birds fed diets with garlic was not different when compared to those on the control treatment. The proportions of minor VFA (isovaleric and isobutyric acids) were increased in the birds fed diets with grapeseed CT, compared to those fed diets with cranberry and mimosa CT's, garlic, rosemary, thyme EO and yarrow (P=0.002). The birds fed the control diets also had a greater proportion of isovaleric and isobutyric acids, compared to those fed diets with yarrow, thyme, rosemary and garlic (P=0.002). However, the birds on the control treatment diets were not significantly different compared to those fed cranberry CT in the diet in relation to their caecal proprtions of isovaleric and isobutyric acids.

Figure 5.1 *Effect of dietary supplementation with various phytochemicals, either in the form of herbs or extracted active compounds on the caecal VFA concentrations (g kg^{-1}) at 21 and 42 days of age in broilers*

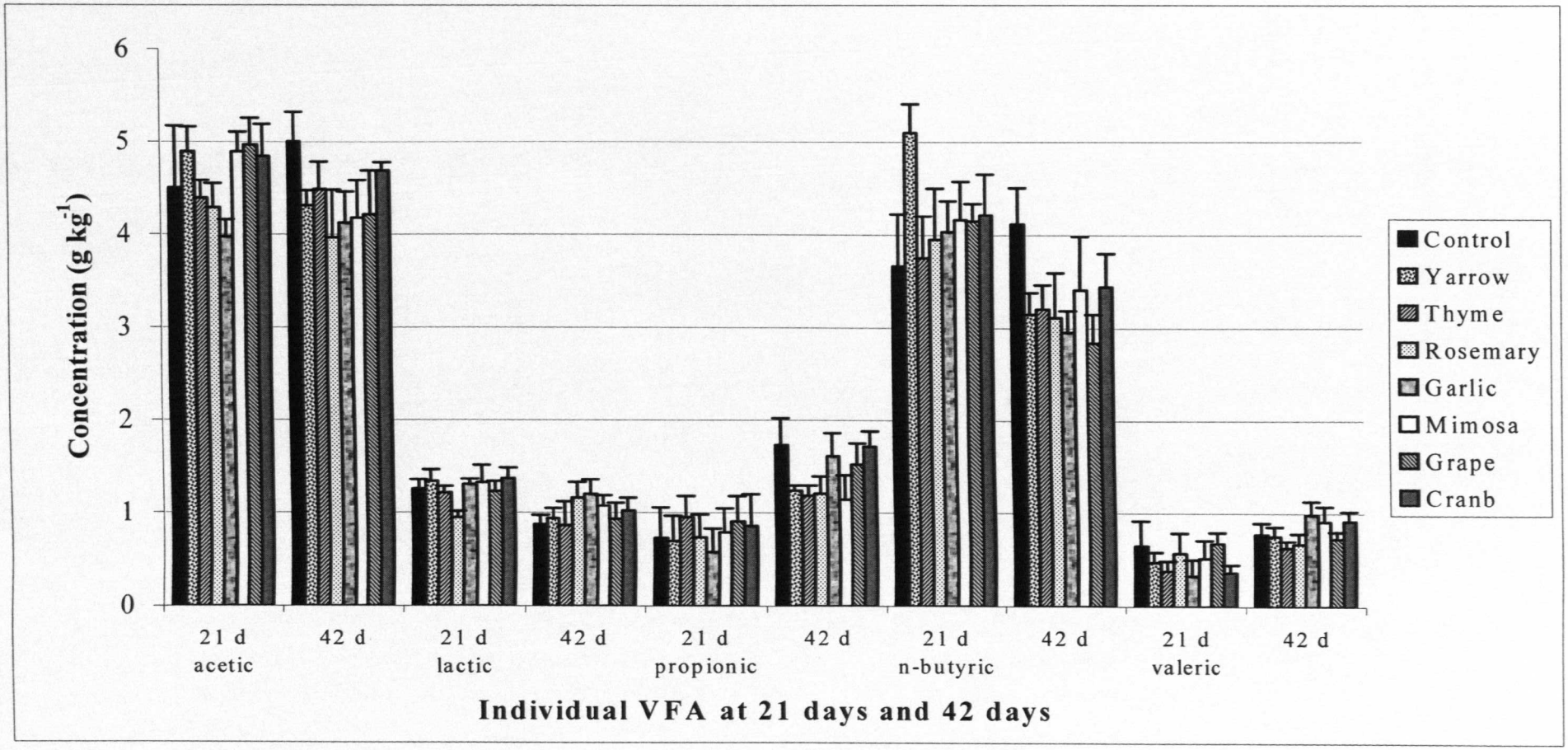


Table 5.22 *Effect of inclusion of dietary phytochemicals on individual concentrations of caecal VFA in broilers at 21 days, where each is presented as a percentage of the total*

	Proportions (%) of individual VFA in relation to total VFA concentration						*Total VFA conc(gkg^{-1})*
Treatment	*Acetic*	*Lactic*	*Propionic*	*n-Butyric*	*Valeric*	*Other*	
(1) Control	42.18	13.75	6.08	34.73	3.23	1.03^{ab}	10.54
	(3.08)	*(3.27)*	*(2.45)*	*(1.63)*	*(2.03)*	*(0.47)*	*(1.45)*
(2) Yarrow	38.92	10.65	5.35	41.10	3.68	0.30^{c}	12.53
	(1.22)	*(1.07)*	*(1.92)*	*(3.25)*	*(0.78)*	*(0.15)*	*(0.41)*
(3) Thyme	41.28	11.38	8.78	34.85	3.47	0.22^{c}	10.71
	(1.91)	*(0.61)*	*(1.86)*	*(3.53)*	*(0.91)*	*(0.11)*	*(0.49)*
(4) Rosemary	41.90	9.03	7.27	37.78	5.20	0.18^{c}	10.36
	(2.66)	*(1.03)*	*(2.23)*	*(3.71)*	*(2.06)*	*(0.12)*	*(0.63)*
(5) Garlic	38.92	12.77	5.35	39.87	3.03	0.03^{c}	10.21
	(1.45)	*(0.49)*	*(2.21)*	*(3.56)*	*(1.48)*	*(0.03)*	*(0.33)*
(6) Mimosa	41.67	11.35	6.67	35.40	4.28	0.60^{bc}	11.76
	(1.59)	*(1.74)*	*(2.14)*	*(3.28)*	*(1.57)*	*(0.17)*	*(0.31)*
(7) Grapeseed	41.03	10.17	7.12	34.85	5.43	1.45^{a}	12.10
	(1.49)	*(0.85)*	*(1.98)*	*(2.74)*	*(0.75)*	*(0.33)*	*(0.57)*
(8) Cranberry	41.57	11.87	7.50	36.03	2.99	0.47^{bc}	11.65
	(2.65)	*(1.13)*	*(2.95)*	*(3.17)*	*(0.75)*	*(0.25)*	*(0.32)*
s.e.d. 1	3.001	2.026	1.550	2.835	1.304	0.337	0.866
s.e.d. 2		2.124	1.626		1.699		
s.e.d. 3					1.594		
s.e.d. 4					1.766		
	NS	NS	NS	NS	NS	P=0.002	P=0.073

Significant differences are indicated in each column by non-identical superscripts. NS (P>0.05)
Data was tested at the P<0.05 level, but the largest differences are significant to the level shown.
Data are means (SEM) for 6 replicates of each treatment, unless corrected for missing data points.
Unless otherwise stated, s.e.d. 1 was used for treatment comparisons. For lactic acid, s.e.d. 2 refers to the comparison between treatment 4 and the rest. For propionic acid, s.e.d. 2 refers to the comparison between treatment 1 and the rest. For valeric acid, s.e.d. 1 refers to comparisons between treatments 2, 3, 4, 5, 6 & 7 with each other, s.e.d. 2 refers to comparisons between treatment 1 and treatments 2, 3, 4, 5, 6 & 7, s.e.d 3 refers to comparisons between treatment 8 and treatments 2, 3, 4, 5, 6 & 7 and s.e.d. 4 refers to the comparison between treatments 1 & 8.

There was no effect of dietary treatment on either the total VFA concentration in the caeca at 42 days, nor was there an effect of treatment on the proportions of any of the individual caecal VFA in relation to the total VFA concentration (**Table 5.23**).

Table 5.23 *Effect of inclusion of dietary phytochemicals on individual concentrations of caecal VFA in broilers at 42 days, where each is presented as a percentage of the total*

	Proportions (%) of individual VFA in relation to total VFA concentration							*Total*
Treatment	*ACET*	*LACT*	*PROP*	*n-BUT*	*VAL*	*ISOB*	*ISOVAL*	*VFA* ($g\ kg^{-1}$)
(1) Control	39.37	7.08	13.27	31.98	6.12	0.37	1.27	12.78
	(0.95)	*(1.06)*	*(1.81)*	*(1.47)*	*(0.79)*	*(0.07)*	*(0.32)*	*(0.89)*
(2) Yarrow	40.17	8.75	11.58	29.07	7.00	0.47	1.62	10.76
	(1.36)	*(0.97)*	*(0.50)*	*(1.51)*	*(0.94)*	*(0.03)*	*(0.19)*	*(0.38)*
(3) Thyme	42.07	7.70	11.15	29.93	6.03	0.35	1.72	10.69
	(2.02)	*(2.02)*	*(0.87)*	*(1.47)*	*(0.88)*	*(0.07)*	*(0.40)*	*(0.62)*
(4) Rosemary	38.58	11.35	11.67	29.72	7.08	0.38	1.35	10.43
	(1.14)	*(0.85)*	*(1.11)*	*(1.74)*	*(0.85)*	*(0.06)*	*(0.17)*	*(1.41)*
(5) Garlic	36.80	10.85	14.03	26.47	8.77	0.40	1.63	11.20
	(0.44)	*(1.20)*	*(1.10)*	*(1.20)*	*(0.95)*	*(0.07)*	*(0.38)*	*(0.86)*
(6) Mimosa	39.57	9.28	10.18	30.87	8.73	0.39	1.86	10.77
	(2.37)	*(1.06)*	*(1.79)*	*(2.66)*	*(1.35)*	*(0.08)*	*(0.74)*	*(1.21)*
(7) Grapeseed	39.80	8.57	14.08	27.10	7.08	0.42	1.82	10.59
	(0.99)	*(1.07)*	*(0.80)*	*(1.30)*	*(0.63)*	*(0.05)*	*(0.23)*	*(1.15)*
(8) Cranberry	38.90	8.40	14.22	28.05	7.62	0.45	1.32	12.12
	(1.34)	*(1.13)*	*(1.31)*	*(2.15)*	*(0.80)*	*(0.07)*	*(0.15)*	*(0.45)*
s.e.d. 1	2.054	1.752	1.709	2.470	1.331	0.084	0.468	1.305
s.e.d. 2		1.838			1.396	0.088	0.491	
s.e.d. 3						0.094		
s.e.d. 4						0.098		
	NS	NS	NS	NS	NS	NS	NS	NS

Significant differences are indicated in each column by non-identical superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates for each treatment, unless corrected for missing data points.
In the table, ACET=Acetic Acid, LACT=Lactic Acid, PROP=Propionic Acid, n-BUT=n-Butyric Acid, VAL=Valeric Acid, ISOB=Isobutyric Acid, ISOVAL=Isovaleric Acid
Unless otherwise stated, s.e.d. 1 was used for treatment comparisons. For lactic acid, s.e.d. 2 refers to the comparison between treatment 6 and the rest. For valeric acid, s.e.d. 2 refers to the comparison between treatment 4 and the rest. For isovaleric acid, s.e.d. 2 refers to comparisons between treatment 6 and the rest. For isobutyric acid, s.e.d. 1 refers to comparisons between treatments 2, 3, 5, 7, & 8, s.e.d. 2 refers to comparisons between treatments 1 & 4 and treatments 2, 3, 5, 7, & 8, s.e.d 3 refers to comparisons between treatment 6 and treatments 2, 3, 5, 7, & 8 and s.e.d. 4 refers to the comparison between treatment 6 and treatments 1 & 4.

5.3.8 Effect of dietary supplementation with various herbs on the organoleptic properties of cooked chicken.

Carcasses from birds fed on the diets with yarrow, thyme EO, rosemary, garlic and the control treatment were thawed and cooked on a commercial rotisserie to an internal temperature of 80°C. They were then cut into joints of white and red meat and served hot to a panel of trained meat assessors. Other portions were allowed to cool, stored overnight at a temperature of 4°C, and served cold to the assessors. The assessors performed the test in isolation and had no knowledge of which samples they were evaluating. On each of 5 separate occasions, meat samples taken from the 5 treatments were subjected to an assessment by the panel, and the

dataset was analysed by the KwikSense© routine. The effect of feeding mimosa, cranberry and grapeseed CT's on the organoleptic properties of poultry meat was not assessed.

A summary of the results is presented (**Table 5.24**). As anticipated, the most striking differences were between the dark and white meat samples, shown as 'meat type' in the table. However, the temperature of the assessment also influenced the perception of the sensory character for 5 flavour attributes (abnormal, sweet, synthetic, garlic and bitter) and for oily mouth feel. Three flavour attributes were influenced by diet, namely flavour intensity, abnormal flavour and garlic flavour. Interactions were observed between meat type and temperature for the overall flavour of the chicken ($P<0.01$), and rosemary flavour ($P<0.05$). There was also an interaction between diet and temperature for garlic flavour ($P<0.05$).

Table 5.24 A *summary of discriminant effects on the various organoleptic characteristics of poultry meat samples measured by the KwikSense analysis*

		Main effects			*1st Order interactions*		
Attribute	Discriminant	Diet (D)	Meat type (M)	Temp (T)	D*M	D*T	M*T
Appearance	Y		***				
Hue	Y		***				
Aroma intensity	Y		***				
Flavour							
Intensity	Y	*	***				
Abnormal	Y	***		***			
Sweet	Y			*			
Chicken	Y		***				**
Synthetic	Y			**			
Salty	N						
Garlic	Y	***		**		*	
Rosemary	Y		*				*
Bitter	Y		**				
Astringent	Y			***			
Thyme	N						
Other	Y		*				
Aftertaste							
Intensity	Y		***				
Mouth feel							
Juicy	Y		***				
Tender	Y		***				
Oily	Y		***	*			
Fibrous	Y		***				
Melt-in-mouth	Y		***				
Moist	Y		***				
Slimy	Y		***				

Significance comparisons are as follows: * ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$)

The differences in flavour intensity were relatively small and were most marked between chickens receiving supplements of rosemary and yarrow (**Table 5.25**). The differences in both abnormal and garlic flavour were associated with the dietary garlic supplement, producing an increase in the intensity of these flavour attributes (P<0.001). These differences were similar for both white and dark meat. However, the effect of the garlic supplement was most marked for the meat served cold (**Table 5.26**). The interaction between temperature and treatment for the 'abnormal meat flavour' attribute was most marked for birds fed on thyme and garlic (P<0.05; **Table 5.27**).

Table 5.25 *Effects of dietary phytochemical inclusion on organoleptic properties in broiler meat*

Meat flavour			*Least Square Mean Values*					
Attribute	Discriminant	Diet (D)	Control	Garlic	Rosemary	Thyme	Yarrow	SED*
Intensity	Y	*	49.9	51	47.8	50	50.6	1.02
Abnormal	Y	***	2.8	6.4	2.5	4	2.7	0.77
Garlic	Y	***	2.1	4.5	1.4	1.8	2.2	0.73

Significance comparisons are as follows: * (P<0.05); ** (P<0.01); *** (P<0.001)

Table 5.26 *Interactions between meat type and temperature in poultry meat for the 'garlic flavour' attribute in meat sourced from birds fed diets with herbal supplements during growth*

Meat type	*Temperature*	*Rating*
Control	Hot	1.6
Garlic	Hot	2.3
Rosemary	Hot	1.2
Thyme	Hot	2.1
Yarrow	Hot	1.3
Control	Cold	2.6
Garlic	**Cold**	**6.7**
Rosemary	Cold	1.6
Thyme	Cold	1.6
Yarrow	Cold	3.2
s.e.d.*		0.77

* (P<0.05)

These tests concluded that the inclusion of garlic in the diet of growing chickens resulted in a significant change in the organoleptic properties of the chicken meat. The tests for acceptability did not identify that these changes were detrimental to the flavour of the product. Other herbal treatments, such as thyme, may also influence the flavour of the meat. However, it was suggested that further modifications to the diet of the birds may change this effect, either enhancing it or detracting from it, as there was no evidence to suggest that this was an optimal effect.

Table 5.27 *Interactions between meat type and temperature on the 'abnormal flavour' attribute in meat sourced from broilers fed various dietary herbal supplements during growth*

Meat type	*Temperature*	*Rating*
Control	Hot	2.7
Garlic	Hot	7.6
Rosemary	Hot	4.2
Thyme	Hot	6.0
Yarrow	Hot	2.8
Control	Cold	0.8
Garlic	Cold	3.4
Rosemary	Cold	2.0
Thyme	Cold	1.9
Yarrow	Cold	2.8
s.e.d.*		0.77

* ($P<0.05$)

5.3.9 Principal components analysis (PCA)

As previously described in Chapter 3, PCA was included in this experiment to compare the results of the separate analyses of variance with a different statistical methodology, due to the large numbers of experimental variables involved in this experiment. The aim was to reduce the number of variables by grouping (correlating) related variables together to explain the sources of variation within the dataset by regression. An example correlation used in the initial stages of PCA was included (**Appendix 8**).

5.3.9.1 The correlation of related data variables associated with nutrient digestibility in broilers when phytochemicals were added to broiler diets

At 21 days, the weightings or "loadings" used in the calculation of the first 2 principal components explained 97.1% of the total variation in the dataset associated with nutrient digestibility (**Table 5.28**). Analysis of variance carried out on the PC1 component showed that cranberry CT supplementation in the diet affected nutrient digestibility ($P=0.003$), but no other treatment had an effect at 21 days (**Table 5.29**). In the PC2 component, the inclusion of rosemary, mimosa, grapeseed and cranberry CT supplements in diets had an effect on nutrient digestibility in the birds, when compared to those birds fed the diets with yarrow and the non-supplemented control diet ($P=0.008$). However, there was no effect observed in the birds fed diets with thyme or garlic supplements when compared to any other treatment on nutrient digestibility in these birds. Generally, these results are in agreement with those of the individual ANOVA's carried out for nutrient digestibility in these birds at 21 days.

Table 5.28 *The loadings calculated for insertion into the correlation matrix in the calculation of principal components for variables relating to nutrient digestibility at 21 days, when phytochemical supplements were included in broiler diets*

Loadings used in PCA for nutrient digestibility at 21 days		
Variable	*PC1*	*PC2*
ADE (MJ kg^{-1} DM)	-0.355	0.372
DE:GE	-0.361	0.284
Coefficient AND	-0.346	-0.494
Retention of dietary nitrogen (g kg^{-1})	-0.345	-0.507
ADEn (MJ kg^{-1} DM)	-0.350	0.470
Coefficient ADMD	-0.360	0.118
Coefficient DOMD	-0.361	-0.070
Digestible OM content of diet (g kg^{-1})	-0.350	-0.201
% of variation explained	**92.5**	**4.6**
Cumulative variation explained	**92.5%**	**97.1%**

There were 7 missing values in this analysis

Table 5.29 *Results of the analysis of variance on the calculated principal components of the correlation matrix of variables associated with nutrient digestibility in broilers at 21 days, when the diets of the birds were supplemented with phytochemicals*

Nutrient digestibility as analysed by PCA		
Treatment	*PC1*	*PC2*
Control (1)	-0.88^{a} *(1.08)*	-0.55^{b} *(0.22)*
Yarrow (2)	-0.57^{a} *(0.41)*	-0.56^{b} *(0.30)*
Thyme (3)	-0.59^{a} *(0.15)*	-0.05^{ab} *(0.39)*
Rosemary (4)	0.54^{a} *(0.71)*	0.45^{a} *(0.24)*
Garlic (5)	-1.45^{a} *(0.36)*	-0.11^{ab} *(0.21)*
Mimosa (6)	0.39^{a} *(1.04)*	0.50^{a} *(0.18)*
Grapeseed (7)	-0.36^{a} *(1.57)*	0.09^{a} *(0.18)*
Cranberry (8)	4.22^{b} *(1.71)*	0.18^{a} *(0.19)*
s.e.d. 1	1.231	0.301
s.e.d. 2	1.291	0.316
s.e.d. 3	1.377	0.337
s.e.d. 4	1.431	0.350
s.e.d. 5	1.349	0.330
s.e.d. 6	1.508	0.369
	P=0.003	P=0.008

Treatments were compared at the P<0.05 level, but the largest differences are as shown.
Significant differences are illustrated in each column by the presence of non-identical superscripts.
Data are means (SEM) of 6 replicates of each treatment, unless corrected for missing data points.
Treatment comparisons are as follows: s.e.d. 1 refers to the comparisons of treatments 1, 4 & 7 with each other, s.e.d. 2 refers to comparisons between treatments 1, 4 & 7 and treatments 2, 5 & 8, s.e.d. 3 refers to comparisons between treatments 1, 4 & 7 and treatments 3 & 6, s.e.d. 4 refers to comparisons between treatments 2, 5 & 8 and treatments 3 & 6, s.e.d. 5 refers to the comparisons of treatments 2, 5 & 8 with each other, and s.e.d. 6 refers to the comparison between treatments 3 & 6.

At 42 days, the loadings used in the calculation of the first 2 principal components explained 98.9% of the total dataset variation associated with the analysis of nutrient digestibility in the birds (**Table 5.30**).

Table 5.30 *The loadings calculated for insertion into the correlation matrix in the calculation of principal components for variables relating to nutrient digestibility at 42 days, when broiler diets were supplemented with dietary phytochemicals*

Loadings used in PCA for nutrient digestibility at 42 days		
Variable	*PC1*	*PC2*
ADE (MJ kg^{-1} DM)	-0.360	-0.225
DE:GE	-0.360	-0.230
Coefficient of AND	-0.335	0.609
Retention of dietary nitrogen (g kg^{-1})	-0.335	0.614
ADEn (MJ kg^{-1} DM)	-0.355	-0.321
Coefficient ADMD	-0.363	-0.100
Coefficient DOMD	-0.361	-0.132
Digestible OM content of diet (g kg^{-1})	-0.359	-0.134
% of variation explained	**93.6**	**5.3**
Cumulative variation explained	**93.6%**	**98.9%**

Analysis of variance carried out on the PC1 component showed no treatment differences, thus the bulk of the digestibility information was similar between treatments (**Table 5.31**). There was a tendency (P=0.058) for an effect of dietary treatment in the birds on nutrient digestibility in the analysis of variance on the PC2 component, but this component represented only a small part of the variation within the dataset. These results agree well with the results of the individual analyses of variance on nutrient digestibility at 42 days in these birds.

Table 5.31 *Results of the analysis of variance on the calculated principal components of the correlation matrix of nutrient digestibility variables in broilers at 42 days, when the diets of the birds were supplemented with various phytochemicals*

Nutrient digestibility at 42 days as analysed by PCA		
Treatment	*PC1*	*PC2*
Control (1)	-0.13 *(0.86)*	-0.51 *(0.16)*
Yarrow (2)	0.81 *(0.40)*	0.54 *(0.49)*
Thyme oil (3)	1.02 *(1.70)*	-0.14 *(0.27)*
Rosemary (4)	-1.57 *(0.49)*	-0.45 *(0.05)*
Garlic (5)	-1.74 *(0.48)*	-0.13 *(0.14)*
Mimosa (6)	1.75 *(1.14)*	0.32 *(0.19)*
Grapeseed (7)	-0.70 *(1.58)*	0.26 *(0.23)*
Cranberry (8)	0.45 *(1.20)*	0.07 *(0.32)*
s.e.d. 1	1.608	0.349
s.e.d. 2	1.686	0.366
s.e.d. 3	1.797	0.391
s.e.d. 4	1.868	0.406
s.e.d. 5	1.761	0.383
	NS	P=0.058

Significant differences are illustrated in each column by non-identical superscripts. NS (P>0.05)
Data are means (SEM) of 6 replicates of each treatment, unless corrected for missing data points.
Treatment comparisons are as follows: s.e.d. 1 refers to comparisons between treatments 3, 5, 7 & 8, s.e.d. 2 refers to comparisons between treatments 3, 5, 7 & 8 and treatments 1, 2 & 6, s.e.d. 3 refers to comparisons between treatment 4 and treatments 3, 5, 7 & 8, s.e.d. 4 refers to comparisons between treatment 4 and treatments 1, 2 & 6 and s.e.d. 5 refers to comparisons between treatments 1, 2 & 6.

5.3.9.2 Amino acid digestibility

At 21 days in these broilers, 97% of the variation between the coefficients of ileal digestibility of AA could be explained by calculation of the first 2 principal components, and the loadings used in their calculation are presented (**Table 5.32**). The individual analysis of variance on the PC1 component showed that birds fed diets with cranberry CT affected the AA digestibility variables, when compared to those birds fed diets with yarrow, thyme EO, rosemary, garlic, grapeseed CT and the controls (P=0.014; **Table 5.33**). The birds fed diets with garlic had an effect on the variables associated with the coefficients of AA digestibility, compared to those birds fed diets with mimosa CT (P=0.014). There were no treatment effects on AA digestibility coefficients in broilers at 21 days of age, when analysis of variance was carried out on the PC2 component. The results of this analysis are in general agreement with those of the individual analyses of variance on the AA digestibility coefficients as presented earlier in this chapter.

Table 5.32 *The loadings calculated for insertion into the covariance matrix in the calculation of principal components for variables relating to the coefficients of amino acid digestibility at 21 days, when phytochemicals were included as dietary supplements*

Loadings used in PCA for amino acid digestibility in broilers at 21 days of age		
Variable	*PC1*	*PC2*
Aspartic acid	-0.213	0.035
Glutamic acid	-0.132	-0.194
Serine	-0.231	0.023
Histidine	-0.219	-0.051
Glycine	-0.370	0.668
Threonine	-0.365	0.109
Arginine	-0.193	-0.035
Alanine	-0.310	0.255
Tyrosine	-0.213	-0.504
Valine	-0.238	-0.274
Phenylalanine	-0.187	-0.100
Isoleucine	-0.221	-0.219
Leucine	-0.229	-0.168
Lysine	-0.236	-0.061
Total dispensible AA	-0.183	-0.095
Total indispensible AA	-0.243	-0.042
Total AA	-0.213	-0.069
% of variation explained	**94.3**	**2.7**
Cumulative variation explained	**94.3%**	**97.0%**

Table 5.33 *Analysis of variance on the principal component scores PC1 and PC2 for the covariance matrix, based on the amino acid digestibility coefficients at 21 days when broilers were fed diets including various phytochemicals*

Amino acid digestibility in broilers at 21 days as analysed by PCA		
Treatment	*PC1*	*PC2*
Control	-2.94^{ab} *(0.08)*	-0.66 *(0.01)*
Yarrow	-2.87^{ab} *(0.13)*	-0.64 *(0.01)*
Thyme EO	-2.87^{ab} *(0.08)*	-0.63 *(0.01)*
Rosemary	-2.81^{ab} *(0.13)*	-0.61 *(0.04)*
Garlic	-3.05^{a} *(0.06)*	-0.63 *(0.01)*
Mimosa CT	-2.77^{bc} *(0.10)*	-0.61 *(0.02)*
Grapeseed CT	-2.92^{ab} *(0.11)*	-0.67 *(0.01)*
Cranberry CT	-2.55^{c} *(0.09)*	-0.66 *(0.01)*
s.e.d.	0.1214	0.0242
	P=0.014	NS

Significant differences in each column are indicated by the presence of non-identical superscripts. NS (P>0.05)
Data are means (SEM) of 6 treatment replicates used in the calculation of each principal component.

At 42 days of age in these broilers, 98.1% of the dataset variation could be explained in the calculation of the first 2 principal components, and the loadings used in their calculation are presented (**Table 5.34**). Analysis of variance on each PC showed no treatment differences on the variables associated with AA digestibility (data not shown).

Table 5.34 *The loadings calculated for insertion into the covariance matrix in the calculation of principal components for variables relating to the coefficients of amino acid digestibility in broilers at 42 days, when the diets were supplemented with phytochemicals*

Loadings used in PCA for amino acid digestibility in broilers at 42 days of age		
Variable	*PC1*	*PC2*
Aspartic acid	-0.215	0.022
Glutamic acid	-0.136	-0.113
Serine	-0.237	0.631
Histidine	-0.233	0.090
Glycine	-0.329	0.361
Threonine	-0.346	0.100
Arginine	-0.224	-0.303
Alanine	-0.293	0.224
Tyrosine	-0.255	-0.274
Valine	-0.241	-0.268
Phenylalanine	-0.185	-0.188
Isoleucine	-0.226	-0.278
Leucine	-0.227	-0.144
Lysine	-0.247	-0.057
Total dispensible AA	-0.185	0.022
Total indispensible AA	-0.245	-0.112
Total AA	-0.214	-0.041
% of variation explained	**97.4**	**0.7**
Cumulative variation explained	**97.4%**	**98.1%**

No significant differences between treatments were found when using ANOVA (p>0.05) for PC1 and PC2 (Not shown).

5.4 Discussion

The discussion is split into several sections, firstly dealing with the compositional analyses for the phytochemical contents of the dietary supplements used. The effect of these phytochemical supplements on broiler performance characteristics, dietary nutrient and AA digestibility measurements at 21 and 42 days of age are considered. Some discussion is given to the use of VFA concentrations as a measure of the microfloral activity and finally the results of the organoleptic assessment of the meat samples. The discussion section concludes with a summary of the main findings in this study.

5.4.1 Supplementary phytochemical composition

The composition of the thyme EO was the same as that used in the experiment reported in Chapter 4, and is not discussed here. The level of activity reported for this garlic against *Candida albicans* (69%) is considered fairly average for a spray-dried garlic powder, but activity values may reach 100% for some garlic samples (Philip Jones, Enterprise, 2003, Pers. comm.). Thus, each garlic sample may also have a variable activity, as this may be affected by the processing conditions employed by the manufacturers in the preparation of the different types of garlic products available. Commercial manufacturers and suppliers of phytochemical products will only perform a quality test on garlic when requested by the consumer in relation to its level of bioactivity *in vitro*. A distillation of the EO of garlic was not undertaken in order to measure its terpene composition, as this will be different chemically to the composition of the garlic powder, due to the distillation process and the unstable nature of these extracted compounds. In general, the literature on garlic has no information on any *in vitro* testing for its efficacy as a supplement, or on its chemical composition when used in studies, which may be due to the unstable nature of its sulphide compounds. However, the extreme complexity of its chemistry would suggest that it is necessary to test for bioactive efficacy before using garlic as a feed supplement, and this test represents a suitable method. However, this method does not take into account discrepancies between alliin and allicin. There may be an HPLC method available to test the garlic, but if garlic EO is to be used in a feeding trial, the measurement of its terpene composition by gas chromatography of the distilled EO may be more appropriate.

The distilled EO from the yarrow herb used in the present experiment had a different chemical composition to that used in the experiment reported in Chapter 3. In the present experiment,

the concentration of chamazulene was higher, at 27.94% of the EO, and the compositions of both camphene and β-pinene were reduced. It has been suggested that the yarrow plant takes around 3 years to establish itself in the field, and the chemical composition will not be stable until this stage. The yarrow used in the present study was first planted in 2000, and was collected when the plants were in bloom (K. Svoboda, 2002, Pers. comm.). Thus, the yarrow used in the present experiment may have an improved antimicrobial quality over that used in the experiment reported in Chapter 3, due to the increased concentration of the sesquiterpene chamazulene. However, Rohloff *et al.* (2000) reported that the terpene composition in yarrow was very much affected by the maturation stage of the plants during harvesting, with lower contents of sesquiterpenes when the plants were in full bloom as opposed to monoterpenes, which were highest just before flowering. The concentration of linalol in the yarrow EO may also have some antimicrobial effect. The EO of basil, which contained 54.95% linalol, was reported to exhibit considerable *in vitro* antimicrobial activity against multidrug-resistant bacteria from *Enterococcus*, *Staphylococcus* and *Pseudomonas* spp. (Opalchenova & Obreshkova, 2003). When purchased, the rosemary herb used in the present experiment was of a dusty, powdered nature, when compared with that used in the experiment reported in Chapter 3. This is not normally the form of a good quality herbal material, as the material should be composed of the dried, spiked leaves of the plant. The composition of the EO distilled from the rosemary herb material was also different chemically to that used in the experiment reported in Chapter 3, with a higher percentage of borneol and reduced α-pinene fractions, although the camphor fraction was consistent at 16.5%. The camphor content of the EO agrees well with the concentration used in the EO of rosemary used by Piccaglia *et al.*, (1993). Camphor may be the terpene responsible for any antimicrobial activity within this EO of rosemary, as the contents of both 1,8-cineole and α-pinene were low in the oil. The EO of *Artemisia annua*, consisting of 44% camphor as the principal component, exhibited strong antimicrobial activity *in vitro* against the Gram-positive bacterium *Enterococcus hirae*, and also had anti-fungal activity (Juteau *et al.*, 2002).

The CT contents of the herb plant material supplements were very low in the present study at <1 g kg^{-1}. As a result, it is considered that all of the effects of these fractions as bioactive supplements were due to their terpene composition. Surprisingly, the CT content within the cranberry extract was very low at 24 g kg^{-1} DM in the present experiment, when measured by the butanol/HCl method. Cranberries are considered to have a high content of flavonoid

compounds, some of which are proanthocyanidins or CT, but these may be conjugated to sugar molecules. Foo *et al.* (2000) measured a high concentration of proanthocyanidins in cranberry, which were mainly epicatechin units. However, Chen *et al.* (2001) determined the composition of the flavonoids and phenolic compounds in freshly squeezed cranberry juice to be around 44% phenolic acids and 56% flavonoids, which would therefore include a mixture of phenolic components and not just CT's. Wilson *et al.* (1998) determined the polyphenolic content in cranberries using gallic acid equivalents, and reported that the CT content of cranberries were mainly composed of the flavanols quercetin and myrecetin, as well as anthocyanidins. The low content of polyphenolic compounds measured in the cranberry CT extract used in the present experiment suggests that this extract may have contained a higher proportion of hydrolysable tannins (HT) or alternatively a mixture of phenolic compounds. Cranberry juice was also reported to contain around 68 g kg^{-1} total carbohydrate, of which 37 g kg^{-1} was composed of sugars (Lowe & Fagelman, 2001). The technical data sheet of the cranberry extract used in the present experiment states that the sugar content was in the region of 394 g kg^{-1}. However, this cranberry was obtained as a concentrated CT extract and the sugar contents were not determined. Clearly, some more analysis should have been carried out on the composition of CT within this cranberry extract used here, as the measurement of tannin contents will differ depending on the methods of analysis used. If there was an appreciable concentration of HT or alternatively simple phenolic components contained within this cranberry CT extract, then this may have affected its action in the gastrointestinal tract of the birds as different types of tannins have different binding affinities to dietary components. The composition of cranberry CT powder extract has been reported to differ from the composition of fresh cranberry juice, where myricetin and quercetin flavonol aglycones were present only after processing (Vvendenskaya *et al.*, 2004). Most of the compositional studies in the literature report the composition of cranberry juice or of the berries, rather than those of concentrates of extracted compounds within the plant material. Vvedenskaya *et al.* (2004) have stated that little information exists on the bioactivity of individual flavonol glycosides or methyl ethers of flavonols within the cranberry, after they identified several new compounds within the plant extract material. This would suggest that more research should be done on the potential binding activity of CT to dietary components for the polyphenolic compounds present within the CT extract. It also underlines the requirement for quality testing any of the supplemented phytochemical materials fully for their composition before they are used in growing trials.

The grapeseed and mimosa CT extracts were also obtained from commercial sources, but these two extracts contained much higher concentrations of CT within the material, when measured by the butanol/HCl method. Grape seeds are known to contain a high concentration of CT phytochemicals such as gallic acid, catechin and epicatechin. Yilmaz & Toledo (2004) determined concentrations of epicatechin CT of 960, 4210 and 1150 mg kg^{-1} within the seeds of Muscadine, Chardonnay and Merlot grapes respectively, and also indicated that the antioxidant capacity of these grape seeds were due to dimeric, trimeric, oligomeric or polymeric procyanidins. The content of CT within both the grapeseed and mimosa powdered extracts were considered sufficient for the purposes of the present experiment. It is possible that the slightly lower CT content in the mimosa extract may have been due to some impurities within this material also, which may have been responsible for some of its effects in reducing performance and digestibility in these birds. The mimosa CT extract may also have contained some mixtures of phenolic compounds or alternatively HT, and in future work, it may be helpful to quantify these materials also. Alternatively, using other methods for analysis of the tanniniferous material would expand the available information when considering using these compounds in poultry diets.

In the present experiment, only the CT content of the plant extracts was measured. However, Vitti *et al.* (2004) reports that it is impossible to assess the ability of any tannin to produce beneficial or detrimental effects in the digestibility of ruminant diets by measuring the concentrations of the tannin supplemented into the diet alone. This has previously been the main basis of assessment when incorporating tannins into animal diets, where it is considered that low concentrations are beneficial and high concentrations detrimental, especially in non-ruminant species. It may have been erroneous to assume that low concentrations of CT supplements in the diets of poultry may have a beneficial effect on the populations of intestinal microflora in the birds, while minimising any negative effects on digestibility, without first carrying out a rigorous testing procedure. Previously, it has been reported that the higher the dietary tannin concentration, the higher was the level of inhibition of intestinal enzymes, and the greater the increase in the size of secretory structures such as the pancreas in broiler cockerels (Ahmed *et al.*, 1991). As a screening procedure, it was suggested that the measurement of total phenolics, radial-diffusion, adsorption to polyvinyl pyrrolidine and also the protein-precipitation capacity of a tannin for bovine serum albumin were necessary when

assessing the suitability of a tannin for dietary supplementation (Vitti *et al.*, 2004). These authors observed that different types of condensed tannin had very different abilities to precipitate proteins and affect digestibility in sheep (Vitti *et al.*, 2004). Although this was beyond the scope of the work carried out in the present experiment, it should be considered in future research.

The diets fed to the birds in the present experiment contained an emulsifier, marketed as Lysoforte™ Booster Dry. This emulsifier was present in all of the treatment diets. Lecithin, the main component of the emulsifier, is present within all living cells, particularly as a component of cell membranes. In poultry, lecithin is present within brain, nerve and muscle cell tissue. All cells in the body require lecithin, in order to allow a more effective passage of nutrients through the cell membrane. Lecithin contains a complex mixture of essential fatty acids, such as linoleic acid, which is needed to metabolise cholesterol. It contains phosphorous, which has an important role in the structure and function of DNA, and also the B-vitamins choline and inositol. Choline is transformed into the neurotransmitter acetylcholine, which is vital for the efficient transmission of brain signals. In this experiment, the lecithin was added to the treatment diets at the request of the commercial sponsor. The lecithin acts to reduce the surface tension of water, allowing it to combine more completely and more effectively with fats and oils. As a result of the presence of the lecithin, fats and oils in the diet may be digested more efficiently and absorbed more completely from the intestine. It also combines with iron, iodine and calcium. Lecithin may interact with known antioxidants within fats and oils, and is normally used in combination with these in the food industry. As a consequence, it may have acted preferentially and synergistically in this study in combination with the garlic and thyme oil supplements in the treatment diets, when compared to the other treatments. The effects of both these treatments may be magnified as a result of the presence of this emulsifier, but the extent to which this may be the case is uncertain and could only be assessed more fully in a separate experiment.

5.4.2 Effects of the phytochemical supplements on broiler performance

The highest BM was observed at day 7 in the birds fed garlic supplements in their diet, when compared to all other supplements used in this study. Although the other performance characteristics were numerically higher for the birds fed the diets with garlic, these were not significantly different from the control treatments at any time point. There was no effect on

any feed supplement on performance after 21 days of age in the birds. Chowdhury *et al.* (2002) reported no effect on feed consumption, feed conversion efficiency (FCE), body weight gain, egg weight or egg mass, when the diets of laying hens were supplemented with sun-dried garlic paste, but they did not measure early growth of the birds. However, garlic was observed to increase the average daily weight gain during the first 21 days of broiler chick growth, although there were no treatment effects on weight gain, feed intake or feed efficiency when the study was terminated at 35 days (Horton *et al.*, 1991a). These results indicate that the positive effect of garlic may be limited to the first few weeks of life in the broiler chick, and are in agreement with the results of the present study. However, Lewis *et al.* (2003) observed an improvement in broiler FCE and a tendency for increased weight gain when feeding garlic between 17-27 days of growth in broiler diets. Average increases in weight gain of 40-55% were reported in broiler chickens fed both diets with garlic supplements and the controls without supplement over the growth period, where both treatments were equivalent at the end of the growth period (Qureshi *et al.* 1983). Qureshi *et al.* (1983) also reported no differences in feed intakes in broiler chickens fed diets including several types of garlic supplements. However, the method of garlic extraction used had a slight effect on the end result in the study of Qureshi *et al.* (1983), and no information was given regarding the effects of supplementation on the various growth stages within the study birds. The effects of garlic supplementation on performance are also variable between different animal species. The inclusion of garlic was observed to increase feed palatability in horses, but did not influence daily feed consumption or performance in either sheep or pigs (Horton *et al.*, 1991b). No effect on the performance of broiler chickens was reported over a 9 week period after the birds were fed diets including yarrow, but there was an increase in body weight gain after 9 weeks in the birds fed yarrow-supplemented diets (Fritz *et al.*, 1993). Lewis *et al.* (2003) reported an improvement in broiler FCE between 17-27 days of growth when feeding diets with 1.8g kg^{-1} yarrow herb. The inclusion level of yarrow in the present study was much greater at 10g kg^{-1} than in the study of Lewis *et al.* (2003), although this was not associated with any positive changes in bird performance when compared to the controls. There was a beneficial effect of dietary yarrow herb inclusion on the growth performance of the birds in the experiment reported in Chapter 3, where this treatment in the study was the second best overall. The diets in the present study were supplemented with yarrow at the same concentration as in the experiment in Chapter 3. Lewis *et al.* (2004) indicated that the effect of supplementation of the broiler chick diet with yarrow depends on the diet composition, when they observed a

positive effect on growth performance in a diet diluted by 10% in its nutrient composition. However, in the present experiment, the inclusion of the emulsifier or carbohydrase in the diets may have been of greater benefit in their utilisation, and may have overshadowed the effects of using yarrow as a supplement.

In the present study, the inclusion of mimosa CT in broiler diets was associated with the poorest values for feed consumption in the first 7 days, when compared to the birds fed all other diets except grapeseed CT and yarrow. The birds fed mimosa CT in the diet also had the lowest average weight gains in the first 7 days, when compared to the birds fed all of the other diets except thyme EO and rosemary. After day 7, the effects of treatment gradually diminished as the birds increased in age. There may be some problems with an astringent taste for either the CT compounds in the mimosa supplement, or the supplement may have contained a mixture of phenolic compounds, which resulted in a decrease in the nutrient utilisation in the birds. The birds fed diets supplemented with thyme EO also had a low initial weight gain within the first 7 days, compared to the birds from all other treatments except those with rosemary and mimosa CT. In the experiment reported in Chapter 4, the birds fed the diets with thyme EO had a reduced feed intake within the first 14 days of the study, especially when the concentration of thyme EO was increased in the diet. The level of thyme EO inclusion in the present study was less than that used in the experimented reported in Chapter 4, but this treatment still had a negative effect on weight gain within the first 7 days. It is possible that the thymol within the thyme EO used was associated with an astringent taste in the birds, or alternatively some other of the phenolic terpenes contained affected the dietary intake levels. However, the inclusion of thymol of 99% purity in diets was reported to have no effect on broiler performance (Lee *et al.*, 2003). Lee *et al.* (2003) used concentrations of thymol about 5 times more dilute than the thyme EO used in the present experiment at 1g kg^{-1}, which was composed of 44.1% thymol. No effect on the performance of either chickens or turkeys was observed when oregano with high thymol content was supplemented in diets (Botsoglou *et al.*, 2002; Papageorgiou *et al.*, 2003). After the initial few weeks in the present experiment, no reduction in performance was observed. Additionally, there were no effects of dietary thyme EO supplementation observed on the BM of mature rats, except at age 22 months, when compared to the rats fed the non-supplemented control diet (Youdim & Deans, 1999a). It would appear that an adaptation period to the presence of thyme EO may be necessary within the diet, and that thyme EO supplementation in diets does not appear to

increase broiler weight gain. As such, thyme EO may be better added in diets within a phytochemical blend.

The results of including tannins in the diet are variable in their effects on growth. The inclusion of mixtures of phenolic compounds, and both HT and CT together, are often associated with a reduction in both growth and in the retention of nitrogen by chicks (Dale *et al.*, 1980; Elkin *et al.*, 1990; Ahmed *et al.*, 1991; Mahmood & Smithard, 1993; Majumdar & Moudgal, 1994). Mixtures of phenolics have also been associated with reduced weight gain in rats (Tebib *et al.*, 1996). Martin-Carron *et al.* (1999) observed no reduction in the weight gain or food intake of rats when feeding diets supplemented with dietary fibre and a polyphenol-rich grape product, either with or without cholesterol supplementation. No depression in growth was reported after the inclusion of high contents of sorghum meal CT in diets fed to ducks (Elkin *et al.*, 1990). In the case of the present study, most of the birds culled for under-performance were from the mimosa and grapeseed CT treatments, but not from the cranberry CT treatment. However, this initial increase in variability was not associated with a reduction in the performance of the remaining birds fed either the grapeseed or cranberry CT treatments in the study, when compared to the birds from the control treatment in the first 7 days. The phenolic content of the diet would appear to be responsible for this initial increase in variability, and as such the presence of CT in the diet may also require an initial adaptation period. However, the composition of the supplement has an unclear effect, as the lower CT concentration in the cranberry CT supplement at 24 g kg^{-1} did not reduce the performance of the birds on this diet, when compared to those on the control treatment at any time. Only 1 bird was culled for poor performance from those fed diets with cranberry CT during the growth period. There may be some effects of the tannins from mimosa in binding dietary components, especially proteins or certain AA, which led to the reduced performances of the birds fed diets with this supplement in the first 7 days of the study. These results would suggest that both cranberry and mimosa should be better supplemented within a blended compound, where other compounds may alleviate this increased variability in the growth response. In general, the tannins may be better fed after day 7, when the birds have a more developed gastro-intestinal tract.

5.4.3 Effects of phytochemical supplements on nutrient digestibility

In the present experiment, there was no effect of garlic as a supplement on the digestibility of energy from the diet by the birds, either at 21 days or 42 days. These measurements were based on the analysis of samples from the ileum, and as such are not an accurate measure of total gastrointestinal tract metabolism. In future experiments, it would be better to collect excreta samples and measure AME in the birds during growth, as the small amount of material available for ileal digestibility analysis in broilers will have reduced the value of the energy utilisation data in this experiment. There were several missing data points from the energy analysis, and the standard errors of the difference between means had to be corrected manually. However, it suggests that the main action of the garlic may be in regulating the activity of the microflora. It is possible that the benefits of garlic supplementation in the diet may be associated with some improvement in the utilisation of the fatty acid components, or their metabolism, which was not assessed in the present experiment. Garlic inclusion in the diets of laying hens was reported to decrease the concentrations of cholesterol in both serum and in the egg yolk (Chowdhury *et al.*, 2002). Aouadi *et al.* (2000) reported that garlic inclusion in the diets of rats lowered the cholesterol levels, decreased low-density lipoprotein and increased high-density lipoprotein levels in serum. Qureshi *et al.* (1983) also observed that various forms of garlic reduced the synthesis of cholesterol in the liver, the serum cholesterol and the low-density lipoprotein concentrations in both chickens and laying hens. After the broiler chick hatches, lipids are rapidly transported by lipoproteins from the yolk sac into the body circulation, and the concentration within the plasma does not change over the first 7 days post-hatch (Sklan, 2003). However, the rate of turnover and utilisation of the fatty acids from the plasma was observed to decrease with age (Sklan, 2003). This suggests that garlic may either slow down the decrease in efficiency of fatty acid turnover, or alternatively increase the early efficiency of fatty acid absorption from the body circulation, and thus reduce or influence the proportion or type of fat deposition within the body. Sklan (2003) also mentions that the uptake of glucose into blood plasma is increased with age in post-hatch broilers, as glucose digested from dietary sources rather than lipids from the yolk become the major energy source for broiler chicks. Garlic may have an effect on the utilisation of dietary glucose, but this effect may be a positive or a negative one, and further studies would be required to ascertain this. When feeding supplements of allicin from garlic in fructose-enriched rat diets, the allicin prevented the rats from gaining weight whereas those fed only diets with fructose continued to gain weight (Elkayam *et al.*, 2003). The dietary consumption

for both groups was similar, and Elkayam *et al.* (2003) suggested that garlic supplements could be used to help prevent weight increases in adult humans, and could therefore be used in the prevention of hypertension. This result was not observed in the present study, and thus effects of garlic on the utilisation of dietary components in growing birds needs to be established more clearly, as these may differ from the effects observed with its supplementation in the diets of mature individuals. Roman-Ramos *et al.* (1995) reported that garlic supplementation in the diets of glucose-tolerance tested rabbits decreased the hyperglycaemic peak by 17.4%, and onion inclusion in the diets decreased this peak by 18%. This suggests there is some effect of these diallyl-disulphide compounds on glucose metabolism. Other spices, such as turmeric, have also been reported to affect the enzymes associated with the metabolism of glucose, resulting in a normalised weight gain in diabetic rats (Narayannasamy *et al.*, 2002).

It is possible that the action in the binding of the cranberry CT to the gut walls may cause a decrease in the absorption of some of the dietary components. Dietary CT's are known to be able to inhibit the intestinal absorption of AA, peptides, lipids and carbohydrates (Waghorn, 1996). This takes place when the CT either bind to digesta proteins or intestinal enzymes, thus reducing the activity of these enzymes (Waghorn, 1996). In the present experiment, there was no reduction in energy utilisation in any birds fed with the CT treatments, when compared to those birds fed the control treatment diets. However, the coefficients of ADMD and DOMD from the diet, and the digestible OM content in the diets were reduced in birds fed the diet with cranberry CT, compared to those fed all of the other treatments except for the diets with yarrow herb. Previously, the addition of carbohydrate was observed to affect the formation of insoluble tannin-protein complexes *in vitro* when testing 3 different types of grapeseed CT, where the disruptive effect of the carbohydrate was different depending on the complexity of the CT structure (Mateus *et al.*, 2004). The differential effects of cranberry CT on digestibility in the present experiment may have been due to its phenolic composition resulting in different interactions with other dietary components, either in binding to carbohydrate, protein or lipid fractions, thus affecting their metabolism or absorption. The inclusion of a combination of dietary fibre and a polyphenol-rich grape product in the diets of hypercholesterolemic rats increased the concentration of HDL cholesterol and lowered the concentrations of LDL and total serum cholesterol, when compared to those diets fed without supplement (Martin-Carron *et al.*, 1999). Martin-Carron *et al.* (1999) stated that this effect of dietary supplementation in

the rats on the cholesterol metabolism was mainly due to the polyphenol component of the fraction and was independent of the amount of soluble fibre in the ration. The negative effects of CT in the diets appeared to decrease with increasing age in the birds, as no effects of any CT treatment were observed on the measurement of any digestibility coefficients at 42 days in the present experiment.

5.4.4 Effect of dietary phytochemicals on enzymes and digestive secretions

The positive early effect of garlic in the present study on BM and AA digestibility may also have been due to some enhancement of the activity of endogenous enzymes or digestive secretions, resulting in a greater utilisation of dietary substrate. Platel & Srinivasan (1996, 2000 & 2001) have demonstrated that garlic and other herbs and spices increase the secretion of endogenous enzymes, bile acids and pancreatic juices. This has previously resulted in a shorter time for the passage of digesta in experimental rats (Platel & Srinivasan, 2001). Additionally, Lee *et al.* (2003) observed that supplementary thymol increased the liver mass and the pancreatic mass in broiler chickens aged 21 days, which suggests that the activities of these organs may also have been increased. The masses of these organs were not measured in the present experiment due to the complicating effect of several people taking the intestinal samples from the birds. However, the stimulation of endogenous secretions may decrease over time, since Lee *et al.* (2003) did not observe an increase in organ size at 40 days of age in broilers supplemented with dietary thymol. In the present study, there were no effects of any supplement on the digestibility of energy, protein or OM at 42 days of age in the birds. Future experiments should focus on the potential for various phytochemical supplements to increase the production of endogenous enzymes or other secretions. There were no increases relating to treatment in the sialic acid concentrations from these birds in the present study, which may suggest that no stimulatory effect occurred with these treatments. In future, it may be beneficial to assess alternative methods of measuring endogenous losses, in order to compare the results of different techniques, especially if ileal samples are used. In previous chapters of this thesis, changes in the concentration of sialic acid have been reported with the dietary inclusion of different herbs or their extracts, suggesting a definite influence of herb or EO terpene compounds on endogenous secretions in broilers. In Chapter 3, the inclusion of oregano, marjoram and yarrow herbs and also oregano EO in broiler diets reduced the sialic acid concentrations in broiler excreta.

Majumdar & Moudgal (1994) reported that precision-feeding tannic acid increased the nutrient uptake at 15 days of age in broiler chickens, by increasing the activity of various digestive enzymes, including alkaline phosphatase. The review of a number of studies reported that dietary CT inclusion consistently reduced the activity of endogenous α-amylase by 65-80% in chickens, and the activity of trypsin by 18-19% in pigs and 55-77% in chickens (Waghorn 1996). When faba bean CT was included in broiler chick diets, there was no increase in endogenous pancreatic enzyme secretion, but instead there was a reduction in the activity of trypsin, α-amylase and to a lesser extent lipase in the digesta (Longstaff & McNab, 1991). This resulted in a decreased AA digestibility, as well as the digestibility of starch and lipids (Longstaff & McNab, 1991). In the bovine rumen, supplementation of tannins from oak leaves decreased the activities of urease, carboxymethylcellulase, glutamate dehydrogenase and alanine aminotransferase, but increased the activities of glutamine synthetase (Makkar *et al.* 1988). In situations where there is an increased digestibility of dietary components, the inclusion of CT may cause increased endogenous nitrogen or cell loss, as endogenous enzyme production may be stimulated. In the present experiment, the mimosa, cranberry and grapeseed supplements were chosen on the basis of their CT content. However, a more complete screening procedure will be necessary on the type and structure of tannin used as a supplement in order to reduce any negative effects on endogenous enzyme activity as far as possible in future studies.

5.4.5 Effect of phytochemical inclusion on the populations of intestinal microflora

The inclusion of 2 different blends of EO components, including thymol, eugenol, curcumin and carvacrol terpenes, and the alkaloid piperine, in the diets of broilers, were reported to inhibit the proliferation and colonisation of *C. perfringens* in the intestinal tract (Mitsch *et al.* 2004). This bacterium utilises nitrogen and may be a predisposing cause of necrotic enteritis in high numbers. Losa & Köhler (2001) also observed a reduction in *C. perfringens* colonisation in broilers with the commercial blend of EO known as CRINA POULTRY. Sims *et al.* (2004) observed a reduction in the intestinal lesions caused by *C. perfringens* in challenged broilers, when the diets were supplemented with either CRINA POULTRY or zinc bacitracin.

Tannins fed in animal diets have been reported in several studies to inhibit the activity of various populations of intestinal microflora. Tebib *et al.* (1996) observed a reduction in the

activities of bacterial enzymes tested after feeding diets with polymeric CT from grapeseed to rats. Makkar *et al.* (1988) reported that oak tannins inhibited most of the microbial activity in the bovine rumen, and suggested that oak tannins may decrease the RNA but not the DNA synthesis in rumen microbes and also inhibit microbial attachment to the feed particles. Various phenolic acids, namely hydroxybenzoic acids and hydroxycinnamic acids, inhibited the growth of *Oenococcus oeni* and *Lactobacillus hilgardii in vitro*, which are 2 of the lactic acid bacteria associated with wine (Campos *et al.* 2003). However, the presence of CT in animal diets may select for bacteria resistant to the presence of tannin, which may be dependent on the dietary concentrations used. Over a 3-week period, the inclusion of *Acacia angustissima* CT in rat diets increased the proportions of CT-resistant bacteria present in the excreta, from 0.3-25.3% at 7 g kg^{-1} CT, up to 47% with the inclusion of CT at 20 g kg^{-1} (Smith & Mackie, 2004). The presence of *A. angustissima* CT in diets resulted in the selection of Gram-negative *Enterobacteriaceae* and *Bacteroides* species in the rat intestine, which were resistant to the tannins (Smith & Mackie, 2004). These CT-resistant bacteria decreased after CT removal from the diets fed to the rats, but were still distinguishable at reduced levels even after 3 weeks in the excreta (Smith & Mackie, 2004). This suggests that CT have a definite role in modifying populations of the microflora within the intestine, but that these compounds should be used carefully as they subject the intestinal microflora to the same pressures as synthetic antimicrobials. Unfortunately, in the research of Smith & Mackie (2004), the CT selected for potentially pathogenic Gram-negative microflora. This may have been due to the presence of the cell envelope structure, which gives these bacteria a greater resistance against the entry of foreign molecules into the cell. In contrast, when calves were fed green tea extracts in their diet, which contained various catechin polyphenols, these compounds inhibited the growth of 7 strains of *Staphylococcus* spp., 7 strains of *Streptococcus* spp., 19 strains of *E. coli* and 26 strains of *Salmonella* spp. (Ishihara *et al.*, 2001). At the same time, the green tea extract catechins maintained high counts of *Bifidobacterium* spp. and *Lactobacillus* spp. in the excreta of the calves, and decreased the frequency of occurrence of scouring (Ishihara *et al.*, 2001). This suggests that CT may be suitable compounds for selective improvement of the resident species of intestinal microflora, but that the CT used should be chosen carefully. It may be more beneficial for tannins to be fed in conjunction with a prebiotic supplement, such as a fermentable carbohydrate, to promote the growth of probiotic bacterial species in the intestine of chicks. Smith & Mackie (2004) indicated that the time lag between withdrawal of CT in the diet and the decrease in levels of tannin-resistant

bacteria suggested that a previous exposure to the presence of tannins in the diet could result in a shorter adaptation period with any subsequent tannin exposure. The ellagitannin compound punicalagin, isolated from *Combretum molle,* has been reported to have anti-mycobacterial activity against *Mycobacterium tuberculosis in vitro* (Asres *et al.*, 2001), which these authors have stated is the first observation of this type of bioactivity. These studies suggest that further research should be carried out into the effects of CT into the activity of the microflora within chickens, and their association with the digesta or the brush border membrane.

Different types of CT may potentially also be fed in combination with each other, where a combination or rotation of these supplements in diets may help to minimise the creation of resistant bacterial populations in the poultry gut. Cranberry CT have been shown to bind to various bacterial species, which then prevent these bacteria from binding to the intestinal surface, a necessary pre-requisite for the initiation of infection. The CT and fructose content in cranberries and other members of the genus *Vaccinium* are suggested to be responsible for preventing uropathogenic phenotypes of P-fimbriated *E. coli* from adhering to the urinary tract (Howell *et al.*, 1998). The consumption of cranberry juice has been shown to reduce bacterial infections and the concentration of bacteria in the urinary tract of elderly women, from an infection level of 28% in the control group to 15% in the group drinking cranberry juice supplements daily (Avorn *et al.*, 1994). However, although it did not produce any negative effects on broiler performance in the present study, cranberry CT may potentially bind to dietary components, especially nitrogenous components, and may lead to these being excreted without being digested. The extracted cranberry CT powder used in the present experiment appeared to absorb water easily, which may be due to its content of fructose or other sugars. The free-flowing powder extract of cranberry CT was observed to clump together quickly after opening. However, the reduced efficacy of the cranberry CT in the present experiment may be related to the molecular weight of the tannins, in conjunction with the degree of polymerisation. Alternatively, the cranberry CT may have been bound to the digestive enzymes, or have interfered with their binding to dietary components. As a result, the combination of this supplement may be unsuitable unless included in diets with higher protein levels to compensate for this effect.

The active constituents of all berries from the genus Vaccinium are of a similar type, namely the anthocyanins. Anthocyanins from bilberry (*Vaccinium myrtillus* L.) were perfused into the jejunum and ileum of rats, where a high proportion of anthocyanin glycosides were absorbed and metabolised from the intestine, depending on the chemical structures (Talavera *et al.*, 2004). Talavera *et al.* (2004) observed that only glycosides remained in the intestinal lumen, irrespective of the type of anthocyanins introduced into the intestine, but that methylated or glucuronidated derivatives were recovered from urine, plasma from the aorta and mesenteric vein and also from bile. Thus, it may be possible that some of the phenolic components from cranberry are metabolised within the intestinal tract. If so, it would be important to elucidate the site of absorption of the anthocyanins and any other components, or determine how much of the active compound is excreted at the end of the digestive process. A more complete picture of their action in the gut would be helpful in determining the suitability of these compounds for dietary inclusion in birds.

5.4.6 Effects of phytochemical supplements on crude protein and amino acid digestibility

In the present study, the coefficients for the apparent digestibility of N were extremely low in all the treatment diets, when compared to the amount of CP in the diet. The apparent digestibility coefficients for the AA were also low. The reductions in both these measurements were in agreement, and were especially pronounced in the birds at 42 days of age. Thus, the reasons for the poor AA digestibility were considered to be due either to formulating or processing conditions in the diet. Although the dietary contents of both N and AA were adequate, there were high concentrations of N/AA in the ileal digesta, which were responsible for the poor digestibility coefficients. For the measurement of the digestibility of AA, these low values were confirmed by repeating several samples. These differences are difficult to explain, especially as these diets were mixed at commercial premises. When the diets were formulated, they were balanced for energy and protein, and the nutrient requirements were met. However, the concentrations of N/AA in the ileal digesta were around double the concentrations expected. In the present study, the digesta samples were taken from the contents of both the jejunum and the ileum. It is possible that the AA components of the digesta had not yet been fully broken down and absorbed, but this should not explain the presence of concentrations of each AA in the digesta at these levels.

The protein used in these diets was a high protein soybean meal (48%), obtained from a commercial feed mill. Although the content of protein is high, the availability of protein to the birds was obviously reduced. It is unlikely that this soybean meal should have contained appreciable amounts of antinutrient factors such as trypsin inhibitors or lectins, as these should have been removed by heat treatment. It is still possible that this should have been the case in these diets, or the temperature during heat processing of the soybean meal may alternatively have been too high, which may have reduced the digestibility of the protein fraction. However, this soybean meal may have contained other anti-nutritional factors, such as oligosaccharides, which may have been bound to the protein fraction, reducing the digestibility or absorption values for AA or protein. Of a range of soybean meal types, diets fed to broilers containing heated soybean meal were reported to have the highest coefficients of AA digestibility, even though others were bred for a lower content of anti-nutrients (Batal & Parsons, 2003). These authors also measured higher coefficients of AA digestibility at 14 and 21 days of age, when feeding diets with both the Kunitz inhibitor and lectin antinutrients removed, compared to those meals with only one type of anti-nutrient removed (Batal & Parsons, 2003).

The effect of age on the digestibility of soybean meal is variable, and appears to depend on the type of soybean fed. In the present study, the discrepancy in the digestibility coefficients between 21 and 42 days of age suggests that the soybean meal in the present study may have come from two separate sources, and that the soybean used in the finisher rations may have been poorer. No further processing of these diets was done at SAC Ayr. The low digestibility coefficients for energy in the present study may therefore be partly due to the reduced value of protein to the study birds. Wheat varies in quality, with an energy content of between 9.5-16.5 MJ kg^{-1} DM, but an acceptable AME value for wheat would be in the region of 14.5-15.5 MJ kg^{-1} DM in broilers (Choct *et al.*, 1995). As these diets were fed with a carbohydrase, the ADE values should have been higher, and the reduced availability of protein may have left the energy and protein unbalanced in the diet. Angkanaporn *et al.* (1994) reported that dietary NSPs isolated from wheat significantly increased the loss of endogenous AA in broilers, and decreased the extent of digestibility of the protein. This may have accounted for some of the increase in AA concentrations in the ileal digesta in the present study, but it is notable that the sialic acid concentrations were not appreciably higher in these birds than in those of an equivalent age in the experiment reported in Chapter 4. No measurements of endogenous

protein loss were made in these birds. Hew *et al.* (1998) reported that the digestibility of AA in broilers was significantly improved when the diets were fed with a xylanase, as should have been the case in the present study. The efficacy of the enzyme used in the present study was not measured, as the diets were prepared off-site before the start of the experiment. Care was taken to ensure that the diets were balanced when they were formulated. There may have been some discrepancy between the nutrient composition of the ingredients used in the formulation, and those actually mixed into the diets. This is unclear, however, as these diets were not sent for detailed nutrient analyses.

It is generally assumed that the supply of protein in its content of AA is additive, thus the digestible AA content of a diet can be predicted by the digestibility coefficients of the individual AA ingredients (Angkanaporn *et al.*, 1996). In the present experiment, the digestibility coefficients were particularly poor for threonine and glycine, at both 21 and 42 days of age. However, using a range of diets with soybean of differing quality, Batal & Parsons (2003) reported AA digestibility values for threonine to vary between 46-86% in broiler chicks at 21 days of age. When feeding diets to broilers with both constant and varying ratios of essential AA: dietary CP, there was a reduced feed consumption with increasing CP concentration, which suggests that one or several AA's limited the consumption of feed by the birds (Sklan & Plavnik, 2002). It is also possible that some non-essential AA's were available in insufficient quantities to allow an optimal rate of protein synthesis, which may partially explain the decreased protein and energy efficiency in the birds. As the sulphur AA were destroyed during the acid hydrolysis procedure, and these are generally the first to be deficient, it is not possible to obtain a full picture of which AA may have limited the performance of the birds in the present study. However, the sulphur AA, threonine and glycine all require to be supplied at adequate concentrations in the diet, as these are essential for growth and cannot be synthesised by the body from other AA's. It is known that inter-relationships exist between different AA, which may be either positive or negative when one or the other is supplied at a higher concentration (Angkanaporn *et al.*, 1997). Alternatively however, there may have been an excess amount of AA provided to the birds for protein synthesis. Excess availability of AA may have increased the essential AA requirement from the diet, which may have resulted in a decreased bird performance because of an inefficiency of AA utilisation (Sklan & Plavnik, 2002).

The digestibility coefficients in the ileum are supposed to be a more accurate representation of the digestion of AA by the birds, as these are free from the complication of the activity of the microflora in the lower tract. The measurement of apparent AA digestibility coefficients in broilers fed diets with soybean meal were previously determined at between 0.700-0.887 (Angkanaporn *et al.*, 1996), although these measurements were based on the ileal digestibility alone, without the digesta contents of the jejunum. These coefficients more accurately reflect the AA digestibility coefficients reported in the other experimental chapters in this thesis. Likewise, experimental data presented by NRC (1994) reports that the true digestibility coefficients of AA lie between 82-93% when soybean meal (48% protein) is fed. As the AA digestibility coefficients were so much lower in the present experiment and the final extent of AA absorption is unknown, it must be suggested that the higher concentrations of AA in the gastrointestinal tract may have created conditions suitable for growth of bacteria such as *C. perfringens*. However, the presence of the enzyme may have ameliorated some, if not all of these effects, especially at 21 days of age in the birds, by breaking down the NSP fraction of the diet.

In the present study, the highest digestibility coefficients for each AA were observed in those birds fed diets with garlic, when compared to those birds fed rosemary, mimosa and cranberry CT supplements at 21 days of age. However, these digestibility coefficients in the birds fed diets with garlic were not significantly greater than those fed the control diets at 21 days. By 42 days of age, there were no effects of dietary supplementation on the coefficients of AA digestibility in these birds, which indicates that the effects of these phytochemical supplements in the birds may decrease over time. Several AA are present in garlic powder, including arginine, lysine, alanine, glutamic acid, serine and histidine (Das *et al.*, 1996). Das *et al.* (1996) state that the inclusion of 1 g of garlic powder in the diet should increase the circulating arginine concentration in human blood by 10%, when an absorption efficiency of 50% was assumed. In diets fed to rats with high protein levels (40%), the additional inclusion of garlic at 8 g kg^{-1} decreased the plasma concentrations of corticosterone and increased the testosterone concentrations, when compared to those diets fed with high protein but no garlic (Oi *et al.*, 2001). In these rats, the urinary excretion of 17-ketosteroid, the nitrogen balance and the hepatic arginase activity were all increased when garlic was included in the high protein diets, compared to those without garlic (Oi *et al.*, 2001).

When EO or herbs were included in the diets of the broilers in the present study, there were no effects in the digestibility of protein, energy, DM or OM in these birds, when compared to those fed the control treatment. Other studies have reported effects on digestibility when herb or EO compounds were used as supplements in animal diets. When a commercial blend of EO, known as CRINA RUMINANTS, was fed as a supplement in sheep diets, the degradation of soybean meal in the rumen was inhibited, and the deamination of AA in the rumen was inhibited by 25% (Newbold *et al.*, 2004). This suggests that these compounds suppressed the activity of micro-organisms involved in protein turnover, potentially the hyper-ammonia producers, which are related to *C. perfringens* in the intestine of broilers. However, the small intestinal mucosa catabolises a significant proportion of the dietary AA, at a different rate for each AA, which has a significant effect on the quantity and efficiency of intestinal utilisation and metabolism of dietary AA, and their subsequent absorption into the tissues (Wu, 1998). In both the present experiment and in that reported in Chapter 4, there was no effect of thyme EO on AA digestibility at either 21 or 42 days in broilers. The experiment in Chapter 4 reported that carbohydrase inclusion increased the digestibility of some AA at 42 days of age. In the present study, the inclusion of carbohydrase in all the treatment diets meant that this effect could not be confirmed. Several studies have reported that terpenes have the potential to affect the digestibility of the proteinaceous dietary components. The digestibility coefficients of asparagine, threonine, serine, glutamic acid and tryptophan were increased in broilers fed dietary terpenes as supplements, including cinnamaldehyde, capsaicin and carvacrol (Jamroz *et al.*, 2003). In dairy cattle, a commercial blend of EO inhibited the rate of deamination of AA's, by controlling the populations of ruminal hyper-ammonia-producing microflora (McIntosh *et al.*, 2003). When sheep were fed on low-protein concentrate diets, the inclusion of EO in diets reduced the numbers and diversity of hyper-ammonia-producing bacteria in the rumen, along with the rate of ammonia production from AA, which corresponds to the activity of these ammonia-producing bacteria (McEwan *et al.*, 2002b). A decreased degradability of certain protein sources in the sheep rumen, along with a decrease in the attachment and subsequent colonisation of these feeds by proteolytic bacteria, was reported when the diets were supplemented with EO (McEwan *et al.*, 2002a). On nutrient agar, the inclusion of mint EO significantly altered the AA production of both *S. enteritidis* and *S. aureus in vitro* (Tassou *et al.*, 2000).

In the present study, the inclusion of cranberry in the diet had a negative effect on the digestibility coefficients of each AA in the birds at 21 days of age, when compared to the birds fed the control diets. The precipitation of plant proteins by tannins has been observed to vary depending on the type of tannin used (Perez-Maldonaldo *et al.*, 1995). In the present study, the diets with cranberry CT reduced the digestibility coefficients of most AA's in broilers to a greater extent, when compared to those birds fed diets with grapeseed CT. There was no reduction in the digestibility coefficients of AA in birds fed diets with mimosa CT, when compared to those birds fed the control treatment. However, the digestibility coefficients of AA were reduced in the birds fed mimosa CT in their diet, compared to those birds fed the diets with garlic. The negative effects of these CT supplements may be due to their polyphenolic content or the composition of the material, or the presence of a mixture of types of phenolic compounds. The AA digestibility coefficients were not reduced when grapeseed CT was included in the diets, and this CT extract may be considered the most suitable for broilers based on these results, in allowing the maximum utilisation of dietary protein. This grapeseed CT supplement also contained the highest concentration of CT from the three tested. Mateus *et al.* (2004) observed that the extent of the disruptive effect of the presence of carbohydrate on tannin-protein complex formation differed in relation to both the structures of the carbohydrate and of the CT. The ability of the carbohydrate to disrupt tannin-protein bonds was reduced when increasingly polymerised CT were bound to proteins (Mateus *et al.*, 2004). However, when any of the tested carbohydrate compounds were present in solution, this reduced the formation of insoluble protein-CT complexes (Mateus *et al.* 2004). Tannin supplementation in the diets is known to have a variable effect on the digestibility of protein in the literature. Hossain (1997) reported an increase in the digestibility coefficients of histidine, alanine, tyrosine, phenylalanine, leucine and lysine AA when feeding 10 g kg^{-1} rapeseed meal CT, indicating that the effects of tannins at this supplementation level were not negative and may be positive. However, the digestibility coefficients of all AA were reduced in broiler chicks when faba bean CT at 24 g kg^{-1} was included in the diets (Ortiz *et al.*, 1994). In piglets fed diets with a high content of CT at 200 g kg^{-1}, there was a reduction in the AA, DM and OM digestibility coefficients, and also reduced N retention and trypsin and chymotrypsin activity (Jansman *et al.* 1993).

5.4.7 Effect of supplements on the bacterial fermentation activity

The results of the present experiment showed an overall increase in the concentrations of caecal VFA with age in broilers for both propionic and valeric acids between the sampling points at 21 days and at 42 days. However, the concentrations of lactic and n-butyric acids in the caeca were significantly lower at 42 days compared with that determined at 21 days. Bacterial populations may change with age, but this result was surprising as the populations of microflora within the intestine are supposed to be stabilised by 7 days of age (Ewing & Cole, 1994). However, the fermentative activity of the bacteria may be dependent on the diet, and may alter when a different dietary phase is fed during growth, such as the transition from starter to grower to finisher treatment rations respectively. The presence of any dietary supplements, such as phytochemicals or enzymes, may also shift bacterial fermentation and favour some VFA over others. The inclusion of garlic in broiler diets tended to decrease the concentration of total VFA in the broiler caeca, and garlic may therefore have inhibited microbial activity or population numbers within the intestine, but this is not clear in the results from the present experiment. The antimicrobial nature of garlic is well established (Rees *et al.* 1993; Naganawa *et al.*, 1996; O'Gara *et al.*, 2000; Ross *et al.*, 2001). The lack of an observed effect with the other dietary supplements on the concentrations of the major VFA (acetic, lactic, propionic, valeric and n-butyric acids) was surprising. However, the minor VFA (isovaleric and isobutyric acids) were decreased in the caeca of the birds fed diets with yarrow, thyme EO, rosemary and garlic supplements, when compared to the birds fed the control treatment. It is possible that the activity of garlic may have been enhanced due to the presence of the emulsifier. There may not have been a sufficient bacterial challenge in the present experiment to affect caecal fermentation, as the carbohydrase will have decreased the relevance of NSP within the diets. However, thyme EO, thymol and carvacrol have previously reduced the fermentation of VFA *in vitro* (Varel & Miller, 2001; Shanmugavelu *et al.*, 2004). When supplementing both monomeric flavonoids from grapeseeds and polymeric grapeseed CT into diets fed to rats, there was no difference between the control group and those fed the monomeric flavonoids in the caecal concentrations of VFA (Tebib *et al.*, 1996). However, those rats fed supplements with polymeric CT had a higher total VFA concentration in the caeca, and higher individual concentrations of acetic acid, propionic acid and butyric acid when compared to those rats fed the control treatment (Tebib *et al.* 1996). This suggests that the presence of the CT caused an increase in the caecal fermentation, or this increase may

have been due to a delayed absorption of nutrients due to the formation of tannin-nutrient bonds as suggested by Tebib *et al.*, (1996).

The supplementation of EO in a commercial product known as CRINA RUMINANTS had no effect on inhibiting the VFA or ammonia concentrations within the rumen of sheep (Newbold *et al.*, 2004). The inclusion of yarrow increased the production of acetate at the expense of butyrate when used in an *in vitro* microbial culture system with rumen fluid (Broudiscou *et al.*, 2000). Broudiscou *et al.* (2000) also used several other plant extracts in the *in vitro* system, where all had varying effects on the fermentation of rumen microbes.

5.4.8 Effects of dietary supplementation on the gastrointestinal mucosa

Despite the positive effects observed with its inclusion in the present study, garlic may also have negative effects in the intestine, depending on the form of supplementation of the material. The introduction of raw garlic powder directly into the intestine of dogs using an endoscopic air-powder delivery system caused severe damage and intestinal erosion, whereas an extract of ethanol-soaked aged garlic extract had no undesirable effects (Hoshino *et al.* 2001). The supplementation of boiled garlic powder caused a reddening of the intestinal mucosa, and all supplements were introduced at the inclusion rate of 1 g powder in 10 ml 5% trichloroacetic acid (Hoshino *et al.*, 2001). However, the inclusion of garlic within a dietary ration at the concentrations used in the present study should not cause any intestinal problems. Aged garlic extract has also been shown to be an effective antioxidant, suppressing the generation of free radicals from H_2O_2 and O_2^- and enhancing the activity of antioxidant enzymes in the intestinal cells (Wei & Lau, 1998). It is possible that any negative effects may be removed by using a cooked form of supplementary garlic, which suggests that potential negative effects could be removed by heat treatment during processing or pelleting of the diet. Lectins are proteins or glycoproteins, which bind to carbohydrates, and are the major constituents of a wide variety of fruits, vegetables and cereals. However, a few of these are toxic to animals and humans, although the mechanism of their toxicity is unclear and may be due to their binding to mucosal cells and potential interference with the digestive functions of the intestine. One of these toxic lectins in garlic, known as ASA_{110}, binds to the brush border membrane and the basal region of the intestinal villi in rats by means of mannose residues, and it is resistant to all forms of intestinal enzyme activity (Gupta & Sandhu, 1997). When intubated directly into the rat jejunum, garlic ASA_{110} lectin altered the activity of Na/K

ATPase, and crude garlic extracts increased the levels of nucleic acids and enhanced the activities of disaccharidase enzymes (Gupta & Sandhu, 1998). Gupta & Sandhu (1998) reported differences between the effects of garlic extracts and ASA_{110} garlic lectins in their activity on alkaline phosphatase and nucleic acids, and suggested that other components of garlic should be examined for their effect on the brush border membrane. Modification of the intestinal brush border with other phytochemical extracts has also been observed. The alkaloid piperine has been shown to enhance the bioavailability of intestinal drugs, by altering the membrane lipids in the intestine, changing the conformation of intestinal enzymes and increasing the length of the microvilli to enhance the small intestinal surface (Khajuria *et al.*, 2002). *Quercus suber* and *Q. coccifera* leaves and their associated CT contents were reported to have a protective effect against intestinal lesion development in mice (Khennouf *et al.*, 2003). Khennouf *et al.* (2003) suggested that this protective effect (68-91%) was related to the antioxidant properties of the CT extracts.

5.4.9 Organoleptic assessment of the chicken meat

When broiler chickens were fed diets with supplementary XTRACT, a commercial product containing the terpenes capsaicin, carvacrol and cinnamaldehyde, it was reported that this supplement improved the organoleptic properties of meat colour, taste and smell (Jamroz *et al.*, 2003). Supplementary yarrow herb in broiler diets was reported to enhance the organoleptic perception of the chicken meat (Fritz *et al.*, 1993). This was enhanced still further when the yarrow was fed along with St. John's wort as a dietary supplement in the birds (Fritz *et al.*, 1993). In the present experiment, the garlic produced a change in meat flavour, which was not necessarily detrimental. However, the taste assessment panel suggested that this effect could be dependent on the dietary composition fed to the birds. Other odorous compounds such as fishmeal may impart their own flavour to the meat produced. When samples from the birds were removed on the days of collection, the smell of garlic was noted to permeate throughout the carcass of the birds and was very pungent on opening the intestine. The results of the present experiment would suggest that the inclusion of some plant herbs and EO in the diets may be used to influence chicken meat flavour. This may have potential in the market place to enhance the value of the meat produced, and improve the profitability margin on chicken products, although further testing would be required.

In summary, the results from the present experiment indicated both positive and negative effects of each phytochemical supplement in broilers during their period of growth. In terms of bird performance, garlic inclusion in the diets improved BM when compared to all other supplements in the diets at 7 days ($P<0.001$), but not after this time. At 42 days of age, the eviscerated carcass weights were similar, between the birds fed the control diets and those fed supplements of yarrow, thyme EO, rosemary and garlic. Garlic inclusion in the diet resulted in the greatest weight gains in the birds during the first 7 days of the experiment, compared to the birds fed all other diets apart from the non-supplemented controls ($P=0.008$). There was no effect on weight gain after this time. The inclusion of garlic as a supplement in the diet increased its consumption, compared to those diets fed with yarrow, mimosa and grapeseed CT between days 0-7 ($P=0.008$). Diets with mimosa CT were consumed least in the study between days 0-7, compared to all other diets except those supplemented with grapeseed CT and yarrow ($P=0.008$). Between 8-21 days in the study, diets with garlic were consumed in greatest quantities, and those with mimosa CT were consumed least ($P<0.05$). The birds fed diets with garlic had an improved FCR between 0-7 days, compared to those diets with thyme EO and rosemary ($P<0.05$). However, between days 8-21, the FCR values were best for those birds fed diets with rosemary, compared to those fed diets with thyme EO, and mimosa, grapeseed and cranberry CT's ($P=0.013$). There was no effect of any dietary treatment in the ileal measurements in these birds on the digestibility of energy within the dietary ration at 21 days. However, there was a tendency ($P=0.067$) for the digestibility of energy within the ration to be lower in the birds fed diets with cranberry CT than those with garlic at this time. Both the coefficients of ADMD ($P=0.018$) and DOMD ($P<0.05$) were reduced in the birds fed diets with cranberry CT, compared to all the other treatment diets except for those fed with yarrow herb at 21 days. Likewise, the digestible OM content in the diet was reduced in the birds fed diets with cranberry CT, compared to those fed all other diets except for yarrow herb ($P<0.05$). At 21 days, the birds fed diets with garlic had the highest coefficients of AND ($P<0.05$) and content of N retained from the diet ($P=0.021$), compared to those fed diets with rosemary, as well as mimosa and cranberry CT's. However, the coefficients of N and AA digestibility were low at both sample points in the experiment, especially at 42 days. The birds fed diets with cranberry CT had the lowest coefficients of AND, compared to those fed diets with rosemary, garlic and mimosa CT ($P<0.05$). The birds fed diets with cranberry CT also had the poorest digestible N content from the diet, compared to those fed diets with yarrow herb, thyme EO, garlic and grapeseed CT ($P=0.021$). At 42 days, there were no

treatment effects on the digestibility of energy, DM, OM or N in these birds. There was no effect of treatment on the concentration of sialic acid produced at either 21 or 42 days. Generally, for each AA, the inclusion of garlic in the diet resulted in the highest digestibility coefficients in the birds, whereas the diets with cranberry CT had the lowest digestibility coefficients, compared to those birds fed with rosemary and mimosa CT ($P=0.012$ for the total AA digestibility). There were no effects of dietary treatment on the digestibility of AA at 42 days and the coefficients calculated indicate a problem with the dietary protein content. There were no treatment effects on the caecal VFA concentrations at either 21 or 42 days of age in the birds. The concentrations of lactic ($P=0.002$) and n-butyric ($P<0.001$) acids decreased over time in the caeca, whereas those of propionic ($P<0.001$) and valeric ($P<0.001$) acids increased, and acetic acid was unchanged. At 21 days of age, the total VFA concentration tended ($P=0.073$) to decrease in the caeca of the birds fed diets with garlic, but there was no effect of treatment on total VFA concentrations at 42 days. The birds fed the control treatment diets had a greater proportion of isovaleric and isobutyric acids in the caeca, when compared to those fed diets with yarrow, thyme, rosemary and garlic supplements, but were not significant when compared to the cranberry CT treatment. The results of the organoleptic assessment indicated that the meat from birds fed the garlic-supplemented treatments had some changes in their organoleptic properties, which were not necessarily detrimental to the meat product flavour.

5.5 Conclusions

Generally, the inclusion of garlic in broiler diets was observed to have the best effects of the supplements tested, on bird performance and on the dietary utilisation of N and AA over the first 21 days in the present experiment. This may be due to a decrease in the activity of microflora within the intestine, or alternatively this supplement may have some effect on protein turnover within the gut. The digestibility coefficients of AA were much lower than those normally associated with diets using high protein soybean meal, and should be repeated. It is necessary to fully investigate the role of garlic with regards to lipid metabolism, the length of time the supplement should be included in the diets and also whether its effects on the organoleptic properties of the meat are positive or negative. As the gut microflora mature, the effects of phytochemical supplementation become less clear. The lack of an effect of garlic or any other supplement on any measurements taken after 21 days suggests that the composition of a phytochemical supplement may need to be changed over the growth period

to maximise its effects in the birds. There does not appear to be a negative effect of supplementary yarrow herb on any measurement when it is fed to birds at these concentrations, when compared to the birds on the control treatment. Dietary yarrow may have an effect on the microflora, along with rosemary and thyme EO supplements, suggesting that the main action of the terpene compounds appears to be in reducing intestinal microfloral activity in broilers. There was some variation in the potential for dietary inclusion of the CT compounds, with grapeseed CT maintaining performance and dietary digestibility to the same level as that observed in the control treatment birds in the present study. This may have been due to the composition or degree of polymerisation of the tannins in the grapeseed CT extract, but it is notable that it did not have a negative effect in any measurement in the birds. The cranberry CT appeared to bind to the nitrogenous components of the diet and may prevent these from being digested by enzymes. There may have been some impurities within the mimosa CT used in the present study, which compromised its performance as a supplement. Both mimosa and cranberry CT supplements may be better suited within a phytochemical blend. There appears to be potential for CT as dietary supplements in broilers, provided they are used as purified compounds, although they may be more suitable if fed after day 7 when the birds have a more developed gut. However, it is unlikely they will improve broiler performance over that of the non-supplemented control treatment. A more comprehensive screening procedure is required for the CT compounds before they can be included in diets. The inclusion of phytochemicals in broiler diets at different ages may show promise in improving health and influencing the intestinal microflora, but care must be taken to avoid detrimental effects on the digestibility of nutrients. There is definite potential for some dietary phytochemical supplements added during the growth period to subtly modify the organoleptic properties in chicken meat, which may potentially be useful if the meat was to be marketed as a flavoured or value added product.

CHAPTER 6

6. GENERAL DISCUSSION

Concerns over the microbiological safety of foodstuffs and the use of drugs in the diets of growing animals have become more prominent in consumer focus in recent years. In the last 2 decades, a number of disease outbreaks caused by virulent microbial pathogens, such as *E. coli* O157, *Campylobacter* and *Salmonellosis* have been reported in humans. These disease outbreaks have resulted in fatalities, as there is no established treatment for the infection except with antimicrobial drugs. Strains of bacteria have developed resistance to various antimicrobial drugs used in human medicine, and outbreaks of disease are becoming more frequent in hospitals, particularly those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and enterococci that are resistant to vancomycin (VRE). Resistance in these strains of bacteria may occur either against one antimicrobial or against several drugs. However, there is no effective agent available against either MRSA or VRE in human medical therapy. There have been proven links between bacterial resistance development in animals and its transfer, either between animals and humans or from animal to animal. This may take place through the food or when animals are in close contact with each other. Using an *in vitro* system to simulate the ileum in pigs, genetic material inducing resistance to antimicrobials was found to be readily transferred between various *Enterobacteriaceae* spp. under ileal conditions (Blake *et al.*, 2003). The intestinal tract of both animals and humans represents a high-risk area for bacterial resistance transfer from one species to another, especially if bacteria that are resistant to antimicrobials have contaminated their food.

For over 50 years, antimicrobials have been used to control intestinal microflora in intensive agriculture. The use of antimicrobials may lead to an improved uniformity of growth and improved bird performance through a reduction in the availability of nutrients for the microflora. Antimicrobial use may be responsible for reducing the energy costs associated with immune responses of the birds to the presence of the microflora in intensively housed domestic species. The implications of their removal due to the potential development of antimicrobial resistance in bacteria have had a great impact on the agricultural industry. As a predominantly precautionary measure, the European Union government have already banned several antimicrobial drugs from being used in poultry feeds, and will remove remaining compounds by 2006. This removal of antimicrobial drugs from poultry feeds leaves poultry

produced within the EU at a disadvantage when compared to that produced in countries allowing antimicrobial usage, with higher costs of production and potentially a greater degree of risk from infection. Since 1999, most of the financial disadvantages have been removed by a greater emphasis on biosecurity, good management procedures and changes in nutrition. Hooge *et al.* (2002) states that the losses to poultry producers have now been reduced from much higher estimates to around £10 million, where the removal of antimicrobial growth promoters (AGP) has resulted in occasional outbreaks of bacterial overgrowth resulting in wet litter problems. In 2002, the use of AGP in UK poultry diets decreased to 27 tonnes, or around 6% of total antimicrobial use (VMD, 2003). The Soil Association, the UK's organic farming body, has disputed these usage figures for AGP, and they have suggested that the actual sales figures are much higher. Certainly, the data from the VMD for UK antimicrobial sales in 1999 and 2000 has some missing data, but this has not been the case for 2001 and 2002. The removal of the remaining AGP in 2006 may not cause nearly as severe an impact economically as the 1997 and 1999 bans on the use of these drugs. However, it is still important to find suitable natural bioactive substances to include in poultry diets, which may confer advantages during growth. This work attempted to assess the suitability of different types of phytochemical compounds for dietary inclusion, in association with carbohydrases, which are normally included in broiler diets as supplements.

In this thesis, the first of the three experiments presented focussed on the inclusion of several dried herbs and their distilled essential oils (EO), from marjoram, oregano, thyme, yarrow and rosemary plants in broiler diets. The second experiment looked at the effects of including several concentrations of commercially purchased thyme EO in the diets with a carbohydrase, and investigated the presence of interactions between the two supplements. In the third experiment, the results of feeding yarrow, thyme EO, rosemary, garlic and 3 types of condensed tannins (CT) to poultry were reported. In these three experiments, the performance characteristics, intestinal microfloral activity, nutrient and amino acid (AA) digestibility, volatile fatty acid (VFA) production and ileal morphology were measured in broilers. These measurements were taken in order to assess the impact of the phytochemical supplements on the activity of the microflora, and the effect of any changes in the intestinal microflora on the digestibility and utilisation of the diets by the birds. There may also have been negative effects in the birds associated with the inclusion of these supplements in the diets, although the supplements were selected carefully. These measurements were taken to gain the maximum

amount of information possible relating to the action of these compounds in the birds on their health and performance. Although this thesis had the primary aim of looking at a range of phytochemicals, it is suggested that any future work should study less compounds in greater detail. In this way, the effects of including the same plant compounds with different chemical compositions could be assessed. Also, a more detailed study of the inclusion concentrations could be studied to examine the most efficacious for the birds, and further interactions with commonly used exogenous supplements such as carbohydrase and other enzymes, as well as the various dietary ingredients.

6.1 Taste perception of aromatic and flavour compounds by poultry

Some phytochemicals may be regarded as toxic or anti-nutrient substances, whereas others may stimulate or enhance digestion and performance characteristics, but this can be related to the concentrations in which they are included in diets. The experiments reported in this thesis have demonstrated that plant material from different sources may display either positive or negative properties in poultry, primarily as a result of differences in their chemical composition. The extent of a preference for different flavour compounds over others is relatively poorly understood in chickens. Although previously dismissed as being non-functional, it is now accepted that the olfactory senses of the domestic chicken are moderately well developed, when compared to other avian species, but their function remains unclear. Domestic chickens have taste receptors at the base of their cornified tongue and also 3 olfactory organs (Kare & Rogers, 1976; Jones & Roper, 1997). Jones & Roper (1997) present evidence that the olfactory response in domestic chickens serves to control both feed and water consumption, and also the production of alarm signals in response to fear stimuli, predator avoidance and toxic substances. If this is correct, then the smell or taste of the phytochemical compounds tested in this work may have either encouraged or restricted broiler feed consumption, which will in turn affect nutrient utilisation and bird performance.

In Chapter 3, the oregano herb supplement reduced feed consumption in the birds fed this treatment, when compared to those birds fed the control treatment. This may have been due to an astringent taste, as the oregano herb contained CT at just under 16 g kg^{-1} DM, or to the presence of another unmeasured compound. The mimosa CT used in the experiment reported in Chapter 5 also restricted feed consumption initially in the birds, as did the thyme EO used in both the experiments in Chapter 4 and Chapter 5, within the first 1-2 weeks, which may also

have been due to an olfactory response. This thyme EO was selected for its antimicrobial nature, with a high content of thymol in the chemical components. Thymol has previously been shown to be highly antimicrobial, and it acts by disrupting the cell wall structure in bacteria (Helander *et al.*, 1998; Dorman & Deans, 2000; Lambert *et al.*, 2001). Around 100 years ago, plants from the families Asclepiadaceae (*Gymnema sylvestre* R. Br.) and Rhamnaceae (*Ziziphus jujuba* P. Miller) were identified, which suppress or enhance the sensation of the sweet taste of the food, but not other organoleptic properties. Certain triterpenoid saponin compounds, such as gymnemic acid from *Gymnema sylvestre* and ziziphin from *Ziziphus jujuba,* act by altering the chemical and physical nature of the taste receptor surface, which may lead to a limited dietary intake by removing the attractive sweet tastes (Suttisri *et al.*, 1995). These compounds are used in human weight control, and may also be important when feeding medicinal plants to chickens, as an inhibition of the sweetness taste receptors may increase the sensitivity to compounds with an astringent nature, thus limiting feed consumption levels. However, there is an unknown impact of such compounds in their effects on growing birds. Garlic has also been suggested to be useful in weight control in humans (Aouadi *et al.*, 2000; Elkayam *et al.*, 2003), for its inhibitory effects on lipid metabolism and decreasing the synthesis of cholesterol. However, it promoted an increase in body mass (BM) at day 7 in the experiment reported in Chapter 5. Domestic chickens have been shown to use odour and colour of their drinking water as a learning mechanism for food avoidance, using food colourings and the aromatic oils of almond and vanilla as supplements (Roper & Marples, 1997). These authors reported that there was no preference for or against vanilla, thus it was a non-toxic compound, but the almond oil was reported to reduce feed consumption. It was suggested that birds may be predisposed to more readily remember secondary compound odours traditionally associated with chemical defences, such as almond (presence of prussic acid) or methylalkylpyrazines (insect prey defence mechanism), and that this was genetically imprinted (Marples & Roper, 1996; Roper & Marples, 1997). If so, it may be worth considering the inclusion of medicinal plants in broiler diets from this perspective. Changing the composition of phytochemical compounds in a supplement may change its overall organoleptic properties to be more pleasant, thus promoting rather than restricting feed intake. When stimuli sourced from various anti-feedant aromatic compounds, including the terpenes carvacrol and camphor, increased the production of β-waves in the brain tissue of rats and water voles, this suggested that the presence of the aromatic

compounds may produce an avoidance response with a neurological basis (Vanderwoolf *et al.* 2002).

In comparison with the antimicrobial thyme EO, the thyme EO used in the experiment reported in Chapter 3 had positive effects on BM after 21 days in broilers. This thyme EO also had positive effects on feed consumption and weight gain in broilers from 15-21 days and on average weight gain from 7-28 days, when compared to those on the control treatment. There was no negative effect of this supplement at any time on any of the performance measurements. This thyme EO supplement had an atypical terpene composition to that normally required for an antimicrobial EO, with intermediate levels of both thymol and carvacrol terpenes, and a high proportion of α–terpineol. Several of the EO supplements used in the diets had an atypical composition to that normally regarded as good quality for medicinal use. In future experiments, it may be useful to observe the different effects in poultry of using supplements with a varying chemical composition on productive responses and FCR. The yarrow used in the experiments reported in Chapter 3 and Chapter 5 resulted in no negative effects in bird performance. It may be the case that there may be some added nutritional benefit of feeding a less antimicrobial plant or its extract rather than a strongly antimicrobial one, in some stimulation of feed consumption, which may result in an enhanced productive response. The synthetic sweetener aspartame was determined to be acceptable to broilers or undetectable with regards to feed consumption, but it was preferred more readily than saccharin, salt and quinine in the dietary ration (Balog & Millar, 1989). This means that there is considerable scope for the testing of phytochemical compounds in poultry diets, depending on the desired effect. Beneficial aromatic compounds in poultry diets may help to disguise others that have negative organoleptic properties, such as those in rapeseed meal or fishmeal, in order to improve the consumption of the diet. It may also be possible to tailor dietary supplements by their chemical composition to produce an effect on feed consumption. Alternatively, the use of acceptable phytochemical supplements may encourage feed consumption in under-performing birds, in order to achieve a compensatory growth response. Therefore, in future, it would seem imperative to focus on the quality of the phytochemical supplements in terms of their chemical composition, which can be the only method of consistently assessing results between different studies. The variation in the chemical composition of plant material over different years, suppliers and seasons will complicate the search for a phytochemical compound with a consistent effect in the birds.

Even when both supplements were obtained from the same plant material, the experiment reported in Chapter 3 indicated that there may be considerable differences between the supplementation of plant herbs or EO in poultry diets. In this experiment, the birds fed diets with yarrow herb were second best of all treatments in terms of the performance characteristics overall, while those fed diets with yarrow EO were among the poorest. This difference also appears to be unique for each material tested. There was a considerable variation between thyme herb and its EO in broiler diets and their effects on bird performance, where the thyme EO was the best supplement overall in the experiment, but the birds fed thyme herb were mediocre in terms of performance. However, only BM was increased in the birds fed thyme EO when compared to the control treatment at 21 days. The birds fed diets with marjoram herb or its EO had equivalent levels of performance in all measurements, and neither of these treatments produced different responses to those birds fed on the control treatment. The birds fed diets with rosemary herb had an equivalent feed consumption when compared to those on the control treatment from 22-28 days of age, while those fed diets with rosemary EO had a decreased feed consumption compared to those fed the control treatment. Thus, the composition of each individual supplement would appear to give it a different effect in terms of stimulating or restricting feed intake and weight gain in broilers. Dorman & Deans (2000) have suggested that terpene phytochemicals may be trapped within the secretory structures in an herb plant, and thus the presence of bioactivity may be dependent on the use of the EO fraction. In contrast, Shelef *et al.* (1984) reported a greater inhibitory effect of sage herb than the EO from this plant against bacteria when incubated in meat broth. Thus, the effect is unclear and may depend on individual circumstances.

6.2 Experimental techniques

Overall, it is considered that the growth trials used in the work carried out for the preparation of this thesis are the best form of testing these phytochemical compounds, as this environment represents the one that would be in use in the commercial situation. However, there may be some benefits in screening EO and herbs initially to a greater extent *in vitro*. While the *in vitro* technique on culture media we used gave an indication of the inhibitory effect of the EO against different bacteria, it only allowed the testing of one bacterial strain, which is not representative of the many strains or species present in the intestinal tract, and undefined mixtures of strains may be more suitable. Plating methods under *in vitro* conditions select for

one bacterial species of interest, providing ideal conditions for their growth. Only 5% of bacteria are culturable from the resident species in the intestine, and it is inevitable that symbiotic or antagonistic relationships will exist between different bacterial species, which may affect their viability. In future work, it may be more helpful to assess the activity of plant compounds by using an *in vitro* technique more closely related to the conditions present in the intestinal environment. Alternatively, the use of molecular biology techniques such as RT-PCR will allow the identification of much more of the microbial species resident in the intestinal tract. Shanmugavelu *et al.* (2004) have developed a fermentation technique for *in vitro* purposes using broiler intestinal contents, and have demonstrated its efficacy as a screening test for the activity of different plant compounds. Likewise, Blake *et al.* (2003) developed an *in vitro* simulated pig gut system, enabling daily monitoring of microfloral population changes for its resident species, which may enable the effects of plant compounds to be tested over time spans such as a growth period of 42 days for chickens. In future work, it may be beneficial to screen any potential bioactive compounds using this type of methodology, rather than by direct plating methods.

The measurement of the bioactive potential of the CT compounds also requires some reassessment. The variable properties of the structures and binding potentials of each type of CT compound needs to be assessed before these compounds are included in poultry diets. In future, a measurement of both the concentrations of total phenolics within these materials and the concentration of hydrolysable tannin (HT) may be necessary. It may also be beneficial to assess the binding potential of the tannin structure to proteins when screening them initially, as suggested by Vitti *et al.* (2004). Vitti *et al.* (2004) disputed the fact that it is the concentration of CT alone in the diet that is responsible for any adverse nutritional effects. In the experiment reported in Chapter 5, the grapeseed, mimosa and cranberry CT were all included at the same concentrations in the diets, but grapeseed and cranberry CT did not negatively affect bird performance compared to that of the birds on the control treatment. However, the inclusion of mimosa CT in the diets resulted in negative effects on broiler weight gain and feed consumption between 0-7 days of age, when compared to the birds on the control treatment. The inclusion of cranberry CT in the diets reduced the digestibility coefficients of amino acids (AA) in broilers, when compared to those birds fed the control treatment. From the results in this thesis, the inclusion of CT in broiler diets shows potential, but a much more comprehensive screening procedure is required, to prevent an increased requirement for

protein provision in the diet. It may be the case that the CT compounds may be better fed in the diets after 7 days of age, where the gut of the birds are more developed.

6.3 Use of enzymes in conjunction with phytochemicals

Of the various feed additives available, enzymes have been established over the years as the supplements most likely to improve the productivity and nutrient digestibility of the diet, by reducing the anti-nutritive effects of the non-starch polysaccharides (NSP) in wheat and barley. NSP-degrading enzymes act against an increased viscosity of the digesta, which is caused by polysaccharides leaching from the bran and endosperm cells of wheat grains (Chesson, 2000). At the same time, they simultaneously release other nutrients previously trapped inside the cell walls of the grain, by breaking down the indigestible fibre component of the cereal cell walls (Chesson, 2000). In the experiment reported in Chapter 3, the poor quality of the wheat and the absence of an enzyme or antimicrobials in the diet may have resulted in the low AME in the birds fed these diets. The presence of a commercial carbohydrase has previously been observed to significantly improve the AME in wheat of low quality fed to broilers (Choct *et al.*, 1995), and it may have been beneficial to include an enzyme such as this in the diets from this experiment. The carbohydrase used in the experiment reported in Chapter 4 was shown to have a much greater effect in improving the quality of the dietary ration than the thyme EO. However, there was a significant interaction between both supplements in the AME, both with and without a correction to nitrogen equilibrium, as well as in all the measurements of nutrient digestibility. It may be that the carbohydrase inclusion reduced the concentrations of phytochemicals required to produce an optimal effect on dietary digestibility in these birds. At 42 days of age, the birds fed the treatment with 1 g kg^{-1} thyme EO and carbohydrase had the highest coefficients of ADMD, DOMD and AMN, as well as the highest concentrations of digestible OM and metabolisable N in the diet. The birds fed this treatment also had the largest contents of AME and AME:GE, both with and without a correction for nitrogen equilibrium, where the benefit was mainly due to the dietary carbohydrase inclusion.

The carbohydrase also had a positive effect throughout the first 3 weeks of the experiment on all measurements of bird performance in Chapter 4. The presence of an exogenous enzyme has been reported to have an age-dependent effect, with the best responses observed over the first few weeks (Almirall & Esteve-Garcia, 1994). Older birds gain less of a benefit from the

presence of enzymes, especially carbohydrase (Bedford, 1996), as they become more able to tolerate the quantity of anti-nutrient NSP in the diet. In chapter 4, the carbohydrase also numerically reduced the numbers of caecal *C. perfringens*, suggesting that it may have had a beneficial effect on the intestinal microflora composition of these birds. The role of enzymes have previously been suggested to become more important after the removal of AGP, due to their ability to increase the rate of digestion, and reduce bacterial numbers in the ileum by depriving them of substrate (Bedford, 2000). In Chapter 4, the carbohydrase did not appear to be antimicrobial in its action, as it did not affect the intestinal coliform numbers. As such, the combination of the antimicrobial thyme EO, which reduced intestinal coliforms and the carbohydrase in broiler diets appeared to have a dual effect, both in improving intestinal health and in the provision of a greater nutrient supply to the bird.

The carbohydrase in these diets also improved the digestibility coefficients of several AA, in agreement with the work of Hew *et al.*, (1998). This may be due to the liberation of these nutrients from the indigestible grain structure by the carbohydrase, due to its action on the arabinoxylan components in the cereal grain. However, the carbohydrase may also reduce the extent of endogenous AA loss as a result of the reduction in the anti-nutritive activity of NSP (Larsen *et al.*, 1993; Hew *et al.*, 1998). The presence of the thyme EO in the diets had no effect in increasing AA utilisation in the birds in this experiment. Thus, the experiment suggests the inclusion of a carbohydrase along with thyme EO may have a synergistic effect in the birds. However, the action of the carbohydrase appears to take place through different mechanisms on all measurements other than nutrient digestibility, when compared to that of the thyme EO. The inclusion of both supplements together would also suggest a reduction in the cost of supplementing the diets with phytochemicals, which is likely to be their major limiting factor for commercial consideration. In Chapter 5, the carbohydrase was included in all the treatment diets, so it is impossible to assess its contribution compared to that of the various phytochemicals. Further experiments similar to the one described in Chapter 4 would be necessary in order to test the most suitable inclusion levels of these compounds, but it is suggested that the enzyme will have a much greater effect in improving the nutritional quality of the diet. It is considered that it would be beneficial to validate the action of other enzymes in the diets along with phytochemical supplements, or to assess a mixture of carbohydrase and protease with phytochemicals.

The action of the carbohydrase in breaking down the NSP component of the dietary fraction should allow a more complete mixing of terpene components with the digesta, although this has not been demonstrated. This in turn would give these compounds greater access to control undesirable microflora within the intestinal lumen, and may be one of the mechanisms by which the thyme EO and the carbohydrase work together to increase nutrient digestibility. However, the experiment reported in Chapter 4 provides evidence that the thyme EO itself increases AME and AMEn, independently from the carbohydrase as a supplement. This experiment also suggested that thyme EO increased AME and AMEn to a greater extent than the amount of energy directly provided by the EO as a supplement in the diet. This may have been due to the independent action of the thyme EO in decreasing intestinal viscosity, as has been reported previously with other EO supplements (Francesch *et al.*, 1999; Williams & Losa, 2001; Losa, 2001). However, there may be a direct effect of thyme EO on the digestibility of dietary nutrients also. Jamroz *et al.* (2003) measured a tendency for an improvement in the utilisation of dietary energy and dry matter when broilers were fed diets including the commercial compound XTRACT. At this point, it is unclear whether or not terpene compounds such as thyme EO can directly affect the digestibility of dietary nutrients, and it is more realistic to suggest that they may have increased the utilisation of energy in the birds indirectly. By controlling the activity or reproductive potential of the intestinal microflora, EO may decrease the requirement of the microflora for energy and protein. The principal components analysis section in Chapter 3 also supported a minor effect of the inclusion of dietary terpenes on altering the energy and protein supply to the birds in the analysis of PC2, but indicated that this action may be independent for each dietary terpene supplement.

The carbohydrase and the thyme EO appear to act independently on the intestinal microflora. In the broilers at 42 days of age in Chapter 4, there was a conflicting action of the carbohydrase and the thyme EO on the caecal microflora, where the carbohydrase increased the concentrations of several VFA. The thyme EO decreased the concentrations of these VFA, but increased the concentrations of lactic acid, enhancing the overall antimicrobial action. The action of the carbohydrase increased the digestive breakdown of dietary components into smaller units, which may then be more easily fermented in the caeca. However, when these units reach the caeca, the antimicrobial action of thyme EO may have already selectively influenced the populations of bacterial species present. In the caeca, the antimicrobial efficacy

of thyme EO should be increased due to the anaerobic conditions, as was confirmed with *Salmonella typhimurium* by Juven *et al.*, (1994). As a result, some of this fermentable energy may become available to the birds rather than the microflora. The energy value of fermented substrate from the caecal microflora has previously been suggested (Jamroz *et al.* 2002), but the influence of the microflora is unclear, and may be either positive or negative in the birds (Muramutsu *et al.* 1994).

6.4 Effects of phytochemical supplements on the microflora

Phytochemicals would appear to have a generally selective inhibitory effect on the microflora when included as dietary supplements *in vivo* over the course of these experiments. In this respect, they behave similarly to the antimicrobials used previously in broiler production when added in the diets, except that they do not appear to act indiscriminately. However, the action of these supplements is not always consistent and their inclusion in the diet does not always produce a direct improvement on bird performance. In the experiment reported in Chapter 3, there was no significant effect of any treatment on the microfloral populations, either in the excreta or the caecal samples. However, there were numerical reductions in both the caecal populations of *C. perfringens* (around 2 $\log_{10}$ c.f.u.g^{-1}) and similar reductions (1.5-1.75 $\log_{10}$ c.f.u. g^{-1}) in the proportions of caecal *C. perfringens*: total anaerobes, when thyme and yarrow herbs were included in the diets of the birds. Further tests carried out using an increased number of sample replicates would be required to confirm these differences. Several of the extracted EO tested in the original *in vitro* screening analysis also reduced the growth of several bacterial species, namely *E. coli*, *S. aureus*, *B. subtilis* and to a lesser extent *L. monocytogenes.* These extracts included the EO from *Origanum vulgare*, *O. majorana*, *Thymus spicata* and *Satureja hortensis* (Appendix 4), but these EO and the others also tested had a lesser or only a minor effect on the other bacterial populations. These EO samples were not tested for their chemical composition, as they were not to be used in the feeding trial.

In the birds suffering from colisepticaemia in Chapter 4, the thyme EO decreased the numbers of intestinal coliforms directly, which should have reduced the length of the infective period. The measurement of lactic acid in the caeca tended to be higher at 19 days of age in the birds fed thyme EO within their diets in Chapter 4, which would suggest that this EO did not reduce the concentrations of lactic acid bacteria. By 42 days of age in this experiment, this increased concentration of lactic acid in the caecal contents with the inclusion of thyme EO in the diets

was significantly different, while this supplement also tended to reduce the propionic acid concentration and isobutyric acid proportions in the caeca. The fermentation of these two VFA are associated with various bacterial species, so it is unclear which were affected. Lactic acid is used in the food industry and is known for its antimicrobial properties, which may be partly due to its effect in lowering pH (Shelef, 1994), as this would inhibit the growth of most bacteria. Thyme EO, or its components carvacrol and thymol, have also inhibited bacterial fermentation previously *in vitro* (Varel & Miller, 2001; Shanmugavelu *et al.*, 2004). Yarrow herb may also have potential in controlling the intestinal microflora *in vivo*. However, this does not appear to be as strong an effect as that observed with the thyme EO. In Chapter 5, the proportions of isovaleric and isobutyric acids in relation to the total concentrations of caecal VFA were reduced in the birds fed diets including yarrow herb, when compared to those fed the control diets. This would support the numerical reductions in bacterial populations observed with this herb in Chapter 3. The inclusion of yarrow increased the production of acetate at the expense of butyrate when used in an *in vitro* microbial culture system with rumen fluid (Broudiscou *et al.*, 2000), demonstrating that it has the potential to modify fermentation properties. Broudiscou *et al.* (2000) also used several other plant extracts in an *in vitro* system, where all had varying effects on the fermentation of rumen microbes. The concentrations of both isobutyric and isovaleric acids were also reduced in the caeca of the birds fed diets with thyme, rosemary and garlic, compared to those birds fed the control diets. However, these VFA are normally found in much lower quantities in the caeca and are therefore of lesser importance, compared to acetic, propionic, valeric and n-butyric acids.

6.5 Effects of phytochemical supplementation on nutrient digestibility

The effects of phytochemical inclusion in the diets on the coefficients of digestibility of AA may be indirectly related to the activity of the microflora, by bacterial AA metabolism and conversion processes. There was no effect of any dietary treatment on the digestibility coefficients of AA in the experiment reported in Chapter 3, nor any treatment effect on the digestibility of the other nutrients. This may be partially due to the poor digestibility of the diets, which resulted in the low AME measurement and coefficients of ADMD observed in these birds. These low values for digestibility coefficients may have been increased by the inclusion of a dietary enzyme, to increase the accessibility of nutrients from the cereal grains. There was no effect of thyme EO inclusion in the experiment in Chapter 4 on the coefficients of AA digestibility, although the inclusion of carbohydrase increased the digestibility of

several AA at 42 days. However, some plant extracts and supplements appear to have an effect on the AA digestibility coefficients. The inclusion of EO or their terpene components have previously been reported to inhibit the degradation of soybean meal and deamination of AA in sheep (McEwan *et al.*, 2002a&b; Newbold *et al.*, 2004) and dairy cattle (McIntosh *et al.*, 2003). The digestibility coefficients of several AA were increased in broilers when terpenes were included in their diets (Jamroz *et al.*, 2003). Mint EO has also significantly altered AA production by *S. enteritidis* and *S. aureus in vitro* (Tassou *et al.*, 2000). In Chapter 5, the presence of garlic in the diets increased the AA digestibility coefficients, compared to those in birds fed diets including mimosa and cranberry CT, but not in comparison with those fed the control diet. The presence of most AA have been determined in garlic powder, especially arginine at 81 mmoles l^{-1}, which should increase the concentration of circulating arginine by 10% when included in the diet (Das *et al.* 1996). Das *et al.* (1996) also determined the presence of significant amounts of lysine, alanine, glutamic acid, serine and histidine in garlic powder. Garlic inclusion was also observed to decrease concentrations of plasma corticosterone and increase the testosterone concentrations in rats fed on higher levels of dietary protein (Oi *et al.*, 2001). However, the control treatment birds had a greater digestibility of AA than those birds fed the diets with cranberry CT. The grapeseed CT treatment diet did not reduce the digestibility coefficients of any AA, and was equivalent to both the birds fed the diets with garlic and the non-supplemented control diets. However, the precipitation of plant proteins by tannins has been observed to vary depending on the tannins used (Perez-Maldonaldo *et al.*, 1995).

In the digestibility analyses carried out in Chapter 5, feeding broilers with cranberry CT supplements in the diets resulted in a lower coefficient of AND and reduced the concentration of digestible N in the diet at 21 days, when compared to the control birds. The birds fed mimosa CT supplemented diets had a reduced concentration of digestible N in the diets, when compared to those fed the control treatment. ADMD, DOMD and the digestible OM content of the diets were reduced in the birds fed diets with cranberry CT, when compared to those fed the control diets at 21 days. In addition, the birds fed mimosa CT and thyme EO supplements in their diets had a lower digestible N concentration in the diet at 42 days of age, but this was not significantly reduced when compared to any other treatment. These results would tend to suggest that the cranberry and mimosa CT in these supplemented diets may have bound to the various dietary components, especially proteins, making them unavailable for digestion by the

birds. The structures of the CT or their protein-binding potentials were not determined in this experiment. Additionally, various other compounds may be present as well as the CT, such as simple phenolic components or HT. Tannins of this type may be more suitable if fed within a blended phytochemical compound, or when the birds have a more mature gut. However, the results of the experimental analysis do not suggest that these compounds are altogether unsuitable, as the birds fed diets with grapeseed CT maintained the coefficients of nutrient digestibility to the same extent as the birds fed the control diets. Grapeseed CT compounds appear to be of a suitable type for inclusion in future feeding trial studies. The cranberry CT in diets did not reduce broiler performance to any degree in this study, and these birds performed equally well when compared to those fed the control treatment diets. It may be beneficial to assess this compound in the diet when an additional tannin-binding agent has been added, such as polyethylene glycol (PEG) or polyvinylpyrrolidone (PVPP). These compounds may disrupt the formation of tannin-protein complexes, and may be useful in assessing the extent to which these compounds bind to the digestive enzymes in the birds.

6.6 Principal Component Analysis

Principal component analysis (PCA) represented a useful check on the validity of the statistical testing of the experimental data. Due to the nature of the experiments reported in this thesis, there were substantial numbers of data variables, up to 100 in each experiment. The PCA allowed grouping of related data variables. When the results of the analysis of variance were cross-checked against those of the PCA, an agreement between the two sets of results indicated that the statistical tests were correct. Unfortunately, the PCA could not be performed on all the data, but the agreement of the tests indicated that the statistical techniques employed were in agreement and thus were successful.

It is possible that further experiments may isolate other supplements with an inhibitory effect on the activity of intestinal microflora. A blended product using phytochemical compounds of different types would probably be most effective in the birds for continued efficacy as a supplement. It is considered that plant extracts and EO's exhibiting an antimicrobial effect should not be used alongside probiotic bacteria in a supplement, as they have both demonstrated equally bactericidal effects against gram positive bacteria in probiotic blends, as well as gram negative pathogenic bacteria (Kamel, 2001). The action of phytochemicals in the birds may additionally be more holistic in nature than that of the previously used synthetic

AGP. Some have been shown to have effects in improving the organoleptic quality of broiler meat (Fritz *et al.*, 1993; Jamroz *et al.*, 2003), which may be useful in improving the market value of the product. EO, herbs and individual monoterpenes have been reported to have effects against the resorption of bone when included in the diets of mature rats (Muhlbauer *et al.*, 2003). Individual EO's have different effects in influencing the contractural properties of intestinal and skeletal muscle (Lis Balchin & Hart, 1997), which may lead to some having a positive effect on certain intestinal functions, such as mixing of the digesta. The antioxidant potential of garlic (Yin & Cheng, 1998), EO (Botsoglou *et al.*, 2002; Papageorgiou *et al.*, 2003) and CT (Cao *et al.*, 1998; Serafini *et al.*, 2000) have been shown in animal tissues. Garlic is known to have positive effects in lipid metabolism (Aouadi *et al.*, 2000; Chowdhury *et al.*, 2002), which may result in a more efficient utilisation of dietary fat. Topical application of garlic to the skin has also shown effects against northern fowl mite infestation in laying hens (Birrenkott *et al.*, 2000). There are therefore several ways in which these phytochemical supplements may affect the growth, productivity and health of broilers. However, a careful assessment of dosage levels and the chemical composition is required for potential phytochemical supplements. It is also important to establish the relationship of these supplements with other dietary components, in order that the digestibility of the dietary ration is not reduced.

6.7 Recent developments in legislation of feed additives

In light of the crisis in consumer confidence regarding the safety and quality of human foods, the EU government will introduce and enforce new animal feed legislation for the inclusion of all feed additives into animal diets. This will come into force in October 2004, and will have considerable impact on the development of suitable alternatives to antimicrobial drugs. The legislation, known as the General Food Law, was first discussed in 2002 and requires that all feed ingredients, supplements and commercial animal feeds be completely transparent for the purposes of traceability through the food chain as of January 1st, 2005. It covers all issues pertaining to food safety and hygiene, and also issues relating to animal welfare. Under the General Food Law, Regulation (EC) 1831/2003, all feed additives to be marketed in animal diets must previously have been through an authorisation procedure. Authorisation will be granted for each feed additive for 10 years, and procedures will be specific to each animal species and will govern specific conditions of use. This legislation also phases out the

remaining antimicrobials in animal feeds as of January 1st, 2006. Under this new legislation, there are 5 categories of feed additives:

- Technological – incorporating preservatives, antioxidants, emulsifiers, stabilisers and acidity regulators
- Sensory additives – flavours and colorants
- Nutritional additives – including vitamins, minerals, AA and trace elements
- Zootechnical additives – Digestibility enhancers and gut flora stabilisers
- Coccidiostats and histomonostats

With the state of the current research on phytochemical compounds, it is unclear exactly how to classify phytochemical additives, as they appear to fall into several of these categories. Variations in the quality of the source material for phytochemicals and of their constituent chemical compounds may determine how they should be classified. However, the inherent variability of these phytochemicals will require that suitable material should be sourced carefully, and may mean that the plant material should always be sourced from a single supplier for consistency of product quality. For scientific purposes, reporting the chemical composition of the material in the research literature is paramount to an understanding of the action of these phytochemical compounds and their potential for inclusion into animal diets.

6.8 Conclusions from the work in this thesis

Several conclusions became apparent as a result of the work in this thesis, with regards to the inclusion of phytochemical compounds as supplements in poultry diets:

- Phytochemical compounds in terpenes and tanniniferous material have the potential to influence performance and digestibility measurements in broilers, either positively or negatively, depending on their chemical composition. However, when feeding diets of an average quality, their influence on the performance and nutrient digestibility of the diet is much less than that of carbohydrases.

- One phytochemical compound obtained from two different sources may have a completely different effect in the performance characteristics of broilers, as a result of a changed chemical composition. It is therefore important to fully characterise the chemical composition of the material before use.

- EO and carbohydrase can be supplemented together in broiler diets to improve nutrient digestibility synergistically. This work supports an effective inclusion level of 1 g kg^{-1} for an EO when a carbohydrase is included at commercial levels in the diet.

- None of the phytochemical supplements tested had an adverse effect in the birds, at these inclusion levels in the diets. However, some require an initial period of adaptation in the birds, which may be due to the composition of their primary constituent phytochemicals. As such, these components may be better fed within a blended supplement of various phytochemicals, or they may be better supplemented in the diets of older birds with a more developed gut.

- The measurement of the content of CT in a tannin-containing material is insufficient when assessing it as a phytochemical supplement in the diet, even when these products are obtained commercially as CT extracts from the various plants. They must also be assessed for their ability to bind proteinaceous compounds and for the concentrations of the other types of phenolic components, in order to determine potential positive or negative effects in the birds.

- The action of the various phytochemical supplements would appear to change over the course of the growth period. Some supplements have a positive effect in the initial few weeks, such as garlic. However, others appear to have a negative effect initially and a more beneficial effect as the birds matured, such as the thyme EO. It may therefore be important to tailor the composition of a supplement over the course of the growth period to produce the best results.

- The inclusion of thyme EO, with a high content of the phenolic terpene thymol, appears to demonstrate antimicrobial properties *in vivo* in broilers. Supplementary thyme EO in the diets had a direct antimicrobial effect in reducing the caecal coliform concentrations when compared to the birds on the control treatment at 19 days of age. At the same time, throughout the growth period, the caecal concentration of lactic acid remained elevated in those birds fed thyme EO, when compared to those without.

- The inclusion of an herb supplement may have different properties on broiler performance, when compared to those of an EO, even when both components are obtained from the same plant. However, it is not necessarily the case that an extracted EO will produce better effects than the whole plant material, and this may be dependent on the EO quality.

- Garlic showed potential as a supplement in the diets of young birds, up to 21 days of age, improving the measures of bird performance and AA digestibility.

- The inclusion of phytochemical compounds in broiler diets may have positive or negative effects on the organoleptic properties of the meat produced. The effect of including garlic as a dietary supplement may not necessarily be negative, but requires more testing to assess its potential.

6.9 Further opportunities for research into phytochemicals

- It is important to elucidate the site of action for phytochemical compounds within the gastrointestinal tract of poultry, in order to determine where they are exerting the greatest effect in the birds. It may be beneficial to radio-label these compounds, in order to determine their recovery in the excreta and thus the extent of their metabolism in the gastrointestinal tract. It is also possible that two different phytochemical supplements may have a different site of action within the gastrointestinal tract.

- More research is required on the interactions between different supplements, whether of the same type, or supplements with a different mode of action to display the potential for their combined use in phytochemical blends. Different terpene components within the EO may have a synergistic or antagonist mode of action when supplemented together. However, little is known concerning the effects of supplementation of EO in combination with tannins or some other compound.

- The astringent nature of these compounds within the diet may either have a positive or a negative effect when they are included in low concentrations. It is hypothesised that, when included in low concentrations, they should have a positive effect. By measuring the water intake of the birds when including these supplements in the diet, it may be possible to establish the astringency of these compounds, especially the CT. The level of water intake in the birds may affect the litter quality within the housing, in terms of the consistency of the droppings.

- By using a range of different enzymes in combination with phytochemical supplements, or an enzyme blend, it may be possible to identify other effects and mechanisms for their action in the birds. Use of a protease enzyme in connection with these supplements may help to establish clearer effects in relation to the dietary digestibility of nitrogen and AA. The synergistic action of the carbohydrase and thyme EO in this work on nutrient digestibility would suggest that the development of a phytochemical/ carbohydrase blend may be the most promising opportunity for further assessment of phytochemical compounds in poultry.

- The sialic acid determination in the experiments in this thesis demonstrated some effects of terpenes on the endogenous losses. However, it is unclear whether or not these mechanisms were positive or negative. Assessment of the various phytochemical compounds for their action on the endogenous enzyme systems, or on other digestive secretions, would be necessary to determine whether these compounds have a positive or negative effect on endogenous secretions, especially when used in conjunction with enzymes, which will themselves influence endogenous losses.

- The literature reports clear effects of garlic on lipid metabolism. With the beneficial response of garlic supplementation in the diet on early BM, it may be useful to measure concentrations of cholesterol or serum lipids in birds fed these or other diets.

- In order to reduce the numbers of birds necessary for work on phytochemicals, it would be advantageous to develop an *in vitro* continuous fermentation system, in order to establish whether or not these compounds have an effect on the intestinal microfloral populations or on the concentrations of intestinal VFA.

- An assessment of the effects of phytochemicals on the immune system of the bird should be carried out. Any stimulatory effect on the production of antibodies *in vivo* when these phytochemicals are supplemented into the diets may indicate that they have an immune-enhancing effect. In particular, including herbs containing polysaccharides at low levels in the diets may act in a similar way to other fermentable polysaccharides like MOS or FOS.

- Some assessment of the potential of the intestinal microflora to develop resistance to phytochemical compounds should be made, and an assessment of the rate of development of any resistance, in order that treatment strategies could be developed or whether phytochemicals should be rotated as supplements within the dietary ration.

- It may be advantageous to measure the effects of phytochemical supplements on other physiological parameters, such as bone density, which may affect skeletal conformation and influence current welfare problems, for example leg health. EO have been shown to

prevent the re-absorption of bone in mature monogastric animals, and it may be useful to assess their effects on bone density in growing birds.

- Bacterial challenge testing is required to determine the antimicrobial efficacy of different phytochemicals *in vivo*, in order to determine whether or not these compounds have potential in helping the birds to recover from various bacterial or coccidial infections. The development of alternative strategies to treat coccidial infections in poultry would be beneficial if the use of coccidiostats is threatened.

CHAPTER 7

7. BIBLIOGRAPHY

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CHAPTER 8

8.1 APPENDIX 1

Range of mono- and sesqui-terpene components of the essential oil ofThyme (*Thymus vulgaris* spp.) (after Lawrence, 1976-1994)

MAIN TERPENE COMPONENTS	Range of % Composition
Borneol	0.5-26.2
Camphene	0.1-12.8
Camphor	Trace-16.3
Carvacrol	0-43.0
Caryophyllene	0.2-6.4
1,8-cineole	0.2-7.4
p-cymene	2.2-59.0
Geraniol	0.1-5.8
Limonene	0.3-7.9
Linalol	0-32.8
Methyl carvacrol	0.1-3.0
Myrcene	0.3-6.3
α-pinene	0.4-3.7
β-pinene	0.2-4.7
α-terpinene	0.3-4.4
γ-terpinene	0.3-19.4
Terpinene-4-ol	0.2-9.5
α-terpineol	0.3-12.3
α-thujene	0.5-2.3
Thymol	0-58.6

Range of mono- and sesqui-terpene components in essential oils of Oregano (*Origanum vulgare* spp)
(after Lawrence, 1976-1994)

MAIN TERPENE COMPONENTS	Range of % Composition
Borneol	1.6-2.8
Camphene	0.2-1.7
Carvacrol	60.0-73.7
Caryophyllene	2.1-17.5
1,8-cineole	0.3-1.4
p-cymene	3.1-36.0
Germacrene D	Trace-9.5
Limonene	0.2-6.0
Linalol	0.3-76.6
Linalyl acetate	0.4-2.3
Myrcene	1.0-1.8
Ocimene	1.0-13.5
α-phellandrene	Trace-10.6 (normally under 5)
β-phellandrene	0.6-2.9
α-pinene	0.5-1.6
β-pinene	0.1-3.9
Sabinene	Trace-13.5
α-terpinene	0.1-7.4
γ-terpinene	1.9-24.3
Terpinene-4-ol	0.5-4.6
α-terpineol	Trace-3.3
α-terpinyl acetate	Trace-2.7
Thymol	Trace-30.9

Range of mono- and sesqui-terpene components in essential oils of Marjoram *(Origanum majorana* spp)
(after Lawrence, 1976-1994)

MAIN TERPENE COMPONENTS	Range of % Composition
Carvone	1.2
Caryophyllene	3.5-5
cis-sabinene hydrate	Trace-15
cis thuyan-4-ol	9
p-cymene	Trace-14
Geranyl acetate	Trace-1.2
Limonene	0-2.32
Linalol	Trace-20 (normally up to 10)
Myrcene	0-7.56
α-phellandrene	1-5
β-phellandrene	2.8
α-pinene	3-7
β-pinene	7.25 (up to 9)
Sabinene	0-9
α-terpinene	5-14
γ-terpinene	5-20.8
Terpinene-4-ol	20-45.5
α-terpineol	Trace-8
Terpinolene	1.6-5
α-thujene	1-5
Trans-sabinene hydrate	1.0

Range of mono- and sesqui-terpene components in essential oils of Yarrow *(Achillea millefolium* spp)
(after Lawrence, 1976-1994)

MAIN TERPENE COMPONENTS	Range of % Composition
Borneol	0.0-19.01
Bornyl acetate	Trace-2.2
Camphene	0.1-6.2
Camphor	Trace-20.6
Caryophyllene	1.6-32.0
Chamazulene	5.0-33.2
1,8-cineole	0.0-14.2
p-cymene	0.1-7.3
Furfuryl alcohol	11.4
Germacrene D	9.3-13.6
Isoartemesia ketone	8.6
Isobutyl acetate	1.4
Limonene	Trace-10.7
Menthol	1.0
Myrcene	0.6-2.0
α-pinene	0.0-23.8
β-pinene	5.5-23.0
Sabinene	7.5-41.3
α-terpinene	0.5-1.3
γ-terpinene	1.3-3.7
Terpinen-4-ol	2.1-5.6
α-terpineol	0.8-4.3

Range of mono- and sesqui-terpene components in essential oils of Rosemary *(Rosmarinus officinalis* spp) (after Lawrence, 1976-1994)

MAIN TERPENE COMPONENTS	Range of % Composition
α-anorphene	7.0
Borneol	0.4-17.3
Bornyl acetate	0.5-21.0
Bornylene	6.0-8.0
Cadinene	12.0
Camphene	Trace-19.2
Camphor	0.0-56.5
δ-3-carene	0.0-13.0
β-caryophyllene	0.0-17.1
Carvacrol	Trace-2.0
1,8-cineole	3.0-60.1
p-cymene	Trace-11.4
Fenchone	2.5
Geraniol	0.0-9.2
α-humulene	0.0-4.0
Isobornyl acetate	0.0-7.3
Limonene	0.2-10.6
Linalol	0.0-19.8
Linalyl acetate	Trace-1.4
γ-muurolene	3.1-7.0
Myrcene	0.2-52.1
Nerol	0.0-3.6
α-phellandrene	0.2-4.2
β-phellandrene	0.9-4.5
α-pinene	1.4-57.5
β-pinene	Trace-9.1
Sabinene	Trace-6.4
trans-ocimene	1.3
α-terpinene	0.3-1.2
γ-terpinene	0.1-4.2
α-terpineol	Trace-10.0
Terpinene-4-ol	0.2-6.8
Terpinolene	0.0-7.28
α-thujene	Trace-12.5
α-thujone	0.2-4.2
β-thujone	1.1
Thymol	Trace-1.2
Verbenone	0.3-29.0

8.2 APPENDIX 2

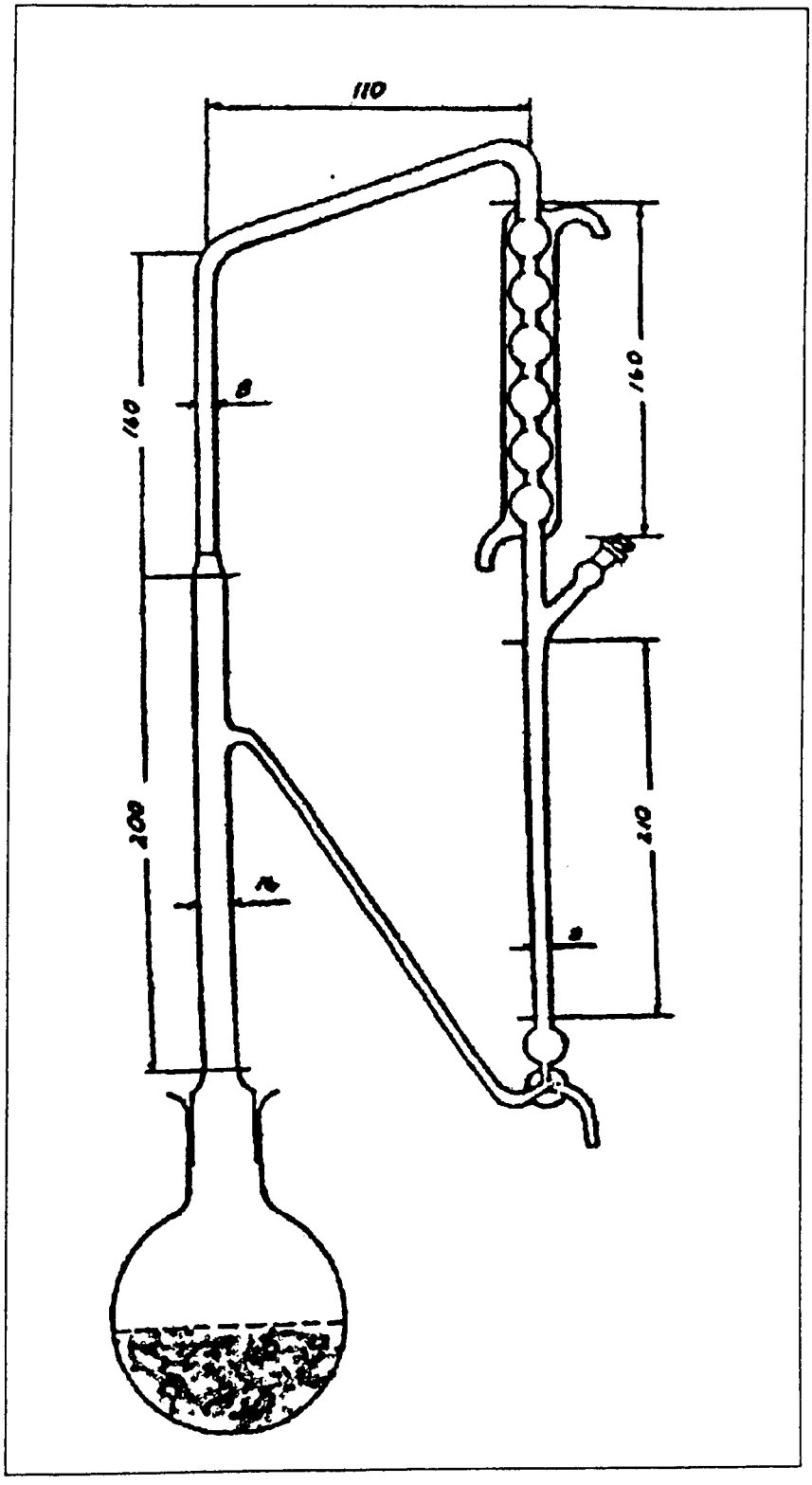
Methodology used in the British Standard distillation procedure of essential oils

(Adapted from British Standards, 1985)

1. Around 100g of dried herb sample should be prepared for distillation
2. Flasks previously soaked in Decon 90 (5%) should be rinsed with tap water initially, and then a further 3 rinses with distilled water.
3. The flask should be filled with sample and the distilled/deionised water added, so that the dried sample is completely covered.
4. The flask should be secured by fixing a clamp around its neck.
5. The distillation apparatus should be rinsed out with tap water before use.
6. Attach the distillation apparatus to the flask and connect the water supply for the condensed to the tap. Turn on the water so that there is a reasonable pressure.
7. Rinse out the collection tube of the apparatus 3 times with distilled/deionised water.
8. Switch on the electromantle. This should be turned up for initial heating and down again when the oil starts to appear in the collection tube.
9. The apparatus should be left for 2-3 hours until all the oil has been distilled.
10. Measure the amount of oil from the graduation.
11. Run off any collected water from the gathering tube and collect the oil sample in a labelled vial.
12. Rinse the distillation apparatus under the tap. Replace the stopper, refill the apparatus with 5% Decon 90 and leave to steep.
13. Once cool, empty the distillation flask, refill with 5% Decon 90 and leave to steep for at least 3 hours.
14. Rinse flask with deionised water ready for next distillation.

(A diagram of the distillation apparatus is shown on the following page)

(APPENDIX 2b) British Pharmacopoeia Clevenger System apparatus



8.3 APPENDIX 3

Identification of terpene composition in the experimental plant supplements by GC

These tables represent the major terpene constituents of the plant essential oils used in the feeding trials as measured by gas chromatography and identified by known reference standards. All plant material purchased as herb plants was then distilled at SAC Ayr.

Experiment 1

All plant materials with the exception of yarrow were purchased as dried plant herbs from an herbal wholesaler (Green City, Glasgow, UK). The yarrow seeds used in this feeding trial were imported from Slovakia and grown at SAC Ayr.

(a) Thyme essential oil

Main component	*% composition*
α- pinene	4.30
β- pinene	8.35
γ-terpinene	5.18
Linalol	3.98
*	1.67
Terpinene-4-ol	2.79
*	4.69
α-terpineol	29.69
*	9.93
Thymol	10.21
Carvacrol	7.32
Total number of peaks	112
Total % of oil identified	71.82

* corresponds to peaks not identified from database standards

(b) Oregano essential oil

Main component	*% composition*	
	Trace 1 (29/05/02)	Trace 2 (24/09/02)
α- pinene	1.01	1.98
*	---	1.02
β- pinene	---	1.74
α-phellandrene	---	1.18
Myrcene	---	1.42
α-terpinene	---	1.62
1,8-cineole	---	6.17
Limonene	1.78	---
*	---	4.14
*	2.85	---
*	1.42	---
*	1.5	---
*	1.34	---
γ-terpinene	19.85	17.27
p-cymene	1.34	1.28
*	1.51	2.1
*	1.1	2.24
*	1.05	1.23
*	1.14	1.24
4-terpineol	40.25	35.11
*	1.04	1.08
α-terpineol	4.89	5.36
Terpinyl acetate	7.30	---
Total number of peaks	60	99
Total % of oil identified	76.42	73.13

* corresponds to peaks not identified from terpene standards

(c) Marjoram essential oil

Main component	*% composition*	
	Trace 1 (29/05/02)	Trace 2 (24/09/02)
Myrcene	1.62	1.21
γ-terpinene	5.91	4.48
p-cymene	8.15	8.49
Linalol	1.25	1.19
*	1.61	1.52
Terpinene-4-ol	7.02	7.55
*	---	1.04
α-terpineol	1.05	1.08
Trans-cinnemaldehyde	---	5.98
Carvacrol	54.39	56.35
Total no of peaks	122	114
Total % of oil identified	79.39	86.33

* corresponds to peaks not identified from terpene standards

(d) Rosemary essential oil

Main components	*% composition*	
	Trace 1 (29/05/02)	Trace 2 (24/09/02)
α-pinene	12.38	11.04
Camphene	3.77	3.4
β-pinene	2.8	2.6
*	1.01	---
1,8-cineole	46.61	47.1
*	1.6	1.6
Camphor	16.02	17.7
*	---	1.1
*	---	1.1
Borneol	6.67	4.4
α-terpineol	---	3.1
Total number of peaks	75	96
Total % of oil identified	88.25	89.34

* corresponds to peaks not identified from terpene standards

(e) Yarrow essential oil

Main components	*% Composition*	
	Trace 1 (29/05/02)	Trace 2 (24/09/02)
α-pinene	2.5	5.1
Camphene	---	35.4
β-pinene	18.47	18.6
Sabinene	9.88	---
*	---	1.1
Myrcene	---	1.1
α-terpinene	---	5.9
Limonene	3.49	2.6
Ocimene	1.6	1.2
Linalol	16.78	10.3
*	---	1.2
Terpinene-4-ol	---	1.1
Linalyl acetate	4.82	---
Borneol	1.11	3.0
α-terpineol	6.29	---
Terpinyl acetate	1.07	---
*	1.68	---
*	2.13	1.2
Thymol	1.81	2.4
Chamazulene	6.9	2.7
Total number of peaks	125	120
Total % of oil identified	74.72	89.40

* corresponds to peaks not identified from terpene standards

Experiment 2

Gas chromatograph trace of thyme essential oil used in the second experiment (purchased from Essentially Oils Ltd., UK)

Main Component	*% Composition*
α- pinene	2.11
p-cymene	32.04
Linalol	4.62
α-terpineol	9.59
Thymol	44.08
Total number of peaks	46
Total % oil identified	92.44

Experiment 3

a) Rosemary essential oil

Main component	*% Composition*
α-pinene	2.17
Camphene	1.11
1,8-cineole	2.38
Camphor	16.5
Pinocamphone	1.5
Linalol	2.04
Linalyl acetate	2.7
Bornyl acetate	1.37
*	1.47
Borneol	21.96
γ-terpineol	1.18
Terpinyl acetate	1.1
*	1.6
*	4.94
*	2.89
Thymol	1.58
*	2.6
*	4.96
*	1.53
Total number of peaks	86
Total % oil identified	55.59

* corresponds to peaks not identified from terpene standards

At the time of analysis, only a very low yield of essential oil was obtained from this plant. Rosemary normally produces a good quantity of essential oil, at levels of 1-3%, but in this case, 131.79g of dried rosemary herb material yielded 0.03 ml oil after distillation, or 0.06 % v/w. The extremely dusty nature of the plant material suggested that the plant herb may have been dried at too high a temperature (>38°C), which may have affected the quality of the final terpene composition in the essential oil. This low volume of oil yield would suggest that the bioactive components were much diluted in the plant. However, the harsh chemotype of the oil would suggest that this oil is very antibacterial in nature, due to the presence of the high camphor, borneol and camphene fractions.

b) Thyme essential oil

Main Component	*% Composition*
α- pinene	2.11
p-cymene	32.04
Linalol	4.62
α-terpineol	9.59
Thymol	44.08
Total number of peaks	46
Total % oil identified	92.44

* corresponds to peaks not identified from terpene standards

This thyme oil was purchased from Essentially Oils Ltd., UK and was the same as that used in the 2nd experiment.

c) Yarrow essential oil

Main components	*% Composition*
α- pinene	1.15
Camphene	10.09
*	1.94
β-pinene	5.06
Limonene	4.53
1,8-cineole	1.88
Linalol	15.13
Linalyl acetate	2.67
Terpinene-4-ol	1.9
*	1.5
Borneol	4.68
*	4.03
Chamazulene	27.94
Total number of peaks	86
Total % of oil identified	75.03

* corresponds to peaks not identified from terpene standards

For this analysis, 129.38 g of ground dried yarrow herb was distilled to yield 0.235 ml essential oil (a yield of 0.2% v/w). This perennial plant was grown at SAC Ayr, having been especially chosen from Slovakia for its high content of the bioactive terpene chamazulene. It is known that only the precursors of the terpene chamazulene are present in the dried plant material, whereas chamazulene itself does appear in the GC trace of the essential oil. At the time of analysis, this plant had been growing in its environment for about 2 years, so the terpene constituents may not yet be settled in their composition within the plant and this may vary from year to year.

8.4 APPENDIX 4

Results of the assessment of growth inhibition in vitro against a range of bacterial species to measure the bioactivity of the essential oils

Table Inhibition of the growth of 8 bacterial species *in vitro* by essential oils obtained from a Turkish university using seeded Iso-sensitest agar.

	Area of inhibition (cm^2) for each bacterial species							
Plant oil	*L. monocytogenes*	*S. enteritidis*	*E. coli*	*E. faecium*	*B. subtilis*	*P. aeruginosa*	*S. aureus*	*Y. enterocolitica*
S. hortensis	3.66	2.86	6.62	1.15	*4.05	*0.40	4.47	---
O. majorana	3.08	*1.07	9.15	4.67	*3.76	*0.35	4.99	---
T. spicata	3.28	7.86	7.36	4.17	*4.78	*0.27	5.49	---
O. vulgare	3.02	*1.97	9.59	4.35	*4.82	0.43	6.76	4.74
R. officinalis	0.58	0.74	1.30	0.79	*0.46	---	*0.70	4.74
E. tenuifolia	0.30	0.55	0.67	---	0.47	0.22	0.63	---
M. spicata	1.07	0.62	1.23	---	1.21	---	1.12	---
M. communis	0.37	0.21	0.43	---	*0.11	---	*0.25	0.47
L. nobilis	---	0.76	0.85	0.22	*1.20	0.23	*0.74	1.18
F. vulgare	---	0.67	0.37	---	0.36	0.18	0.44	---
C. cyminum	1.73	0.81	*0.83	0.83	2.28	0.35	2.95	3.43
S. fruticosa	0.31	0.67	0.94	---	*2.86	---	0.37	1.33
Leaves	---	1.16	1.07	---	0.74	---	0.58	*0.83
leaves/branches	0.18	0.79	0.96	0.65	1.08	0.28	0.44	0.92
Fruit	---	0.92	*0.62	0.85	*0.38	---	*1.86	---

Calculation of the area for bacterial growth inhibition was taken after the exclusion of the paper disk used for impregnation (0.6 cm diameter). N=2 for each test. * describes the presence of a ring of partial bacterial inhibition located outside the measured ring zone of inhibition, where it is possible that the bacterial strain was only partially inhibited.

8.5 APPENDIX 5

Method for Alliin bioassay by well diffusion to determine the bioactivity of garlic

(as carried out by Interprise Ltd., Port Talbot, West Glamorgan)

This is a method used to determine the alliin activity in alliin concentrate and alliin containing products (expressed as % and compared to the Interprise bioassay standard, which is 100%).

Methodology Details

1 Prepare the bioassay plates by pouring 20 ml sterile MRS agar (DeMann, Rogosa & Sharpe, Oxoid, UK) into 90 mm plastic single vent Petri dishes. Leave to dry overnight.

2. The following day aseptically cut four 8 mm equidistant wells in the agar using the wide end of a sterile Pasteur pipette.

3. The test organism (*Candida albicans* NCYC 1470) is grown up overnight using 1 ml of NCYC 1470 culture freezer stock into 20 mls MRS broth.

4. Add 1 ml of the NCYC 1470 broth to flood the bioassay plates. Gently rotate to ensure an even coverage. Carefully draw off the excess culture using a sterile Pasteur pipette and leaves the plates to dry in a sterile air-flow cabinet.

5. Prepare solutions of Interprise freeze dried 'bioassay standard' in sterile water to give a range of alliin concentrations 10 mg ml^{-1} to 25 mg ml^{-1} in increments of 5 mg ml^{-1} as shown in the table.

6. Also prepare test sample solutions at 10, 20, 50 and 100 mg ml^{-1} concentrations as shown in the table.

STANDARD	*Concentration (mg ml^{-1})*	*Garlic or solution*	*H_20 (ml)*	*Solution code*
	25	0.25g garlic	9.75	[A]
	20	4 ml [A]	1	[B]
	15	3 ml [A]	2	N/A
	10	1 ml [B]	1	N/A
TEST GARLIC	100	1g	9	[A]
	50	1 ml [A]	1	N/A
	20	1 ml [A]	4	N/A
	10	0.5 ml [A]	4.5	N/A

7. Pipette 50µl of each concentration of the bioassay standard and test samples into the four wells of a plate and leave to dry completely.

8. Invert and incubate the plates at 37°C for 24 hours, putting them in a sealed plastic bag with a moistened piece of cotton wool in the bottom.

9. Measure the diameter of the inhibition zones and record the average.

10. Plot results and determine alliin activity of sample from standard curve, before autoclaving used bioassay plates and NCYC 1470 culture prior to disposal.

8.6 APPENDIX 6

Amino Acid analysis by HPLC

Preparation of standards and solutions for use in the analysis:

1. *Hydrochloric Acid HCl (6N)* was prepared by measuring 516 ml concentrated HCl accurately into a 1 l volumetric flask, adding 400 ml distilled water with stirring to dissipate the heat produced. When cool, the solution was diluted to volume and mixed thoroughly.
2. *Acetic Acid (25mM)* was prepared by adding 1.45 ml glacial acetic acid to 500 ml distilled water in a 1 l volumetric flask, then diluting to volume and mixing thoroughly.
3. A 250 mM solution of *di-Sodium Hydrogen Phosphate* was prepared by adding 1700 ml distilled water to a beaker containing 179.07g *di*-sodium hydrogen (ortho) phosphate, dodecahydrate. The powder was allowed to dissolve and then the solution was diluted to a volume of 2 l with distilled water in a volumetric flask.
4. *Propionic Acid* (250mM) solution was prepared by adding 1700 ml distilled water into a beaker containing a weight of 37.04g propionic acid. The contents were diluted to volume in a 2 l volumetric flask and mixed thoroughly.
5. An *Internal Standard* solution was prepared by accurately weighing around 25.78 mg DL-α-amino-n-butyric acid (Sigma Chemicals, UK) and dissolving this in about 50 ml of distilled water, before making the solution up to volume in a 100 ml volumetric flask.

Several standard solutions were also prepared for the running of the HPLC machine:

- *Mobile Phase A*

About 2000 ml of mobile phase A was required per 100 samples during HPLC. A quantity of 400 ml propionic acid and 400 ml *di*-sodium hydrogen phosphate (Solutions 3 & 4) were measured and placed in a 2 l beaker. The beaker contents were mixed thoroughly and the solution pH adjusted to 6.5 with 4.2M NaOH. At pH 6.5, further volumes of 140 ml acetonitrile, 4 ml tetrahydrofuran and 1020 ml distilled water were added. The final solution was passed through a 0.22 μm membrane filter under vacuum, and purged with helium to remove atmospheric gases.

- *Mobile Phase B*

Around 5 l of mobile phase B was prepared for every 100 samples on the HPLC machine. Volumes of 800 ml di-sodium hydrogen phosphate and 800ml propionic acid (Solutions 3 & 4) were placed in a 5 l beaker. The beaker contents were mixed thoroughly and the solution pH adjusted to 5.75 with concentrated propionic acid in the fume cupboard. At pH 5.75, 280 ml acetonitrile, 8 ml tetrahydrofuran and 2040 ml distilled water were added and the solution mixed thoroughly. The final solution was then passed through a 0.22μm membrane filter under vacuum, and purged with helium to remove atmospheric gases.

- *Mobile Phase C*

Volumes of around 240 ml methanol, 50 ml dimethylsulphoxide and 430 ml distilled water were placed into a 2 l beaker. The resulting solution was mixed thoroughly and then passed through a 0.22μm membrane filter under vacuum, before finally de-gassing by purging with helium.

8.7 APPENDIX 7

Sialic acid analysis

Solutions of the following were prepared for each day of the analysis.

1. Around 0.4559g periodic acid (VWR Ltd, UK) was weighed accurately, transferred to a 50 ml volumetric flask and made up to volume with distilled water to prepare a 0.04M solution.
2. A solution of concentrated hydrochloric acid (37%; VWR Ltd, UK) was diluted to 25%. Using a measuring cylinder, 60 ml hydrochloric acid was measured in the fume cupboard and then transferred to a 100 ml volumetric flask, before diluting it to volume with distilled water.
3. A solution of resorcinol (Fisher Certified Reagent, VWR Ltd, UK) was also prepared by accurately weighing around 25μmoles (7 mg) of $CuSO_4.5H_20$ (VWR, Ltd., UK) was weighed accurately and placed into a beaker along with 0.6g resorcinol granules. Using a measuring cylinder, 40 ml distilled water and 60 ml of 25% hydrochloric acid (Solution 3) were each dispensed, and these were also added to the beaker and mixed thoroughly.
4. A 95% tert-butyl alcohol solution (2-methyl-propan-2-ol; VWR Ltd, UK) was prepared daily in a 100 ml volumetric flask, adding 95 ml of 2-methyl-propan-2-ol and a further 5 ml distilled water to make up to volume. Due to its temperature-sensitive nature, at temperatures below 20°C the tert-butyl alcohol container was placed in warm water before use to prevent crystal formation.

8.8 APPENDIX 8

Minitab printout to show correlations between the various amino acid variables in Principal Components Analysis as a multivariate statistical tool

Correlations (Pearson)

	His	Thr	Arg	Val	Phe	Iso	Leu	Lys
Thr	0.872							
Arg	0.814	0.868						
Val	0.908	0.876	0.850					
Phe	0.896	0.931	0.907	0.935				
Iso	0.895	0.893	0.850	0.949	0.977			
Leu	0.899	0.939	0.903	0.931	0.992	0.969		
Lys	0.906	0.948	0.908	0.914	0.977	0.940	0.985	
Tyr	0.880	0.930	0.894	0.942	0.976	0.953	0.971	0.965
Ala	0.867	0.930	0.922	0.895	0.954	0.918	0.962	0.951
Gly	0.803	0.826	0.797	0.892	0.861	0.890	0.845	0.814
Glu	0.878	0.953	0.927	0.889	0.964	0.909	0.967	0.971
Ser	0.889	0.899	0.907	0.918	0.968	0.939	0.973	0.968
Asp	0.891	0.929	0.894	0.903	0.976	0.942	0.982	0.973
Tot AA	0.915	0.946	0.927	0.949	0.990	0.966	0.990	0.982
Disp AA	0.898	0.942	0.924	0.922	0.984	0.947	0.989	0.984
Indisp AA	0.921	0.947	0.920	0.963	0.986	0.974	0.984	0.974
	Tyr	Ala	Gly	Glu	Ser	Asp	Tot AA	Disp AA
Ala	0.936							
Gly	0.862	0.851						
Glu	0.955	0.961	0.827					
Ser	0.959	0.932	0.827	0.950				
Asp	0.953	0.953	0.831	0.975	0.967			
Tot AA	0.979	0.969	0.888	0.978	0.978	0.982		
Disp AA	0.973	0.970	0.849	0.986	0.984	0.991	0.994	
Indisp AA	0.976	0.961	0.911	0.964	0.963	0.968	0.996	0.982

Principal Component Analysis

Eigenanalysis of the Covariance Matrix

Eigenvalue	0.3178	0.0144	0.0048	0.0030	0.0021	0.0017
Proportion	0.915	0.041	0.014	0.009	0.006	0.005
Cumulative	0.915	0.957	0.971	0.979	0.985	0.990
Eigenvalue	0.0012	0.0008	0.0005	0.0003	0.0002	0.0002
Proportion	0.003	0.002	0.002	0.001	0.001	0.000
Cumulative	0.994	0.996	0.997	0.998	0.999	1.000

Variable	PC1	PC2	PC3
Histidine	-0.223	-0.093	0.523
Threonine	-0.295	-0.112	-0.210
Arginine	-0.167	-0.112	-0.210
Valine	-0.299	0.100	0.567
Phenylalanine	-0.193	-0.098	0.035
Isoleucine	-0.222	0.019	0.228
Leucine	-0.206	-0.142	0.028
Lysine	-0.205	-0.202	0.050
Alanine	-0.276	-0.148	-0.292
Glycine	-0.429	0.848	-0.222
Glutamic Acid	-0.232	-0.204	-0.228
Serine	-0.137	-0.096	0.054
Aspartic Acid	-0.220	-0.175	-0.047
Total AA	-0.218	-0.076	-0.001
Dispensible AA	-0.195	-0.139	-0.057
Indispensible AA	-0.238	-0.017	0.046