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**NUTRITIONAL STRATEGIES TO IMPROVE ENTERIC
HEALTH AND GROWTH PERFORMANCE OF
POULTRY IN THE POST ANTIBIOTIC ERA**

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**UNIVERSITY
of
GLASGOW**

**A thesis submitted to the College of Medical, Veterinary and Life
Sciences, University of Glasgow for the Degree of
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August 2012

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

**In the Name of Allah,
the Most Gracious, the Most Merciful**

Abstract

Four studies consisting of 6 experiments were conducted to investigate the likely mechanisms of actions of feed additives used in the place of antibiotics to improve performance and enteric health in broilers. In the first study, the relationship between digesta pH, body weight and nutrient utilisation in broilers of the same breed but with different propensity for weight gain at different ages were investigated. It was noted that birds in group H (heavy) consumed more feed ($P < 0.001$) than those in group L (light) during the starter (day 10 to 14) and grower (day 15 to 28) phases. Birds in group H had lower ($P < 0.05$) caecal pH in the starter and grower phases and lower ($P < 0.05$) proventriculus pH in grower and finisher (day 29 to 42) phases. In the grower phase, caecal pH was correlated ($r = 0.553$) with total tract retention of DM and energy utilisation at both the ileal and total tract levels, whereas during the finisher phase, crop pH was correlated with ileal nutrient utilisation, and jejunum pH was correlated with total tract energy utilisation. The data showed that differences in body weight are also reflected in differences in gut pH which is likely indicative of differences in intestinal condition between birds with heavier or lighter body weight. The differences in the gut pH explained about half of the variations in total tract nutrient and energy utilisation. Lower gut pH is advantageous for beneficial bacteria colonization but disadvantageous for pathogenic ones colonization and hence it is likely that birds with the same genetic potential may have differences in growth performance based on the type of bacteria colonizing their gut.

In the second study, the response of broiler chickens to the supplementation of benzoic acid (BA) was investigated using growth performance, nutrient and energy utilization, intestinal acidity and histomorphology of the intestine as response criteria, using 945 Ross 308 male broilers in 3 treatments with 7 replicates each for 42 days. In the grower (day 11 to 21) phase, BA supplementation at 0.53 g/kg (BA1) increased ($P < 0.001$) body weight gain and reduced ($P < 0.01$) FCR, whereas supplementation at 3.20 g/kg (BA2) reduced ($P < 0.005$) the feed intake without affecting the body weight gain, resulting in a better FCR. Compared with the control, BA supplementations increased ($P < 0.001$) the protein and energy efficiency ratios in starter and grower phases and tended ($P < 0.10$) to increase the energy efficiency ratio in finisher phase (day 22 to 42) or the overall experimental period (day 0 – 42). BA supplementations at both rates reduced the caecal pH. Supplementation of 3.20 g/kg BA stimulated the proliferation of the absorptive cells in

the jejunum, as shown in the improvement of the villus and crypt dimensions. The data from this study indicated that dietary supplementation of BA beneficially modified intestinal milieu and improved the growth performance of broiler chicks at 42 d of age.

In third study, two experiments were conducted to investigate the benefit of using BA and turmeric meal (TM) individually or in combination using growth performance, nutrient utilization, and intestinal health as response criteria. A total of 300 male one-day old broilers (Ross 308) were assigned in 5 treatments in randomized complete block design with $2 \times 2 + 1$ factorial arrangement, with 6 replicate pens and 10 birds each pen. Combination of 1 g/kg BA and 5 g/kg TM improved ($P < 0.05$) body weight gain relative to the control. Supplementation of 2 g/kg BA reduced the pH in the crop ($P < 0.001$) and jejunum ($P < 0.01$), whereas combination of BA and TM at 2 and 10 g/kg respectively reduced digesta pH in the crop ($P < 0.001$), jejunum ($P < 0.01$), and caeca ($P < 0.05$). All of the dietary treatments increased ($P < 0.005$) villus height, crypt depth and width relative to the control. All dietary treatments increased ($P < 0.001$) AME and AMEn relative to the control diet, whilst supplementation of 10 g/kg TM only increased energy digestibility ($P < 0.05$) and ileal digestible energy ($P < 0.01$). Orthogonal contrasts showed that BA and TM were additive in their effects on the growth performance, digesta pH in the proventriculus, jejunum, and ileum, and energy utilization, but associative on the energy digestibility, as well as the crop and caecal pH. None of the treatment altered the relative weight and length of the digestive tract of 21 days old broiler. These studies pointed out that BA and TM can be used in the diet individually or in combination to improve the enteric health, nutrient and energy utilization, and growth performance of broiler chickens.

In the fourth study, two experiments were designed to investigate the efficacy of TM and garlic meal (GM) using growth performance, intestinal pH, and energy and nutrient utilisation as response criteria. Three hundreds male one-day old broilers (Ross 308) were assigned in 5 treatments in randomized complete block design with $2 \times 2 + 1$ factorial arrangement, with 6 replicate pens and 10 birds each pen. Results showed that combination of GM and TM at 10 g/kg each increased ($P < 0.05$) the body weight gain, final body weight, and gain to feed ratio relative to the control and the diet with GM supplementation alone. The crop and caecal pH were reduced ($P < 0.05$) when the diets were supplemented with TM alone at 10 g/kg. The proventriculus pH also dropped ($P < 0.05$) following GM and TM mixture supplementation at 10 g/kg each relative to the control. Supplementation of 10 g/kg TM alone or in combination with GM at 5 g/kg each

increased ($P < 0.05$) the apparent ileal energy digestibility and ileal digestible energy. All of the dietary treatments increased ($P < 0.001$) both AME and AMEn compared with the control. Orthogonal contrasts showed that GM and TM were additive for feed intake, nutrient and energy utilization at both the ileal and total tract levels, but associative in their effects on body weight gain and gain to feed ratio. These studies indicated that GM and TM can be used alone or in combination to support intestinal health, improve energy and nutrient utilization, and stimulate growth performance of broiler chickens. Combination of GM and TM at the rate of 5 g/kg each was optimum for enhancing nutrient and energy utilization and promoting growth performance of broiler chickens.

Taken together, these studies showed that benzoic acid and herbal products (garlic and turmeric meal) can be supplemented in the diet alone or in combination to improve the enteric health, nutrient and energy utilization, and growth performance of broiler chickens. Improvements on the growth performance might be attributed to the reduction of the entero-pathogens in the gut, enhancement of intestinal health, alteration of the absorptive cells in the intestinal wall, and improvement in the nutrient and energy utilization.

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Publications

Part of the work reported in this thesis has been submitted for publication in peer-reviewed journals and some have been presented and published in scientific conferences.

In peer reviewed scientific journals:

1. Dono, N.D., Sparks, N.H.C., and Olukosi, O.A., 2012. Association between digesta pH, body weight, and nutrient utilization in chickens of different body weights and at different ages. *Journal of Poultry Science*. – Under Review

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2. Dono, N.D., Sparks, N.H.C., and Olukosi, O.A., 2012. Benzoic acid and turmeric (*Curcuma longa* L) meal supplementation altered the gut pH without influencing the growth performance or gross profile of the gastrointestinal tract of broilers. Presented at the Poultry Science Association Meetings, July 9 – 12, 2012, Athens, Georgia, USA. *Poultry Science*, Volume 91, Supp. 1, pp 67.

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Author's 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.

Nanung Danar Dono

August 2012

List of Abbreviations

µm	:	micrometer
AGP	:	antimicrobial growth promoter
AME	:	Apparent Metabolizable Energy
AMEn	:	nitrogen-corrected AME
BA	:	benzoic Acid
C	:	carbon
Ca	:	calcium
CD	:	crypt depth
CW	:	crypt width
DFM	:	direct-fed microbial
DM	:	dry matter
DMR	:	dry matter retention
DNA	:	deoxy ribonucleic acid
ED	:	energy digestibility
EER	:	energy efficiency ratio
EM	:	energy metabolizability
FOS	:	fructo oligosaccharide
g	:	gram
GE	:	gross energy
GI	:	gastrointestinal
GIT	:	gastrointestinal tract
GM	:	garlic meal
GOS	:	gluco oligosaccharides
GRAS	:	generally recognized as safe
H	:	heavy
HCl	:	hydro chloride
HDL	:	high-density lipoprotein
HMG-CoA	:	3-hydroxy-3-methylglutaryl coenzyme A
IDE	:	ileal digestible energy
IU	:	International Unit
Kcal	:	kilo calorie
KJ	:	kilo joule
Kg	:	kilo gram

L	:	light
LDL	:	low-density lipoprotein
MBC	:	Minimum Bactericidal Concentration
ME	:	metabolizable energy
MJ	:	mega joule
MOS	:	mannan oligosaccharides
N	:	nitrogen
ND	:	nutrient digestibility
NE	:	necrotic enteritis
NR	:	nutrient retention
NSP	:	non-starch polysaccharides
OA	:	organic acid
OM	:	organic matter
P	:	phosphorus
PER	:	protein efficiency ratio
pH	:	potential of hydrogen
pKA	:	acid dissociation constant
ppm	:	part per million
SEM	:	Standard Error of the Mean
TM	:	turmeric meal
TMT	:	total mucosal thickness
Ti	:	titanium
TiO ₂	:	titanium dioxide
TOS	:	galacto oligosaccharides
VFA	:	volatile fatty acid
VH	:	villus height
VH:CD	:	ratio of villus height to crypt depth
VW	:	villus width

Dedication

This thesis is dedicated to the memory of my late father H. Sarju Budiman, who guided me on how to be the very best that I could be. This thesis is also dedicated to my mother, wife, and daughters who light up my life.

Abu Hurairah ra. narrated that Prophet Muhammad *shallallaahu 'alaihi wa sallam* said, “*Whoever adopts the path of seeking knowledge, Allah eases for him the way to Paradise.*” (Related by Ahmad, At-Tirmidzi, Abu Dawud, Ibnu Majah, and Al-Darami).

1. LITERATURE REVIEW

Feed plays an important role in the poultry production system and represents about 70-75% of total cost of productions for meat and egg production (Martinez, 1999). Feed contains nutrients as a vital component that can be used to optimize growth and health. Through the gastrointestinal (GI) tract, feed can expose the birds to a wide variety of conditions (Yegani and Korver, 2008). The GI tract also acts as a major site of potential exposure to pathogens. Consequently, the health condition of the GI tract plays significant role in achieving optimum productivity and welfare in poultry production. The health of the GI tract affects feed digestion, nutrient absorption and metabolism, energy and protein utilization, immune response, and diseases resistance (Kelly and Conway, 2001; Yegani and Korver, 2008). Providing birds with appropriate feed is believed to have beneficial effects on their health, since inappropriate maintenance of the gut health depresses immune system and productivity (Dibner and Richards, 2004).

1.1. Gut Development and Health

The gut is a barrier between the birds and the environment, and its function is to digest feed and selectively absorb the nutrients required for health at the same time ensuring toxic substances remain out. Maintenance of gut development and health is very important to support development and health of the entire organism (Choct, 2009). Gut health itself is defined as a dynamic equilibrium of complex interaction between diet quality, microflora population, and intestinal mucosa, ensuring proper function of the digestive system and lack of pathology (Conway, 1994). Consequently, maintaining overall health requires a healthy gut that free from many diseases, such as dysbacteriosis, infections, inflammatory (Mead, 1997; Wilson *et al.*, 2005).

1.1.1. Gut Development

In poultry production, feed is closely related to the health condition of the birds in order to optimize growth performance. Chickens which are maintained with appropriate diet and management will grow properly. Currently, a newly hatched chick improves its body weight by 25% overnight and 5000% by 5 weeks, to 2 Kg. As the growth period is progressively shortened and feed efficiency continuously improved, the health care and nutrition of the bird are becoming more demanding. This makes it more essential to pay attention to the minute changes that occur in the gut, whereas the damage is often slight

and usually characterised by microscopic changes in the mucosal layer. These minute changes affect the efficiency of nutrient assimilation because underneath the mucosa is a vast surface of epithelial cells of the absorptive type essential for the transport of nutrients into the enterocytes (Choct, 2009).

Apajalahti *et al.* (2004) showed that the best performance will be achieved when the birds are in a healthy state and supported by high quality diet. An optimal quality of diets will significantly improve the development of the gut. The growth of the gut and digestive organs are affected by the growth and establishment of the populated microflora. Kraehenbuhl and Neutra (1992) described that the gut colonizes more than 640 microflora, so gut development and health may be interrupted by microflora imbalances. The condition of the gut microflora also influences the host's gastrointestinal development, biochemistry, immunology, physiology, and nonspecific resistance to infection. Microflora that colonizes the gut during the early post-hatch period forms a synergistic relationship with their host (Torok *et al.*, 2009).

The gut starts to develop when the fertilized egg is brooded and continues after the egg is hatched. Suitable condition and uninterrupted process are strictly needed to support the gut development. Intake of good quality feed is accompanied by rapid development of the GI tract and associated organs. Because initial growth is critical for intestinal development, the chicks require appropriate timing and form of nutrient available to grow. It is reported that early access to nutrients and water stimulate the activity of the GIT and digestive organs (Uni, 1998; Noy and Sklan, 2001). Development of the GIT is an important aspect of growth, especially during the first week post-hatching period (Sell *et al.*, 1991). Close to and shortly after hatch, segments of the GIT and digestive organs increase in size and weight more rapidly in relation to body weight than other organs and tissues (Noy and Sklan, 2001).

As described by Potturi *et al.* (2005), early access to *ad libitum* feed in poultry stimulated growth and development of villi and the absorptive surface in the small intestine. The number of villi per cross-section absorptive area of intestine in the duodenum and jejunum and the total segment villus surface area increased considerably following early access to quality feed (Geyra *et al.*, 2001). Bigot *et al.* (2003) conveyed that earlier feeding in the post-hatch period is not only able to stimulate the growth of GI tract, but also important to promote the growth of entire organism. Early access to feed could minimize retardation of body weight gain at later stages of life. Irrespective to the

development of GI tract, delayed access to feed resulted in delayed enterocyte proliferation and greater enterocyte apoptosis during the first week post-hatch. Greater numbers of aerobic bacteria are also present in the small intestine shortly after hatch, caused by postponement of access to feed (Potturi *et al.*, 2005).

1.1.2. Gut Health

Healthy animals are generally characterized as having a well functioning intestinal tract. This is fundamental for the efficient conversion of feed for maintenance and for growth or production. The most important characteristic of a well-functioning intestinal tract is the balance of its bacterial population. This equilibrium within the intestinal tract is upset when the animal is subjected to stressful conditions such as bacterial infections, high temperature and humidity, change of feed, and transportation (Fuller, 1977; Jin *et al.*, 1997). Any gut damage caused by pathogens will lead to poor intestinal health, which will depress efficiency in nutrient utilisation. Subclinical forms of infection with no obvious signs of lesions – such as in Necrotic enteritis (NE) – are often financially more destructive than acute, short-term infections. Therefore, the health of the gut may affect the way nutrients are partitioned, mobilized and utilised for organ development, tissue growth and immune system maturation (Kelly and Conway, 2001).

Establishment of the microbial community in the gut begins in the early stage of the post-hatch period. At the moment of hatching, the environment of the GI tract is in a sterile condition and free from exogenous pathogens (Lan *et al.*, 2005; Richards *et al.*, 2005). When the chick starts to consume feed, the composition of the microflora starts to change. One day after hatch, bacterial densities in the GI tract in the broiler chickens attain 10^8 per gram of ileal digesta and 10^{10} cells per gram of cecal digesta. During the first 3 days post-hatch, the numbers of microbes reach 10^9 per gram of ileal digesta and 10^{11} per gram of cecal digesta and remain relatively stable for the following 30 days. Therefore, maintaining gut health is essential for the birds. Provision of intestinal microflora of healthy adult birds to newly hatched chicks resulted in protection against intestinal infections including different types of *Salmonella* and also had positive impact on growth rate (Goren *et al.*, 1984).

In general, intestinal bacteria may be divided into species that exert potentially harmful (pathogenic) or beneficial on the host. Pathogenic effects include diarrhoea, localized or systemic infections, liver damage, carcinogenesis, intestinal putrefaction, and

toxin formation. Beneficial effects may be caused by the inhibition of growth and establishment of harmful bacteria, stimulation of immune system, lowering of gas distension problems, improved digestion and absorption of essential nutrients, and synthesis of vitamins (Gibson and Roberfroid, 1995; Jeurissen *et al.*, 2002). Microbial population in the gut is dependent on the breed, age of the chickens, dietary factors, and geographic location (Apajalahti *et al.*, 2001; Knarreborg *et al.*, 2002).

1.1.3. Factors Affecting Gut Development and Health

Feed ingested by a bird can contain nutrients, non-nutrients, and beneficial and potentially harmful organisms. In this way, the gut is become a major site of potential exposure to pathogens. Normally, the lumen of the gut contains feed and its constituents, endogenous nutrients, resident and transitory microflora, and also secretions from the gut and its accessory organs such as the liver, gall bladder, and pancreas. The digestive tract should selectively consent to the absorption of the digested nutrients across the intestinal wall into the body and evade the harmful components of the diet from crossing the intestinal barrier. Any deleterious component that is ingested together with or attached in the feed might depress the development and health of the gastro-intestinal tract (Mead, 1997; Dibner and Richards, 2004; Korver, 2006).

The development and health state of the enteric health of poultry might be interrupted by multi-causal factors, i.e. physical, chemical, and biological factors (Dekich, 1998). Conway (1994) described that gut development and health are affected by complex interaction between the diet, the microbial colonization, and the state of intestinal mucosa. Type, structure, quantity, and quality of the diets that the bird consumes have significant effects on the growth and proliferation of intestinal cells. The development of the intestinal cells and nutrient utilisation are intricately related. Hydrolysis of macromolecules in the small intestine is achieved, to a large extent, by pancreatic enzyme activities, which are corresponded with body weight and intestinal weight (Sklan and Noy, 2000). Yegani and Korver (2008) described that development and health state of the gut are effected by ingested diets and incursion of infectious agents. Non-starch Polysaccharide (NSP), physical texture and form of feed probably become the most important factors of diets that affect gut development and health. On the other hand, infectious agents, such as bacterial infections, parasites, viruses and toxins obviously depress the development and health of the gut.

1.1.3.1. Diets

Poultry diet is composed of feedstuffs which currently dominated by cereal grains. All cereals used in the diets contain various levels of NSP, such as β -glucans and arabinoxylans (Pettersson and Åman, 1989; Choct and Annison, 1992a). NSP resists to the digestive enzymes and affects viscosity in the environment within the lumen of the gut. High viscosity of the intestinal contents due to the presence of high NSP in the feed has been reported to cause digestive problems and reduce health status (Choct and Annison, 1992b). Waldenstedt *et al.* (2000) reported that NSP reduce the rate of digesta passage and availability of nutrients in the gut, while extension of digesta retention time facilitates bacterial colonization and activity. Wheat, barley, rye, and oats contain NSP in high levels which are known to lead to increased digesta viscosity, decreased digesta passage rate, digestive enzymatic activities and nutrient digestibility, depressed feed efficiency, and inhibit the growth of the birds (Alimrall *et al.*, 1995; Choct *et al.*, 1996; Jørgensen *et al.*, 1996; Bedford and Schulze, 1998).

Physical texture and form of feed may also affect intestinal development and health. Physical stimulation by hard and solid material of feed, such as fibre, is required by intestinal cell wall to develop (Dibner and Richards, 2004). Engberg *et al.* (2004) showed that the physical form of cereal grain diets may affect the morphological and physiological characteristics of the gut. Feeding birds with coarsely ground feed results in lower mortality rate than those of with finely ground feed. Similarly, Branton *et al.* (1987) showed that mortality rate associated with NE of broiler chicken fed coarse ground diets was lower than that of fed finely ground diets. Other studies have shown that dietary whole wheat may contribute to the gut development, stimulate absorption of digested nutrients (Hetland *et al.*, 2002; Taylor and Jones, 2004), reduce the number of exogenous pathogens (Engberg *et al.*, 2004; Bjerrum *et al.*, 2005), and improve feed efficiency (Plavnik *et al.*, 2002). In a more specific study, Gabriel *et al.* (2003) observed that broiler fed whole wheat diets have lower weight gain than those of fed ground wheat diets. In addition, some studies have shown that the health of the intestine was not only related to the physical development as a result of stimulation by feed ingredients and solid particles, but was also determined by the organisms harboured in the gut (Gibson and Roberfroid, 1995; Apajalahti *et al.*, 2004; Jia *et al.*, 2009b).

1.1.3.2. Infectious Agent

The presence of complex infectious agents, such as bacterial infections, parasites, viruses, and other infectious agents can disturb the enteric health of poultry (Reynolds, 2003). Severe enteric damages by bacterial infections will result in visual disease and a high mortality rate (Porter Jr, 1998). Lesions caused by NE might be is the most severe of any disease that occurs in the chicken intestine (Long *et al.*, 1974; Kaldhusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 2001).

Among parasitic disorders, probably histomoniasis or Blackhead, caused by *Histomonas meleagridis*, is the worst problem caused by intestinal protozoa. Mortality rate caused by this inflammatory disease is quite high in turkeys, sometimes approaching infection of 100% of a flock. In chickens, the mortality rate may be 10 to 20% with high morbidity. Lesions of histomoniasis were more severe in turkeys while *C. perfringens* was more specific in chickens (McDougald, 1998; McDougald, 2003; McDougald, 2005).

Development and health state of digestive tract might be also decreased by viral infections. Viral infections of the gut occur in all age of chickens and turkeys, but tend to predominate in young birds. Clinically, these infections result in a wide range of outcomes from unclear, economically insignificant effects to those that are severe and economically devastating (Guy, 1998; Reynolds, 2003). Several types of viruses have been identified as causes of gastrointestinal tract infections in poultry. These include avian rotaviruses (Yason and Schat, 1986; Theil *et al.*, 1986; Yason *et al.*, 1987), coronaviruses (Wege *et al.*, 1982), enteroviruses (Swayne *et al.*, 1990; Guy and Barnes, 1991), adenoviruses (Wigand *et al.*, 1982), astroviruses (Reynolds and Saif, 1986; Reynolds *et al.*, 1987), and reoviruses (Joklik, 1981; Wickramasinghe *et al.*, 1993). Typical impacts of these gastrointestinal infections on poultry are reduced weight gain, depressed feed efficiency, decreased flock uniformity (Guy, 1998), depressed immune status of affected birds (Guy, 1998) and may exacerbate other diseases (Sponenberg *et al.*, 1985; van den Hurk *et al.*, 1994).

In poultry, feedborne toxins might also cause enteric disease. Probably, mycotoxins and biogenic amines are among the most common types of feedborne toxins in poultry (Dekich, 1998). The impacts of mycotoxins have been recognized as a widespread cause of economic losses due to reduction in performance and depression in health status. Histopathology of the gut lesions associated with the acute intoxication is characterized by haemorrhage, necrosis, and inflammation, which occur with the shortening of villi and

reduction in the proliferation activity in crypt of the intestinal epithelium (Sklan *et al.*, 2003). Hoerr *et al.* (1981) reported that necrosis sometimes also occurs in the mucosa of the proventriculus and gizzard. Clinical cases of NE in poultry are mostly attributed to extreme growth of *Clostridium perfringens* in the intestine. Naturally, *C. perfringens* are found in low numbers in the intestinal tract of healthy birds and considered to be part of the normal gut flora. However, excessive growth of these bacteria in the gut can lead to toxin production, which in turn, can result in gut lesions and restriction of the growth and health of intestinal cells (Kaldhusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 2001; Craven *et al.*, 2001). Wise and Siragusa (2005) underlined that severe case of clinical NE can leads to an increase in mortality rate of the birds. In addition, intestinal mucosal damage by coccidiosis in chickens is usually considered as one of the most important predisposing factors because coccidiosis is often seen to occur just before or concurrent with outbreaks of NE in the field (Truscott and Al-Sheikhly, 1977; Baba *et al.*, 1997).

1.1.4. Problems Associated with the Gut Development and Health

There are several problems associated with the health of the intestine which results in growth depression. These problems might be summarized as higher colonization of pathogenic bacteria, higher incidences of sub-clinical (bacterial) infections and dysbacteriosis, lower absorptive cell proliferation, and lower nutrients uptake and utilization (Sklan *et al.*, 2003; Van Immerseel *et al.*, 2006; Gholamiandehkordi *et al.*, 2009; Sarson *et al.*, 2009).

In young chicks, normal proliferation of cells in the GI tract might be inhibited by higher colonization of exogenous pathogenic microflora. Evidence revealed that there was a strong relationship between certain feed ingredients and the incidence of NE. Some studies have shown that outbreak of NE can be exacerbated by high levels of wheat (Riddell and Kong, 1992; Kaldhusdal and Skjerve, 1996; Branton *et al.*, 1997; Annett *et al.*, 2002), barley (Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996; Annett *et al.*, 2002), rye (Riddell and Kong, 1992) or fishmeal (Truscott and Al-Sheikhly, 1977) inclusion in the diets.

It has been reported that development of the GI tract might be obstructed by the presence of high levels of fishmeal, particular amino acids, and NSP. High level of dietary fishmeal and methionine in the diets were reported to stimulate overgrowth of *C. perfringens* and induce the clinical NE in the lower small intestine (Drew *et al.*, 2004). The

presence of high levels of NSP in the diets was also reported to increase viscosity of digesta and decrease digesta passage rate. Extension in the digesta retention time stimulates bacterial growth and colonization in the small intestine. The increased in clostridial proliferation due to these dietary factors might result in the higher incidence of NE that results in the inhibition of the mitotic proliferation and growth of absorptive cells in the small intestine (Waldenstedt *et al.*, 2000; Annett *et al.*, 2002). Research showed that inhibition in the growth of intestinal absorptive cells resulted in the reduction nutrient uptake and utilization (Sklan *et al.*, 2003).

In addition, incidence of NE or clostridial infection, caused by *Clostridium perfringens* types A and C, have been shown to generate serious problems related to animal welfare and precipitated economic losses (McDonel, 1986). Outbreaks of NE worldwide are intermittent but sporadic, and result in high mortality, impaired feed conversion, reduced growth performance, increased product refusal rates and severe economic loss (Ficken and Wages, 1997). It has been calculated that total global economic loss of the poultry industry per year due to NE incidence (either clinical or sub-clinical) can be estimated 0.05 US dollars per bird (Van der Sluis, 2000b). Furthermore, economic impact of undiagnosed bacterial enteritis and coccidiosis can cost up to 0.09 US dollars per bird due to loss of gut integrity (Van der Sluis, 2000a).

It has been hypothesized in some studies that pathogenic microflora in the gut decrease nutrient absorption by increasing intestinal thickness, digesta passage time, increase nutrient requirements of the host by increasing turnover of the gut mucosa and also by competing for a portion of the dietary energy and protein with the host (Ravindran *et al.*, 1984; Dibner and Buttin, 2002; Apajalahti *et al.*, 2004). These enteric problems associated with the presence of pathogenic microflora can be prevented and/or cured with the addition of antibiotics. Evidences have shown that inclusion of antibiotics in the diets inhibits the growth of exogenous pathogens which results in the improvement of feed efficiency, growth performance and health status of the birds (Coates *et al.*, 1955; Stutz and Lawton, 1984; Uni *et al.*, 1998; Engberg *et al.*, 2000; Miles *et al.*, 2006). Thomke and Elwinger (1998) described that all antibiotics control growth and proliferation of microflora in the GI tract, but all antibiotics do not accomplish this control by the same mechanism. Antibiotics differ according to their ability to influence certain disease states or improve growth and feed efficiency.

1.2. Antibiotics as Growth Promoter

Antibiotics are chemical substances derived initially from certain fungi, bacteria, and other organisms that can inhibit the growth of, and even destroy, harmful microorganisms (Davey, 2000). With the advances in medicinal chemistry, antibiotics can be produced synthetically. According to their origin-based classification, antibiotics can be categorized into natural, semi-synthetic, and synthetic (Rinderknecht *et al.*, 1947; Edwards *et al.*, 1975; Herrlich and Schweiger, 1976; Edwards *et al.*, 1976). According to their effect on microorganisms, antibiotics might be classified into bacteriocidal, which kill bacteria, and bacteriostatic, which only inhibit bacterial growth (Hinton, 1988; Norcia *et al.*, 1999).

As antimicrobial growth promoters (AGP), antibiotics have been practiced in poultry feed for about 60 years in the United States and other countries. Early findings of beneficial effects of AGP were reported in poultry diets by Moore *et al.* (1946) and in swine diets by Jukes *et al.* (1950). Initial report was also showed by Starr and Reynolds (1951) after feeding trial of streptomycin in turkeys. Other preliminary reports were made by Barnes (1958) and Elliott and Barnes (1959) that showed the use of tetracycline as AGP in poultry diets. As from that time, antibiotics have been used consistently in animal diets. In the United States, the use of antibiotic as feed additives was approved in 1951 by the United States Food and Drug Administration, but without any veterinary prescription. European states followed to authorize antibiotics in the 1950s and 1960s (Jones and Ricke, 2003).

Antibiotics have been added mainly during the grow-out period to protect poultry from pathogenic organisms, maintain health, promote growth, facilitate better feed efficiency, and improve meat quality. For instance, two of the more popular broad spectrum antibiotics utilized within the poultry industry, i.e. virginiamycin and bambarmycins, have been reported to improve the growth and performance of broilers and turkeys (Johnston *et al.*, 1983; Miles *et al.*, 1984; Waldroup *et al.*, 1985; Salmon and Stevens, 1990). Subtherapeutic levels of avoparcin, bacitracin methylenedisalisyllic acid, efrotomycin, lincomycin, penicillin G procaine, and virginiamycin in the diets have been also reported to improve rate of weight gain and feed efficiency of male broiler chickens (Feighner and Dashkevich, 1987). In a more recent study, Miles *et al.* (2006) showed that addition of virginiamycin to a corn-soybean meal diet stimulated improvement in total body weight and the number of absorptive cells per unit length in the intestine of male and

female broiler chickens. This physical improvement facilitates better nutrient absorption, thus promotes growth performance stimulation.

Moreover, it has been reported that virginiamycin controlled microbial growth within the lumen of the gastrointestinal tract by disrupting bacterial protein synthesis (Parfait *et al.*, 1978). In other studies, it has been reported by Engberg *et al.* (2000) that zinc bacitracin significantly reduced the number of coliform bacteria in the ileum and increased the activities of amylase and lipase in pancreas homogenates. Supplementation with salinomycin and zinc bacitracin, alone or in combination, resulted in significant reduction of the growth of *C. perfringens* and *Lactobacillus salivarius* in the gut of broiler chickens. High numbers of these lactobacilli may depress the growth performance of broiler chickens related to competition in nutrient uptake or impaired fat absorption due to bile acid deconjugation.

Numerous studies have reported that growth enhancement properties of antibiotics are closely related to interactions with the microbes in the gut. AGP can help control disease by selectively modifying and improving the gut microflora, reducing bacterial fermentation and preventing infectious diseases, and results in health status improvement. All these changes lead to an increase in nutrient availability for the animal, allowing enhanced feed efficiency and being able to achieve better growth performance (Dibner and Buttin, 2002; Hernández *et al.*, 2006). Moreover, Donoghue (2003) showed that the use of antibiotic in poultry diets gives significant economical advantages as it facilitates better production efficiency, thus allowing consumer to purchase high quality poultry products at lower price.

1.2.1. Main Mechanisms of Action

Antibiotics were needed because enteropathogenic microbes disturb the balance of microflora populations, compete for nutrients available in the gut, and thus inhibit animal growth. The use of antibiotics in the feed is intended to inhibit the growth of exogenous microflora, hence promotes growth performance of the birds. The growth-promoting effects of antibiotic are closely related to their inhibitory effect on pathogenic microbes in the GI tract. Reduction of the population of intestinal harmful microflora have beneficial effects, including a reduction in the incidence of sub-clinical diseases (thus results in reducing nutritional costs of the immune mechanisms), reduction in the quantity of growth-depressing metabolites produced by intestinal microbes, a reduction in the competition

between microbes and hosts for available nutrients, and improve nutrient uptake by absorptive cells of the gut (Niewold, 2007).

Some studies showed that antibiotics control growth and proliferation of exogenous pathogens in many ways (Engberg *et al.*, 2000; Ferket, 2004). The mechanism vary regarding to their ability to affect certain disease states of animals. For instance, virginiamycin controls microbial growth within the lumen of the gastrointestinal tract by disrupting bacterial protein synthesis (Parfait *et al.*, 1978), whereas the mode of action of moenomycin inhibits bacterial cell wall synthesis (Huber and Neemann, 1968). More over, effectiveness of antibiotics treatments were dependent on the condition of management. In many countries where poultry are raised in intensive systems with thousands of birds living under confinement on single premises, antibiotics were needed in very small amount. But, in other countries, where production is less intensive, the incidences and spectrum of infectious diseases are larger, consequently antibiotics are needed in larger amounts to treat diseases (Castanon, 2007).

Besides controlling proliferation and growth of pathogenic microflora, the addition of antibiotics in the diets was also intended to reduce quantity of microbial metabolites that depress growth and to reduce competition for available nutrients between microbes and the host (Thomke and Elwinger, 1998). Some bacterial metabolites, e.g. ammonia (Visek, 1978; Anderson *et al.*, 1999), amines (Anderson *et al.*, 1999; Gaskins *et al.*, 2002; Gaskins *et al.*, 2002), and lipopolysaccharides (Roura *et al.*, 1992) have been reported as toxic substances that can reduce growth efficiency of the host. Consequently, inhibition of the growth of opportunistic pathogens and reduction of toxic bacterial metabolites due to antibiotic supplementation stimulate digestive efficiency and promote growth acceleration of the birds.

Moreover, the mode of action of antibiotics to promote better growth rate has also been attributed to the stimulation in absorptive cells growth and improvement in nutrient absorption in the gut (Anderson *et al.*, 1999). In other studies, it has been reported that treating the diets with antibiotics stimulates proliferation of absorptive cells in the intestine. Greater villus height and crypt depth in the duodenum, jejunum, and ileum were observed following antibiotics medication (Iji *et al.*, 2001; Xia *et al.*, 2004). Miles *et al.* (2006) stated that improvement in intestinal morphology stimulates better nutrient absorption, resulting in more sparing energy for tissue maintenance that can be used instead for growth, or improving the absorption of various nutrients.

1.2.2. The Ban of Antibiotics

In order to limit the spread and development of antibiotic resistant microflora, the authorisation of several antibiotics (avoparcin, zinc-bacitracin, spiramycin, tylosin and virginiamycin) and growth promoters (carbadox and olaquinox) as feed additives has been withdrawn in European Union since 1997 (Dibner and Richards, 2005). The first nation to eliminate the use of AGPs in feedstuffs was Sweden in 1986 (Aarestrup, 2003), followed by Denmark (avoparcin) on May 20, 1995, Germany (avoparcin) on January 19, 1996, Finland (spiramycin) on January 1, 1998 and Denmark (virginiamycin) on January 15, 1998. The authorisation of these antibiotics as feed additives has been withdrawn because the contribution to bacterial resistance is of a significantly larger extent than from the benefit of these antibiotics for veterinary therapy (World Health Organization, 2003).

An increase in the resistance pool at the animal level might create risks to humans. In view of their possible adverse effects on human health, the authorisation for those 5 antibiotics and those 2 growth promoters has been withdrawn. Avoparcin was banned because this glycopeptides class of antimicrobial produces microflora which is resistance to glycopeptides used in human medicine (Aarestrup, 1995). Bates *et al.* (1994) reported that in 1993 glycopeptide-resistant enterococci (GRE) were found present in feed animals. It was surprisingly and unexpected, as these antimicrobials were not allowed to be used in the feed as a part of disease remedy (Aarestrup *et al.*, 2001). Thus, avoparcin was banned in order to avoid the possibility of an occurrence of an animal reservoir of GRE (vancomycin resistant enterococci or VRE), which potentially posed risks to public health (Aarestrup, 2003; World Health Organization, 2003).

Spiramycin, virginiamycin, tylosin, and zinc-bacitracin were banned because they belonged to classes of antimicrobials that are used in human drugs. Virginiamycin was banned because this streptogramins class antimicrobial was clinically important in human medicine, whereas zinc-bacitracin was important to treat skin infection. On the other hand, carbadox and olaquinox were considered unacceptable due to their toxicity risks. The authorisation of these antimicrobials in the feed has been withdrawn to reduce potency of creating microflora that resistant to the drugs, which was also exhibited a potential hazard to public health (World Health Organization, 2003).

The removal of AGPs authorisation resulted in substantial increase in infection in poultry (Knarreborg *et al.*, 2002; Casewell *et al.*, 2003). As a consequence, the poultry

industry has needed to find alternatives to AGP in order to stem the spike in infection rates. These alternatives are required to be environmental friendly and safe for both animal and humans who consume animal products (Cabuk *et al.*, 2006).

1.2.3. Alternatives to Antibiotics

Various types of feed additives have been evaluated under commercial conditions and in experimental trials with the objective to achieve improvements on growth performance and the best economic return (Bozkurt *et al.*, 2009a). Some of the alternatives that have been studied include herbs, spices and various plant extracts/essential oils, probiotics or direct-fed microbials (An *et al.*, 2008), prebiotics (Dahiya *et al.*, 2006), synbiotics (Ghasemi *et al.*, 2010), organic acids, and dietary enzymes (Cowieson *et al.*, 2006; Dahiya *et al.*, 2006). Combinations of two or more of these additives have been used in various trials in order to maximize the benefits from using them (Hofacre *et al.*, 2003; Choi *et al.*, 2010).

Herbs, spices and various plant extracts/essential oils can be use as alternatives to replace AGP as they rich of phytochemicals (active compounds) that can be used to stimulate growth and health of the animals. *In vitro* studies showed that active compounds in herbs and spices have beneficial effects, such as antimicrobial, antifungal, antihelminthic, and anticoccidial properties (Tabak *et al.*, 1999; Araújo and Leon, 2001; Tzakou *et al.*, 2001; Lee *et al.*, 2003b; Brr and Mahmoud, 2005; Fernandes *et al.*, 2005; Mekala *et al.*, 2006). Animal studies showed that active compounds in these green additives stimulated growth, improved feed efficiency, enhanced nutrient digestibility, lowered mortality, increased immunity, increased liveability, reduced cholesterol level, increased carcass yield and improved meat quality of poultry (Tabak *et al.*, 1999; Lewis *et al.*, 2003; Lewis *et al.*, 2004; Alcicek *et al.*, 2004; Hernández *et al.*, 2004; Cross *et al.*, 2007; Onimisi *et al.*, 2007; Rizzo *et al.*, 2008; Windisch *et al.*, 2008; Rahmatnejad *et al.*, 2009). More over, active biological compounds in garlic, ginger, turmeric, oregano, and cinnamon in various type and amount reduced cholesterol levels in the blood and meat of meat and egg-type chickens (Konjufca *et al.*, 1997; Bampidis *et al.*, 2005; Kim *et al.*, 2005; Lijuan *et al.*, 2007).

Probiotics or direct-fed microbials have been reported to have properties to replace AGP in feed. In available studies, it has been reported that probiotics improved feed intake, stimulated digestive enzymes, promoted gut performance, reduced population of feed-borne pathogens, reduced bacterial metabolites, improved intestinal balance of microflora,

improved gut health, and promoted body immune system (Zacconi *et al.*, 1995; Mead, 2000; Chen and Stern, 2001; Simmering and Blaut, 2001; Schneitz, 2005; Johny *et al.*, 2008; Huff *et al.*, 2008; Chemaly *et al.*, 2008; Awad *et al.*, 2009; Nakazato *et al.*, 2009; Ozaki and Murase, 2009; Feng *et al.*, 2010).

Prebiotics were proposed to replace AGP as they had significant effects on growth promotion and health maintenance in poultry. Several studies have shown that prebiotics have significant effect to lower mortality caused by necrotic enteritis, improved body weight and feed consumption, stimulated better feed efficiency, improved apparent metabolizable energy (AME), and reduced the coliform bacteria in the gut (Acamovic and Brooker, 2005; Dahiya *et al.*, 2006). Spring *et al.* (2000) reported that some oligosaccharides stimulate the immune system by blocking pathogens binding to oligosaccharides receptors on the mucosal surface. Additionally, Patterson and Burkholder (2003) suggested that combinations of prebiotics and probiotics are known as synbiotics.

Organic acids can be used as alternatives to replace AGP as they promoted overall growth performance and health status of poultry. As weak acid, several organic acids significantly controlled microbial growth in the gut, lowered nutrients competition, reduced microbial metabolites, decreased sub-clinical infections, stimulated the growth of intestinal absorptive cells (villus and crypt), and stimulated secretion of digestive organs of broiler chickens (Vogt *et al.*, 1982; Patten and Waldroup, 1988; Roy *et al.*, 2002; Dibner and Richards, 2004; Pelicano *et al.*, 2005; Buhler *et al.*, 2006; Kluge *et al.*, 2006; Józefiak *et al.*, 2007; Viola and Vieira, 2007; Paul *et al.*, 2007; Chotikatum *et al.*, 2009; Chowdhury *et al.*, 2009; Józefiak *et al.*, 2010).

Dietary enzymes have also been reported to have properties to replace AGP in poultry feed. It was reported that enzyme supplementation regulated nutrient supply, promoted growth performance, improved feed efficiency, improved nutrient digestibility and retention, developed digestive and capacities of the intestine, improved carcass yield, and improved meat quality (Esteve-Garcia *et al.*, 1997; Ravindran *et al.*, 1999; Zyla *et al.*, 2000; Angel *et al.*, 2006; Olukosi and Adeola, 2008; Choct, 2009). Other studies showed that the used of dietary enzymes in the diets improved Apparent Metabolisable Energy (AME) and AMEn, and digestibility of dry matter, starch, beta-glucans, and lipid (Wu *et al.*, 2004b; Juanpere *et al.*, 2005).

1.3. Feed Additives that Support Enteric Health

There is information in available studies on cross-talk between exogenous pathogens and enteric health of poultry (Fuller, 1977; Jin *et al.*, 1997). It has been shown that pathogens depressed microflora balance in the intestine. The concept of a balanced intestinal microflora improving resistance to infection and reduction in resistance when the intestinal microflora is disrupted is necessary in understanding the microbe-host interrelationship. The growth and population of exogenous pathogens must be inhibited to ensure intestinal balance of microflora. Patterson and Burkholder (2003) suggested that inhibition of pathogens by intestinal microflora has been called bacterial antagonism, bacterial interference, barrier effect, colonization resistance, and / or competitive exclusion.

Mechanisms of pathogen inhibition by the intestinal microflora include competition for nutrients (substrates), production of toxic conditions and compounds (volatile fatty acids, organic acids, low pH, and bacteriocins), competition for attachment sites on the intestinal epithelium, and stimulation of the immune system (Fuller, 1989; Rolfe, 2000; Gibson and Fuller, 2000). Consumption of fermented feed has been linked with health improvement. Lactic acid bacteria (Lactobacilli and Bifidobacteria) have been identified as causative agents for this health improvement. Lactic acid bacteria and certain other microflora have been reported to improve disease resistance (Patterson and Burkholder, 2003).

1.3.1. Direct Fed Microbials

In 1973, Nurmi and Rantala (1992) introduced direct-fed microbial (DFM) or probiotics as a means of maintaining gut health, by controlling endemic and zoonotic agents in poultry (La Ragione and Woodward, 2003). In many studies, the live microbials are known as probiotics (Morishita *et al.*, 1997). Initially, probiotic, which means ‘for life’ in Greek, has been defined by Parker (1974) as “Organisms and substances which contribute to intestinal microbial balance”. This definition was subsequently refined as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance of microflora” (Fuller, 1989). Probiotics must be of host origin, withstand of processing and storage, resist of gastric acid and bile, persist in the intestinal tract and adhere to epithelium or mucus (Simmering and Blaut, 2001). Probiotics aimed to produce a beneficial effect on the host by administration of viable organisms (Gibson and Fuller, 2000). For a feed supplement to be classified as probiotic, it must produce inhibitory

substances, alter microbial activities, and modulate immune response (Jin *et al.*, 1997). The direct-fed microbial concept was originally designed to restore microflora population in the gut by stimulating activities of bacteria which have beneficial effects on the host and depressing activities of those bacteria that have adverse effects on the host (Simmering and Blaut, 2001; Schneitz, 2005).

The main proposed mode of actions on DFMs are attributed to maintaining beneficial microflora in the alimentary tract and alteration of bacterial metabolisms. Direct-fed microbials maintain the population of normal microflora by antagonistic activity and competitive exclusion. Alteration of bacterial metabolisms of DFMs is attributed to the reduction of bacterial activity and ammonia production (Fuller, 1977; Jin *et al.*, 1997). *In vitro* studies have demonstrated that DFMs are able to inhibit the growth of poultry pathogens, such as *Salmonella enteritidis* (Nurmi *et al.*, 1992; Johny *et al.*, 2008; Sillankorva *et al.*, 2010), *S. typhimurium* (Nurmi *et al.*, 1992), *S. kedougou* (Zacconi *et al.*, 1995), *Escherichia coli* (Nakazato *et al.*, 2009; Ozaki and Murase, 2009), *Clostridium perfringens* (Timbermont *et al.*, 2009; Feng *et al.*, 2010), *Clostridium botulinum* (Mead, 2000), *Listeria monocytogenes* (Chemaly *et al.*, 2008; Huff *et al.*, 2008), *Campylobacter jejuni* (Aho *et al.*, 1992; Nurmi *et al.*, 1992; Chen and Stern, 2001; Schneitz, 2005), and *Yersinia enterocolitica* (Mead, 2000).

The antagonistic activity of DFMs towards pathogens can be attributed to the production of bactericidal substances, including bacteriocins and organic acids. Bacteriocins are active proteinaceous substances produced by a variety of Gram-negative and Gram-positive bacteria that have a bactericidal action (Tagg *et al.*, 1976). The bactericidal properties of DFMs are species specific and have been considered as antibiotics with a narrow bacterial-host range of activity. They exert their lethal activity through adsorption to specific receptors located on the external surface of sensitive exogenous bacteria, followed by metabolic, biological and morphological alterations resulting in the killing of bacteria (Daw and Falkiner, 1996; Jin *et al.*, 1997). Antagonism by organic acids produced by DFMs has also been associated with major end products of their metabolism. Bactericidal action of organic acid is associated with pH reduction in the intestinal that results in an inhibition of the growth of exogenous pathogens (Eklund, 1985; Adams and Hall, 1988).

Direct-fed microbials maintain intestinal health by competitive exclusion to prepare population of live obligate and facultative anaerobic bacteria (Schneitz *et al.*, 1989),

originating from normal, healthy adult individuals of an avian species, which is free from specific pathogenic microorganisms (Nurmi *et al.*, 1992). This treatment is intended to reduce the populations of feed-borne pathogens in the alimentary tract (Daw and Falkiner, 1996). Microflora that used in the DFMs can be classified as undefined preparations of cultures microflora and defined specific microflora. Undefined DFM was expected to prepare normal gut microflora with various viable unspecific cultures which derived directly from chicken intestines (Cameron and Carter, 1992; Spencer *et al.*, 1998). Defined DFM products were intended to prepare the intestinal environment with a single or small number of specific bacterial strains, such as *Bacillus subtilis* (La Ragione and Woodward, 2003), *Lactobacillus acidophilus*, *Streptococcus faecium* (Morishita *et al.*, 1997). Many strains of microflora have been used commercially in poultry diets, including *Lactobacillus* spp., *Bacillus* spp., *Enterococcus* spp., and *Saccharomyces* spp. (Simon *et al.*, 2001; Lan *et al.*, 2003; Patterson and Burkholder, 2003). Undefined complex cultures were more effective than defined DFM treatments under laboratory conditions (Stavric, 1992; Stavric and D'aoust, 1993).

Alteration of bacterial metabolism by DFMs is also attributed to the production of digestive enzymes, reduction in bacterial enzymes, and the suppression of ammonia production (Jin *et al.*, 1997). *Lactobacillus* spp. has been shown to produce digestive enzymes that may enrich the concentration of digestive enzyme in the intestine. In different experiments, Moon and Kim (1989) and Magboul *et al.* (1997) reported that *Lactobacillus* spp. was found to have amylolytic, proteolytic and lipolytic activities. In addition, Jin *et al.* (1997) showed that all 12 *Lactobacillus* spp. isolated from chicken intestine were found to secrete amylase, protease and lipase, either extracellularly or intracellularly, or both extracellularly and intracellularly. Improvements in digestive enzymes in the intestine are important to increase the digestion of nutrients (Philips and Fuller, 1983; Sissons, 1989). On the other hand, Chiang and Hsieh (1995) reported that DFMs (containing *L. acidophilus*, *B. subtilis* and *S. faecium*) suppressed ammonia concentration in the excreta and litter of broilers.

Another proposed mode of actions of DFMs is closely related to bacterial enterotoxin neutralization and immune system stimulation. Enterotoxin produced by exogenous pathogen may be neutralized by a substance produced by a probiotic. As shown by Mitchell and Kenworthy (1976), *L. bulgaricus* produces a metabolite that has a neutralizing effect on enterotoxin released from coliforms. In addition, DFMs also

stimulate the immune system by elevating immunity response. In a study, Pollmann *et al.* (1980) reported that oral inoculation of germ-free animals with *L. acidophilus* led to elevated levels of total serum protein, globulin rather than albumin, and increased white blood cell counts.

Many studies have demonstrated that DFMs showed beneficial effects on lowering nutritional competition in the gut for nutrients and mucosal binding sites, producing substances that kill or inhibit the growth of pathogenic bacteria (Schneitz, 2005), maintaining normal gut microflora (Barnes *et al.*, 1980; Walker and Duffy, 1998; Mead, 2000), improving growth performance and intestinal health (Chichlowski *et al.*, 2007; Awad *et al.*, 2009), improving feed intake, feed efficiency and digestion (Jin *et al.*, 1998; Cavazzoni *et al.*, 1998; Zulkifli *et al.*, 2000; Kabir *et al.*, 2004), neutralizing adverse effect of enterotoxins (Castagliuolo *et al.*, 1996; Rolfe, 2000), and promoting body immune system (Rolfe, 2000; Simmering and Blaut, 2001; Dahiya *et al.*, 2006). Other studies have shown that supplementations of DFMs give a better growth performance of intestinal absorptive cells. Histomorphology measurement showed that probiotic supplementation can increase villus height and crypt depth of 5-wk broiler chickens (Chichlowski *et al.*, 2007; Awad *et al.*, 2009).

1.3.2. Prebiotic

Prebiotic has been defined as “a non-digestible feed ingredient that give beneficial effects on the host intestinal health by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacterial species already resident in the large intestine” (Gibson and Roberfroid, 1995). A prebiotic is a dietary supplement that intended to reach the large intestine in an intact form and has a specific metabolism therein. In this target site (large intestine), prebiotics was directed toward beneficial rather than harmful bacteria. The preferred target organisms for prebiotics are beneficial bacterial species belonging to the genera of *Lactobacillus* and *Bifidobacterium* (Gibson and Fuller, 2000). For a feed supplement to be classified as a prebiotic, it must be neither hydrolyzed by digestive enzymes nor absorbed in the stomach or small intestine. Prebiotics must be selective for one or a limited number of potentially beneficial commensal bacteria in the large intestine and induce luminal or systemic effects that are beneficial to the host health (Gibson and Roberfroid, 1995). Prebiotics must be able also to stimulate the growth and metabolism of beneficial bacteria in the gut, and thus shifts the intestinal microflora ecology towards a healthier composition (Collins and Gibson, 1999; Simmering and Blaut, 2001).

Non-digestible oligosaccharides are dietary substrates that have been reported to possess good prebiotic potential. These are oligomeric carbohydrates, whose osidic bond allow resistance to digestive enzymes in the upper gastrointestinal tract but can be fermented and metabolized by colonic bacteria (Van Loo *et al.*, 1995; Roberfroid, 1997). Among non-digestible oligosaccharides, fructo oligosaccharides and mannan oligosaccharides are predominantly used as prebiotics. They have been reported to stimulate the growth of endogenous microflora in the gut (Gibson and Roberfroid, 1995). Similarly, Patterson and Burkholder (2003) stated that fructo oligosaccharide products (FOS, oligofructose, inulin) are mostly used in poultry diets. Fructo oligosaccharides are available naturally as inulin, which is the storage carbohydrate in many thousands of plants, or can be synthesized enzymatically from sucrose (Van Loo *et al.*, 1995). However, other products such as trans-galacto oligosaccharides, gluco oligosaccharides, glycol oligosaccharides, lactulose, lactitol, malto oligosaccharides, xylo oligosaccharides, raffinose, stachyose, and sucrose thermal oligosaccharides have also been investigated as the candidate of prebiotics (Patterson *et al.*, 1997; Orban *et al.*, 1997; Piva, 1998; Collins and Gibson, 1999). Several samples of oligosaccharides that have been investigated are presented in Table 1-1.

The proposed mode of action of prebiotics to stimulate growth development and health of poultry is mostly attributed to growth inhibition of potentially harmful intestinal microflora (through competition for substrates and mucosal attachment sites) (Oyofe *et al.*, 1989; Wang and Gibson, 1993; Newman *et al.*, 1994; Grizard and Barthomeuf, 1999; Spring *et al.*, 2000), increased intestinal acidity (through production of short-chain fatty acids) (Campbell *et al.*, 1997), growth stimulation of intestinal absorptive cells (Treviño *et al.*, 1990; Choi *et al.*, 1994; Iji and Tivey, 1999), and stimulation of the enteric immune system (Yamazaki *et al.*, 1985; Ueda, 1986; Parks *et al.*, 2001), thus facilitating better performance and health status of the birds. It has been reported in many studies that supplementing diets with probiotics stimulates the growth of beneficial microflora in the gut (Gibson and Roberfroid, 1995). Supplementing diets with FOS improved enteric health of poultry by reducing colony-forming units of *S. typhimurium* and stimulating the mitotic division of absorptive cells in the gut (Choi *et al.*, 1994). The length of the ileal microvilli in birds challenged with *S. typhimurium* were found greater following dietary FOS supplementation. Enhancements in the jejunal villi length (Iji and Tivey, 1999) as well as the length of the small intestine (Treviño *et al.*, 1990) have also been reported for birds fed FOS. Moreover, dietary treatment of FOS lowered triacylglycerol and plasma cholesterol

concentration in human (Tokunaga *et al.*, 1986) and reduced plasma lipids and fatty acid synthase activity gnotobiotic in rat (Agheli *et al.*, 1998) subjects.

Table 1-1. Several examples of *in vitro* and *in vivo* studies of prebiotics

Source of prebiotic	<i>In vitro</i> study ¹	<i>In vivo</i> study ²
Fructo oligosaccharides (FOS)	Wang and Gibson (1993) Gibson and Wang (1994) McBain and Macfarlane (1997)	Tokunaga <i>et al.</i> (1986) Hidaka <i>et al.</i> (1986) Bailey <i>et al.</i> (1991) Choi <i>et al.</i> (1994) Bouhnik <i>et al.</i> (1996) Luo <i>et al.</i> (1996) Garleb <i>et al.</i> (1996) Campbell <i>et al.</i> (1997) Djouzi and Andrieux (1997) Agheli <i>et al.</i> (1998) Iji and Tivey (1999) Fukata <i>et al.</i> (1999)
Gluco oligosaccharides (GOS)	Djouzi <i>et al.</i> (1995)	Valette <i>et al.</i> (1993) Djouzi <i>et al.</i> (1995) Djouzi and Andrieux (1997) Iji and Tivey (1999) Chung and Day (2002)
Galacto oligosaccharides (TOS)	Tanaka <i>et al.</i> (1983) Durand <i>et al.</i> (1992) Bouhnik <i>et al.</i> (1997)	Tanaka <i>et al.</i> (1983) Hidaka <i>et al.</i> (1986) Mitsuoka <i>et al.</i> (1987) Matsumoto <i>et al.</i> (1989) Ito <i>et al.</i> (1990) Andrieux and Szylit (1992) Kikuchi <i>et al.</i> (1992) Rowland and Tanaka (1993) Djouzi and Andrieux (1997) Bouhnik <i>et al.</i> (1997)
Mannan oligosaccharides (MOS)	Fernandez <i>et al.</i> (2002) Smiricky-Tjardes <i>et al.</i> (2003) Çetin <i>et al.</i> (2005)	Oyfo <i>et al.</i> (1989) Newman <i>et al.</i> (1994) Schoeni and Wong (1994) Olsen (1995) Savage <i>et al.</i> (1996) Fairchild <i>et al.</i> (1999) Spring <i>et al.</i> (2000) Parks <i>et al.</i> (2001)

¹The *in vitro* studies cited contained both pure and mixed culture fermentation data.

²The *in vivo* studies taken from both animal and human study, with various treatment and condition.

From a microbial examination, Bouhnik *et al.* (1996) suggested that ingestion of FOS stimulated enhancement of colonic *Bifidobacterium* population and activity of β -fructosidase enzyme. Campbell *et al.* (1997) reported that dietary treatment of FOS increased butyrate concentration, reduced pH, stimulated the growth of *Bifidobacterium* and total anaerobes bacteria in the gut. Wang and Gibson (1993) suggested that enrichment of bifidobacterial population in the gut is preferable as *Bifidobacterium* species have an

inhibitory effect on the potentially pathogenic bacteria *Escherichia coli* and *Clostridium perfringens*. Enhancement of short-chain fatty acid concentration, reduction in intestinal acidity, and growth stimulation of *Bifidobacterium* due to dietary FOS supplementation are preferable in the need to improve enteric development and health.

In one study, Spring *et al.* (2000) reported that an oral challenge of 4,000 ppm of dietary MOS reduced cecal *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella montevideo*, *Salmonella give*, and *Salmonella dublin* population in broiler chickens, while populations of *Salmonella choleraesuis* and *Salmonella pullorum* were not affected. In different *in vivo* trials, mannose has also been shown to decrease cecal colonization of *S. typhimurium* in young chicks (Oyofe *et al.*, 1989). Schoeni and Wong (1994) also reported a reduction in *Campylobacter jejuni* colonization when birds were fed MOS. Fairchild *et al.* (1999) showed that under *E. coli* challenge, dietary MOS significantly improved body weight and body weight gain of 3 week birds. Mechanism of action of MOS as prebiotics is not attributed to enrichment of beneficial bacterial populations (Spring *et al.*, 2000) but to removal of pathogen species from the intestinal tract (Ofek *et al.*, 1977; Newman *et al.*, 1994). Removal of pathogenic species that could attach to the lumen of the intestine due to the presence of MOS in the gut will stimulate the immune system of the birds (Parks *et al.*, 2001) and lead to a more favourable environment for nutrient utilization by the host (Fairchild *et al.*, 1999).

Other study using yeast as prebiotics showed that colonization of *S. typhimurium* decreased following dietary *Saccharomyces boulardii* challenge (Line *et al.*, 1995). However, Girard (1996) suggested that live yeast cells used different mode of action to improve enteric health. Live yeast cells excrete metabolites, which are known to stimulate the growth and population of endogenous microflora in the intestine. In blood biochemical examination, Djouzi and Andrieux (1997) reported that dietary treatment of FOS, GOS, and TOS significantly reduced plasma cholesterol concentrations and stimulate short chain fatty acids production. Those three oligosaccharides also found to reduce intestinal pH of the caecal contents of gnotobiotic rats.

In more extensive research involving prebiotics, it has been shown that prebiotics alone or in combination with a lactic acid producing bacterial culture are effective to improve enteric health. Patterson and Burkholder (2003) reported that prebiotic substrates enrich certain bacterial population in the gut, such as lactic acid bacteria. Prebiotic products might also be effective to improve enteric health by reducing disease problems,

including NE lesions. Hofacre *et al.* (2003) suggested that lactic acid producing bacterial culture alone or in combination with MOS, reduced *C. perfringens*-associated mortality and increased feed efficiency, but there were no mortality incidences found caused by NE on both FOS or MOS supplementation. Results of these studies reveal that prebiotics can be used as a growth promoter in broiler diets as an alternative to antibiotics.

1.3.3. Dietary Enzymes

Poultry diets are mostly composed of cereal feedstuffs in large amounts. These cereal feedstuffs contain macro nutrients like starch, protein and fats, and many other components such as β -glucans, xylans, arabinoxylans, pentosans, pectins, mannans, cellulose, lignin, mucilage and phytic acid, which cannot be digested properly by poultry digestive organs (Adams, 2004) and reduced nutrient digestibility by interfering with interactions between digestive enzymes and their substrates (Bedford and Schulze, 1998). Those of which are mentioned as non-digestible feed components possibly increase intestinal viscosity and generate stress in the digestive tract which in turn cause reduction in nutrient utilization in the gut (Adams, 2004).

Intestinal viscosity is a major factor limiting bird performance, especially in cereal-based fed birds (Bedford and Morgan, 1996). Research by Fengler and Marquardt (1988) indicated that even small increases in the solution viscosity have a serious impact on the rate of nutrient diffusion. The feed retention time in this first third of the small intestine, where 85% of the nutrient absorption occurs (Imondi and Bird, 1966; Isshiki *et al.*, 1989), is only approximately 2 hours (Isshiki *et al.*, 1989). Consequently, increased intestinal viscosity due to high-fibre diets depress feed passage rate (Salih *et al.*, 1991; Bedford and Morgan, 1996) and increase pathogenic microbial population in the small intestine (Feighner and Dashkevich, 1988; Langhout *et al.*, 2000). Uncontrolled growth of pathogenic species in the gut, that requires energy and protein from intestinal digesta, can compete to the nutrients available for the host. Excessive amount of these pathogens in gut will lead to depression in enteric development and health of the birds. Since AGPs were no longer allowed in the diets, other substances such as various exogenous enzymes, must be used to overcome these problems (Bedford and Morgan, 1996; Mathlouthi *et al.*, 2002).

In many studies, it has been shown that supplementation of various exogenous enzymes improve growth performance of animals (Denbow *et al.*, 1995; Denbow *et al.*, 1998; Augspurger *et al.*, 2003; Adedokun *et al.*, 2004). Since it is likely that cereal diets

contain starch that is protected in the cell walls, the use of dietary enzymes has become an important factor to help improve starch digestion (Pettersson and Åman, 1989). It has been suggested by Pluske *et al.* (2002) that dietary enzymes fasten nutrient digestion by breaking open endosperm cell walls. The benefits in this enzymatic reaction can be almost entirely expected to decrease digesta viscosity, and thus achieve better digestion/diffusion (Choct and Annison, 1992a). Accordingly, the main proposed mode of action of dietary supplementary enzymes in cereal-based diets include lowering viscosity in the small intestine (Bedford and Morgan, 1996), reduced feed passage time (Salih *et al.*, 1991), which allows large amount of macro nutrients to be hydrolyzed, digestion of cell walls and creation of holes large enough to allow enzymes molecules to penetrate into the cells (Vahjen and Simon, 1999), and generating carbohydrate fragments capable of supporting beneficial bacterial population in the gut (Pluske *et al.*, 2002). The use of specific enzyme, like amylase, as a puncturing agent, is favourable to improve nutrient digestibility. It also allows the digested nutrients to be diffused by absorptive cells in the intestinal wall (Chesson, 1994).

Evidence shows that the use of supplementary enzymes as alternatives for AGPs in poultry diets modulate the gut microflora and performance of broiler chickens (Choct, 2009). It has been demonstrated that enzyme supplementation mitigates the negative effect of *C. perfringens* (Sinlae and Choct, 2000) and *Eimeria* sp. (Jackson *et al.*, 2003) challenges, improves nutrient digestibility (Choct *et al.*, 1999), reduces pathogenic fermentation in the gut (Langhout *et al.*, 2000), reduces ileal viscosity (Shakouri *et al.*, 2009), increases ileal digestibility (Bedford, 2000; Kiarie *et al.*, 2007), as well as increases the number of total anaerobic bacteria in the gizzard (Shakouri *et al.*, 2009) and increasing beneficial microflora fermentation in the ceca (Jia *et al.*, 2009a). The improvement in fermentation of beneficial bacteria in the ceca might result in the enhancement of volatile fatty acid (VFA) production. These changes in VFA profile may serve to favour the beneficial organisms (for example *Bifidobacteria*) and suppress populations of deleterious bacteria (*Salmonella*, *Campylobacter* and *Clostridium*), which in turn improve growth performance (Bedford, 2000).

Experiments have shown that β -mannans present in the intestinal mucosa are potent stimulators of the innate immune system (Wannemacher Jr, 1977), resulting in the improvement of macrophages and monocytes proliferation and resultant cytokine production (Zhang and Tizard, 1996; Duncan *et al.*, 2002). The use of β -mannanase

supplementation in corn-based diets significantly improved body weight gain and reduced intestine lesions (Sinlae and Choct, 2000; Jackson *et al.*, 2003). Other studies using carbohydrases showed that supplementary xylanase and β -glucanase enzymes improved nutrient utilization in the diets containing high levels of xylans and β -glucans from wheat and barley, respectively (Bedford and Classen, 1992; Jackson *et al.*, 1999; Mathlouthi *et al.*, 2002). In a serial study, Shakouri *et al.* (2009) have investigated that supplementary enzymes beneficially modify the microstructure of the gut by inducing an increase in villus height and villus:crypt ratio of birds receiving wheat-based diet, as well as increasing crypt depth in birds receiving barley-based diet and a reduction in crypt depth of chickens on the sorghum-based diets. In this study, intestinal morphology, enzyme activities, gut microflora, and nutrient digestibility have been found to be improved, resulting in more nutrients being available to support poultry growth development and health.

Experiments using corn-soybean meal based diets have demonstrated that supplementary phytase in various levels increased body weight gain, feed intake, feed efficiency, tibia ash weight and percentage (Denbow *et al.*, 1995; Augspurger *et al.*, 2003; Augspurger and Baker, 2004), toe ash weight and percentage (Denbow *et al.*, 1998), digestibility of phosphorus (P) and nitrogen (N) in total intestine tract (Denbow *et al.*, 1998; Adedokun *et al.*, 2004; Dilger *et al.*, 2004), retention of dry matter (DM) (Dilger *et al.*, 2004), C (Onyango *et al.*, 2005), P (Broz *et al.*, 1994; Sebastian *et al.*, 1996), N (Ravindran *et al.*, 1999; Cowieson *et al.*, 2004), and several amino acids (Sebastian *et al.*, 1997; Ravindran *et al.*, 1999; Onyango *et al.*, 2005) of broiler chickens. Olukosi *et al.* (2007; 2008) indicated that supplementary phytase alone or in combination with other enzymes (xylanase, amylase, and protease) significantly improved body accretion rates of nutrients (DM, protein, fat, ash, Ca, P), improved total tract N and P retention, as well as improved metabolizable energy and DM retention. Results of this study showed that phytase was efficacious in hydrolyzing phytate P for bone mineralization, nutrient utilization, nutrient accumulation in the carcass and growth performance of broiler chickens. Simons *et al.* (1990) demonstrated that when microbial phytase was added to low-P diets for broilers, the availability of P increased to over 60% and the amount of P in the droppings decreased by 50%. On the low-P diets, supplementation of microbial phytase increased the growth rate and feed conversion ratio of broiler chickens. In other studies using diets based on fibre-rich feedstuffs like wheat bran, rice bran, coconut meal, and palm kernel meal, Onilude and Oso (1999) showed the positive effects of cellulase,

amylase and pectinase to improve proportions of both protein and ash in the carcass of broiler chickens.

In pig diets, dietary supplementation with graded amounts of *E. coli*-derived phytase increased weight gain, feed efficiency, plasma Ca and P concentrations in 10-kg pigs, improved P digestibility and retention in the 13-kg pigs, increased weight gain and feed efficiency, as well as improved Ca and P digestibility and retention in 19-kg pigs (Adeola *et al.*, 2004).

1.3.4. Organic Acids

Organic acids are considered to be any organic carboxylic acid with the general structure of R-COOH, and hence include fatty acids and amino acids. Organic acids are short-chained acids (C1–C7) and are either simple monocarboxylic acids such as formic, acetic, propionic and butyric acids, or are carboxylic acids bearing an hydroxyl group (usually on the α carbon) such as lactic, malic, tartaric, and citric acids (Dibner and Buttin, 2002). These weak acids are added to the feed in sub-therapeutical dosage and are believed to have the capacity to improve growth performance (Vogt *et al.*, 1982), nutrient utilization (Runho *et al.*, 1997), and health (Canibe *et al.*, 2001). In addition, because organic acids are only partly dissociated, not all of them influence intestinal microflora (Byrd *et al.*, 2001; Roy *et al.*, 2002). Most of the organic acids with antimicrobial activity have a pKa value between 3 and 5 (Dibner and Buttin, 2002; Pölönen and Wamberg, 2007). Growth inhibitory properties of some organic acids are presented in Table 1-2.

1.3.4.1. Modes of Action of Organic Acids in Poultry Nutrition

Organic acids are known to have strong antibacterial properties which are important to support enteric health and growth performance of poultry. The proposed mode of action of organic acids is related to the reduction of intestinal pH (Waldroup *et al.*, 1995), which might followed by alterations in the intestinal ecosystem (Canibe *et al.*, 2001). The main objective of supplementing the organic acids is to lower intestinal pH (Ricke, 2003), with the intention of controlling microbial growth and colonization (results in the lowering nutrients competition (Partanen *et al.*, 1998; Ricke, 2003), reduction of microbial metabolites (Gabert *et al.*, 1995), and decreasing of incidence of sub-clinical infection) (Fuller, 1977; Russell, 1992), as well as stimulating the growth of intestinal absorptive cells (Visek, 1978; Loddi *et al.*, 2004), stimulating pancreatic secretion (Schulz *et al.*,

1971; Dibner and Buttin, 2002), and promoting the whole growth performance of broiler chickens (Roy *et al.*, 2002; Dibner and Richards, 2004; Pelicano *et al.*, 2005).

Table 1-2. Several organic acids used in animal nutrition

Organic acids	Properties ¹		Growth inhibitory ²		
	Molecular formula	Acid dissociation constant (pKA)	Bacteria	Yeast	Mould
Formic acid	HCOOH	3.75	++++	+	+
Lactic acid	CH ₃ CHOHCOOH	3.86	+	-	-
Acetic acid	CH ₃ COOH	4.76	++	+++	+++
Propionic acid	CH ₃ CH ₂ COOH	4.87	++	+++	+++
Citric acid	C ₃ H ₅ O(COOH) ₃	3.10-5.40	n.a.	n.a.	n.a.
Sorbic acid	C ₆ H ₈ O ₂	4.76	+++	++++	++++
Benzoic acid	C ₆ H ₅ COOH	4.20	+++	++++	++++

¹Adapted from Pölönen and Wamberg (2007); ²adapted from Lassén (2007)

Organic acids have been shown to have the ability to penetrate the cellular membrane of bacteria. Inside the cell, they release protons in the more alkaline cytoplasm, resulting in the reduction of intracellular pH (Baronofsky *et al.*, 1984; Biggs and Parsons, 2007) and hence are bactericidal (Russell, 1992). The RCOO⁻ anions produced from the acid can interrupt DNA and protein synthesis. This condition is unfavourable for enteropathogenic bacteria colonization, such as *E. coli*, *Campylobacter*, and *Salmonellas* (Humphrey and Lanning, 1988; Dibner and Buttin, 2002) that are sensitive to acid pH (Waldroup *et al.*, 1995). These acid-intolerant bacteria are under stress and unable to replicate more rapidly (Hernández *et al.*, 2006), resulting in bacteriostatic and bacteriocidal activity of organic acids (Russell, 1992; Vale *et al.*, 2004).

Activity of organic acids to reduce pH in the lower gut is highly desirable, because the lower gut is colonized by many anaerobic opportunistic pathogens. Reduction of the intestinal pH might depress the growth of pathogenic bacteria, reduce subclinical infections (Humphrey and Lanning, 1988), and support the proliferation and growth of beneficial bacteria (Eklund, 1983). Reduction of subclinical infections and stimulation of the growth of beneficial bacteria may contribute to increase nutrient digestibility and a reduction in nutrient demand by the gut-associated immune tissue and microorganisms (Dibner and Buttin, 2002).

1.3.4.2. The Role of Organic Acids in Poultry Diets

The primary expected beneficial effect of organic acids supplementation is enhanced growth performance. It has been shown in many studies that inclusion of a single or combination of organic acids improved growth performance of broiler chickens. Supplementation of fumaric acid in the diets significantly improved average daily gain and feed efficiency (Patten and Waldroup, 1988; Skinner *et al.*, 1991) and depressed the growth of lactic acid and coliforms bacteria in the ileum and ceca (Pirgozliev *et al.*, 2008). Similarly, Runho *et al.* (1997) showed an evidence that in Hubbard broilers dietary fumaric acid didn't give any effect on growth rate, but reduced feed consumption, resulting in a better feed conversion ratio. It has also been reported that fumaric acid supplementation improved metabolizable energy. In recent study using organic acids, Chotikatum *et al.* (2009) reported that combination of lactic acid, citric acid, ascorbic acid, and propionic acid benzoic acid in the diets have significant effect on growth performance improvement of broiler chickens.

Vogt *et al.* (1982) showed that body weight gain and feed efficiency of broiler chickens improved significantly when the diets were supplemented with malic, sorbic, and tartaric acids. Other studies using citric acid have shown that supplementary of organic acids in broiler ration significantly improved weight gain (Afsharmanesh and Pourreza, 2005; Chowdhury *et al.*, 2009), increased feed consumption (Moghadam *et al.*, 2005; Chowdhury *et al.*, 2009), improved feed efficiency (Abdel-Fattah *et al.*, 2008), increased retention of phosphorus (Brenes *et al.*, 2003; Liem *et al.*, 2008), improved bone ash (Atapattu *et al.*, 2005; Rafacz-Livingston *et al.*, 2005; Martinez-Amezcuca *et al.*, 2006; Chowdhury *et al.*, 2009), decreased the pH of cecal digesta, crop and gizzard (Andrýs *et al.*, 2003), and the intestine (Denli *et al.*, 2003), as well as depressed the growth of *Escherichia coli*, *Salmonella*, and other gram-negative bacteria in broiler chicks (Chowdhury *et al.*, 2009). In addition, dietary citric acid supplementation also improved immune status (Rahmani and Speer, 2005; Abdel-Fattah *et al.*, 2008; Chowdhury *et al.*, 2009) and carcass yield (Chowdhury *et al.*, 2009) of broiler chickens. Better growth performance in these studies might be attributed to the reduction in pathogenic bacterial colonization in the GIT accompanied by reduced competition for nutrients between the host and exogenous pathogen microflora (Partanen *et al.*, 1998; Rieke, 2003; Józefiak *et al.*, 2010).

Dietary inclusion of organic acids has beneficial effects on intestinal acidity and histomorphology (Geyra *et al.*, 2001; Loddi *et al.*, 2004). Reduction of intestinal pH is one of many ways to control pathogenic bacteria population that are pH-responsive. A reduction in intestinal acidity has a substantial role with organic acids to reduce enteropathogenic bacteria colonization that is sensitive to acid pH (Jensen, 2001), even though could be favourable to stimulate growth of beneficial bacteria (Eklund, 1983; Eklund, 1985). The lower number of those acid-intolerant microflora may indirectly stimulate nutrient digestibility improvement (Dibner and Buttin, 2002).

Sub-therapeutic doses of organic acids modulate the natural microbial population in the gut by reducing the adhesion of bacteria such as: *Escherichia coli*, *Campylobacter*, *Clostridium perfringens* (Roy *et al.*, 2002), *Salmonella kedougou*, and *Salmonella gallinarum* (Hernández *et al.*, 2006). For instance, population of Coliform bacteria in the gut of broiler chickens depressed significantly following benzoic acid (Józefiak *et al.*, 2007) or fumaric acid (Knarreborg *et al.*, 2002) supplementation. In Dibner and Buttin (2002)'s study, dietary organic acid supplementation reduced population of *E. coli*, *Salmonella*, *Campylobacter*. In pigs, Mroz *et al.* (2000) and Buhler *et al.* (2006) reported evidence of bacteriostatic action of organic acids due to observations of reduced ammonia levels with the dietary inclusion of benzoic acid.

Alongside the properties of organic acids to stimulate growth performance, inclusion of organic acids seems to have direct effects on the histomorphology of the gut. Organic acids stimulate intestinal growth by increasing the height and width of villus and the depth of crypt. Paul *et al.* (2007) showed that ammonium formate or calcium propionate supplementation promoted villus height, whilst Senkoğlu *et al.* (2007) showed that combination of formic and propionic acid stimulated villus height and width. In another study, Garcia *et al.* (2007) showed that formic acid supplementation stimulated villus height and crypt depth of broiler chickens. The higher villus and greater crypts indicated high proliferative cellular activity due to organic acid inclusion, expecting to provide an adequate epithelium turnover rate. Geyra *et al.* (2001) pointed out that the increase in the number and size of crypts has two direct effects, which are to provide enterocytes for the increasing intestinal absorptive surface area as the villus to grow and to increase cell renewal rate. Improvement of the intestinal histomorphology in birds fed organic acids is believed to account for some of the growth-promoting properties of organic acids. On the other hand, reduction of the growth and population of *Salmonella*, *E.*

coli, and *Campylobacter* in the gut might also indirectly stimulate mitotic division of absorptive cells in the gut wall (Xia *et al.*, 2004; Pelicano *et al.*, 2005). The reduction of these organisms, which produce toxins that can disrupt the villi and crypt structure, helps to account for the improvement in gut structure (Dibner and Buttin, 2002). Supplementation of organic acids must be done in low dosage, because excessive dosage resulted in growth depression in intestinal villus height and width, as well as crypt depth (Smulikowska *et al.*, 2010).

1.3.4.3. Benzoic Acid

Benzoic acid is one of the oldest safe chemical preservative which is used in the food, beverages, drug, and cosmetics likely because of its low cost, ease of incorporation into products, and relatively low toxicity (Chipley, 1993). Benzoic acid has GRAS (Generally Recognized as Safe) status and sodium benzoate was the first chemical preservative approved by the US Food and Drug Administration for use in foods (Jay, 1992). The acid (C_6H_5COOH , molar mass 121.11) is a colourless crystalline solid or white granular powder with pKA of 4.19 (Baird-Parker, 1980) that has been known to inhibit fungal and bacterial growth (Chipley, 1993).

Benzoic acid inhibits aflatoxin growth by blocking an enzyme in its biosynthetic pathway (Chipley, 1993). Krebs *et al.* (1983) suggested that the antifungal property of benzoic acid is based on the accumulation of benzoate at low external pH which the intracellular pH into the range where phospho-fructokinase enzyme works. Not only inhibiting fungal growth, benzoic acid also depressed the growth of pathogenic bacteria. An *in vitro* study by Friedman *et al.* (2003) showed that benzoic acid has antibacterial properties to lower the growth and population of many pathogenic bacteria as *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Reduction in these pathogenic organisms by lowering intestinal pH possibly results in more nutrients being available in the digesta content, which might stimulate absorptive cell proliferation (Pelicano *et al.*, 2005; Vieira *et al.*, 2005; Viola and Vieira, 2007).

In a field study using cockerels, Józefiak *et al.* (2010) reported that the inclusion of 1.0 g/kg benzoic acid improved body weight gain in starter and grower periods, improved feed conversion ratio in the starter period, as well as depressed the population of lactic acid bacteria in the caeca and coliform bacteria in the ileum. Increased supplementation up to 2.0 g/kg significantly increased dry mater of the digesta content in the crop and caeca, and

decreased pH of the caecal contents. The authors showed no differences on the coliform bacteria populations in the caeca. Previous study by Józefiak *et al.* (2007) demonstrated a bacteriostatic effect of benzoic acid against coliform bacteria in the GIT as a result of 2.5 g/kg inclusion. In this study, the authors also showed 5.0 g/kg supplementation tended to decrease acidity of the caecal contents.

Other studies using dosage of 6.0 g/kg (Lu-nu *et al.*, 2009) showed that benzoic acid inclusion in the diets significantly improved growth performance, reduced abdominal fat percentage, increased glycogen content in the muscle, and increased water-holding capacity of muscles, resulting better carcass quality of broilers. The authors suggested that the acid acts by regulating energy metabolism and therefore enhance the water holding capacity of muscles. The results provided in available papers showed evidences that benzoic acid can be used as an alternative to AGP to improve growth performance and meat quality of broiler chickens. However, higher inclusion of 7.5 g/kg is not recommended as it depressed body weight gain and increased feed conversion ratio of broiler chickens (Józefiak *et al.*, 2007).

1.3.4.4. Formic Acid

Formic acid is one of the weak acids which are known to have an antibacterial effect (Pölönen and Wamberg, 2007). The strength of the effect of this acid is attributed to its high dissociation constant (pKA 3.75) and hence is suitable for the preservation of highly acidic products in the pH range below 3.5 (Lassén *et al.*, 2007). As organic acids have high solubility, it can be absorbed through the skin and mucous membranes. The acid (HCOOH, molar mass 46.03) is a colourless, transparent liquid with a pungent odour, an irritant to eyes, skin, and mucous membranes and is also miscible with water (Lassén, 2007).

Formic acid supplementation has been reported to improve growth in broilers (Senkoylu *et al.*, 2007; Paul *et al.*, 2007). Improvements in performance likely resulted from the beneficial effect of the acid on the intestine. Supplementation of formic acid stimulated cell proliferation of villus and crypt in different segments of small intestine of broiler chickens (Hernández *et al.*, 2006; Senkoylu *et al.*, 2007; Paul *et al.*, 2007). Furthermore, inclusion of 10.0 g/kg formic acid in the diet reduced pH of ileum, colon (Roth *et al.*, 1992), and caeca (Waldroup *et al.*, 1995) and this is important for controlling microflora population in the GIT. In Hinton and Linton (1988) study, it has been reported that 6.0 g/kg formic acid supplementation reduced *Salmonella* infection in the gut of

broiler chickens. Similarly, Humphrey and Lanning (1988) reported that 5.0 g/kg formic acid supplementation significantly reduced transmission of Salmonellas in broiler chickens. In other studies, the growth and population of *Escherichia coli* K12 and *Salmonella* spp. at pH 5.0 were reduced dramatically following formic acid treatment (Cherrington *et al.*, 1991). In the same way, incorporation of 10.0 g/kg formic acid into the diet resulted in total inhibition of all pathogenic bacteria, including Salmonellas and Lactobacilli (Impey and Mead, 1989). Izat *et al.* (1990) also reported a reduction on Salmonellae in carcass and caecal of broiler chickens following 10.0 g/kg supplementation. Study with lower dose has shown that 3.0 g/kg formic acid depressed the growth of *Escherichia coli* in gut of broiler chickens (Paul *et al.*, 2007). In breeding hens, Humphrey and Lanning (1988) reported that 5.0 g/kg of formic acid in the diet reduced the isolation rate of salmonella and was associated with a reduction in the incidence of infection in newly hatched chicks.

In Hernández *et al.* (2006) study, it has been reported that the jejunum histomorphology of broiler chickens were stimulated by 5.0 g/kg formic acid inclusion, resulted in higher villus, deeper crypt, larger villus surface area, and better dry matter digestibility. Similarly, Garcia *et al.* (2007) have shown that the growth of absorptive cells at the jejunum are stimulated by 5.0 g/kg formic acid supplementation, resulted in greater villus height, crypt depth, and villus surface area. Apparent ileal digestibility and better feed conversion rate were also promoted by formic acid inclusion. In this experiment, the authors demonstrated the effects of formic acid to stimulate the proliferation of normal crypt cells, enhancing healthy tissue turnover and maintenance, as well as improving nutrient utilization.

1.3.4.5. Propionic Acid

Propionic acid is a weak acid which have antifungal properties against an unwanted growth of yeast and moulds (Pölönen and Wamberg, 2007). The acid ($\text{CH}_3\text{CH}_2\text{COOH}$, molar mass 74.08) is a colourless liquid, transparent liquid with a pungent odour, unlimited miscibility with water and a boiling point of 141°C. Many studies have shown that propionic acid, which has pKA value of 4.87 (Pethybridge *et al.*, 1983), has antibacterial properties against pathogen species in the GIT, including *Bacillus subtilis* (Sheu and Freese, 1972), *Escherichia coli* (Hinton and Linton, 1988), *Salmonella typhimurium* (Hume *et al.*, 1993; Corrier *et al.*, 1995; Matlho *et al.*, 1997; Kwon *et al.*, 1998), *S. enteritidis*, and *S. agona* (Iba and Berchieri, 1995) species. In addition, Izat *et al.* (1990) showed that 4.0 g/kg

propionic acid supplementation reduced the total number of coliforms and of *Escherichia coli* in the duodenum, whilst the growth performance, feed utilization, or abdominal fat of broiler chickens were not affected. Antimicrobial properties of propionic acid might be attributed to its ability to penetrate the bacterial membrane. Hinton *et al.* (1990) described that when propionic acid infiltrate the bacterial membrane, its undissociated lipophilic form easily penetrates bacterial cell walls. Inside the cell, the acid may dissociate and kill the cell. However, Hume *et al.* (1993) showed that the effectiveness of propionic acid to control *Enterobacteriaceae* such as *Salmonellae* depends on the severity of *Salmonellae* challenge.

Recent experiment in Japanese quail (Bonos *et al.*, 2010) showed that supplementation of 6.0 g/kg calcium propionate effective to improve body weight and to reduce feed intake, resulting in a better feed conversion ratio. The authors also reported an improvement of carcass quality due to dietary calcium propionate inclusion.

In a broiler study, significant improvements in body weight gain and feed conversion ratio were observed in 35-d broiler chickens receiving diets supplemented with combination of propionic and formic acid (Senkoylu *et al.*, 2007). In this study, intestinal viscosity was significantly affected by organic acid inclusion. In addition, the growth of absorptive cells in the intestine was also significantly stimulated by the inclusion, resulting in the improvement of the villus height. Improvement of the growth performance of poultry receiving propionic acid alone or in combination likely due to reduction in host-microbe competition for nutrients (Thomke and Elwinger, 1998; Partanen *et al.*, 1998), reduction in incidence of sub-clinical infections (Russell, 1992), secretion of immune mediators (Rahmani and Speer, 2005), reduction in ammonia production (Mroz *et al.*, 2000) and other growth-depressing metabolites produced by bacteria in the gut (Gabert *et al.*, 1995). Improvements in nutrient digestibility observed in the study also corresponded with improvements in pancreatic secretion which are likely to directly enhance nutrient and energy utilization (Dibner and Buttin, 2002).

1.3.5. Herbs, Spices, and Essential Oils

Herbs, spices, and essential oils, contain active compounds (phytochemicals) which can be used as alternatives to AGP in poultry diets. Phytochemicals feed additives consists of many phytochemicals that can be categorized as: phenolics and polyphenols (simple phenols and phenolic acids, quinones, flavones, tannins, and coumarins), terpenoids and essential oils,

alkaloids, and lectins and polypeptides (Cowan, 1999; Hernández *et al.*, 2006). Windisch and Kroismayr (2007) described that classification of phytobiotics can be categories by herbs (leaves, flowering, non-woody, and non-persistent plants), spices (herbs with an intensive smell or taste), essential oils (volatile lipophilic phytochemicals derived by cold expression or by steam or alcohol distillation methods), or oleoresins (derived by non-aqueous solvents extraction). Essential oils are volatile natural complex compounds formed by aromatic plants as secondary metabolites. Essential oils mostly concentrated in particular organ such as leaves, bark (stem), fruit, or seed, and when they occur in various organs in the same plant, they frequently have different composition profiles (Singh *et al.*, 1995; Jayaprakasha *et al.*, 1997; Hili *et al.*, 1997; Dhuley, 1999).

Table 1-3. List of various main phytochemicals contained in common herbs and spices

Herbs/Spices	Phytochemicals	References
Garlic (<i>Allium sativum</i>)	allicin, diallyl sulfide, diallyl trisulfide, ajoene	Apitz-Castro <i>et al.</i> (1983); Ahsan <i>et al.</i> (1996); Adibmoradi <i>et al.</i> (2006); Taylor <i>et al.</i> (2006); Maluf <i>et al.</i> (2008); Kim <i>et al.</i> (2009); Choi <i>et al.</i> (2010)
Ginger (<i>Zingiber officinale</i> Rosc.)	gingerol, gingerdiol, gingerdione, dehydroshogaol, paradol, curcumene, zingerone, zingiberene, camphene, β -phellandrene, β -sesquiphellandrene,	Kikuzaki and Nakatani (1996); Ji <i>et al.</i> (1997); Surh (1999); Agarwal <i>et al.</i> (2001); Jolad <i>et al.</i> (2005); Gupta and Ravishankar (2005); Ali <i>et al.</i> (2008)
Turmeric (<i>Curcuma longa</i> L.)	curcumin, demethoxycurcumin, bisdemethoxycurcumin, zingiberene, curcumenol, curcumol, eugenol, tetrahydrocurcumin, triethylcurcumin, turmerin, turmerones, turmeronols, curcuminoids, curcumene, turmerol, curlone, curdione, germacrone, bisabolene, <i>p</i> -cymene, cineole	Araújo and Leon (2001); Jayaprakasha <i>et al.</i> (2005); Aggarwal <i>et al.</i> (2007)
Oregano (<i>Origanum vulgare</i> Linn.)	carvacrol, thymol, <i>p</i> -cymene, γ -terpinene, β -caryophyllene, α -terpinene, etc.	Aligiannis <i>et al.</i> (2001); Lee <i>et al.</i> (2003b); Baydar <i>et al.</i> (2004); Bampidis <i>et al.</i> (2005)
Cinnamon (<i>Cinnamomum zeylanicum</i>)	eugenol, carvacrol, cinnamaldehyde, <i>trans</i> -cinnamaldehyde, sesquiterpenes (α -bergamotene and α -copaene), camphor, diterpenes, etc.	Jayaprakasha <i>et al.</i> (2003); Negi <i>et al.</i> (2007); Johny <i>et al.</i> (2008); Cheng <i>et al.</i> (2008); Al-Kassie (2009); Zita <i>et al.</i> (2009)

There are numbers of herbs and spices that can be used as phytobiotic feed additives, such as garlic (Cavallito and Bailey, 1944; Qureshi *et al.*, 1983), turmeric (Ammon *et al.*, 1993; Kumari *et al.*, 2007), ginger (Surh, 1999), curcumin (Al-Sultan, 2003), oregano (Aligiannis *et al.*, 2001), thyme (Oussalah *et al.*, 2007), rosemary (Lopez *et al.*, 2005), cinnamon (Tabak *et al.*, 1999), clove (Lopez *et al.*, 2005), basil (Lopez *et al.*, 2005), and *Carica papaya* (Fouzder, 1999). Efficacy of phytobiotics as feed additives and

the content of the phytochemicals (relative composition and concentration) may vary due to differences in part of the plant in which the chemical is located (Jayaprakasha *et al.*, 1997; Lambert *et al.*, 2001), geographical origin (Rhyu, 1979; Vokou *et al.*, 1993; Kokkini *et al.*, 1994), genotype (Aligiannis *et al.*, 2001), seasonal variations (McGimpsey *et al.*,

Table 1-4. List of various efficacies of phytochemicals in some herbs/spices

Herbs/Spices	Properties or efficacies	References
Garlic (<i>Allium sativum</i>)	Growth promoter; antimicrobial; immunostimulator; hypolipidemic or antihyperlipidemic; hypocholesterolemic; antioxidant, meat improver agents	Ahsan <i>et al.</i> (1996); Choi <i>et al.</i> (2010); Chowdhury <i>et al.</i> (2002); Dorhoi <i>et al.</i> (2006); Gardzielewska <i>et al.</i> (2003); Habib <i>et al.</i> (2008); Kim <i>et al.</i> (2007); Kim <i>et al.</i> (2009); Konjufca <i>et al.</i> (1997); Lijuan <i>et al.</i> (2007); Mahmood <i>et al.</i> (2009); Mottaghitalab and Taraz (2002); Qureshi <i>et al.</i> (1983); Soliman <i>et al.</i> (1999); Surh <i>et al.</i> (1999); Togashi <i>et al.</i> (2008); Yalçin <i>et al.</i> (2006); Yalçin <i>et al.</i> (2007);
Turmeric (<i>Curcuma longa</i> L.)	Growth promoter; digestive stimulant; antimicrobial; hypocholesterolemic; egg and meat quality improver; antioxidant, immunostimulator agents	Al-Sultan (2003); Gautam <i>et al.</i> (2007); Masuda <i>et al.</i> (2004); Motterlini <i>et al.</i> (2000); Osawa <i>et al.</i> (1995); Piper <i>et al.</i> (1998); Platel and Srinivasan (2000); Platel <i>et al.</i> (2002); Pulla Reddy and Lokesh (1994); Rao <i>et al.</i> (2003); Toennesen (1992); Yadav <i>et al.</i> (2005)
Ginger (<i>Zingiber officinale</i> Rosc.)	Growth promoter; antimicrobial; antioxidant; hypolipidemic or antihyperlipidemic; hypocholesterolemic; meat and carcass quality improver agents	Ademola <i>et al.</i> (2009); Aeschbach <i>et al.</i> (1994); Agarwal <i>et al.</i> (2001); Fuhrman <i>et al.</i> (2000); Halliwell and Gutteridge (1989); Kikuzaki and Nakatani (1996); Krishnakantha and Lokesh (1993); Naveena and Mendiratta (2001); Rababah <i>et al.</i> (2004); Sharma <i>et al.</i> (1996); Smith-Palmer (1998); Syed Ziauddin <i>et al.</i> (1995); Zhang <i>et al.</i> (2009)
Oregano (<i>Origanum vulgare</i> Linn.)	Growth promoter; antimicrobial; insecticidal; hypocholesterolemic; and antioxidant agents	Adam <i>et al.</i> (1998); Aligiannis <i>et al.</i> (2001); Bampidis <i>et al.</i> (2005); Case <i>et al.</i> (1995); Daferera <i>et al.</i> (2000); Friedman <i>et al.</i> (2002); Karpouhtsis <i>et al.</i> (1998); Kikuzaki <i>et al.</i> (1994); Kikuzaki and Nakatani (1993); Kim <i>et al.</i> (1995); Lagouri <i>et al.</i> (1993); Lagouri and Boskou (1996); Lambert <i>et al.</i> (2001); Lee <i>et al.</i> (2003b); Marino <i>et al.</i> (2001); Milos <i>et al.</i> (2000); Ultee (1998); Vekiari <i>et al.</i> (1993)
Black seed (<i>Nigella sativa</i>)	Growth promoter; antimicrobial; meat and egg quality; immunostimulator agents	Denli <i>et al.</i> (2004a); El-Sheikh <i>et al.</i> (1998); Mahmood <i>et al.</i> (2009); Nasir and Grashorn (2010); Osman and Barody (1999); Tollba and Hassan <i>et al.</i> (2003)
Cinnamon (<i>Cinnamomum zeylanicum</i> Blume)	Growth promoter; hypocholesterolemic; antioxidant; antiallergenic, antitumor; immunostimulator agents	Al-Kassie (2009); Ciftci <i>et al.</i> (2010); Dhuley (1997); Dragland <i>et al.</i> (2003); Dugoua <i>et al.</i> (2007); Dusan <i>et al.</i> (2006); Faix <i>et al.</i> (2009); Jayaprakasha <i>et al.</i> (2007); Lee <i>et al.</i> (2003b); Mancini-Filho <i>et al.</i> (1998), Ranjbar <i>et al.</i> (2006); Sarica <i>et al.</i> (2009); Tomaino <i>et al.</i> (2005)

1994), climatic variations (Salgueiro *et al.*, 1997), and processing methods (Venskutonis, 1997; Delespaul *et al.*, 2000).

1.3.5.1. Herbs and Spices as Alternatives to AGP

The main phytochemicals contained in common herbs and spices (garlic, ginger, turmeric, oregano, and cinnamon) which are commonly used in poultry diets are presented in Table 1-3. *In vitro* studies showed that phytochemical substances present in herbs and spices have antibacterial (Deans and Ritchie, 1987; Juven *et al.*, 1994; Hammer *et al.*, 1999), antifungal (Conner and Beuchat, 1984; Benjilali *et al.*, 1984; Daouk *et al.*, 1995; Hammer *et al.*, 1999), antiparasitic (Anthony *et al.*, 2005), antihelminthic (Chatterje *et al.*, 1982), and anticoccidial (Giannenas *et al.*, 2003) properties. For instance, active compounds carvacrol and thymol derived from oregano and thymus exhibit antimicrobial and antifungal properties (Basilico and Basilico, 1999). Essential oils derived from sage (*Salvia ringens*) have antimicrobial properties, as they inhibit the growth of intestinal bacteria and fungi (Tzakou *et al.*, 2001). *In situ* studies have demonstrated growth-promoting properties (Cross *et al.*, 2007; Rizzo *et al.*, 2008; Rahmatnejad *et al.*, 2009) and showed beneficial effects of essential oils from several herbs and spices to lower pathogenic microbes in the GIT (Sokmen *et al.*, 1999; Kamel, 2001; Cabuk *et al.*, 2003).

There is an evidence to suggest that herbs, spices and various plant extracts have broad properties to improve growth performance and health of poultry (Table 1-4). It has been shown that in broiler chickens inclusion of essential oils in the diets stimulates feed intake (Halle *et al.*, 1999; Durrani *et al.*, 2006; Al-Kassie, 2009), body weight gain (Lewis *et al.*, 2003; Durrani *et al.*, 2006), feed efficiency (Halle *et al.*, 1999; Tollba and Hassan, 2003; Demir *et al.*, 2003), nutrient digestibility (Hernández *et al.*, 2004; Jamroz *et al.*, 2005), as well as appetite and digestion (Kamel, 2001; Alcicek *et al.*, 2004; Zhang *et al.*, 2005). Improvement in feed efficiency were also found in layer chickens (El-Sheikh *et al.*, 1998; Akhtar *et al.*, 2003), in Japanese quail (Denli *et al.*, 2004a), and in ducklings (Mandour and Rady, 1997). Histomorphological examination showed that inclusion of phytochemicals in the diet stimulates proliferation and growth of absorptive cells in the GIT, resulting greater villus height and deeper crypt (Jamroz *et al.*, 2006).

1.3.5.2. Mode of Action of Phytochemicals in Poultry Nutrition

The primary mode of action of phytochemicals as growth-promoter is attributed to the growth inhibition of harmful intestinal microflora in the GIT (Benjlali *et al.*, 1984; Juven *et al.*, 1994; Hammer *et al.*, 1999; Lopez *et al.*, 2005; Islam *et al.*, 2006) and by stimulating function of digestive organ, e.g. the pancreas and small intestine (Jang *et al.*, 2004). Windisch and Kroismayr (2007) reported that reduction on the population of enteropathogens results in a more stabilized microflora that will indirectly stimulate functions of digestive organs and reduce microbe-host competition for nutrients. The mechanism by which the phytochemicals exert their antimicrobial activity consists of interactions with the microbial cell membranes of microorganisms by changing permeability for cations such as H⁺ and K⁺ (Cabuk *et al.*, 2006). The antimicrobial compounds are quickly exerted by determining structural alterations of the cell envelope. Population of enteropathogen microbes which are known to less resistant to this antimicrobials activity will decreased, while many beneficial microbes, such as *Bifidobacterium* spp. and *Lactobacillus* spp. are relatively resistant (Di Pasqua *et al.*, 2007; Ouwehand *et al.*, 2010).

Another mechanism of actions which proposed for active compounds in herbal products as growth promoters are related to their oxidation-resistant activity (Faix *et al.*, 2009; Zhang *et al.*, 2009) and improvement of the immune system (Emadi and Kermanshahi, 2007; Yarru *et al.*, 2008; Najafi and Toriki, 2010), thereby stimulating animal's growth.

1.3.5.3. Garlic

Garlic (*Allium sativum* L.; Liliaceae) is widely used and distributed in most parts of the world because of its many beneficial properties. Garlic is rich in organosulfur substances, such as allicin, diallyl sulfide, and diallyl trisulfide. The allicin gives garlic its characteristic odour and flavour as well as most of its biological properties (Chowdhury *et al.*, 2002). Takhtajan (1997) described taxonomic position of garlic as follows:

Class : Liliopsida
Sub Class : Lillidae
Order : Amaryllidae
Family : Alliaceae

Sub Family : Allioideae
Genus : Allium
Species : *Allium sativum*

In general, the primary sulfur-containing constituents in whole garlic are the γ -glutamyl-S-alk(en)yl-L-cysteines and S-alk-(en)yl-L-cysteine sulfoxides, including alliin. Whole garlic normally contains approximately 1% alliin, together with (1)-S-methyl-L-cysteine sulfoxide (methiin) and (1)-S-(trans-1-propenyl)-L-cysteine sulfoxide. Garlic cloves contain S-(2-Carboxypropyl)glutathione, γ -glutamyl-S-allyl-L-cysteine, γ -glutamyl-S-(trans-1-propenyl)-L-cysteine and γ -glutamyl-S-allyl-mercapto-L-cysteine. A garlic bulb contains approximately 0.9% γ -glutamylcysteines and up to 1.8% alliin. The S-allyl cysteine is formed from γ -glutamyl cysteine catabolism and is thought to contribute to the health benefits of some garlic preparations (Amagase *et al.*, 2001; Amagase, 2006).



Figure 1-1. Garlic (*Allium sativum* L.)

Adapted from Woodville (1793)

<http://www.motherearthnews.com/uploadedImages/articles/issues/2009-10-01/Grow%20garlic.JPG>

Several phytochemicals of garlic, mainly polyphenols such as flavonoids and sulfur-containing substances, have been revealed antioxidative properties in meat-type (Kim *et al.*, 2009) and egg-type (Gorinstein *et al.*, 2005) chickens. Furthermore, antioxidative activity of garlic depends on the part of the plant, as they contain different type and amount of phytochemicals. For instance, garlic husk had 7 times greater total polyphenols than garlic bulb, and the non-edible garlic husk had 1.5 times greater radical scavenging activity than the edible part (Kim *et al.*, 2009; Choi *et al.*, 2010).

In poultry nutrition, garlic is known to result in improved growth (Onibi *et al.*, 2009; Mahmood *et al.*, 2009), inhibition of growth of pathogens in the gut (Ahsan *et al.*, 1996; Sarica *et al.*, 2005), enhanced pancreatic function (Adibmoradi *et al.*, 2006), and improved meat and carcass quality (Kim *et al.*, 2009).

Garlic can be used as a feed additive in broiler diets as it able to improve weight gain and reduce feed conversion ratio (Singh *et al.*, 1998; Avato *et al.*, 2000; Lewis *et al.*, 2003; Carrijo *et al.*, 2005; Mahmood *et al.*, 2009). This performance improving property is attributed to the antibacterial properties of allicin and ajoene. Maluf *et al.* (2008) reported that ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide), an organic sulphur compound, has antimicrobial properties. Therefore, feeding diets containing ajoene may inhibit the growth of entero-pathogenic bacteria, thus contributing on the balance of gut microbial populations (Harris *et al.*, 2001) and resulting in a better growth performance (Lewis *et al.*, 2003; Adibmoradi *et al.*, 2006). *In vitro* studies have shown that garlic extract has antimicrobial effects, such as antibacterial and antifungal properties (Indu *et al.*, 2006). Numerous studies reported that garlic can be used effectively to inhibit the growth of enteropathogenic bacteria, including 20 different serogroups of *Escherichia coli*, 8 serotypes of *Salmonella*, and *Aeromonas hydrophila* (Johnson and Vaughn, 1969). Earlier *in vitro* studies have shown that garlic extract have strong antibacterial properties against *Escherichia coli*, *Salmonella typhimurium* (Johnson and Vaughn, 1969), *Vibrio cholerae* (Ahsan *et al.*, 1996), *Shigella dysenteriae*, *Shigella flexneri*, *Staphylococcus epidermidis*, *Enterobacter aerogenes* (Arora and Kaur, 1999), *Bacillus subtilis*, *Micrococcus* (Sharma *et al.*, 1977), *Clostridium botulinum* (De Wit *et al.*, 1979), and antifungal properties against *Aspergillus niger*, *Candida albicans* (Yoshida *et al.*, 1987), *C. tropicalis*, *C. acutus*, and *C. inconspicua* (Arora and Kaur, 1999). In a study using broiler chickens, Sarica *et al.* (2005) have investigated that supplementation of 1.0 g/kg garlic meal reduced the concentrations of total aerobic bacteria and *E. coli* in the small intestine.

Supplementation of garlic meal was reported to improve proliferation of absorptive cells in the gut. Dietary garlic supplementation increased villus height, crypt depth and ratio of villus height to crypt depth (Adibmoradi *et al.*, 2006). Yason *et al.* (1987) stated that the crypt can be regarded as the villus factory, and a large crypt indicates fast tissue turnover and a high demand for new tissue. An improvement in either villus height or crypt depth may lead to an increase in nutrient absorption and better utility.

In a product evaluation study, it was reported that supplementing diets of laying hens with 30 g/kg garlic meal did not affect the egg flavour (Birrenkott *et al.*, 2000). However, a study using male Arbor Acre broiler chickens showed that dietary supplementation with 20 g/kg garlic husk or garlic bulb increased the thigh meat hardness and flavour scores (Kim *et al.*, 2009). Similarly, Onibi *et al.* (2009) reported that dietary supplementation of 0.5 g/kg garlic meal increased the garlic aroma but not the palatability of the meat of 7 weeks old Shaver Starbo broiler chickens. These data suggest that supplementing broiler diets with garlic meal can enhance eating quality because sensory panels found that the meat from chickens fed a garlic-supplemented diet had better texture and flavour than the meat from control diets. On the other hand, no severe toxic side effects were reported in these clinical studies, even at high dosages. Other garlic supplements do not have studies of toxicity or safety, and few have any clinical studies to confirm their efficacy (Amagase *et al.*, 2001).

1.3.5.4. Ginger

Ginger (*Zingiber officinale* Roscoe; Zingiberaceae) is widely used as a spice or condiment and medical treatment for certain diseases (Surh, 1999; Habib *et al.*, 2008). Takhtajan (1997) described taxonomic position of ginger as follows:

Class : Liliopsida
Sub Class : Commelinids
Order : Zingiberales
Family : Zingiberaceae
Genus : Zingiber
Species : *Zingiber officinale* Roscoe

As a medicinal plant, it is widely used in Chinese, Ayurvedic and Tibb-Unani herbal medicines all over the world, for a wide array of unrelated ailments that include

inflammatory, pains, sore throats, cramps, constipation, indigestion, dementia, fever and infectious diseases, and anticancer (Shukla and Singh, 2007; Ali *et al.*, 2008). In human studies, phytochemicals in ginger rhizome have been reported to possess anti-cancer (Shukla and Singh, 2007), anti-inflammatory (Habib *et al.*, 2008), anti-oxidative (Masuda *et al.*, 2004), anti-microbial, anti-emetic, anti-hypertensive (vasodilatator), anti-thrombotic, hypoglycaemic, hypolipidemic, hypocholesterolemic, analgesic, and antipyretic (Ali *et al.*, 2008) properties.



Figure 1-2. Ginger (*Zingiber officinale* Roscoe)

Adapted from Köhler *et al.* (1887)

Phytochemicals of importance in ginger include gingerol, gingerdiol, gingerdione (Kikuzaki and Nakatani, 1996), 6-dehydroshogaol, zingerone, curcumene, zingiberene (Agarwal *et al.*, 2001), β -phellandrene, β -sesquiphellandrene, camphene (Ji *et al.*, 1997) in various amounts according to the preparation process (Gupta and Ravishankar, 2005; Nanasombat and Lohasupthawee, 2005). Jolad *et al.* (2005) noted that ginger rhizome

contains a total of 115 biologically active constituents including the main pungent principles, the gingerols and shogaols.

In animal studies, phytochemicals in ginger rhizome have been reported to stimulate growth performance (Ademola *et al.*, 2009), and inhibit the growth of pathogenic bacteria (Smith-Palmer, 1998; Nanasombat and Lohasupthawee, 2005) of broiler chickens. Supplementing diet with ginger meal resulted in a better growth performance, as shown by the improvement to body weight (Ademola *et al.*, 2009; Zhang *et al.*, 2009), improvement of feed intake (Onimisi *et al.*, 2007), reduction of feed conversion ratio (Moorthy *et al.*, 2009), and stimulation of water consumption of broiler chickens (Onimisi *et al.*, 2007).

In vitro studies showed that phytochemical substances in ginger rhizome inhibit the growth of *Campylobacter jejuni*, *Staphylococcus aureus* (Smith-Palmer, 1998), *Listeria monocytogenes* (Thongson *et al.*, 2004; Ekwenye and Elegalam, 2005), *Escherichia coli* (Gupta and Ravishankar, 2005; Nanasombat and Lohasupthawee, 2005), *Salmonella choleraesuis* (Nanasombat and Lohasupthawee, 2005), *Salmonella enteritidis* (Smith-Palmer, 1998; Nanasombat and Lohasupthawee, 2005), *Salmonella typhimurium* DT104 (Thongson *et al.*, 2004; Ekwenye and Elegalam, 2005), and *Klebsiella pneumonia* (Nanasombat and Lohasupthawee, 2005). Moreover, supplementation of 10.0 mg/kg ginger extract has been reported to inhibit the growth of microflora, such as *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, and *Candida albicans* (Jagetia *et al.*, 2003). Phytochemicals in ginger rhizome were also found to have antifungal properties toward various fungi including *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola* (Wang and Ng, 2005).

1.3.5.5. Turmeric

Turmeric (*Curcuma longa* L. or *C. domestica* Val.; Zingiberaceae) is a perennial herb that is widely used and cultivated in the tropical and sub tropical regions of the world, such as in China, Indonesia, India, Malaysia, Jamaica, and Peru (Govindarajan and Stahl, 1980). It belongs to the genus *Curcuma* that consists of hundreds species of plants, such as ginger, curcuma, white-turmeric, black-turmeric, wild-turmeric, mango-ginger, siam-tulip (hidden-ginger), zedoary, cardamom, and galangal. This medicinal plant possesses rhizomes and underground root-like stems (Araújo and Leon, 2001) that had been originally used as a food additive in curries to improve the storage condition, appearance, flavour, palatability,

and preservation of food (Jayaprakasha *et al.*, 2005). Chattopadhyay *et al.* (2004) described taxonomic position of turmeric as follows:

Class : Liliopsida
Sub Class : Commelinids
Order : Zingiberales
Family : Zingiberaceae
Genus : Curcuma
Species : Curcuma *longa*

Turmeric have also been used as a household remedies in Nepal (Eigner and Scholz, 1999), Indonesia (Kirana *et al.*, 2003; Murnigsih *et al.*, 2005), China, and Japan (Cao *et al.*, 2001) to treat various diseases/disorders, such as inflammation, ulcers, fresh wounds, insect stings, dysentery, diabetes, viral infections, liver obstruction, jaundice (Nadkarni, 1976), cough, biliary disorders, hepatic disorder, anorexia, diabetic wounds, rheumatism, sinusitis (Ammon *et al.*, 1992), anaemia, atherosclerosis, oedema, haemorrhoids, hepatitis, indigestion, skin disease, urinary disease, and liver disorders (Chattopadhyay *et al.*, 2004).

Phytochemicals of turmeric rhizomes consist of volatiles and non-volatiles constituents. The major active substances in the non-volatile components are the colouring agent and are a rich source of phenolic compounds, such as curcumin (Roughley and Whiting, 1973), demethoxycurcumin, bisdemethoxycurcumin, and colourless metabolites tetrahydro-curcumin (Huang *et al.*, 1995). The major active substances in volatile oil are curcuminoids (Toennesen, 1992), ar-turmerone (Govindarajan and Stahl, 1980; Ferreira *et al.*, 1992), zingiberene (Smith and Robinson, 1981), turmerone (Govindarajan and Stahl, 1980; Baik *et al.*, 1993), and curlone (Kiso *et al.*, 1983). Curcumin (diferuloylmethane) is the main yellow bioactive component that has a wide spectrum of biological actions, including antioxidant, antibacterial, antifungal, antiprotozoal, antiviral, antiinflammatory, antihypertensive, and hypocholesteremic activities (Chattopadhyay *et al.*, 2004). Curcumin and curcuminoids posses anti-nematocidal (Kiuchi *et al.*, 1993), anti-inflammatory (Mukhopadhyay *et al.*, 1982; Srivastava and Srimal, 1985; Ammon *et al.*, 1993), antioxidative (Ruby *et al.*, 1995; Osawa *et al.*, 1995; Sugiyama *et al.*, 1996), anti-coccidials (Allen and Fetterer, 2002; Abbas *et al.*, 2010), and imunomodulatory (Kumari *et al.*, 2007; Yarru *et al.*, 2009) properties. Araújo and Leon (2001) summarized the

biological properties of curcumin and other phytochemicals as curcumin (anti-bacteria, anti-virus, antioxidant, anti-inflammatory and anti-tumor), ar-turmerone (anti-snakebite), methylcurcumin (anti-bacteria, anti-protozoal against *Leishmania amazonensis*), curcuminoids (anti-protozoal *Plasmodium falciparum*, and *Leishmania major*). Other bioactive compounds also exhibit beneficial effects, such as demethoxy curcumin (antioxidant), bisdemethoxy curcumin (antioxidant), and sodium curcumin (anti-inflammatory).

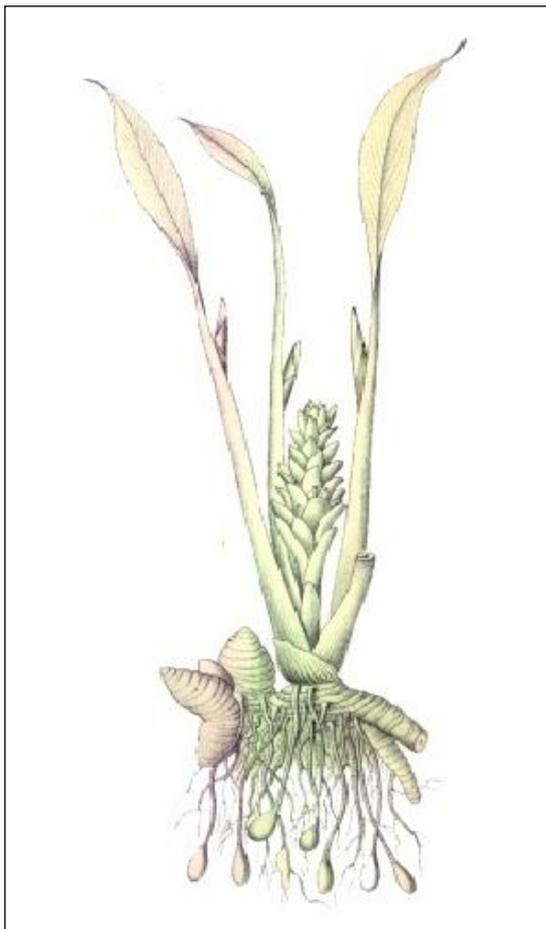


Figure 1-3. Turmeric (*Curcuma longa* L.)

Adapted from Köhler *et al.* (1887)

In a poultry study, Al-Sultan (2003) reported that dietary supplementation of 5.0 g/kg turmeric meal in the diets of broiler chickens improved body weight gain and reduced feed intake, resulted in a better feed conversion ratio. Similar results were found by Durrani *et al.* (2006) that 5.0 g/kg supplementation significantly improved body weight gain and reduced feed consumption of broiler chickens and a better feed conversion ratio. In this study, turmeric meal supplementation also improved carcass quality, reduced fat percentage, increased dressing percentage, as well as improved the weight of breast, thigh,

and gilet. Improvement to body weight gain and carcass quality in these experiments is attributed to the antioxidant activity of turmeric (Osawa *et al.*, 1995; Sugiyama *et al.*, 1996) through stimulation of protein synthesis in the gut by enzymatic system. In Radwan *et al.* (2008) study, it has been investigated that 5.0 g/kg dietary supplementation of turmeric meal significantly reduced feed conversion ratio, improved body weight gain, as well as increased egg production, egg weight, and egg mass of laying hens.

Turmeric is well known to have a property as a safe, natural, and residue free phytobiotics (Wang *et al.*, 1998). The World Health Organization declared turmeric and its yellow coloring agent (curcumin) as safe to be used in human food and animal feed (WHO, 1987). In human and animal studies – so far – turmeric is considered to have low toxicity (Alia *et al.*, 2006), therefore this yellow additive is secure and ideal for poultry. There are no publications as yet that have reported harmful effects of turmeric meal in poultry diets when used at low to moderate concentrations. Consumption of excessive dosage of turmeric is not recommended because it may induce hepatotoxic effect as noted in studies using mice (Kandarkar *et al.*, 1998) and rats (Deshpande *et al.*, 1998). In particular, Al-Sultan and Gameel (2004) recommended not supplementing broiler diets with more than 50.0 g/kg turmeric meal as it contributed on the induction of parenchymal and portal infiltration of mononuclear cells and hyperaemia of portal vessels.

1.3.5.6. Oregano

Oregano (*Origanum* spp.; Lamiaceae) are herb plants member of genus *Origanum*. The genus *Origanum* consists of 38 species widespread in the Mediterranean region, but 75% of them are limited to the eastern Mediterranean area. Some and members of the species are *Origanum scabrum* Boiss. and Heldr, *O. microphyllum* Vogel., and *O. microphyllum*. The most popular species of oregano is *Origanum vulgare* L. and is widely used in food products (Aligiannis *et al.*, 2001). Taxonomic position of oregano is as follows:

Class : Asterids
Sub Class : Euasterids I
Order : Lamiales
Family : Lamiaceae or Labiatae
Sub Family : Nepetoideae
Genus : *Origanum*
Species : *Origanum* Spp.

Many origanum are common as food flavouring as they contain a wide range of volatile secondary metabolites. They have also been used in many places as functional herbal products to preserve food products and to inhibit bacterial and fungal growth on ready-to-cook poultry meat. Marino *et al.* (2001) reported that antibacterial activity of oregano depends on the type, composition, and concentration of the spice or the essential oils, the type and concentration of the target microorganism, the composition of the substrate, the processing, and the storage conditions.



Figure 1-4. Oregano (*Origanum* spp.)

Adapted from König (1888)

There are more than 25 substances presented in oregano (Aligiannis *et al.*, 2001; Bampidis *et al.*, 2005; Karimi *et al.*, 2010), but the most important constituents are the essential oils carvacrol, thymol, γ -terpinene, and ρ -cymene (Sivropoulou *et al.*, 1996). These substances have an important role in the functional properties of oregano (Lee *et al.*, 2003b; Bozkurt *et al.*, 2009b). Carvacrol, an isomer of thymol, is presumed to be responsible for most of the activities of oregano because it is the major phytochemical in the plant (Bampidis *et al.*, 2005). Ouattara *et al.* (1997) described that carvacrol and

thymol account 82.03% of total essential oils in *O. vulgare* and both of them are generally recognized as safe (GRAS) for growth promoter (Ultee *et al.*, 2000).

Phytochemical substances present in oregano are reported to have antimicrobial (Ultee, 1998; Lambert *et al.*, 2001; Marino *et al.*, 2001; Friedman *et al.*, 2002), antifungal (Adam *et al.*, 1998; Daferera *et al.*, 2000; Aligiannis *et al.*, 2001), insecticidal (Karpouhtsis *et al.*, 1998; Lambert *et al.*, 2001), and antioxidative (Lagouri *et al.*, 1993; Vekiari *et al.*, 1993; Lagouri and Boskou, 1996; Milos *et al.*, 2000) properties. Efficacy of oregano as antimicrobials are mainly attributed to carvacrol and thymol (Kim *et al.*, 1995; Lambert *et al.*, 2001; Bampidis *et al.*, 2005), whilst property as antioxidant is attributed to gingerols and shogaols (Kikuzaki and Nakatani, 1993; Kikuzaki *et al.*, 1994).

In-vitro studies have shown that essential oils of oregano exhibited high level of antimicrobial activity against eight strains of Gram-negative and Gram-positive tested bacteria, including two strains of *Staphylococcus aureus* (Sivropoulou *et al.*, 1996). Later study by Dorman and Deans (2000) showed that essential oils of oregano contains a wide range of antimicrobial properties against 25 tested Gram-negative and Gram-positive bacteria, including *Bacillus subtilis*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella pullorum*, *Serratia marcescens*, *Staphylococcus aureus*, and *Yersinia enterocolitica*. Minimum inhibitory concentrations (MICs) of oreganum essential oils range from 100 to 1,000 ppm (Lee *et al.*, 2003b). In the study of Sivropoulou *et al.* (1996) it has been investigated that essential oils of *O. vulgare* ssp. *hirtum* at 1/4000 dilution killed all tested bacteria within 60 minutes after exposure. At 1/50000 dilution, these oils caused significant decrease in bacterial growth rates. Moreover, Kim *et al.* (1995) showed that carvacrol has a high antibacterial effect against *Salmonella typhimurium*, with minimum inhibitory and bactericidal concentrations (MIC and MBC) of 250 µg/mL. Given their antimicrobial activity, it would be expected that thymol and carvacrol could have positive effects on growth performance in broilers.

Poultry studies have shown that oregano has many substantial properties to promote growth performance and health, such as in broiler chickens (Lewis *et al.*, 2003; Demir *et al.*, 2005; Calislar *et al.*, 2009), in broiler turkeys (Bampidis *et al.*, 2005), and in quails (Cetingul *et al.*, 2009). Oregano alone or in combination with other additives improved growth performance of broiler chickens. In Cabuk *et al.* (2006) study, combination between oregano essential oil and other essential oils from 5 herbs reduced feed intake and

improved feed conversion ratio of broiler chickens. Another study by Bozkurt *et al.* (2009b) showed that essential oils of oregano alone or in combination with mannan oligosaccharide (MOS) improved the body weight and feed conversion ratio of male broiler chickens.

Responses of broiler chickens to oregano supplementation seems to be influenced by nutrient composition of the diet (Jamroz *et al.*, 2005), level of supplementation (Alcicek *et al.*, 2003; Ertas *et al.*, 2005), variety of the herb (Halle *et al.*, 2004; Hassan *et al.*, 2004), environmental and sanitary conditions (Jamroz *et al.*, 2005; Karimi *et al.*, 2010), chemical composition of essential oils (Baydar *et al.*, 2004), and interaction with other feed additives (El-Hakim *et al.*, 2009). Chemical composition of essential oils in oregano depends on climatic, seasonal, and geographic conditions, harvest period, and distillation technique (Marino *et al.*, 2001; Baydar *et al.*, 2004).

Efficacy of oregano essential oil as growth promoter in poultry probably is due to its antimicrobial properties. It has been demonstrated that phytochemical in oregano reduced bacterial inhabitants in the intestine, such as *C. perfringens*, *E. coli* (Juneja and Friedman, 2007; Ouwehand *et al.*, 2010), *Streptococcus epidermis*, *Salmonella enterica* serovar. *infantis*, *Salmonella enterica* serovar. *enteritidis*, and *Salmonella enterica* serovar. *typhimurium* (Ouwehand *et al.*, 2010).

1.3.5.7. Cinnamon

Cinnamon (*Cinnamomum zeylanicum* Blume; Lauraceae) is one of the oldest herbal medicines with the botanical synonym *Cinnamomum verum* J.Presl (Tateo and Chizzini, 1989). This herb is more commonly used as a non-essential addition to other remedies than as a remedy by itself. Cinnamon is often used to cover the unpleasant taste of other drugs because it contains several aromatic phytochemicals (Faix *et al.*, 2009). Taxonomic position of cinnamon is as follows:

Class	:	Magnoliopsida
Sub Class	:	Magnoliids
Order	:	Laurales
Family	:	Lauraceae
Genus	:	Cinnamomum
Species	:	<i>Cinnamomum zeylanicum</i>

Cinnamon contains more than 300 volatile compounds (Faix *et al.*, 2009) which are constituents of essential oils, including eugenol, cinnamaldehyde, *trans*-cinnamaldehyde, sesquiterpenes (α -bergamotene and α -copaene), camphor, and diterpenes (Jayaprakasha *et al.*, 1997; Cheng *et al.*, 2004). Composition and quantity of the phytochemicals varies according to the part of the plant in which it is found. Essential oils derived from the leaves are rich in eugenol (about 70% of total volatile), whereas those from the bark are rich in *trans*-cinnamaldehyde (about 75% of total volatile) and those from the buds are rich in sesquiterpenes. Essential oils from the roots are rich in camphor (Senanayake *et al.*, 1978; Jayaprakasha *et al.*, 2002; Jayaprakasha *et al.*, 2003).



Figure 1-5. Cinnamon (*Cinnamomum zeylanicum*)

Adapted from Köhler *et al.* (1887)

Bioactive constituents present in cinnamon, such as cinnamaldehyde, eugenol, and thymol, have been reported by *in vitro* study to inhibit the growth of pathogenic microflora at the concentration range of common antibiotics. Cinnamaldehyde and eugenol have strong antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella*

pneumoniae, *Salmonella typhimurium*, *Vibrio parahaemolyticus* (Chang *et al.*, 2001), *Helicobacter pylori* (Tabak *et al.*, 1999; Hernández *et al.*, 2004), *Bacillus subtilis*, and *Listeria monocytogenes* (Oussalah *et al.*, 2007). *Trans*-cinnamaldehyde also have strong antimicrobial properties toward a wide range of feedborne pathogens, including gram-positive and gram-negative pathogenic bacteria, such as *Salmonella enteritidis* and *Campylobacter jejuni* (Friedman *et al.*, 2002; Johny *et al.*, 2008). Lopez *et al.* (2005) study reported that essential oils of cinnamon inhibit the growth of four Gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Bacillus cereus*), three Gram-negative bacteria (*Escherichia coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis*), and three fungi (a yeast, *Candida albicans* and two molds, *Aspergillus flavus*, *Penicillium islandicum*). Nguyen *et al.* (2009) showed that essential oils of cinnamon inhibit five phytopathogenic fungi *Phytophthora capsici*, *Rhizoctonia solani*, *Fusarium solani*, *Colletotrichum gloeosporioides*, and *Botrytis cinera*. In other studies, it has also been demonstrated that the growth of *Aspergillus flavus* (Hernández *et al.*, 2004), *Saccharomyces cerevisiae*, *Candida albicans*, *Debaromyces hansenii* (Brr and Mahmoud, 2005) were inhibited by essential oils of cinnamon. Antibacterial and antifungal properties of cinnamon are mainly due to its terpenoids content, i.e.: cinnamaldehyde and eugenol (Tabak *et al.*, 1999; Chang *et al.*, 2001; Schmidt *et al.*, 2007; Yen and Chang, 2008; Cheng *et al.*, 2008).

Poultry study have shown that dietary inclusion of 200 ppm cinnamon essential oils improved feed intake, body weight, and feed conversion ratio of 6-weeks broiler chickens (Lee *et al.*, 2003b; Al-Kassie, 2009). Improvement of the growth performance might be due to the bioactive constituents in cinnamon that stimulated enzymatic function of liver and pancreas (Langhout *et al.*, 2000), which increased digestion rate and improved efficiency in the utilization of feed. Perdok *et al.* (2003) reported an appetite and digestion stimulant properties of cinnamon on poultry. Hernández *et al.* (2004) showed that 200 ppm cinnamon inclusion in combination with essential oils from other herbs stimulated ileal digestibility of dry matter and increased apparent digestibility of protein of 6-weeks broiler chickens. Another study showed that 100 ppm cinnamon essential oils could improve the breast weight of broiler chickens (Isabel and Santos, 2009).

Furthermore, it has been reported that the dietary inclusion of cinnamon essential oils reduced plasma triglycerides concentration (Lee *et al.*, 2003b), as well as lowered cholesterol levels of serum (Al-Kassie, 2009), breast and thigh meat (Ciftci *et al.*, 2010) of

broiler chickens. Inclusion of cinnamon essential oils also reduced plasma triglyceride and plasma total cholesterol levels of Japanese quail (Sarica *et al.*, 2009). These results showed that cinnamon oil had hypocholesterolemic properties, which in turn cause improvement on poultry meat quality.

1.3.6. Appropriate Diet Formulation and Ingredient Selection

Selection of ingredient and diet formulation may also stimulate enteric health of poultry. Appropriate feeding strategy is known to improve health status as inclusion of whole grain in the diet, by preventing proventricular dilatation, reducing mortality related to ascites, and improving overall growth performance of broiler chickens (Jones and Taylor, 2001), which may be related to an improvement in digestibility of starch (Hetland *et al.*, 2002). Feeding broilers with barley, which was stored in the airtight containers, improved the metabolizable energy of the grain as compared with dried barley, as well as improved the nutrient digestibility and phosphorus retention in broilers (Perttila *et al.*, 2001), whilst wheat was shown to improve growth performance and feed efficiency (Engberg *et al.*, 2004).

The particle size of ingredients exhibited a strong effect on the physiological function of the broiler gastrointestinal tract. It has been reported that the size of the gizzard increased with an increase in the feed structure size (Nir *et al.*, 1994; Bennett *et al.*, 2002). Increasing particle size in the diet has also been reported to increase acidity of the digesta in the gizzard, as well as an increase acidity of small intestinal pH was observed related to an increased particle size of the grain. Moreover, feeding broiler chickens with mash resulted in a lower population of enterobacteria and higher numbers of lactobacilli and *Clostridium perfringens* in the gut than that of pelleted feed (Engberg *et al.*, 2002).

Some experiments have revealed that dietary factors may also appear to be important in inducing intestinal problems that can lead to the reductions in the intestinal health. The use of energy rich cereal grains in the diets, e.g. wheat, rye, and barley, which are more available in European countries than corn, can reduce health state of the gut by increasing viscosity of the digesta, lengthening intestinal transit time, and increasing incidence of NE. On the other hand, high levels of protein from animal sources also predispose to NE (Dahiya *et al.*, 2006). A study comparing diet types in the formulated diets showed that the difference of diets, physically and chemically, can modify the populations of microorganisms in the gut and the integrity of intestinal epithelium. For instance, feeding

broiler chickens with corn and sorghum-based diets stimulated the growth of *Enterococcus*, barley stimulated *Lactobacillus*, oat stimulated *Escherichia* and *Lactococcus*, and rye stimulated *Streptococcus*. Feeding treatments with another ingredient, such as flaxseed may also impact intestinal microbial colonization (Apajalahti *et al.*, 2004). The excessive increase of the pathogens in the gut will lead to the over stimulation of mucus layer production, which in turn will inhibit the growth and alteration of absorptive cells in the gut, reduce nutrient uptake, lower energy and nutrient utilization, and reduce the growth performance of the birds (Iji and Tivey, 1998; Sklan, 2004). Dietary supplementation of exogenous enzymes in a wheat-soybean based diet improved nutrient and energy utilization of the birds (Olukosi *et al.*, 2007; 2008). In conjunction with dietary enzyme, ileal amino acid digestibility improvement generated by phytase was greater in wheat than corn (Ravindran *et al.*, 1999).

In regards to the effects of diet formulations on the enteric health and growth performance, some studies have shown that intestinal condition and body tissues development of the birds fed wheat-based diets will be more influenced by the NSP content than those of birds fed corn-based diets. The high content of NSP in wheat (Pettersson and Åman, 1989) increases the viscosity of the digesta in the gut, which can cause digestive problems and reduce the health state of the birds (Choct and Annison, 1992a). NSP in the ingested diets increase the digesta passage time in the gut, stimulate pathogens colonization and activity, induce infections and enteric lesion, reduce digestive secretions and nutrient digestibility, reduce feed efficiency, and hence inhibit the growth of the birds (Choct *et al.*, 1996; Jørgensen *et al.*, 1996). Feeding broiler chickens with diet containing high amount of cereals also affect the balance of microflora which in turn will affect intestinal health of the birds. For instance, feeding broiler chickens with barley stimulates the growth and colonization of *Lactobacillus*, while feeding birds with oat or rye stimulates *Escherichia* or *Streptococcus*, respectively (Apajalahti *et al.*, 2004). There are various studies in literature that have reported that outbreak of necrotic enteritis (NE) can be diminished by feeding the birds with high levels of wheat (Kaldhusdal and Skjerve, 1996; Branton *et al.*, 1997), barley (Kaldhusdal and Hofshagen, 1992; Annett *et al.*, 2002), rye (Riddell and Kong, 1992). In a study comparing corn- and wheat-based diets, Untawale *et al.* (1978) reported that corn-based diets reduce the occurrence and severity of NE compared to diets based on wheat, barley, rye or oats. In addition, rye-based diets also increased the number of pathogens in the gut as well as inhibited the growth performance of broiler chickens. The improvement of the growth of the birds might be related to the

improvement of the intestinal health and the nutrient-energy utilization of the birds. In conjunction with the use exogenous enzyme, the nutrient digestibility improvement by phytase was greater in wheat than corn (Ravindran *et al.*, 1999). Furthermore, Wu *et al.* (2004a) reported that feeding broiler chickens with the diets containing whole wheat resulted in the greater improvement on body weight gain, feed efficiency and energy utilization compared to those fed on diets containing ground wheat. However, pre-pelleting inclusion of whole wheat seemed to be useless on relative weight of intestinal organs. Moreover, supplementation of xylanase in the whole wheat-based diets increased the ileal villus height. From those studies it might be hypothesized that in some cases, the use of corn in the formulated diets seems to be more appropriate than wheat in term of the intestinal health maintenance and growth performance improvement of broiler chickens. However, corn is less available than wheat in the European countries (Dahiya *et al.*, 2006).

The sufficient amount of minerals in the formulated diets is also beneficial to improve the enteric health state and overall growth performance of broiler chickens. The adequate supply of minerals or addition of minerals in a form of readily utilisable in the diet is important to increase the capacity of mineral availability for the normal functioning and overall health of poultry (Waldroup, 1974; Adebisi, 2009). Underwood (1999) suggested that phosphorus (P) and calcium (Ca) are the key minerals that are essential in the diets for poultry, most importantly for bone formation and maintenance. However, there may be limitations in regards to the availability of minerals in the diet. For instance, approximately two-thirds of the total P in plant feedstuffs, which are the major constituents of poultry diets, are in the form of phytate P (Punna, 1999). Phytate P (phytate bound P) is a mixed salt of phytic acid in cereal grains, mainly involving Mg, Ca, Na and K, which is poorly digested by poultry digestive system (Selle, 2000). This unavailability is exasperated due to the very low endogenous phytase activity in the digestive tract of poultry (Yu *et al.*, 2004). Some studies suggest that supplementation of exogenous phytase can help improve the P availability for the body requirement (Simons, 1990). Improvement of the P availability in the body will lead to the improvement in the nutrient and energy digestibility, as well as beneficial to recover the growth performance of the birds (Olukosi and Adeola, 2008). Further, calcium uptake in the gut may also be compromised where majority of the P in the diet are present as phytate P because of the importance of maintaining an adequate Ca to P ratio for efficient mineral uptake for greater capacity for growth and body development (Qian *et al.*, 1997). In addition, a study using an avian commercial male broiler chickens by Xia *et al.* (2004) has shown that diet supplementation

with copper-bearing montmorillonite (Cu-MMT) significantly improved the growth performance, reduced the total viable counts of *Escherichia coli* and *Clostridium* in the small intestine and cecum, improved the activities of total protease, amylase, and lipase in the small intestinal contents, as well as improved the intestinal mucosal morphology.

1.4. Aims and Objectives of the Thesis

The overall objective of this study is to investigate nutritional factors that support enteric health of poultry. The uses of organic acids and phytochemicals derived from herbs, spices, or plant extracts in the diets have been observed to improve growth performance, nutrient digestibility, and intestinal health of broiler chickens. Organic acid which used in this study is benzoic acid, while phytochemicals which used are derived from turmeric acid and garlic acid.

The main objectives of this study are:

1. To examine the potential relationship between digesta pH, body weight, and nutrient and energy digestibility of different body weights and at different ages.
2. To investigate the response of broiler chickens to acidified feed using growth performance, nutrient and energy utilization, intestinal acidity, and intestinal histomorphology as response criterion.
3. To investigate the role of organic acids and phytochemicals from herbal product to stimulate enteric health and growth performance of poultry.
4. To examine the possible correlativity among organic acid and herbal products supplementation on the performance characteristics, intestinal pH, gut profile and micropathology of broiler chickens.

2. RELATIONSHIP BETWEEN DIGESTA pH, BODY WEIGHT, AND NUTRIENT UTILISATION IN CHICKENS OF DIFFERENT BODY WEIGHTS AND AT DIFFERENT AGES

2.1. INTRODUCTION

A healthy intestine is one of the most important requirements in enhancing the productivity of broiler chickens. Favourable changes in the intestinal environment may provide a more optimal condition for the bird to more approach its optimum performance level. Reduction of the digesta pH in the gut is associated with the reduction of the growth and colonization rates of intestinal pathogenic microflora (Ferket, 2004) and such a reduction in pH might stimulate growth and proliferation of beneficial species (Partanen *et al.*, 1998; Apajalahti *et al.* 1999), reduce competition for nutrients between intestinal pathogens and the host, stimulate intestinal absorptive cells proliferation (Visek, 1978; Thomke and Elwinger, 1998), as well as trigger pancreatic secretion (Schulz *et al.*, 1971; Dibner and Buttin, 2002). Hinton Jr *et al.* (2000) observed that a lower intestinal pH stimulated the growth of beneficial bacteria and inhibited the growth and colonization of enteropathogens especially *Salmonellae* and *Enterobacterium*. A more favourable intestinal environment will be expected to enhance nutrients uptake by absorptive cells in the gut (Niewold, 2007) and hence engender greater nutrient digestibility (Lewis *et al.*, 2003; Adibmoradi *et al.*, 2006) and also aid in shunting more nutrient towards growth rather than maintenance activities. All of these should result in better growth performances.

Nevertheless, there is a considerable animal to animal variation both in intestinal environment, nutrient utilisation, and consequently growth rate. It is generally expected that birds with greater rates of growth utilized their nutrients more efficiently. There is also the possibility that these birds may have an intestinal environment that is more favourable for proliferation of beneficial microflora. Several studies indicated that variation in growth response of individual birds was closely related to differences in nutrient retention efficiency (Punna and Roland Sr, 1999; Dänicke *et al.*, 2000). It is thought that conditions that favour lower intestinal pH which is usually associated with colonisation by beneficial microbes might also correlate well with greater energy and nutrient utilization efficiency and ultimately growth (Apajalahti *et al.*, 1999; Apajalahti *et al.*, 2004). It is essential to investigate whether such a relationship exists as it will aid in understanding the relationship between intestinal environment, growth rate and nutrient utilization.

The current experiment was designed to relate the variation in digestive tract milieu using pH as an index in two groups of birds to their growth performance and nutrient utilization.

2.2. MATERIALS AND METHODS

2.2.1. *Birds and Housing*

A total of 90 male one-day old broiler chickens (Ross 308) were used in this study. All animal experiment work was approved by the SAC Animal Experimentation Committee.

All the chicks were raised together from day old in a floor pen (200 cm x 200 cm) equipped with feeders and bell drinkers until day 10 when the grouping of the birds began as detailed below. The pens and individual metabolism crates were situated in a poultry house with facilities to control temperature, light, and humidity. Temperature was gradually reduced from 32°C on day 1 to 20°C on day 42. The chicks were vaccinated at the hatchery against Infectious Bursal Disease and no additional vaccinations were administered during the study neither were coccidiostat or enzymes added to the diet. Exogenous enzymes were not added in the diets in order to remove confounding effect of increased nutrient availability from enzyme supplementation which in itself usually produce changes in performance and sometimes gut environment.

2.2.2. *Diets*

A single starter and grower wheat-soybean meal based diets based (Table 2-5) were used for all the birds. The diets were formulated to meet the Ross 308 specifications for each of the rearing phases. The starter (23% CP; 3100 kcal ME per kg) and grower (21% CP; 3200 kcal ME per kg) diets were given from 1 to 10 and from 11 to 42 days old, respectively. Titanium dioxide (0.3 %) was added to all diets as an indigestible marker. Water and feed were offered *ad libitum*.

2.2.3. *Grouping of Birds and Sampling Procedures*

On day 10, all the birds were individually weighed, wing-tagged, and divided into 3 batches of 30 birds each with all the batches having the same average body weight (241.35±4.70 g). One batch of 30 birds were used for the study starting on day 10, the

second batch of 30 birds were used for the study starting on day 15 whereas the last batch of 30 birds were used for the study starting on day 29.

The first batch of 30 birds was further divided to two groups of 10 birds each. The grouping of the birds to two groups of 10 was done by ranking the birds according to weight and choosing the 10 heaviest and the 10 lightest birds, the 10 birds with median weights were not used for the study. The 10 lightest birds were allocated to the light (L) group and the 10 heaviest birds were allocated to the heavy (H) group. The birds were individually housed in metabolism crates and provided with grower diet and water for *ad lib* consumption. On days 13 to 14, total excreta voided were collected from each individual bird for nutrient utilisation evaluation. On day 15, the birds and feed were weighed and the birds were subsequently euthanized by an overdose of intravenous sodium pentobarbital injection.

The pH of the digesta from 4 different intestinal segments namely crop, mid jejunum, mid ileum, and mid right caeca was determined immediately by inserting the needle tip of an Orion 9863BN pH meter (Micro-pH Electrode with 1.7 mm tip diameter) into the specific GIT sites. Two independent pH readings were taken from each site. The ileal content from all the birds were removed by gently flushing with distilled water.

On days 24 and 38 respectively, the same procedures as were done on day 10 were carried out for the second and third batches of 30 birds, respectively. For the second batch, excreta collection was done on days 27 and 28, and the birds were euthanized on day 29. Whereas for the third batch, excreta were collected on days 41 and 42, and the birds were also euthanized on day 43.

The diets, ileal digesta and excreta were analysed for Ti, dry matter, energy and nitrogen to determine ileal and total tract nutrient and energy utilisation.

2.2.4. Calculations and Statistical Analyses

The dry matter (DM), organic matter (OM), N, and gross energy (GE) values were determined in samples of diet, ileal digesta, and excreta. DM content was determined by drying the samples in the oven (Uniterm, Russel-Lindsey Engineering Ltd., Birmingham, England, UK) at 105°C for 24 hours (Method 934.01; AOAC, 2006). Organic matter was determined by ashing the samples in the muffle furnace (Carbolite Furnace, Bamford,

Table 2-5. Ingredient composition (g/kg, as-fed basis) and calculated nutrient and energy content of the diets used in the study

Item	Starter	Grower
<i>Ingredients composition, g/kg</i>		
Wheat, White	633.5	650.0
Gluten meal	17.0	26.5
Soybean oil	24.0	41.5
Soybean meal -48%	215.5	200.0
Casein	70.0	39.0
Limestone	17.5	17.5
Dicalcium phosphate ¹	13.0	13.0
Common salt	3.5	3.5
Vitamin/mineral premix ²	3.0	3.0
DL-Methionine	1.5	1.5
L-Lysine HCl	1.5	1.5
Titanium dioxide marker ³	0.0	3
Total	1000.0	1000.0
<i>Calculated nutrients and energy</i>		
Protein, g/kg	242.99	216.02
ME, kcal/kg	3076.70	3151.67
Ether Extract, g/kg	38.54	56.17
Ash, g/kg	23.97	23.82
Calcium, g/kg	10.76	10.53
Total Phosphorus, g/kg	6.52	6.21
Ca:P	1.65	1.70
Potassium, g/kg	6.98	6.77
Sodium, g/kg	1.71	1.71
Lysine, g/kg	15.22	12.43
Methionine, g/kg	6.04	5.33
Threonine, g/kg	9.40	8.01
Isoleucine, g/kg	11.00	9.46
Arginine, g/kg	12.89	11.48
Valine, g/kg	12.56	10.57
Tryptophan, g/kg	3.13	2.74

¹Contained 21.3% Ca, 18.7% P

²Vitamin A, 16,000 IU; vitamin D₃, 3,000 IU; vitamin E, 25 IU; vitamin B₁, 3 mg; vitamin B₂, 10 mg; vitamin B₆, 3 mg; vitamin B₁₂, 15 µg; hetra, 5 mg; nicotinic acid, 60 mg; pantothenic acid, 14.7 mg; folic acid, 1.5 mg; Biotin, 125 µg; choline chloride, 25 mg; iron, 20 mg; copper, 10 mg; manganese, 100 mg; cobalt, 1.0 mg; zinc, 82.222 mg; iodine, 1 mg; selenium, 0.2 mg; molybdenum, 0.5 mg

³Prepared as 3 g titanium dioxide (TiO₂) added to 12 g gluten meal.

Sheffield, England, UK) at 500°C overnight (Method 934.01; AOAC, 2006). Total nitrogen content was determined by combustion method (Method 968.06; AOAC, 2006). Gross energy value was determined in an adiabatic oxygen bomb calorimeter using benzoic acid as an internal standard (Model 6200, Parr Instruments, Moline, Illinois, USA).

The ileal digestibility was calculated using the index method, with Ti as an indigestible marker. Ileal digestible and metabolizable energy were calculated by multiplying ileal energy digestibility and energy metabolizability with feed gross energy. Ti concentration in samples of diets and ileal digesta were determined for index method digestibility calculations as per method of Short *et al.* (1996).

The apparent ileal nutrient digestibility or total tract nutrient retention (ND) was calculated using the following relation:

Equation 2-1. Formula for calculating apparent ileal nutrient digestibility

$$ND = \left\{ 1 - \left(\frac{C_i}{C_o} \times \frac{N_o}{N_i} \right) \right\} \times 100\%$$

where ND = nutrient digestibility (%); C_i and C_o = concentration of Ti in the diet and digesta or excreta (%), respectively; N_i and N_o = concentration of nutrient in the diet and digesta or excreta (%), respectively.

The apparent ileal digestible energy (IDE) or apparent metabolizable energy (AME) was calculated using this relation:

Equation 2-2. Formula for calculating apparent ileal digestible energy or apparent metabolizable energy

$$IDE \text{ or } AME = \left\{ GE_i - \left(GE_o \times \frac{C_i}{C_o} \right) \right\}$$

where GE_i = gross energy (kcal/kg) in the diet; GE_o = gross energy (kcal/kg) in the ileal digesta or excreta; C_i and C_o = concentration of Ti in the diet and digesta or excreta (%), respectively.

The apparent total tract nutrient dry matter (DMR) or nitrogen (NR) retention was calculated using following formula:

Equation 2-3. Formula for calculating apparent total tract nutrient retention

$$DMR = \left(\frac{DM_i - DM_o}{DM_i} \right) \times 100\% \quad \text{or} \quad NR = \left(\frac{N_i - N_o}{N_i} \right) \times 100\%$$

where DM_i and DM_o = DM content in the diet and excreta (%), respectively; N_i and N_o = nitrogen content in the diet and excreta (%), respectively.

Nitrogen-corrected AME (AMEn) value was calculated by correction for zero nitrogen retention using 8.22 kcal per g nitrogen retained in the body as described by Larbier and Leclercq (1992).

All the data for each group were compared using T-test. The strength of the relationship between the response criteria was assessed using correlation analyses. GenStat 11th edition (2008) Software was used for all statistical analysis. All statements of significance are based on a probability of less than 0.05.

2.3. RESULTS

2.3.1. Growth Performance

The data for the growth performance in different growth phases are presented in Table 2-6. In all the phases, the birds in H group consumed more feed ($P < 0.05$) and had higher final body weight ($P < 0.05$) than the birds in L group. During the starter and grower phases, the birds in H group gained weight at a faster rate (30% and 53% for starter and grower phases, respectively) than those in the L group. Weight gain was similar for both groups during the Finisher phase and gain:feed was no different for the two groups at any phase during the experiment.

2.3.2. Digesta pH

Table 2-7 shows the data for digesta pH in different sections of the digestive tract of the broiler chickens at different ages. During the starter and grower phases, the birds in the H group had lower cecal content pH ($P < 0.05$) than the birds in L group. The pH of digesta in proventriculus was lower ($P < 0.05$) for birds in the H group during the grower and

Table 2-6. Growth performance of broiler chickens in different growth stages (starter, grower, finisher) on as-fed basis¹

Item	Groups		Statistics	
	L	H	SEM	<i>P</i> -value
Starter phase (day 10 to 14)				
Weight gain, g	134.3	174.0	9.41	0.008
Feed Intake, g	282.4	329.0	7.88	<0.001
Gain:feed, g/kg	472.5	528.6	31.01	0.222
Final weight, g	278.4	353.1	11.59	<0.001
Grower phase (day 15 to 28)				
Weight gain, g	332.1	509.6	21.15	<0.001
Feed Intake, g	742.3	980.9	29.17	<0.001
Gain:feed, g/kg	462.9	534.6	29.68	0.122
Final weight, g	958.8	1432.1	25.54	<0.001
Finisher phase (day 29 to 42)				
Weight gain, g	697.1	771.8	49.88	0.304
Feed Intake, g	1304.8	1519.4	65.05	0.031
Gain:feed, g/kg	526.8	507.2	17.17	0.435
Final weight, g	2232.4	3181.2	60.61	<0.001

¹Data represent means from 10 replicates per treatment.

Table 2-7. Intestinal pH of broiler chickens in different growth stages¹

Item	Groups		Statistics	
	L	H	SEM	<i>P</i> -value
Starter phase (day 0 to 14)				
Crop	4.887	4.909	0.06	0.842
Jejunum	6.215	5.573	0.44	0.328
Ileum	7.369	7.259	0.05	0.143
Caeca	6.486	6.242	0.07	0.016
Grower phase (day 15 to 28)				
Crop	4.985	4.801	0.07	0.089
Proventriculus	2.552	2.112	0.15	0.049
Jejunum	5.323	5.200	0.06	0.146
Ileum	6.413	6.340	0.11	0.636
Caeca	6.006	5.772	0.07	0.021
Finisher phase (day 29 to 42)				
Crop	5.282	4.947	0.10	0.033
Proventriculus	3.835	2.844	0.25	0.012
Jejunum	6.196	5.931	0.10	0.088
Ileum	7.463	7.108	0.21	0.245
Caeca	6.798	6.830	0.06	0.688

¹Data represent means from 10 replicates per treatment.

Table 2-8. Apparent ileal nutrient digestibility and digestible energy of broiler chickens in different growth stages on DM basis¹

Item	Groups		Statistics	
	L	H	SEM	P-value
Starter phase (day 0 to 14)				
DMD, %	55.4	54.3	2.39	0.736
ND, %	83.7	83.7	0.36	0.992
ED, %	60.6	60.2	2.25	0.912
IDE, kcal/kg	2393	2365	95.32	0.834
Grower phase (day 15 to 28)				
DMD, %	68.2	73.1	1.49	0.031
ND, %	84.3	84.9	0.32	0.448
ED, %	73.2	76.3	1.49	0.162
IDE, kcal/kg	2922	3052	64.38	0.171
Finisher phase (day 29 to 42)				
DMD, %	70.3	67.5	0.53	0.002
ND, %	80.8	75.7	0.66	<0.001
ED, %	72.0	69.1	0.70	0.008
IDE, kcal/kg	2890	2771	29.43	0.011

¹Data represent means from 10 replicates per treatment. DMD is ileal dry matter digestibility; ED is energy digestibility; IDE is ileal digestible energy.

Table 2-9. Apparent ileal nutrient digestibility and digestible energy of broiler chickens in different growth stages on intake basis¹

Item	Groups		Statistics	
	L	H	SEM	P-value
Starter phase (day 0 to 14)				
DMD, g/bird/day	26.57	31.74	1.831	0.061
ND, g/bird/day	3.11	3.78	0.129	0.002
IDE, kcal/bird/day	261.64	317.01	16.136	0.026
Grower phase (day 15 to 28)				
DMD, g/bird/day	78.92	107.97	6.025	0.004
ND, g/bird/day	7.48	9.72	0.465	0.004
IDE, kcal/bird/day	765.47	1015.64	55.324	0.006
Finisher phase (day 29 to 42)				
DMD, g/bird/day	143.36	166.45	5.267	0.007
ND, g/bird/day	12.81	14.46	0.465	0.023
IDE, kcal/bird/day	1324.32	1536.06	48.437	0.007

¹Data represent means from 10 replicates per treatment. DMD is ileal dry matter digestibility; ND is nitrogen digestibility; IDE is ileal digestible energy.

Table 2-10. Apparent total tract nutrient retention and metabolizable energy of broiler chickens in different growth stages on DM basis¹

Item	Groups		Statistics	
	L	H	SEM	P-value
Starter phase (day 0 to 14)				
DMR, %	64.1	64.2	0.69	0.973
NR, %	55.7	59.7	2.24	0.255
EM, %	66.7	66.0	2.11	0.815
AME, kcal/kg	3009	2977	95.18	0.815
AMEn, kcal/kg	2868	2848	95.95	0.885
Grower phase (day 15 to 28)				
DMR, %	71.0	66.0	2.25	0.136
NR, %	72.3	60.6	2.74	0.008
EM, %	71.3	67.6	2.16	0.248
AME, kcal/kg	3214	3050	97.16	0.248
AMEn, kcal/kg	3125	2923	102.46	0.182
Finisher phase (day 29 to 42)				
DMR, %	74.2	67.3	1.84	0.016
NR, %	65.1	54.6	2.66	0.013
EM, %	76.7	70.6	1.81	0.030
AME, kcal/kg	3458	3183	81.81	0.030
AMEn, kcal/kg	3347	3039	89.80	0.027

¹Data represent means from 10 replicates per treatment. DMR is total tract dry matter retention; NR is total tract nitrogen retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen corrected AME.

Table 2-11. Correlation coefficient values of intestinal pH in different growing phases and nutrient – energy utilization of broiler chickens¹

Items	DMD	ND	ED	IDE	DMR	NR	EM	AME	AMEn
Starter phase									
Crop	0.135	-0.007	0.132	0.118	0.225	-0.212	0.158	0.158	0.140
Jejunum	0.450	0.089	0.537	0.554	0.098	-0.159	0.093	0.093	0.080
Ileum	-0.027	-0.153	-0.096	-0.084	0.305	-0.034	0.290	0.290	0.286
Caeca	0.166	0.024	0.158	0.145	0.236	-0.166	0.164	0.164	0.150
Grower phase									
Crop	-0.245	-0.194	-0.256	-0.249	-0.094	-0.004	-0.130	-0.130	-0.122
Jejunum	0.158	0.200	0.237	0.247	0.298	0.228	0.317	0.317	0.319
Ileum	0.403	0.354	0.486	0.492	-0.064	-0.265	0.031	0.031	0.002
Caeca	0.495	-0.030	0.488*	0.468*	0.553*	0.193	0.553*	0.553*	0.536*
Finisher phase									
Crop	0.499*	0.518*	0.405	0.390	0.109	0.112	0.095	0.095	0.097
Jejunum	0.278	0.256	0.204	0.203	0.563*	0.597*	0.531*	0.531*	0.540*
Ileum	0.189	0.159	0.002	0.008	-0.163	-0.088	-0.185	-0.185	-0.176
Caeca	-0.048	-0.121	-0.143	-0.128	-0.175	-0.097	-0.185	-0.185	-0.177

¹DMD is ileal dry matter digestibility; ND is nitrogen digestibility; ED is energy digestibility; IDE is ileal digestible energy; DMR is total tract dry matter retention; NR is nitrogen retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen-corrected AME.

*Significantly correlated (P < 0.05)

Table 2-12. Correlation coefficient values of intestinal pH in different growing phases and nutrient – energy utilization in light broiler chickens (L group)¹

Items	DMD	ND	ED	IDE	DMR	NR	EM	AME	AMEn
Starter phase									
Crop	0.251	0.031	0.206	0.190	0.319	-0.303	0.250	0.250	0.213
Jejunum	-0.182	0.152	-0.144	-0.102	-0.260	0.130	-0.326	-0.326	-0.304
Ileum	-0.120	-0.288	-0.199	-0.228	0.055	-0.411	0.116	0.116	0.072
Caeca	0.328	0.058	0.287	0.271	0.338	-0.221	0.250	0.250	0.221
Grower phase									
Crop	-0.201	0.121	-0.306	-0.282	-0.432	-0.372	-0.410	-0.410	-0.415
Jejunum	0.337	0.027	0.404	0.395	0.615	0.396	0.610	0.610	0.608
Ileum	0.114	-0.137	0.124	0.113	0.260	-0.141	0.364	0.364	0.340
Caeca	0.701	-0.259	0.771*	0.753*	0.863*	0.739*	0.804*	0.804*	0.815*
Finisher phase									
Crop	0.484	0.572	0.470	0.436	0.031	0.041	0.012	0.012	0.015
Jejunum	0.524	0.627	0.741*	0.756*	0.759*	0.801*	0.705*	0.705*	0.723*
Ileum	0.280	0.112	0.056	0.041	0.002	-0.006	-0.008	-0.008	-0.008
Caeca	-0.118	-0.115	-0.139	-0.087	0.210	0.226	0.186	0.186	0.192

¹DMD is ileal dry matter digestibility; ND is nitrogen digestibility; ED is energy digestibility; IDE is ileal digestible energy; DMR is total tract dry matter retention; NR is nitrogen retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen-corrected AME.

*Significantly correlated (P < 0.05)

Table 2-13. Correlation coefficient values of intestinal pH in different growing phases and nutrient – energy utilization in heavy broiler chickens (H group)¹

Items	DMD	ND	ED	IDE	DMR	NR	EM	AME	AMEn
Starter phase									
Crop	0.209	-0.105	0.306	0.322	0.067	-0.106	0.001	0.001	-0.002
Jejunum	0.534	0.093	0.645*	0.656*	0.153	-0.446	0.126	0.126	0.116
Ileum	-0.053	-0.159	-0.116	-0.107	0.447	0.308	0.398	0.398	0.411
Caeca	0.033	0.129	0.172	0.178	-0.020	-0.104	-0.038	-0.038	-0.041
Grower phase									
Crop	-0.127	-0.159	-0.187	-0.181	-0.312	-0.254	-0.338	-0.338	-0.344
Jejunum	0.455	0.391	0.396	0.409	-0.069	-0.105	0.017	0.017	0.004
Ileum	0.616	0.512	0.652	0.657	-0.327	-0.385	-0.225	-0.225	-0.255
Caeca	0.491	0.394	0.428	0.422	-0.172	-0.163	-0.138	-0.138	-0.148
Finisher phase									
Crop	0.258	0.516	0.344	0.291	-0.042	-0.140	-0.034	-0.034	-0.044
Jejunum	-0.381	-0.395	-0.291	-0.291	-0.224	-0.132	-0.221	-0.221	-0.214
Ileum	0.500	0.605	0.667	0.677	0.419	0.396	0.453	0.453	0.449
Caeca	0.173	0.325	0.035	0.005	-0.387	-0.366	-0.371	-0.371	-0.372

¹DMD is ileal dry matter digestibility; ND is nitrogen digestibility; ED is energy digestibility; IDE is ileal digestible energy; DMR is total tract dry matter retention; NR is nitrogen retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen-corrected AME.

*Significantly correlated (P < 0.05)

finisher phases. During the finisher phase, the crop digesta pH was lower ($P < 0.05$) for birds in the H group whereas jejunum digesta pH tended ($P < 0.10$) to be lower for birds in the H group. Proventriculus digesta pH during grower phase also tended ($P < 0.10$) to be lower for birds in H group. Digesta pH in the crop was similar during the grower phase, while digesta pH in the caeca was similar during finisher phase. There was no difference found in the ileal digesta pH between both groups at any phase during the experiment, neither was in the jejunum digesta pH in the starter nor grower phases.

2.3.3. Energy and Nutrient Utilization

The apparent ileal nutrient digestibility and ileal digestible energy of the broiler chickens at different phases in the current study are presented in Table 2-8. In the starter phase, there was no difference in ileal nutrient and energy utilisation between the groups. During the grower phase, the ileal dry matter digestibility was 7 % lower ($P < 0.05$) for birds in L group. In finisher phase, the birds in L group had greater ($P < 0.01$) ileal digestibility of DM, nitrogen and energy. However, calculations based on digestible nutrient and energy intake (Table 2-9) showed that birds in H group have greater ileal digestibility of nitrogen ($P < 0.01$) and energy ($P < 0.05$) in starter phase. In grower phase, birds in H group have greater ($P < 0.01$) ileal digestibility of DM, nitrogen, and energy. Similarly, birds in H group have greater higher digestibility of DM ($P < 0.01$), nitrogen ($P < 0.05$), and energy ($P < 0.01$).

The apparent total tract nutrient retention, energy metabolizability and metabolizable energy content of the diets for the broilers at different growth stages are presented in Table 2-10. There were no differences in total tract nutrient and energy utilisation between the two groups in starter phase. However, during the grower phase, birds in L group had greater ($P < 0.01$) total tract N retention. During the finisher phase, the birds in L group had greater ($P < 0.05$) total tract nutrient retention and energy metabolizability than the H group birds. Similarly, both the AME and AMEn were greater ($P < 0.05$) for birds in L group.

Table 2-11 shows the correlation coefficients between intestinal pH and nutrient or energy utilization of broiler chickens at different growth phases. Caecal pH in grower phase was positively correlated with the total tract nutrient and energy retention ($r = 0.553$) as well as apparent ileal digestibility of energy ($r = 0.488$) and ileal digestible energy ($r = 0.468$). In the finisher phase, crop pH was positively correlated with ileal DM digestibility

($r = 0.499$) and ileal N digestibility ($r = 0.518$). Jejunum pH in finisher phase was positively correlated with total tract N retention ($r = 0.597$), total tract DM retention ($r = 0.563$), energy metabolizability ($r = 0.531$), and AMEn ($r = 0.540$). Correlation coefficients between intestinal pH and nutrient or energy utilization of broiler chickens at different growth phases in light and heavy birds are presented in Table 2-12 and 2-13, respectively. In light birds (L group), caecal pH in grower phase was positively correlated with the energy digestibility ($r = 0.771$), ileal digestible energy ($r = 0.753$), dry matter retention ($r = 0.863$), nitrogen retention ($r = 0.739$), energy metabolizability ($r = 0.804$), as well as AME ($r = 0.804$) and AMEn ($r = 0.815$), whereas jejunal pH in finisher phase was positively correlated with the energy digestibility ($r = 0.741$), ileal digestible energy ($r = 0.756$), dry matter retention ($r = 0.759$), nitrogen retention ($r = 0.801$), energy metabolizability ($r = 0.705$), as well as AME ($r = 0.705$) and AMEn ($r = 0.723$). On the other hand, in heavy birds (H group), jejunal pH in starter phase was positively correlated with energy digestibility ($r = 0.645$) and ileal digestible energy ($r = 0.656$).

2.4. DISCUSSION

The objective of the current study was to investigate the relationship between digesta pH, body weight and nutrient utilisation on broiler chickens of different body weights and at different ages. Because the birds were of the same breed and received the same experimental conditions, the different growth rates reflected only the differences in individual genetic potential as well as environmental factors not caused by experiment. In addition, the birds in both the L and H groups received the same diet and hence differences in body weight was not related to nutrient provision but could be related to factors such as intestinal environment which may influence digestive efficiency. This provides an appropriate model for studying how differences in growth rate relate to the nutrient utilisation and gut environment of individual bird.

In general, broiler chickens in H group consumed more feed and grew faster than the birds in L group but only during the starter and grower phases. The lack of difference in growth rates between the two groups at the finisher phase may be because the birds were approaching the limit of body development. Larbier and Leclercq (1992) noted that broiler chickens eat to fulfil their daily requirements. In line with this, birds with greater body weight will be expected to consume more feed first because they will be expected to have greater maintenance requirement and secondly because of the need to sustain a more superior rate of gain. The higher feed intake and the faster growth rate for the birds in H

group in the current study may be attributed to the greater effectiveness of the birds in H group to digest, absorb and utilise dietary nutrients, to meet requirements for maintenance and growth. This supposition is supported by greater ileal DM digestibility in the H group during the grower phase. Nevertheless, the heavier birds are not necessarily more efficient at nutrient utilisation because the total tract retention was consistently lower for the birds in the H group in the current study from the grower phase onwards.

It should be noted though, that the birds in H group retained more nutrients when expressed in absolute terms, primarily because they had greater feed or nutrient intake, but when nutrient retention is expressed as a percentage of nutrient intake, the birds in H group were less efficient than the birds in the L group. The greater digestibility and retention values of the birds in L group are likely a compensatory mechanism to account to counteract their lower level of feed or nutrient intake (Zubair and Leeson, 1996). Furthermore, the superior growth performance of the birds in H group could be attributed to the possible greater capacity for secretion of digestive enzymes. As indicated previously, the birds in H group retained more nutrients and energy than the birds in L group in spite of greater digestibility and retention values of the birds in the L group. These could be attributed to a greater availability of enzymes in the gastrointestinal tract. As indicated by Meng *et al.* (2005) in a study using male Arbor Acres broiler chicken, birds utilized more nutrient and energy when their digestion and metabolism processes are augmented by available enzymes. Availability of digestive enzymes in the small intestine will facilitate more rapid and extensive digestion of starch, protein, and other nutrients, making more nutrients and energy available for the growth of the birds at the critical growing stages (Olukosi *et al.*, 2007). Pettersson and Åman (1989) indicated that improvement in digestion process by nutrient-degrading enzymes enhanced the micronutrient availability for absorption, which in turn stimulated better growth performance. The adequacy of the complex nutrient-degrading enzymes in the digestive tract is a requisite for adequate nutrient utilization. Sufficient amount of complex digestive enzymes in the gut is crucial for enhancing caecal fermentation (Choct *et al.*, 1996) and in increasing the energy and nutrient retention including retention of amino acids (Ravindran *et al.*, 1999; Cowieson *et al.*, 2004; Onyango *et al.*, 2005), nitrogen (Ravindran *et al.*, 2000; Olukosi *et al.*, 2008), and minerals (Broz *et al.*, 1994; Viveros *et al.*, 2002; Olukosi *et al.*, 2007). Enhancement of the utilisation of all the above likely resulted in improvements in feed efficiency and rate of weight gain of the birds in H group. There were no measures of enzyme activities in

the current study, but the greater values for digestible and retained nutrients for birds in H group suggests a greater enzymatic activities in these birds.

In the current study, there were differences in the pH of the digesta contents between the H and L groups. Generally, the digesta for the heavier birds had lower pH values. Angel *et al.* (2010) studied the pH in different gastrointestinal sections at different growing stages and reported that intestinal pH changes with GIT section and age. As Hinton Jr *et al.* (1990) pointed out, a reduction of the pH in the gut is beneficial as a defence mechanism against the growth and proliferation of pathogens in the gut of broilers chickens. The lower pH in the digestive tract is also beneficial because it provides a more optimal intestinal milieu for the growth and colonization of beneficial microflora to the exclusion of the pathogenic organisms. Some studies have reported that reduction of the intestinal pH reduced the growth and population of undesirable microorganisms such as *Escherichia coli* (Izat *et al.*, 1990; Józefiak *et al.*, 2010), *Salmonella typhimurium* (Hume *et al.*, 1993), *S. enteritidis* (Iba and Berchieri, 1995; Chotikatunon *et al.*, 2009), *S. gallinarum* (Hernández *et al.*, 2006), *S. enterica* (Friedman *et al.*, 2003), *Clostridium perfringens* (Roy *et al.*, 2002; Van Immerseel *et al.*, 2004), *Campylobacter jejuni* (Chaveerach *et al.*, 2004), *Listeria monocytogenes* (Friedman *et al.*, 2003). Reduction in the population of entero-pathogens in the intestine might indirectly optimize mitotic division of the cells that have responsibility for micronutrient absorption (Xia *et al.*, 2004; Pelicano *et al.*, 2005). The mechanism should result in a more efficient digestion and reduced maintenance energy requirement (Apajalahti *et al.*, 2004).

Dibner and Buttin (2002) pointed out that reduction of these pathogens might beneficially reduce toxins produced by these organisms, which in turn reduce enteric problems and result in a more conducive gut structure. Therefore, it can be expected that birds with lower intestinal pH have a healthier intestinal environment that is more conducive for optimal performance. In the current study, birds in H group had lower digesta pH in the crop, proventriculus, and caeca when compared to the birds in L group. Digesta pH in crop and caeca of the birds in H group were 4.80 – 4.95 and 5.77 – 6.83, respectively, which were less suitable for the growth and population of *Salmonella*, *E. coli*, and *Campylobacter* species (Banwart, 1979; Foster and Hall, 1991; Murphy *et al.*, 2006). Therefore, the faster growth in the heavier birds in recent study might be related to the lower pH in the digestive tract that inhibited the growth and colonization of enteropathogens. Reduction of pathogenic species alongside the reduction of toxic

metabolites should allow better nutrient utilization for growth (Lewis *et al.*, 2003). Indeed, the birds in H group had a more superior growth performance which lends support to that argument.

In the current experiment, the pH and energy-nutrient digestibility were determined at 3 different growth phases. There was no difference found in the energy and nutrient digestibility during starter phase. In the grower phase, birds in H group had higher apparent ileal DM digestibility when compared to L group. In finisher phase, birds in H group retained lower energy and nutrient than birds in L group, but in absolute values, the birds in H group retained greater amounts of energy and nutrient as they had greater energy and nutrients consumption. Consequently, the lower nutrient utilisation in the H group is an indication of diminishing return on energy utilisation (Kuhi *et al.*, 2009; Kuhi *et al.*, 2011). The data from the current study also suggest that this principle of diminishing returns on energy and nutrients utilisation becomes more apparent as the birds grow older. On the other hand, birds in H group had greater protein efficiency ratio and energy efficiency ratio (data not shown) when compared with the birds in L group thus showing that the birds in the H group were not less efficient in comparison to the birds in L group with regards to utilising protein and energy for growth. The greater efficiency in nutrient and energy utilisation for growth in H group might be also attributed to the difference of the digesta pH between both groups. As mentioned previously, birds in H group had lower pH than L group. This low pH should create better intestinal milieu for the birds, such as depression in the growth and colonization of pathogenic bacteria and reduction in the competition for available nutrients between bacteria and the host animal (Thomke and Elwinger, 1998; Izat *et al.*, 1990; Hume *et al.*, 1993; Roy *et al.*, 2002). Reduction in competition for nutrients in birds with a more optimal intestinal environment will reduce maintenance nutrient and energy costs in such birds thus ensuring that more of the nutrients they consume are available for productive purposes.

In view of the possible relationship between pH and growth and nutrient utilisation, the relationship between individual variations in growth rate, pH and nutrient utilisation was investigated. In the current study there was no correlation between intestinal pH and the apparent ileal digestibility of nitrogen and total tract nitrogen retention during the starter phase. Nevertheless, the caecal pH in grower phase had positive correlation with the apparent ileal digestible energy, as well as with the total tract dry matter retention, energy metabolizability, and apparent total tract metabolizable energy. In finisher phase, crop pH

had positive correlation with ileal dry matter and nitrogen digestibility. Similarly, jejunum pH in this phase had positive correlation with total tract dry matter and nitrogen retention, as well as with the apparent metabolizable energy. However, when the data from both L and H groups were calculated separately, results showed that caecal pH in grower phase and jejunal pH in finisher phase of light birds (L group) had positive correlation with the energy digestibility, ileal digestible energy, dry matter and nitrogen retention, energy metabolizability, as well as the AME and AMEn. In the H group, jejunal pH of birds in H group had positive correlation with energy digestibility and ileal digestible energy. Consequently, it seems that the main driver for the correlation between pH and energy utilization when both population (L and H) were combined was the correlation between pH and energy utilization in the L group.

There are no available data to compare the current results with, but we are able to demonstrate for the first time that differences in intestinal environment in individual birds only explains about 55% of the variability in nutrient utilisation. This therefore implies that nearly half of individual variability in nutrient utilisation and growth can not be explained by the factors that we investigated in the current study. It is likely that other factors not accounted for in our study that may explain the variability in response are heterogeneity in individual genetic potential, micro-environmental factors and possible associative interactions between the two (Brockmann *et al.*, 2000; McKay *et al.*, 2000). Deeb and Cahaner (2001) noted that broiler line with genetic propensity for high growth rate genetic potential appears to be relatively more sensitive to environmental conditions. Although all the birds used in the current study belong to one genetic line, the birds in H and L groups, being of different capacity for growth, might be different also in their response to environmental factors. Carlborg *et al.* (2003) showed that in laying hens, variation in body weight at different ages and growth is caused by epistatic interactions between quantitative trait loci of the birds indicating the role of external environment on turning on or off of genes that may be responsible for growth and nutrient utilisation potential among others. In addition, Lilja (1983) reported that a high growth rate capacity is closely related to a rapid early development of the digestive organ and the liver. This relationship between growth rate and digestive organ development may explain the differences in nutrient utilisation between the two groups in the current study and might be an explanation for why birds in H and L groups had different growth rate.

In conclusion, differences in body weight are reflected in differences in proventricular and caecal pH which is indicative of differences in intestinal condition between birds with heavier or lighter body weight. Furthermore, differences in pH of the some intestinal sections partly explained the variations in total tract nutrient and energy utilisation. Therefore, individual variability in body weight and nutrient utilisation in grower and finisher phases were partly explained by variability in pH of jejunal and caecal content which in turn may be related to the type of microbes colonizing the hind gut.

3. BENZOIC ACID SUPPLEMENTATION IMPROVED GROWTH PERFORMANCE AND BENEFICIALLY MODIFIED INTESTINAL PH AND HISTOMORPHOLOGY OF BROILER CHICKENS

3.1. INTRODUCTION

The ban on the use of antibiotics as growth promoters (AGP) for broilers in the EU from 1997 may result in a substantial increase in enteric health problems which in turn can depress the growth performance of chickens. Knarreborg *et al.* (2002) and Casewell *et al.* (2003) reported that the removal of AGPs authorization resulted in substantial increases in intestinal infections. In order to improve enteric health and growth performance without using AGP, alternative strategies are needed in view of the prevalence of enteric health problem and the associated losses in bird performance and profitability. One of the alternatives to AGP that have been suggested is the dietary supplementation of organic acids (Patten and Waldroup, 1988; Runho *et al.*, 1997; Dibner and Buttin, 2002). Organic acids (OA) are known to have strong antibacterial properties and have been used for years to sanitize feed and consequently ensure optimum health of the gastro-intestinal tract (Canibe *et al.*, 2001).

Understanding the role of dietary inclusion of OA in the gastro-intestinal tract (GIT) is a vital key for ensuring flock health, improving the efficiency of nutrient utilisation, and ultimately improving growth performance. The main proposed mode of action of the dietary OA in this study was to reduce the digesta pH of the GIT that might be useful to inhibit the load, growth, and population of enteropathogenic microflora (Humphrey and Lanning, 1988; Russell, 1992; Waldroup *et al.*, 1995) and therefore is important to reduce the competition for available nutrients, reduce local inflammation, as well as intestinal thickening by the mucin barrier (Thomke and Elwinger, 1998; Apajalahti *et al.*, 2004). Reduction in the pH of sections of the GIT after supplementation of OA has been reported by various authors (Józefiak *et al.*, 2007; Józefiak *et al.*, 2010). Dietary inclusion of OA has been shown to reduce feed conversion and increase body weight (Canibe *et al.*, 2001; Senkoylu *et al.*, 2007). Organic acid inclusion also seems to have direct effect on the absorptive cells of the GIT of broiler chickens. Both higher villus and deeper crypt (Hernández *et al.*, 2006; Paul *et al.*, 2007; Senkoylu *et al.*, 2007) have been reported when the diets of broiler chickens was supplemented with OA. Nevertheless,

dietary supplementation OA at the rate of 5.0 g/kg depressed the growth performance of broiler chicken (Paul *et al.*, 2007; Józefiak *et al.*, 2007). In a later study, Smulikowska *et al.* (2010) reported that 6.0 g/kg OA supplementation decreased the growth and proliferation of absorptive cells in the jejunum of chickens.

There is a dearth of information regarding the mode of action of BA and its effects on acidity and histomorphology in the poultry digestive tract. The objective of the current study was to investigate the effects of supplementing BA on the growth performance, efficiency of energy and nutrient utilization for growth as well as the intestinal acidity and histomorphology of broiler chickens.

3.2. MATERIALS AND METHODS

3.2.1. Birds, Housing, and Experimental Design

A total of 945 day old Ross 308 male broiler chickens were allocated to 3 treatments in a randomized complete block fashion. Each treatment had 7 replicate pens with 45 birds per replicate pen. Power calculation was done using Genstat in order to determine the optimum sample size. The treatments were a control diet (C) which was formulated to meet all nutrients and energy requirements for the birds at each phase (starter, grower and finisher) but without benzoic acid (BA); treatment 2 was the control plus 0.53 g/kg BA whereas treatment 3 had BA supplemented at the rate of 3.20 g/kg. These dose rates were based on the recommendation of the supplier of the acid where the 0.53 g/kg is the optimum dose and the 2.30 g/kg is the maximum dose. The feeding program consisted of a starter diet (0 to 10 days of age), a grower diet (11 to 21 days of age), and a finisher diet (22 to 42 days of age). The diets were formulated to meet the recommendations of the National Research Council (1994) for broiler chickens. The ingredients and chemical compositions of the diets are presented in Table 3-14. All the diets for each period were prepared with the same batch of ingredients. Feed and water were provided for *ad libitum* intake.

Chicks were housed in floor pens (225 cm x 140 cm) equipped with a feeder and nipple drinkers. The pens were in a house with facilities to control temperature, light, and humidity. The temperature gradually reduced from 32°C on d 1 to 20°C on d 42. No coccidiostat or enzymes were added to the experimental diets. The chicks were vaccinated at the hatchery against Infectious Bursal Disease, and no additional vaccinations were administered during the study.

Table 3-14. Composition of experimental basal diets and calculated nutrient content

Attributes	Experimental basal diets		
	Starter	Grower	Finisher
<i>Ingredients (g/kg as fed)</i>			
Wheat	672.5	695.8	753.2
Prairie meal	50.0	30.0	39.0
Soya ext hipro	203.0	209.0	117.0
Extruded fat-free soy	25.0	0.0	27.0
L Lysine HCl	3.2	3.3	5.4
L Threonine	0.3	0.6	1.3
Methionine hydroxy analog acid	1.2	1.5	1.6
Soya oil	18.0	35.0	35.0
Di-calcium phosphate	10.0	7.8	6.5
Limestone	9.5	10.0	7.0
Sodium chloride	3.3	3.0	3.0
Premix vitamin ¹	4.0	4.0	4.0
BA premix ²	To 1000.0	To 1000.0	To 1000.0
Total	1000.0	1000.0	1000.0
<i>Calculated nutrient content (g/kg DM)</i>			
Ether extract	38.5	51.3	55.8
Crude fiber	28.4	27.8	27.7
ME, kcal/kg	3068.9	3138.2	3224.2
Total lysine	12.3	11.8	11.5
Methionine	4.3	4.1	3.9
Methionine + Cysteine	8.8	7.3	6.8
Calcium	8.0	7.6	6.0
Phosphorous	5.6	5.1	4.7
Available phosphorous	3.3	2.8	2.5
<i>Analysed nutrient content (g/kg DM)</i>			
Dry matter	880.6	881.3	877.6
Crude protein	226.0	209.0	180.0
Neutral detergent fibre	92.8	76.8	72.3
Ash	50.8	44.9	38.3
GE, kcal/kg	4565.1	4660.7	4684.6

¹Vitamin mixture provided per kg of diet = vitamin A 13500 IU, vitamin D3 5000 IU, vitamin E 500 mg

²Benzoic acid was added to a portion of wheat to make BA premix.

3.2.2. *Sampling Procedure*

Body weight and feed intake data were taken on d 0, 10, 21 and 42 for calculation of weight gain and feed conversion ratio. The protein efficiency ratio (PER) and energy efficiency ratio (EER) were calculated for each phase using the following formula:

Equation 3-4. Formula for calculating the protein efficiency ratio (PER)

$$PER (g/g) = \frac{Body\ Weight\ Gain\ (g)}{Protein\ Intake\ (g)}$$

Equation 3-5. Formula for calculating energy efficiency ratio (EER)

$$EER (g/100\ kcal) = \frac{Body\ Weight\ Gain\ (g) \times 100}{Gross\ Energy\ Intake\ (kcal)}$$

On d 42, two birds per replicate pen with body weight similar to the mean body weight of the pen were killed by injection with 1.5ml of 6% sodium pentobarbital solution per kg chicken live weight. Digestive tract between the crop and caeca were removed and pH values of the crop, ileum, and caeca were measured using a sterile glass pH electrode (HI 99163, HANNA Instruments, Romania).

Jejunum section was used for the intestinal histomorphology examination. The jejunum was separated from the end duodenal loop up to 1 cm proximal to the Meckel's diverticulum. Sections of approximately 6 cm were taken from the mid-jejunum, gently flushed with phosphate buffer saline (pH 7.2), and cut into 3 equal pieces. The tissue sections were then immediately fixed into 10% buffered Formalin solution, shaken gently, and stored in a bottle with tightly sealed lid until further processing. The fixed intestinal sections were subsequently dehydrated by transferring through a series of ethyl alcohols with increasing concentrations (70, 80, 95, and 100%), cleared with xylene, and embedded in polyfin embedded wax. Tissue sections (2 μ m) were cut by microtome (Leitz-1512 Microtome, Leitz, Wetzlar, Germany), placed on glass slides, and stained with hematoxylin (Gill no. 2, Sigma, St. Louis, MO) and eosin (Sigma).

The measurements for the villus and crypts dimension were carried out using an Olympus BX41 Laboratory Microscope (Olympus UK Ltd., Essex, UK) at 40 x magnification. Pictures of villus and crypts were obtained with a video camera (JVC TK

1085E), connected with a monitor screen (DELL), and computer, with measurements made using the Spot Basic (Diagnostic Instruments, Inc., Sterling Height, MI) imaging software.

Fifteen well-oriented villi and crypts from jejunum were measured along their length (height and depth, respectively) and width. The villus height (VH) was measured from the crypt-villus junction to the brush border at the tip. Villus width (VD) was measured parallel to the adjoining villus. The crypt depth (CD) was measured from the base near the lamina propria to the crypt-villus junction. Crypt width (CW) was measured parallel to the adjoining crypt. Villus height and crypt depth formed total mucosal thickness. Total mucosal thickness (TMT) was measured perpendicular to the muscularis mucosae from the lamina propria towards the brush border at the tip of the villus (including villus height and crypt depth) at all locations of the jejunum. Similar procedures were used by Bollinger (1996). All measurements were made to the nearest micrometer.

3.2.3. *Statistical Analyses*

Growth performance, nutrient and energy utilization, intestinal pH, and histomorphology data were analyzed statistically by Analyses of Variance employing randomized complete block design. Least Significant Difference was used to separately significantly different means (Snedecor and Cochran, 1989). Significance was declared at $P \leq 0.05$. All statistical analyses were performed using GenStat Release 11th edition for Windows (VSN International Ltd., Hemel Hempstead, UK) software.

3.3. RESULTS

3.3.1. *Growth Performance*

The effects of low dose BA supplementation on performance of broiler chickens are summarized in Table 3-15. Broiler chickens fed 0.53 g/kg benzoic acid were 14%, 10% and 3% heavier at 10, 21 and 42 days, respectively ($P < 0.001$) compared with birds receiving the control diet. Supplementation BA at 0.53 g/kg increased body weight gain ($P < 0.05$) of broiler chickens significantly by 17%, 8%, and 3% in the starter, grower, and overall experimental period, respectively. No differences were found in the feed intake between birds receiving BA-supplemented feed and the control in the starter and finisher phases but birds receiving BA at 3.20 g/kg level (BA2) had lower ($P < 0.01$) feed intake than birds on control or 0.53 g/kg BA-supplemental level (BA1) during the grower phase.

Table 3-15. Growth performance responses of broiler chickens to benzoic acid supplementation in different growth phases¹

Growth performance parameter	Dietary treatment ²			Level of significance	
	C	BA1	BA2	SEM	P
<i>Starter phase (day 0 to 10)</i>					
Initial body weight, g	43.18	43.31	43.42	0.209	0.735
Final body weight, g	238.43 ^c	272.37 ^a	262.37 ^b	1.810	<0.001
Body weight gain, g/bird/day	19.52 ^c	22.91 ^a	21.90 ^b	0.174	<0.001
Feed intake, g/bird/day	26.53	27.40	26.66	0.366	0.236
Gain:feed	736.47 ^b	836.15 ^a	822.21 ^a	10.160	<0.001
<i>Grower phase (day 11 to 21)</i>					
Initial body weight, g	238.43 ^c	272.37 ^a	262.37 ^b	1.810	<0.001
Final body weight, g	848.94 ^b	932.31 ^a	872.60 ^b	9.240	<0.001
Body weight gain, g/bird/day	55.50 ^b	59.99 ^a	55.48 ^b	0.730	0.001
Feed intake, g/bird/day	82.32 ^a	80.01 ^a	74.63 ^b	1.308	0.004
Gain:feed	675.12 ^b	751.42 ^a	744.68 ^a	16.160	0.010
<i>Finisher phase (day 22 to 42)</i>					
Initial body weight, g	848.94 ^b	932.31 ^a	872.60 ^b	9.240	<0.001
Final body weight, g	2583.88 ^b	2666.90 ^a	2557.45 ^b	24.500	0.020
Body weight gain, g/bird/day	82.57	82.60	80.23	0.883	0.136
Feed intake, g/bird/day	184.01	186.22	175.84	5.440	0.393
Gain:feed	450.27	451.35	458.31	10.640	0.847
<i>Overall growth phase (day 0 to 42)</i>					
Initial body weight, g	43.18	43.31	43.42	0.209	0.735
Final body weight, g	2583.88 ^b	2666.90 ^a	2557.45 ^b	24.500	0.020
Body weight gain, g/bird/day	60.47 ^{ab}	62.47 ^a	59.86 ^b	0.583	0.020
Feed intake, g/bird/day	116.60	117.40	110.52	2.590	0.163
Gain:feed	519.15	535.78	542.24	9.850	0.270

^{a-c}Means within a row without a common superscript differ significantly (P < 0.05).

¹Means represent 7 pens of 45 birds each per treatment.

²C = Control diet; BA1 = diet with 0.53 g/kg benzoic acid inclusion; BA2 = diet with 3.20 g/kg benzoic acid inclusion

Table 3-16 Energy and protein efficiency ratios of broiler chickens which receiving diets supplemented with benzoic acid¹

Items ²	Dietary treatment			Level of significance	
	C	BA1	BA2	SEM	P
<i>Starter phase (day 0 to 10)</i>					
Protein Intake, g	5.28 ^b	5.58 ^a	5.40 ^{ab}	0.074	0.040
PER, g/g	3.70 ^b	4.10 ^a	4.06 ^a	0.050	<0.001
Energy Intake, kcal/g	106.6	111.2	107.5	1.477	0.111
EER, g/100 kcal	18.32 ^b	20.60 ^a	20.36 ^a	0.252	<0.001
<i>Grower phase (day 11 to 21)</i>					
Protein Intake, g	15.16 ^a	14.88 ^a	13.84 ^b	0.243	0.006
PER, g/g	3.67 ^b	4.04 ^a	4.02 ^a	0.087	0.017
Energy Intake, kcal/g	338.1 ^a	330.4 ^{ab}	311.8 ^b	5.420	0.014
EER, g/100 kcal	16.44 ^b	18.20 ^a	17.82 ^{ab}	0.391	0.019
<i>Finisher phase (day 22 to 42)</i>					
Protein Intake, g	29.07	29.85	28.53	0.872	0.575
PER, g/g	2.85	2.82	2.83	0.067	0.932
Energy Intake, kcal/g	756.5	767.9	722.3	22.400	0.359
EER, g/100 kcal	10.95	10.95	11.16	0.258	0.808
<i>Overall growth phase (day 0 to 42)</i>					
Protein Intake, g	49.51	50.31	47.77	0.876	0.153
PER, g/g	3.19	3.31	3.31	0.048	0.181
Energy Intake, kcal/g	1201.3	1209.5	1141.7	22.300	0.104
EER, g/100 kcal	13.14	13.77	13.83	0.212	0.076

^{a-c}Means within a row without a common superscript differ significantly (P < 0.05).

¹Means represent 7 pens of 45 birds each per treatment.

²Protein efficiency ratio (PER) calculated as weight gain divided by protein intake; Energy efficiency ratio (EER) calculated as weight gain x 100 divided by GE intake.

Gain to feed ratio of the birds in BA1 was 14% and 12% lowered in the starter and grower phases, respectively ($P < 0.01$) compared with birds receiving the control diets. No differences were found in the gain to feed ratio between birds fed BA supplemented and the control diets in finisher phase and in overall experimental period.

In regard to the P content in the diets formulation, the available P (av. P) was lower than the recommendation of ROSS 308, but it was still above the minimum requirement for optimum growth performance of broiler chickens, which is 1.8 g/kg (Bhanja *et al.*, 2005). Our previous study in SAC also showed that the use of 2.2 g/kg av. P did not show any negative effect on growth performance. In addition, all birds in the same period in this study received the same basal diets. Therefore, the comparatively low av. P in this study (2.5-3.3 g/kg) was not expected to inhibit performance.

3.3.2. Efficiency of Energy and Protein Utilization for Growth

Responses of broiler chickens to BA supplementation on efficiency of energy and protein utilization in different growth phases are presented in Table 3-16. In the starter phase, BA supplementation had no effect on energy intake but protein intake was greater ($P < 0.05$) in BA1 compared to the control whereas BA2 was intermediate. Protein and energy efficiency ratio were greater in BA-supplemented diets ($P < 0.05$) than in the control diet in both the starter and grower phases. Protein and energy intakes were lower ($P < 0.01$) in BA2 compared with BA1 and control during the grower phase. Birds in BA supplemented diets (BA1 and BA2) were tended ($P < 0.05$) to have higher protein and energy ratio than those of received control diets. There were no effects of dietary treatments on protein or energy intakes or on protein and energy efficiency ratios in the finisher phase or the overall experimental period with the exception of EER which tended to be greater ($P < 0.10$) in BA supplemented diets compared with the control.

3.3.3. Digestive Tract pH and Jejunal Histomorphology

Table 3-17 shows the pH in different sections of the digestive tract on d 42 and the response to BA supplementation. There were no effects of BA supplementation on pH of the digesta in the crop or ileum. However, the pH of the caecal content of birds on BA1 diet dropped ($P < 0.05$) by 0.33 units relative to the control pH level but the pH of caecal content was similar between BA1 and BA2.

Table 3-17. Intestinal pH in different sections of the digestive tract of broilers at 42 days of age in response to benzoic acid supplementation of wheat-soyabean meal¹

Acidity parameter	Dietary treatment			Level of significance	
	C	BA1	BA2	SEM	<i>P</i>
Crop	4.59	4.42	4.35	0.079	0.147
Ileum	6.16	5.87	5.79	0.172	0.310
Caeca	6.12 ^a	5.80 ^b	5.83 ^b	0.084	0.033

^{ab}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Means represent 7 pens of 2 birds per treatment.

Table 3-18. Jejunal histomorphological responses to supplementation of benzoic acid in wheat-soyabean meal to fed to broiler chickens for 42 days¹

Morphology parameter	Dietary treatment		Level of significance	
	C	BA2	SEM	<i>P</i>
Villus height (µm)	2,215.60	5,276.12	161.913	< 0.001
Villus width (µm)	298.42	405.60	14.411	0.013
Crypt depth (µm)	630.94	998.25	42.570	0.009
Crypt width (µm)	249.79	309.66	13.316	0.050
Ratio ²	3.82	5.84	0.176	0.004
Total mucosal thickness (µm)	2,846.54	6,274.37	190.058	0.001

¹Means represent 15 readings from 4 pens of individual bird per replicate pen.

²Ratio of villus height to crypt depth.

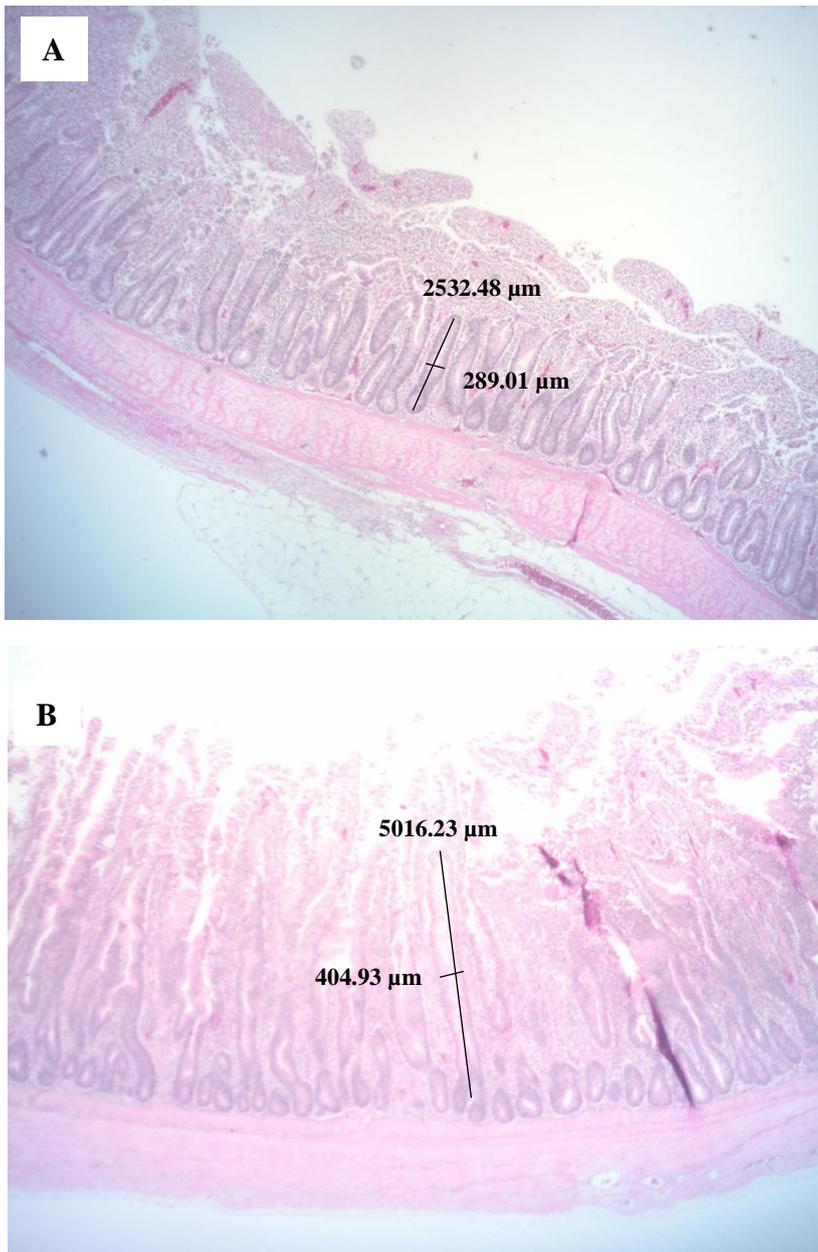


Figure 3-6. Photomicrography of the jejunum of 42-day old broiler chickens showing the villi dimensions of the birds (A) without and (B) with 3.20 g/kg BA supplementation, representing data in Table 3-18. Note the longer villi in the jejunum of BA-supplemented birds (lower panel). The overall architecture of the villi of BA-supplemented bird shows a narrower and well-protruded structure whereas the villi in the control birds were more jagged and runted in appearance.

Supplementation of BA stimulated the proliferation of the absorptive cells in the jejunum (Table 3-18). Compared with the control, the birds receiving BA2 diet had longer ($P < 0.01$) and wider villi ($P < 0.05$). The height and the width of the villi increased by 138 % and 36%, respectively. Supplementation of BA resulted in the deeper and wider crypts ($P \leq 0.05$). The depth and the width of the crypts increased by 58 % and 24%, respectively in broilers receiving BA2 diet. The villus height to crypt depth ratio was lower ($P < 0.01$) in broiler chickens fed the control diet. The thickness of the mucosal layer in the jejunum was 120% lower ($P < 0.01$) in the birds fed control diet compared with those receiving BA2 diet.

Photomicrography examination of the jejunum cell wall (Fig. 3-6) show that 42-day old broiler chickens fed BA-supplemented diet have longer villi and deeper crypts (lower panel) compared with those receiving control diets (upper panel).

3.4. DISCUSSION

It has been demonstrated by various authors that dietary supplementation of OA improved growth performance of broiler chickens (García *et al.*, 2007; Paul *et al.*, 2007; Abdel-Fattah *et al.*, 2008). The beneficial effects of OA supplementation on performance have been attributed to a more efficient use of nutrients, which in turn results in improved feed efficiency. In a study using fumaric acid, Patten and Waldroup (1988) and Skinner *et al.* (1991) showed that OA supplementation improved daily gain and feed efficiency. Chotikatum *et al.* (2009) reported that combination of lactic acid, citric acid, ascorbic acid, propionic acid, and BA in broiler diets improved the growth performance of the chickens.

Observations in the current study are in line with such findings as shown in greater weight gain and final body weight, during starter, grower and for the overall experimental periods. There was also reduced feed intake in BA2 during the grower phase and reduced FCR during starter and grower phases in BA-supplemented diets. The observed reduced feed intake in BA-supplemented diet in the current study is in agreement with the observations of Biggs and Parson (2008), in which 4.0% malic acid supplementation reduced feed intake of broiler chickens in grower phase (8 – 21 days old). Leeson *et al.* (2005) and Pirgozliev *et al.* (2008) also reported a reduction in the feed intake of 21 d old birds when the diets were supplemented by low dose of organic acid. The reduction of feed intake in these studies may be a palatability issue. It may be possible that the birds refused

to eat diets that were too acidic. There are no data to support the views either way and hence it can only be assumed that palatability issue may surface in diet containing high level of organic acid. In a study using formic and propionic acid mixture, Senkoğlu *et al.* (2007) reported that dietary supplementation of organic acids improved body weight gain and feed conversion ratio of 21 d old male broiler chickens. On the contrary, some studies have indicated that dietary supplementation of OA in low dosages had no effects on the performance of broiler chickens in grower phase. Using 5.0 and 10.0 g/kg formic acid, Hernández *et al.* (2006) showed that dietary organic acid did not affect body weight gain, feed intake, and FCR of male broiler chickens. Furthermore, in contrast to the report of Paul *et al.* (2007), results in current study showed that BA supplementations had no effects on growth performance responses in finisher period (d 22 – 42). The observations in the finisher phase in the current study is similar to the reports of Hernández *et al.* (2006) who reported no effect of 5 and 10 g/kg formic acid supplementation on weight gain, feed intake, and feed conversion ratio of male broiler chickens during finisher phase. Similarly, Sun *et al.* (2005) reported that low dose supplementation of citric and sorbic acid mixture did not affect cumulative feed conversion ratio of male and female broiler chickens from 28 to 49 days of age. Supplementation of 7.2 g/kg formic and propionic acid mixture did not affect body weight of 21 and 49 d old male and female broiler chickens (Isabel and Santos, 2009).

The efficiency of energy and nutrient utilization for growth was investigated in the current study using energy and protein efficiency ratios. It can be reasoned that a reduction in bacterial load will enable utilization of more nutrients and energy for productive purposes and decrease nutrient and energy requirements for maintenance. We calculated efficiency using PER and EER to measure the efficiency of protein and energy intake per gram body weight of the birds as a proxy of digestibility. Digestibility is one way of determining nutrient utilisation efficiency whereas EER and PER are another. The condition of the experiment did not enable collection of excreta or ileal digesta and hence we decided to determine nutrient utilisation efficiency by examining the ratios. The observations in the current study lent support to this argument as the efficiency of utilization of dietary protein and energy for weight gain was greater in BA supplemented diets especially during starter and grower phases. Supplementation of BA in current study likely optimized efficiency of nutrient digestion and reduced maintenance needs, resulting in greater PER and EER responses. According to Larbier and Leclercq (1992), in the grower phase birds, nutrient and energy are mostly used to maintain body expenditures,

such as basal metabolism, thermogenesis linked to hyperthermia, thermogenesis associated with adaptation to cold conditions, and intrinsic thermogenesis related to feed intake. In the finisher phase, as the birds have fully recovered with feathers, the digested nutrient and energy are used mostly for meat production. Herewith, dietary BA supplementation did not affect the PER and EER. Improvement of PER and EER in grower phase could also be attributed to a lower competition between pathogenic microbes and the host for the digested nutrients (Dibner and Richards, 2004), providing a more conducive intestinal environment for period (d 22 – 42). The observations in the finisher phase in the current study is similar to the reports of Hernández *et al.* (2006) who reported no effect of 5 and 10 g/kg formic acid supplementation on weight gain, feed intake, and feed conversion ratio of male broiler chickens during finisher phase. Similarly, Sun *et al.* (2005) reported that low dose supplementation of citric and sorbic acid mixture did not affect cumulative feed conversion ratio of male and female broiler chickens from 28 to 49 days of age. Supplementation of 7.2 g/kg formic and propionic acid mixture did not affect body weight of 21 and 49 d old male and female broiler chickens (Isabel and Santos, 2009).

The efficiency of energy and nutrient utilization for growth was investigated in the current study using energy and protein efficiency ratios. It can be reasoned that a metabolism process resulted in greater protein and energy digestibility (Dibner and Buttin, 2002). Kluge *et al.* (2006) had shown in piglets that 5.0 g/kg BA supplemental level reduced the population of pathogenic organisms in the GIT, resulting in more efficient nitrogen utilization. The current study has shown for the first time the possible influence of BA supplementation on efficiency of energy and protein utilization for growth and has demonstrated that improvement in growth performance due to BA supplementation reflects a more efficient nutrient and energy utilization for weight gain.

Supplementing BA in the wheat-soyabean meal in the current study had no effect on pH of the crop and ileum contents but reduced pH in the caeca. Reduction in pH of the caecal content was observed at 0.53 g/kg BA supplemental level but no further reduction was observed at 3.20 g/kg level. The observation was similar to that of Józefiak *et al.* (2010) who noted that 2.0 g/kg BA supplementation reduced caecal pH of 42-d old broiler chickens. It is likely that the reduction in pH was due to the BA supplemented altering the intestinal ecosystems (Canibe *et al.*, 2001), providing an unfavorable acidic environment to pathogenic microflora (Eklund, 1985), reducing ammonia and other growth depressing microbial metabolites (Dibner and Buttin, 2002), and stimulating the growth and

population of beneficial microflora (Partanen *et al.*, 1998) in the GIT. Chaveerach *et al.* (2004) noted that administering drinking water with OA decreased population of pathogenic microflora in the caeca. Reduction in the population of pathogenic organisms in the intestine will reduce microbial metabolites (Gabert *et al.*, 1995) and incidences of sub-clinical infections (Russell, 1992) ultimately stimulating the growth of absorptive cells in the intestine (Vissek, 1978; Loddi *et al.*, 2004). It is worthy of note that the reduction in GIT pH was not BA-dose dependent. A 6-fold supplemental level did not correspondingly decrease the pH in the GIT thus indicating that after BA had reduced pH to a beneficial level, further addition did not reduce the pH further.

The proposed mode of action of OA for controlling microbial growth involves depolarization of the bacterial membrane, an alteration in internal pH, and changes in the nutrient transport and synthesis within the bacterium (Eklund, 1983; Davidson *et al.*, 1997). As weak acids, OA have the ability to penetrate the cellular membrane of bacteria easily. In the cell, they could release protons in the alkaline cytoplasm, resulting in the reduction of intracellular pH. Such reduction is unfavorable for intestinal colonization of pH-sensitive entero-pathogenic bacteria (Pelicano *et al.*, 2005), but at the same time could be favourable for stimulating the growth of beneficial bacteria (Eklund, 1985). The mechanism by which disruption of cytoplasmic pH of the microbes operates is through the need for bacteria to maintain their internal neutral pH environment when there is a decrease in pH (Hernández *et al.*, 2006). In an attempt to maintain the homeostasis in internal pH, there is an unfavourable alteration in enzymatic reactions and the nutrient transport system, forcing the bacterial cell to use ATP to release excess protons (Ricke, 2003). The organism uses its energy in trying to restore the normal balance, causing a reduction in their cellular energy (Biggs and Parsons, 2008). The RCOO^- produced from the acid can interrupt DNA replication and protein synthesis. Consequently, the acid-intolerant bacteria, such as *E. coli*, *Campylobacter* and *Salmonella* (Canibe *et al.*, 2001; Van Immerseel *et al.*, 2006) are put under stress and are unable to replicate rapidly when there is a disruption in internal pH (Hernández *et al.*, 2006). Incidentally, these are the more common pathogenic microbes that tend to populate the intestinal milieu of the chickens and have been reported to be responsible for GIT disorders (Paul *et al.*, 2007).

The gut lumen and mucosal surface of the intestine and caeca are the main locations for the growth and colonization by intestinal microflora, including the pathogenic species (Lan *et al.*, 2005). Higher colonization of pathogenic species in these sites might stimulate

incidences of sub-clinical (bacterial) infections and necrotic tissue damages, lower absorptive cells proliferation, lower immune system, and lower nutrients uptake and utilization (Sklan *et al.*, 2003; Van Immerseel *et al.*, 2006; Sarson *et al.*, 2009). Reduction in the caecal pH as observed in the current study should inhibit intestinal pathogenic colonization competing with the host for available nutrients. It was observed that the pH in the crop and ileum in the current study was not affected by BA supplementation. Similar observation had been made by Smulikowska *et al.* (2009) who noted that 1.0 g/kg OAs inclusion did not affect the acidity of the crop, proventriculus, ileum as well as the concentration of short-chain fatty acids in the GIT. However, OA supplementation at that dose stimulated greater energy and nutrient utilization in 30-d old female broiler chickens. Further study by Smulikowska *et al.* (2010) showed that 6.0 g/kg OAs inclusion had no effect on acidity in the crop, proventriculus, jejunum, ileal, and caeca, but depressed the growth of absorptive cells in the jejunum cell wall of female broiler chickens.

Intestinal pH has been shown to be related to gut health with lower pH indicative of better intestinal environment and hence growth. The additives have been reported to stimulate gut growth and nutrient utilization and one way of studying the effect of the additive on gut growth by examining its morphology. The observation in current study showed that dietary BA supplementation restored the absorptive capacity of the intestinal mucosa by increasing intestinal villi and crypts size, resulted in longer and wider villi as well as deeper and wider crypts. The observed thicker mucosa and greater ratio of villus height to crypt depth is an indication of capacity for greater nutrient absorption. The longer villi will provide greater absorptive surface and deeper crypt indicate a more enhanced capacity for replenishing the enterocytes (Geyra *et al.*, 2001; Mathlouthi *et al.*, 2002). The longer villi in this study may have resulted from the BA contributing to a pH reduction in the intestine and, consequently, reducing colonization of the intestine by enteropathogenic bacteria (García *et al.*, 2007). As described by Sklan (2004), overgrowth of enteropathogens drives the intestine to secrete mucus barrier to protect absorptive surface of the intestine from luminal irritant and lumen bacteria. Therefore, minimization of the growth and population of pathogenic species due to BA supplementation will lead to the reduction of toxic bacterial metabolites which in turn maximize digestive efficiency by facilitating greater proliferation of the absorptive cells (Iji and Tivey, 1998). Improvement of these intestinal histomorphology criteria is believed to account for some of the growth-promoting properties of organic acids supplementation. In addition, reduction of the population or activity and virulence of pathogenic organisms possibly resulted in more

nutrients made available in the digesta content, which also might stimulate absorptive cells proliferation (Apajalahti *et al.*, 1999; Apajalahti *et al.*, 2004). Pelicano *et al.* (2005) and Viola and Vieira (2007) had also shown that longer villi and deeper crypts are indicative of high proliferative cellular activity due to BA supplementation and are expected to provide an increased epithelium turnover rate. Senkoylu *et al.* (2007) similar reported that OA maximized the growth of absorptive cells in the intestinal wall of broiler chickens. Therefore, improvements in the growth performance in current study were likely attributed to the reduction of the pH in the intestine that might reduce the growth and population of the pathogenic microflora.

It is concluded from the current study that dietary supplementation of BA stimulated growth performance, facilitated a more efficient energy and protein utilization for weight gain, decreased cecal pH, and beneficially modified the structure of the jejunum of broiler chickens at 42 d of age. The data on growth performance, energy and protein efficiency ratios and pH indicate that maximum efficiency of BA was attained at less than 3.20 g/kg supplementation. Future studies evaluating the relationship between intestinal pH and efficiency nutrient utilization would be useful to further elucidate the mode of action of OA in promoting health and growth of broiler chickens.

4. GROWTH PERFORMANCE, INTESTINAL pH, GUT PROFILE, AND ENERGY AND NUTRIENT UTILISATION OF BROILERS FED DIETS SUPPLEMENTED WITH BENZOIC ACID AND TURMERIC MEAL INDIVIDUALLY OR IN COMBINATION

4.1. INTRODUCTION

In order to avoid the risk of developing resistant pathogens, as well as to meet the public demand for drug-free healthy food, the use of antimicrobial growth promoters (AGPs) in poultry diets was totally banned in the European Community in January 2006. However, the absence of in-feed AGPs has resulted in health problems in poultry, including substantial increase in infection (Witte, 1998; Casewell *et al.*, 2003). For instance, the incidence of necrotic enteritis (NE) or Clostridial infection has produced serious problems related to animal welfare and resulted in severe economic losses (Hofacre, 2001).

As a consequence, poultry and feed industries have needed to find alternatives to AGP in order to stem the spike in the rate of sub- and clinical infections. These alternatives are required to be environmental friendly, applicable in the diets, easy to administer, generally recognized as safe (GRAS) for both animal and humans who consume animal products, and address organic livestock issue (Cabuk *et al.*, 2006). Some of the dietary alternatives to AGPs are organic acids (OA) and phytobiotics which are derived from herbs, spices, or plant extracts. Both organic acids and phytobiotics from herbs, spices or plant extracts have been documented to possess antimicrobials properties (Deans and Ritchie, 1987), stimulate growth performance (Patten and Waldroup, 1988; El-Hakim *et al.*, 2009), improve immune status (Soliman *et al.*, 1999), and increase carcass yield in poultry (Naveena and Mendiratta, 2001).

Organic acids and phytobiotics have different modes of action on how they stimulate growth of broiler chickens by their antimicrobials action (Ferket, 2004) and digestive stimulation (Dibner and Buttin, 2002). The main purpose of the diet acidification is to reduce the pH (Andryst *et al.*, 2003) because lower pH inhibits the proliferation of acid-intolerant enteropathogenic bacteria such as coliform, Salmonella, Campylobacter (Islam, 2012). Some organic acids, e.g. benzoic acid, fumaric acid, lactic acid, formic acid, butyric acid, sorbic acid, and propionic acid, are commonly used in poultry feed to sanitize

feed and prevent microbial infection (Thompson and Hinton, 1997). Organic acid also have direct positive effect on stimulating the growth of absorptive cells in the intestinal wall (Paul *et al.*, 2007) and can therefore promote nutrient digestion and absorption. Benzoic acid is superior to other acids as it has beneficial effect on reducing coliforms in both the stomach and small intestine (Eklund, 1985), as well as on reducing *Salmonella typhimurium* (Jensen, 2001).

On the other hand, phytobiotics in herbal products are efficacious alternatives to AGPs. *In vitro* studies showed that these active substances found in herbs and spices have antibacterial (Deans and Ritchie, 1987), antifungal (Conner and Beuchat, 1984; Radwan *et al.*, 2008), antiparasitic (Anthony *et al.*, 2005), anticoccidial (Allen *et al.*, 1998; Giannenas *et al.*, 2003), immunomodulatory (Antony *et al.*, 1999; Churchill *et al.*, 2000; Kumari *et al.*, 2007), and antioxidant (Osawa *et al.*, 1995; Masuda *et al.*, 2001) properties. *In situ* studies have also demonstrated growth-promoting (Cross *et al.*, 2007) and digestion stimulation (Lee *et al.*, 2003a; Jang *et al.*, 2007) properties of the phytochemicals.

Although both organic acids and herbal products have been reported to be viable alternatives to AGP, there are no reports of their use in combination. Little to nothing is known about the influence of dietary OA and herbal product alone or in combination in altering digesta pH, profile of digestive tract, gut morphology, and energy and nutrient utilization. Although OA and phytobiotics have similar beneficial effects, if they exhibit their effects via different mechanisms there is the likelihood that a combination of both additives will be more beneficial than if used individually.

The objectives of the current study therefore were to determine the growth performance, nutrient utilization, and intestinal health responses of broilers to supplementation of benzoic acid and turmeric meal. The study is also aimed to assess the impact of the additives on the growth of digestive organs and thus determine how the additives affected whole-body energetic. In addition, possible associative, additivity and interactivity of benzoic acid and turmeric meal were assessed.

4.2. MATERIALS AND METHODS

4.2.1. Birds and Housing

A total of 300 male one-day old broiler chickens (Ross 308) were used in this study which

consisted of two experiments. All animal experiment work was approved by the SAC Animal Experimentation Committee. The chicks were vaccinated at the hatchery against Infectious Bursal Disease and no additional vaccinations were administered during the study.

On day 1, the chicks were wing-tagged, weighed and allocated in 30 floor pens (110 cm x 150 cm) which were equipped with feeders and bell drinkers. The pens were situated in environment-controlled rooms with facilities to control temperature, light, and humidity. The room temperatures on day 0 to 7, day 7 to 14, and day 14 to 21 were kept at 35, 32, and 27 °C, respectively.

4.2.2. Birds Grouping, Diets, and Experimental Design

The chicks were allocated to 5 treatments in randomized complete block design with a 2×2 + 1 factorial arrangement. Each treatment had 6 replicate pens with 10 birds per replicate pen on day 1. The treatments were a wheat-soybean meal based control diet that met the nutrient specification for the broiler breed. Treatments 2 and 3 were the control diet plus benzoic acid (BA) or turmeric meal (TM) added at the rates of 2 or 10 g/kg, respectively. Treatment 4 was the control plus a combination of BA and TM at the rates of 1 and 5 g/kg, respectively whereas treatment 5 was similar to treatment 4 except that the additives were used at the rates of 2 and 10 g/kg of diet. Titanium dioxide (0.3 %) was added to all diets as an indigestible marker. Starting from day 1, the experimental diets were fed to the birds for the whole 21-days experimental period. Birds had free access to feed and water, but no coccidiostat or enzymes were added to the diets. The ingredients and chemical compositions of the diets are presented in Table 4-19.

4.2.3. Sampling Procedures

On day 14, 150 birds were transferred to 30 metabolism cages for digestibility trial (Experiment 2) and the remaining 150 birds continued in previous pens to continue growth performance and intestinal health study (Experiment 1).

4.2.3.1. Experiment 1: Growth Performance and Gut Profiling

The 150 birds that continued in the floor pens were used for this experiment. The birds received experimental diets from day 1. The birds and feed were weighed on day 2, to determine growth performance.

Table 4-19. Ingredients composition and nutrient calculation of the experimental diet

Item	Dietary treatments ¹				
	T1	T2	T3	T4	T5
<i>Ingredients composition, g/kg</i>					
Wheat, White	611.00	611.00	611.00	611.00	611.00
Soybean meal -48%	259.50	259.50	259.50	259.50	259.50
Soybean oil	31.20	31.20	31.20	31.20	31.20
Gluten meal	14.50	14.50	14.50	14.50	14.50
Limestone (38% Ca)	16.00	16.00	16.00	16.00	16.00
Dicalcium phosphate ²	17.00	17.00	17.00	17.00	17.00
Common salt	3.50	3.50	3.50	3.50	3.50
Vitamin/mineral premix ³	4.00	4.00	4.00	4.00	4.00
DL-Methionine	3.00	3.00	3.00	3.00	3.00
L-Lysine HCl	5.00	5.00	5.00	5.00	5.00
Threonine	0.30	0.30	0.30	0.30	0.30
Titanium dioxide premix ⁴	15.00	15.00	15.00	15.00	15.00
Turmeric meal	0.00	0.00	10.00	5.00	10.00
Benzoic acid premix ⁵	0.00	10.00	0.00	5.00	10.00
Gluten meal carrier	20.00	10.00	10.00	10.00	0.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00
<i>Calculated Nutrients and Energy, g/kg</i>					
Protein	223.46	222.22	217.26	219.74	216.02
ME, kcal/kg	3015.89	3008.45	2978.69	2993.57	2971.25
Ether Extract	45.96	45.91	45.71	45.81	45.66
Ash	25.39	25.36	25.26	25.31	25.23
Calcium	10.72	10.72	10.72	10.72	10.72
Total Phosphorus	6.91	6.90	6.86	6.88	6.85
Ca:P	1.55	1.55	1.56	1.56	1.56
Sodium	1.71	1.71	1.71	1.71	1.71
Chloride	3.72	3.72	3.72	3.72	3.72
Potassium	7.88	7.87	7.85	7.86	7.84
Lysine	13.76	13.74	13.66	13.70	13.64
Methionine	6.48	6.45	6.29	6.37	6.25
Threonine	10.72	10.68	10.53	10.61	10.49
Isoleucine	9.13	9.09	8.90	9.00	8.86
Arginine	12.37	12.33	12.18	12.26	12.14
Valine	9.72	9.67	9.45	9.56	9.39

¹T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1 g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM.

²Contain 21.3% Ca and 18.7% P.

³Vitamin/mineral premix supply per kilogram of diet: vitamin A, 16,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin B1, 3 mg; vitamin B2, 10 mg; vitamin B6, 3 mg; vitamin B12, 15 µg; hetra, 5 mg; nicotinic acid, 60 mg; pantothenic acid, 14.7 mg; folic acid, 1.5 mg; biotin, 125 µg; choline chloride, 25 mg; iron, 20 mg; copper, 10 mg; manganese, 100 mg; cobalt, 1.0 mg; zinc, 82.222 mg; iodine, 1 mg; selenium, 0.2 mg; and molybdenum, 0.5 mg.

⁴Titanium dioxide (TiO₂) premix to be added as indigestible marker at a ratio of 1:4 of titanium dioxide:gluten meal.

⁵Benzoic acid premix to be made contain 20% of benzoic acid.

On day 21, the birds were euthanized by cervical dislocation prior to determination of intestinal pH. Digesta pH of the crop, proventriculus, jejunum, ileum, and caeca were measured using a sterile glass pH electrode (HI 99163, HANNA Instruments, Romania). Two independent pH readings were taken from each site. One representative bird with body weight closest to the mean weight of the pen was eviscerated and the digestive organs used for determination of treatment effect on intestinal growth. The gizzard, liver, and pancreas were weighed using a digital-electric balance (Mettler PM6000, Mettler Instruments Ltd., Switzerland). The length and weight of the duodenum, jejunum, and ileum *in-situ* were also measured. After the measurements, the content of those sections were emptied by gently flushing them with distilled water and the weight of empty intestinal parts were measured.

Jejunal section from one bird from each pen was taken for histomorphological examination of the jejunum. The chosen bird in each pen had body weight closest to the median body weight for the pen. The jejunum was separated from the end duodenal loop up to 1 cm proximal to the Meckel's diverticulum. Sections of approximately 6 cm were taken from the mid-jejunum, gently flushed with phosphate buffer saline (pH 7.2), and cut into 3 equal pieces. The tissue sections were then immediately fixed into 10% buffered formalin solution, shaken gently, and stored in a bottle with tightly sealed lid until further processing. The intestinal sections were subsequently dehydrated by transferring through a series of ethyl alcohols with increasing concentrations (70, 90, and 100%), cleared with xylene, and embedded in polyfin embedded wax in a Shandon Excelsior Tissue Processor (Thermo Fisher Scientific, Cheshire, UK). Tissue sections (2 μ m) were cut by a Finesse Rotary Microtome (Thermo Shandon Inc, Pittsburgh, PA), placed on glass slides, and stained with haematoxylin (Gill no. 2, Sigma, St. Louis, MO) and eosin (Sigma). The measurements for the villus and crypts dimension were carried out using a Leica DM4000 B Digital Microscope (Leica Microsystems Imaging Solutions Ltd., Milton Keynes, UK). Images of villus and crypts were captured using a Leica DC480 digital camera with measurements made using the LEICA QWin imaging software.

Fifteen well-oriented villi and crypts from jejunum were measured along their length (height and depth, respectively) and width. The villus height (VH) was measured from the crypt-villus junction to the brush border at the tip. Villus width (VD) was measured parallel to the adjoining villus. The crypt depth (CD) was measured from the base near the lamina propria to the crypt-villus junction. Crypt width (CW) was measured

parallel to the adjoining crypt. Similar procedures were used by Bollinger (1996). All measurements were made to the nearest micrometer.

4.2.3.2. Experiment 2: Digestibility

On day 14, 150 birds were transferred from floor pens to raised floor pens (5 birds per pen) and continued to receive the respective diets they had been receiving from day 1. The metabolism trial consists of 5 days of adaptation and 2 days of sample collection. Excreta were collected on days 20 and 21 and daily excreta collections were pooled within a pen and stored in a freezer at -20°C . All the birds were euthanized on day 21 and the distal sections of ileum (portion of the small intestine from Meckel's diverticulum to approximately 1 cm anterior to the ileo-cecal junction) were removed and contents were gently flushed with distilled water into aluminium containers. Digesta from birds within a pen were pooled, and stored at -20°C .

4.2.4. Chemical analyses

The dry matter (DM), organic matter (OM), ash, N, and gross energy (GE) values were determined in samples of diet, ileal digesta, and excreta. DM content was determined by drying the samples in the oven (Uniterm, Russel-Lindsey Engineering Ltd., Birmingham, England, UK) at 105°C for 24 hours (Method 934.01; AOAC, 2006). Organic matter was determined by ashing the samples in the muffle furnace (Carbolite Furnace, Bamford, Sheffield, England, UK) at 500°C overnight (Method 934.01; AOAC, 2006). Total N content was determined by the combustion method (Method 968.06; AOAC, 2006). Gross energy value was determined in an adiabatic oxygen bomb calorimeter using benzoic acid as an internal standard (Model 6200, Parr Instruments, Moline, Illinois, USA). Titanium concentration in samples of diets and ileal digesta was determined using the method of Short *et al.* (1996).

4.2.5. Calculations and Statistical Analyses

The profile of gastro-intestinal tract was determined as the weight of gizzard, pancreas and liver as well as the length and weight of the small and large intestines. Relative length and weight of each of the sections and the entire intestine were determined.

Relative weight of the intestines was calculated using this relation:

Equation 4-6. Formula for calculating relative weight of intestinal tract

$$RW_B = \frac{W_I}{W_B}$$

where RW_B = relative weight per live body weight (g/g); W_I = weight of the intestinal section (g); W_B = body weight of the live birds (g).

The relative length per weight of the intestinal section was calculated as:

Equation 4-7. Formula for calculating relative length of intestinal tract

$$RL_I = \frac{L_I}{W_I}$$

where RL_I = relative length per weight of intestinal section (cm/g); L_I = length of the intestinal section (cm), and W_I = weight of the intestinal section (g).

Ileal digestibility was calculated using the index method, with Ti as an indigestible marker. Ileal digestible and metabolizable energy were calculated by multiplying ileal energy digestibility and energy metabolizability with feed gross energy. Nitrogen-corrected AME (AMEN) value was calculated by correction for zero nitrogen retention using a value of 8.22 kcal per g N retained as described by Larbier and Leclercq (1992).

Growth performance, nutrient and energy utilization, intestinal pH, and histomorphology data were subjected to an Analyses of Variance employing randomized complete block design. Means were statistically separated by Tukey's test (Snedecor and Cochran, 1989).

Additivity for the effects of dietary BA and TM supplementation on the response criteria was tested as follows. For each response criterion of interest, the response observed in the control (C) treatment was used as a baseline to which other treatments were compared. The difference between the response to other treatments and the response to C was taken as the effect of dietary BA or TM addition. The values were then analyzed using the GLM procedure of GenStat Release 11th edition (2008), and the sum of the means for effects of individual BA and TM supplementation were compared with the mean of the effect of BA – TM mixture using Orthogonal Contrast. The sum of the individual effects of BA and TM supplementation would not be different from the effects of the BA and TM

mixture (in combination) if the effects of individual BA and TM were additive. All statements of significance are based on a probability of less than 0.05.

4.3. RESULTS

4.3.1. Growth Performance

Growth performance responses of the broiler chickens on day 21 to BA or TM supplementations individually or in combination are summarized in Table 4-20. Supplementation of 2 g/kg BA or 10 g/kg TM individually as well as in combination had no significant effect on any of the growth performance response criteria relative to the control. However, supplementation of combination of 1 g/kg BA and 5 g/kg TM improved ($P < 0.05$) body weight gain relative to the control.

4.3.2. Digesta pH

Table 4-21 shows the data for digesta pH in different sections of the digestive tract of the 21 days old male broiler chickens in response to BA or TM supplementation individually or in combination. There were no treatment effects on pH of the digesta in the proventriculus or ileum. pH in the crop were reduced relative to the control ($P < 0.05$) in diets supplemented with 2 g/kg BA, combination of 1 g/kg BA and 5 g/kg TM as well as combination of 2g/kg BM and 10 g/kg TM. Crop digesta pH was lower ($P < 0.05$) in diet supplemented with a combination of 2 g/kg BA and 10 g/kg TM relative to the diet with 10 g/kg TM alone. In the jejunum, supplementation of 2g/kg BA alone or in combination with 10 g/kg TM reduced ($P < 0.01$) digesta pH relative to the control. In the cecum, only the combination of 2 g/kg BA and 10 g/kg TM reduced ($P < 0.05$) digesta pH relative to the control.

4.3.3. Profile of Gastrointestinal Tract

Responses of the broiler chickens to BA and TM supplementations on the profile of gastrointestinal tract are presented in Table 4-22. None of the dietary treatment altered the relative weight of the digestive tract of 21 days old broiler chickens. The absolute length and relative length to the weight of the small intestine were also not affected by the dietary treatments.

Table 4-20. Growth performance responses of broiler chickens to diets containing different levels of benzoic acid and turmeric meal individually or in combination¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
Final Weight, g	767.12 ^b	809.35 ^{ab}	771.31 ^b	964.54 ^a	873.38 ^{ab}	40.82	0.019
Body Weight Gain, g	719.96 ^b	762.58 ^{ab}	724.16 ^b	917.74 ^a	826.39 ^{ab}	40.50	0.019
Feed Intake, g	1044.7	1007.2	1072.4	1131.4	1075.2	30.30	0.199
Gain:Feed	687.30	780.02	672.75	808.77	766.68	50.22	0.219

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens of 5 birds per treatment

²T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1 g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM.

Table 4-21. Intestinal pH in different sections of digestive tract in response to diets containing different levels of benzoic acid and turmeric meal supplementation¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	<i>P</i> -value
Crop	5.95 ^a	5.22 ^{bc}	5.64 ^{ab}	5.36 ^{bc}	4.98 ^c	0.134	< 0.001
Proventriculus	2.67	2.12	2.43	2.30	2.21	0.166	0.195
Jejunum	6.29 ^a	5.87 ^b	6.11 ^{ab}	5.93 ^{ab}	5.86 ^b	0.086	0.008
Ileum	7.20	6.75	7.01	6.86	6.78	0.173	0.374
Caeca	6.42 ^a	6.01 ^{ab}	6.17 ^{ab}	6.01 ^{ab}	5.84 ^b	0.166	0.025

^{a-c}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens of 5 birds per treatment

²T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1 g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM

Table 4-22. Response of broilers to diets containing benzoic acid or turmeric meal individually or in combination on the profile of gastrointestinal tract of broiler chickens (% of body weight)¹

Intestinal part	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
Full weight (g/100 body weight)							
Gizzard	3.93	3.67	3.46	4.04	4.06	0.196	0.185
Liver	3.13	2.95	3.00	2.91	2.92	0.084	0.357
Pancreas	0.45	0.44	0.44	0.44	0.44	0.016	0.989
Duodenum	1.40	1.27	1.32	1.38	1.42	0.062	0.413
Jejunum	3.82	3.30	3.63	3.27	3.66	0.197	0.237
Ileum	3.05	2.81	2.97	3.01	3.07	0.147	0.744
Empty weight (g/100 body weight)							
Gizzard	2.29	2.16	2.18	2.15	2.14	0.072	0.578
Liver	3.13	2.95	3.00	2.91	2.92	0.084	0.357
Pancreas	0.45	0.44	0.44	0.44	0.44	0.016	0.989
Duodenum	1.32	1.21	1.28	1.28	1.27	0.052	0.651
Jejunum	2.42	2.32	2.39	2.38	2.33	0.098	0.952
Ileum	1.74	1.54	1.69	1.69	1.68	0.077	0.462
Length (cm)							
Duodenum	28.25	28.39	27.30	28.85	26.50	1.003	0.292
Jejunum	66.93	68.72	67.20	66.05	62.28	1.821	0.683
Ileum	68.07	66.22	66.53	70.42	64.28	2.005	0.137
Relative length (cm/g intestinal weight)							
Duodenum	2.71	2.75	2.71	2.65	2.70	0.116	0.599
Jejunum	8.50	8.03	8.44	7.77	8.02	0.293	0.377
Ileum	8.62	7.71	8.38	8.25	8.29	0.275	0.252

¹Data represent means from 6 replicates per treatment

²T1=control (C); T2=C+2g/kg BA; T3=C+10g/kg TM; T4=C+1 g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM

4.3.4. Jejunal Histomorphology

Table 4-23 shows the histomorphology data of the intestinal wall of the 21-day old broiler chickens. Supplementation of BA at the rate of 2 g/kg alone or combination of BA and TM at the rate of 1 or 2 and 5 or 10 g/kg, respectively increased ($P < 0.01$) villus height, crypt depth and crypt width relative to the control. Supplementation with 10 g/kg TM alone in the diet increased the crypt depth and width ($P < 0.001$) relative to the control. Crypts were wider ($P < 0.01$) in all the treatments relative to the control but none of the treatment had any effect on the villus height to crypt depth ratio.

Photomicrographical examination of the jejunal cell wall (Fig. 4-8, 4-9, 4-10, and 4-11) show that 21-day old broiler chickens fed BA or TM supplemented diets had longer villi and deeper crypts compared with those receiving control diets (Fig. 4-7). The jejunal villi were predominantly well-proliferated, similar in size, grown horizontal to the height with firm and smooth wall, and covered with thin mucin barrier layers. The crypts were mostly longer and wider, compared with those of control birds.

4.3.5. Energy and Nutrient Utilization

The apparent ileal nutrient digestibility and ileal digestible energy of the broiler chickens fed diet containing different levels of BA and TM supplementation are presented in Table 4-24. Dietary supplementation of BA and TM did not affect the apparent ileal DM and OM digestibility. However, supplementation of BA alone at the rate of 2 g/kg improved ($P < 0.01$) the apparent ileal ash digestibility relative to the control whereas supplementation of 10 g/kg TM alone was intermediate. The other treatments had no effect on ash digestibility. Only the supplementation of TM alone at the rate of 10 g/kg increased ($P < 0.05$) energy digestibility and IDE relative to the control treatment. In addition, N digestibility was lowest ($P < 0.05$) in the treatment with the combination of BA and TM at the rates of 2 and 10 g/kg, respectively and greatest ($P < 0.05$) in the diet with TM supplementation alone whereas the other diets were intermediate.

The apparent total tract nutrient retention and energy metabolisability, as well as the AME of broiler chickens fed diet containing BA or TM supplementation individually or in combination are presented in Table 4-25. There were no dietary treatments effects on total tract DM, OM or N retention. Only the diet supplemented with 2 g/kg BA had higher ($P < 0.01$) total tract ash retention relative to the control diet. Energy metabolisability was

Table 4-23. Broiler jejunal histomorphological responses to supplementation of benzoic acid or turmeric meal individually or in at 21 days of age¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	<i>P</i> -value
Villus height, μm	557.8 ^b	807.1 ^a	787.2 ^{ab}	911.7 ^a	814.4 ^a	54.445	0.003
Villus width, μm	66.00 ^b	76.60 ^{ab}	77.78 ^{ab}	79.91 ^a	75.14 ^{ab}	2.808	0.023
Crypt depth, μm	57.71 ^b	81.13 ^a	83.51 ^a	92.47 ^a	84.94 ^a	3.269	< 0.001
Crypt width, μm	34.50 ^c	40.01 ^b	41.12 ^b	46.37 ^a	43.96 ^{ab}	1.174	< 0.001
Ratio ³	9.669	10.139	9.615	9.945	9.818	0.704	0.984

^{a-c}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Means represent 15 readings from 6 pens of individual bird per replicate pen.

²T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1 g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM

³Ratio of villus height to crypt depth.

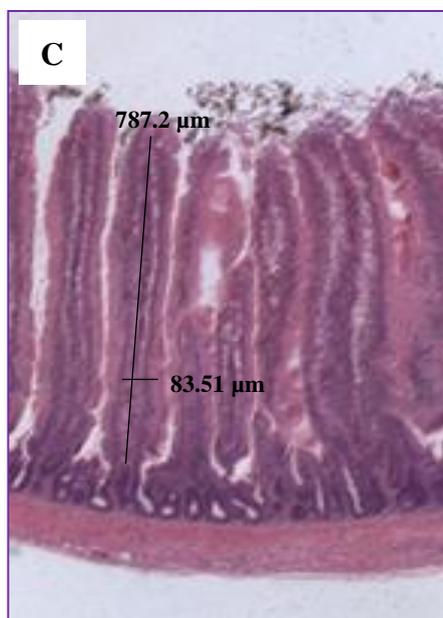
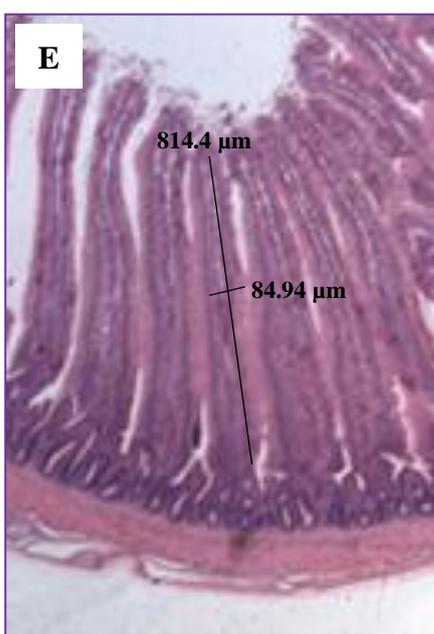
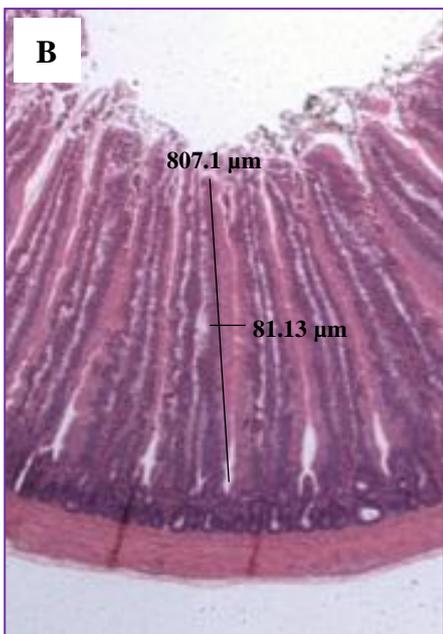
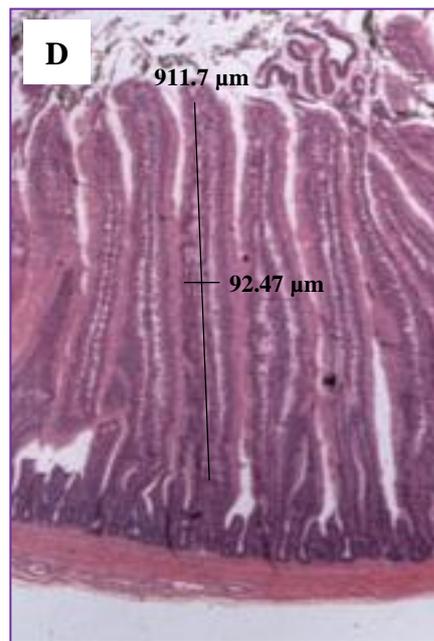
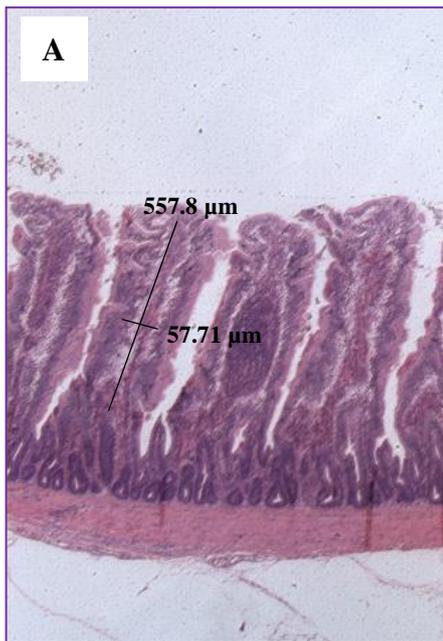


Figure 4-7. Photomicrography of the jejunum of 21-day old broiler chickens fed diet (a) without additive (control), (b) with 2.0 g/kg BA, (c) with 10.0 g/kg TM, (d) with 1.0 and 5.0 g/kg BA and TM combination, and (e) with 2.0 and 10.0 g/kg BA and TM combination supplementation.

The overall architectural of the villi of the control birds were short, appeared coarse, altered inconsistently, serrated, and covered by thick mucin barriers whereas the supplemented birds were long, wide, smooth, well proliferated, and covered by thin mucin barriers. The crypts of the control birds were shallow and their growth appeared stunted and undersized whereas the supplemented birds were deep and well developed.

Table 4-24. Apparent ileal nutrient digestibility and digestible energy of broiler chicken fed diet containing benzoic acid or turmeric meal supplementation on DM basis¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
DMD, %	76.82	75.60	75.04	75.46	74.32	0.974	0.491
OMD, %	80.65	78.80	78.66	79.02	78.19	0.925	0.417
Ash.D, %	33.89 ^b	48.94 ^a	40.15 ^{ab}	36.11 ^b	34.04 ^b	2.397	0.001
ED, %	77.13 ^b	76.50 ^b	79.29 ^a	76.44 ^b	76.67 ^b	0.668	0.038
IDE, kcal/kg	3070.7 ^b	3117.0 ^b	3239.1 ^a	3072.4 ^b	3109.6 ^b	27.008	0.002
ND, %	79.95 ^{ab}	81.27 ^{ab}	82.41 ^a	80.54 ^{ab}	79.76 ^b	0.578	0.028

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens per treatment. DMD is dry matter digestibility; OMD is organic matter digestibility; Ash.D is ash digestibility; ED is energy digestibility; IDE is ileal digestible energy; ND is nitrogen digestibility.

²T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM.

Table 4-25. Total tract nutrient retention and metabolizable energy of broiler chicken fed diet containing benzoic acid or turmeric meal supplementation on DM basis¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
DMR, %	73.59	73.19	73.07	73.68	73.22	0.403	0.777
OMR, %	75.94	75.44	75.51	76.20	75.73	0.400	0.653
Ash.R, %	42.21 ^b	48.40 ^a	41.97 ^b	39.09 ^b	38.45 ^b	0.955	< 0.001
EM, %	74.10 ^b	75.55 ^{ab}	75.31 ^{ab}	76.09 ^a	75.93 ^a	0.409	0.021
AME, kcal/kg	2950.0 ^b	3078.2 ^a	3076.3 ^a	3058.5 ^a	3079.6 ^a	16.530	< 0.001
AMEn, kcal/kg	2841.2 ^b	2968.8 ^a	2969.4 ^a	2952.1 ^a	2979.6 ^a	18.302	< 0.001
NR, %	65.78	65.35	66.73	65.94	66.16	0.871	0.848

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates per treatment. DMR is dry matter retention; OMR is organic matter retention; Ash.R is ash retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen-corrected AME; NR is nitrogen retention.

²T1=control (C); T2=C+2g/kg benzoic acid (BA); T3=C+10g/kg turmeric meal (TM); T4=C+1g/kg BA+5g/kg TM; T5=C+2g/kg BA+10g/kg TM.

greater ($P < 0.01$) in diets with combination of TM and BA relative to the control whereas the diets with individual supplementation of BA and TA were intermediate. Supplementation of BA and TM individually and in combination increased AME and AMEn ($P < 0.01$) relative to the control diet.

4.3.6. Test of Additivity or Interaction between the Feed Additives

Improvements of the growth performance responses, reductions in the digesta pH, and enhancements of the nutrient and energy utilization of broiler chickens due to combination of BA and TM meal supplementation are presented in Table 4-26, 4-27, and Table 4-28, respectively. Results of the orthogonal contrasts showed that BA and TM were additive in their effects on the growth performance responses, digesta pH in the proventriculus, jejunum, and ileum, as well as on the energy metabolizability and apparent metabolizable energy. On the other hand, orthogonal contrasts showed that these additives were associative on the ileal digestible energy ($P < 0.022$) and nitrogen digestibility ($P < 0.025$), but negatively interacted on crop ($P < 0.039$) and caecal ($P < 0.025$) pH.

Table 4-26. Improvement of the growth performance responses of broiler chickens due to benzoic acid and turmeric meal supplementation¹

Item	FW	BWG	FI	Gain:Feed
Diets				
C + 2 g/kg TM (2)	42.22	42.62	-37.53	92.72
C + 10 g/kg TM (3)	4.18	4.20	27.73	-14.55
C + 2 g/kg BA + 10 g/kg TM (5)	106.25	106.44	30.54	79.38
SEM	37.824	37.775	34.054	60.063
<i>P</i> -values for main effect of diets	0.206	0.204	0.320	0.420
<i>P</i> -values for contrast				
2+3 vs. 5	0.103	0.103	0.415	0.596

¹Data represent means from 6 replicates pens of 5 birds per treatment. FW is final weight (g); BWG is body weight gain (g); FI is feed intake (g); Gain:Feed is ratio of gain to feed.

Table 4-27. Reduction of the digesta pH in different segments of gastro-intestinal tract of broiler chicken fed diet containing benzoic acid or turmeric meal supplementation¹

Item	Crop	Proventriculus	Jejunum	Ileum	Caeca
<i>Diets</i>					
C + 2 g/kg TM (2)	-0.73	-0.55	-0.42	-0.45	-0.41
C + 10 g/kg TM (3)	-0.31	-0.24	-0.18	-0.19	-0.25
C + 2 g/kg BA + 10 g/kg TM (5)	-0.96	-0.46	-0.43	-0.41	-0.58
SEM	0.153	0.133	0.054	0.221	0.078
<i>P</i> -values for main effect of diets	0.036	0.283	0.011	0.660	0.039
<i>P</i> -values for contrast					
2+3 vs. 5	0.039	0.668	0.068	0.726	0.025

¹Data represent means from 6 replicates per treatment.

Table 4-28. Improvement of the energy and nutrient digestibility of broiler chicken fed diet containing benzoic acid or turmeric meal supplementation in ileal part and total tract¹

Item	ED	IDE	ND	EM	AME	AMEn	NR
<i>Diets</i>							
C + 2 g/kg TM (2)	-0.63	46.25	1.32	1.45	128.17	127.59	-0.43
C + 10 g/kg TM (3)	1.73	151.19	2.46	1.20	126.28	128.26	0.95
C + 2 g/kg BA + 10 g/kg TM (5)	-0.47	38.93	-0.19	1.82	129.59	138.44	0.38
SEM	0.435	17.748	0.643	0.404	16.470	17.698	0.754
<i>P</i> -values for main effect of diets	0.007	0.003	0.046	0.566	0.990	0.890	0.461
<i>P</i> -values for contrast							
2+3 vs. 5	0.088	0.022	0.025	0.336	0.909	0.638	0.899

¹Data represent means from 6 replicates per treatment. ED is energy digestibility; IDE is ileal digestible energy; ND is nitrogen digestibility; EM is energy metabolizability (%); AME is apparent metabolizable energy (kcal/kg); AMEn is nitrogen-corrected AME (kcal/kg); NR is nitrogen retention (%).

4.4. DISCUSSION

This study was designed to investigate the response of broilers to supplementation of BA and TM and assess possible additivity and interaction between the additives. The potential associativity, additivity or interactivity in the effect of BA and TM were also explored to account the benefit of these additives when they are used in different rates of combination. The level of BA supplementation in this study was based on the results of our previous study (Chapter 2) that showed 0.53 g/kg BA supplementation reduced digesta pH in the caeca and stimulated the growth performance, whereas 3.20 g/kg stimulated the growth of absorptive cells in the jejunum. Therefore, supplementation of the average of both doses (2 g/kg) was intended to approach the optimum dose predicted. This decision was also supported by the findings of Józefiak *et al.* (2007) in which dietary supplementation of BA with the dose of less than 2.5 resulted in better growth performance, digesta pH, and microbial population than the other dose supplementations. On the other hand, the level of 10 g/kg TM supplementation was chosen based on observations of Durrani *et al.* (2006) and Al-Sultan (2003). In addition, two levels of combinations of the additives 2 g/kg BA and 10 g/kg TM or 1 g/kg BA and 5 g/kg TM were used to determine whether combination of 1 g/kg BA and 5 g/kg will have effect equal to 10g/kg TM alone or 2 g/kg BA alone and whether using half the doses of BA and TM will be sufficient for maximum response.

Supplementation of OA and TM in poultry diets are intended to modify gut microflora population (Thompson and Hinton, 1997) and stimulate the digestive system (Tollba and Hassan, 2003), ultimately resulting in promotion of growth and health in the birds. In sub-therapeutical dose, organic acids supplementations have the capacity to stimulate the growth performance (Vogt *et al.*, 1982), nutrient utilization (Runho *et al.*, 1997), and health of the birds (Canibe *et al.*, 2001). Nevertheless, because organic acids are only partly dissociated and dose dependent (Pirgozliev *et al.*, 2008), not all of the acids influence microflora population, stimulate enteric health, and support growth of broiler chickens (Byrd *et al.*, 2001; Roy *et al.*, 2002). However, the cell-growth stimulation might directly improve protein and energy utilisation (Dibner and Buttin, 2002) as a direct result of reduced competition between the host and pathogens for nutrients, by minimizing endogenous nutrient losses or by reducing the production of ammonia and other growth-depressing microbial metabolites (Ferket, 2004).

The data from current study indicate that dietary supplementation of BA or TM individually had no effect on growth performance, but combination of the BA and TM mixture in the diets at the rates of 1 and 5 g/kg respectively, improved the body weight gain and final body weight, without influenced the gain to feed ratio of 21-day old broilers. Results in this growth performance responses was in the line with the finding of Mikulski *et al.* (2008) who reported that a combination of organic acids blend and herbal products at the rate of 1 g/kg increased the body weight of male turkey without affecting the FCR. In another study, dietary supplementation of 0.2 g/kg pure curcumin (phytochemical derived from turmeric) increased the body weight gain and reduced the feed conversion ratio of 42 days old Arbor Acre broiler chickens (Rajput *et al.*, 2013). Improvement in the growth performance responses due to the combination of BA and TM in this study was likely due to the combined effects of BA and TM as antimicrobial and antioxidant agents (Allen *et al.*, 1998; Canibe *et al.*, 2001; Radwan *et al.*, 2008) which may result in stimulation of protein synthesis (Osawa *et al.*, 1995). Increased protein synthesis should result in growth and is beneficial especially during juvenile stage when birds are rapidly growing and are more susceptible to imbalance in gut microflora. Both TM and BA were additive in their effects in stimulating growth in the current study.

Improvement in the growth performance responses may also be attributed to the pH reduction properties of BA. As shown in this study, supplementation of 2 g/kg BA alone reduced the digesta pH in the crop and jejunum. Reduction of the digesta pH in some intestinal sections due to organic acid supplementation in this study was in the line with the findings of previous studies. In a study using corn-wheat-soybean based diets, Józefiak *et al.* (2010) showed that dietary supplementation of 2 g/kg pure benzoic acid reduced digesta pH in the caeca of 21-days old ROSS 308 cockerels, while digesta pH in the crop, gizzard, ileum, and rectum were not affected. In a previous study, Józefiak *et al.* (2007) showed that supplementation of 2.5 g/kg pure benzoic acid in corn-wheat-soybean based diets reduced digesta pH in the crop and caeca of 42 days old COBB 500 cockerels. In another study, Ao *et al.* (2009) reported that dietary supplementation of 2.0 g/kg citric acid in corn-soybean based diets reduced digesta pH in the crop of 22 days old COBB broiler chickens. Chotikatum *et al.* (2009) also showed a reduction in the crop and jejunal pH when the corn-soybean based diets were supplemented with a mixture of lactic, citric, ascorbic, and propionic acids at the rate of 4 g/kg. On the contrary, some authors reported no difference in the intestinal pH when diets were supplemented with formic acid (Hernández *et al.*, 2006) or propionic acid (Thompson and Hinton, 1997). Abdel-Fattah *et al.* (2008) found no

significant difference on the digesta pH of the duodenum, jejunum, and ileum when the diets were supplemented with 15-30 g/kg acetic, citric, or lactic acid. Waldroup *et al.* (1995) also found no significant difference in the caecal pH when the diets were supplemented with 2.5-20 g/kg lactic or formic acids. Paul *et al.* (2007) also reported non-significant difference in the digesta pH of proventriculus and ileum when the diet of Vencobb-100 broiler chicken was supplemented with 3 g/kg OA salt (ammonium formate or calcium propionate). The different results in available literatures indicate that efficacy of organic acids in optimizing intestinal health through pH reduction might depend on the nature of the acids. One of the possible characteristics is the acid dissociation constant (pKa). The difference in the pKa (Table 1-2) might affect digesta pH differently in the gut sections of broiler chickens. Benzoic acid has pKa 4.19 (Baird-Parker, 1980) and showed the strongest killing effect against *Salmonella typhimurium* than that of acetic acid, formic acid, propionic acid, lactic acid, and sorbic acid in pig stomach content at pH 4. In a study using piglets, Maribo *et al.* (2000) reported that benzoic acid has the strongest killing effect against coliform bacteria in both stomach and small intestinal content than propionic acid, formic acid, butyric acid, lactic acid, and fumaric acid.

Reduction of the digesta pH in some intestinal sections in current study can be attributed to the pH reduction property of BA (Dibner and Buttin, 2002). Interestingly, pH was usually lower in diets containing BA compared to the diet containing only TM. Canibe *et al.* (2001) reported that the antimicrobial effect of organic acid in broiler takes place mostly in the upper part of the gut, such as the crop where the acidity is more relevant for the action of the acid. As a weak acid, BA is able to penetrate the cellular membrane of bacteria. In the cell, BA releases protons in the alkaline cytoplasm, resulting in the reduction of the intracellular pH. Reduction of pH is unfavourable for the pH sensitive pathogenic bacteria (Pelicano *et al.*, 2005). This reduction of the cytoplasmic pH makes bacteria unable to perform their enzymatic reactions and nutrient transport systems, forcing the bacterial cell to use ATP to release excess protons (Ricke, 2003). The RCOO⁻ produced from OA can interrupt DNA replication and protein synthesis (Biggs and Parsons, 2008). Therefore, the acid-intolerant pathogenic bacteria are under stress and cannot replicate or grow (Hernández *et al.*, 2006). The limitation of growth of enteropathogen species in the gut is favourable for the growth and colonization of beneficial microflora (Eklund, 1983), reduces microbial metabolites (Gabert *et al.*, 1995; Anderson *et al.*, 1999), reduces incidences of sub-clinical intestinal infections (Russell, 1992), reduces the competition for available nutrients between the microbes and the host (Thomke and Elwinger, 1998; Ricke,

2003), reduce adverse effect of the over-stimulation of intestinal mucus barrier (Ferket, 2004), thus maintaining enteric health and supporting better intestinal milieu for greater nutrient absorption and metabolism (Apajalahti *et al.*, 2004). Reduction of the digesta pH might also stimulate the pancreatic secretion for lipid and starch digestion (Dibner and Buttin, 2002), hence increase the capacity of digestion of some nutrients in the gut. In addition, it was also observed in a study by Maribo *et al.* (2000) that BA could still be found in large amount in the distal parts of small intestine of piglets. This could be an indication that BA might not be metabolized as fast as other organic acids and thus be exerting additional beneficial effect on the terminal section of the gut.

Supplementation of individual BA reduced the pH in some intestinal sections. Interestingly, combination of BA and TM in this study was not only able to reduce digesta pH in the crop and jejunum, but also in the caeca. Achievement in a more intestinal section with lower pH in current study might show the indication that TM augments the pH-reducing action of BA. However, it seems that the effects observed when the both additives combined were principally from BA. It was also observed that TM slightly reduced pH in all intestinal sections measured, but it reduced the pH when both TM and BA were used in combination. Reduction of the digesta pH in the distal part of intestine might show that active substances in TM take a part in the pH lowering action in this additive. As reported by Kamel (2001), herbal products stimulate pepsin activity by reducing pH of the gut to the optimum level for pepsin activity. This improvement in protein digestion should enable greater nitrogen digestibility for greater capacity for growth and body development.

The weight and length of digestive tract can give an indication of its health status. The growth of the gut is affected by the shift in and formation of the microflora that develop a synergistic relationship with the host (Torok *et al.*, 2009). Development and health of the gut may be interrupted by microflora imbalances (Kraehenbuhl and Neutra, 1992). The entero-pathogenic microflora colonizing the gut might attach to the luminal surface of the mucosa (Craven and Williams, 1997), over-stimulate the intestinal epithelial barriers (van der Klis and Jansman, 2002; Ferket, 2004), and restrict the growth of absorptive cell, which in turn physically increases the relative weight of the intestinal segment. On the other hand, reduction in the relative weight and length of the gut are believed to be beneficial for the whole body energetics. Spratt *et al.* (1990) indicated that the gut of broiler chickens only represents around 1.5% of the total body weight, but

consumes approximately 6-8% of the energy derived from the diet. Therefore, the shorter the length of the intestine, the less energy and nutrient required to maintain the section.

In the current study, dietary treatments did not influence the weight and length of the gut. This finding is similar to those previously reported by who used low doses of various organic acids (Denli *et al.*, 2003; Gunal *et al.*, 2006; Abdel-Fattah *et al.*, 2008; Houshmand *et al.*, 2012). In studies with TM, Durrani *et al.* (2006), Gowda *et al.* (2008), Mehala and Moorthy (2008), and Ashayerizadeh *et al.* (2009) reported that dietary supplementation of low dose BA or TM meal did not affect the relative weight and relative length of gastrointestinal tract of broiler chickens. On the contrary, Ademola *et al.* (2009), Dieumou *et al.* (2009), and Raeesi *et al.* (2010) reported that the weight or length of digestive organs of broiler chickens were reduced when the diets were supplemented with low dose of garlic meal. However, it is possible that the variations noted amongst these studies were due to extraneous factors, such as the differences in the experimental methods, breed of the birds, length of rearing period, and basal diets used in the experiments, as well as the difference in the method of TM preparation and the dose of supplementations.

Dietary supplementation of low dose BA and TM in this study stimulated the growth of absorptive cells in the small intestinal wall, as seen in the increase in the length and width of the villus as well the depth and width of the crypts of jejunum. The increase in the absorptive surface in current study was in the line with the findings of Paul *et al.* (2007), Viola and Vieira (2007), and Senkoylu *et al.* (2007). In a recent study, Rajput *et al.* (2013) showed that dietary supplementation of 0.2 g/kg pure curcumin derived from turmeric in a corn-soybean based diet increased the villus height and width of duodenum, jejunum, and ileum of 42 days old Arbor Acre broiler chickens. The villus height to crypt depth ratio in the duodenum and ileum was also significantly increased in curcumin supplemented birds. Contrary to our results, Gunal *et al.* (2006), García *et al.* (2007), and Smulikowska *et al.* (2010) showed that dietary supplementation of low dose organic acids or a blend of plant extracts did not alter the growth of villus and crypts in the small intestinal mucosa of broiler chickens. The improvement of the absorptive sites in the jejunum in current study might be connected to the stimulation of digestion by BA and phytochemicals in the TM. In addition, BA and TM were additive in their effect on the growth of the absorptive cells. Nevertheless, the different responses found between our study and other studies might be caused by the different experimental conditions and the

different level of dietary additives supplementation. The improvement of the absorptive cell in the jejunum cell wall in current study is essential for effective absorption of in-gut available nutrients (Geyra *et al.*, 2001). The treatment-stimulated increase in the villus height and width as well as the crypt depth and width in the current study might be indirect effect of the antimicrobial properties of BA and TM (Araújo and Leon, 2001; Dibner and Buttin, 2002; Chattopadhyay *et al.*, 2004). Reduction of the load and colonization of pathogenic microbes in the gut should reduce the adverse effect of the toxin and harmful substances produced by the microbes (Podolsky, 1993) and hence might prevent over-production of the mucin barrier by the goblet cells (Ferket, 2004). Together with concurrent stimulation of gastric secretions (Platel and Srinivasan, 2000), a more optimal growth of intestinal absorptive cells should allow greater nutrient absorption thus producing a more optimal growth performance (Adil *et al.*, 2006). This mechanism might address the reasons for why the villi in BA or TM supplemented birds were longer and wider with thinner mucus compared to those in control birds.

Dietary supplementation of low dose of BA alone in this study increased the apparent ileal ash digestibility as well as the total tract ash retention and apparent metabolizable energy, while supplementation of TM alone in low dose stimulated the ileal digestible energy (IDE) and apparent metabolizable energy (AME) of broiler chickens. Dietary inclusion of combination of BA and TM increased energy metabolizability and AME. There is insufficient information on the effects of BA and TM on the energy and nutrient utilization in broiler chickens. However, there is evidence to indicate that supplementation of organic acid at the dose of 5 to 10 g/kg improved the AME of broiler chickens (Runho *et al.*, 1997). Improvement of the ash and energy digestibility in this study might be due to the digestive stimulant property of OA and TM. The mechanism by which BA and TM stimulate digestion process is probably due to the stimulation of pancreatic digestive enzymes and bile secretions. Atapattu *et al.* (2005) reported that, as an organic acid, BA may weaken the structure of crude fibre, thus making gastric juices able to penetrate more easily into the cell wall, which in turn will make the macro-nutrients in the diets more susceptible to enzymatic digestion. On the other hand, as a herbal product, turmeric is known to contain phytochemicals that enhance the activities of terminal digestive enzymes of small intestinal mucosa. For instance, Khan *et al.* (2012) reported that curcumin, one of the active compounds in turmeric, increased the activities of pancreatic lipase, amylase, trypsin, and chymotrypsin in broiler chickens. Feeding diets containing curcumin can improve the secretion of digestive enzymes, and so improve the

digestibility of nutrients and energy. In other studies, dietary supplementation of herbal products were shown to stimulate digestion by enhancing the activities of pancreatic α -amylase (Lee *et al.*, 2003a), pancreatic maltase activity (Williams and Losa, 2001; Jang *et al.*, 2007) of broiler chickens. In studies with rat, dietary supplementation of turmeric increased the bile acid flow and secretion (Platel and Srinivasan, 2001; Platel and Srinivasan, 2004) as well as stimulated secretion of pancreatic digestive enzymes, such as amylase, lipase, proteases, and chymotrypsin (Platel and Srinivasan, 1996; Platel and Srinivasan, 2000; Platel *et al.*, 2002; Rao *et al.*, 2003), which play a crucial role in metabolism and accelerating macro-nutrient digestion in the small intestine. As shown in some studies, a greater availability of enzymes in the digestive tract enhances the digestion process. As reported by Noy and Sklan (1995), ileal digestibility of starch in broilers between 4 and 21 days of age rarely reaches 85%. Therefore, increased availability of digestive enzymes could improve the digestion process in the gut (Bedford and Schulze, 1998). For instance, supplementations of exogenous amylase may increase the presence of amylase in the gut and improve the digestibility of robust starch in the jejunum and ileum. A study using a mixture of enzymes including amylase has indicated that supplementation of exogenous enzymes increased digestive process on a corn and soybean meal diet (Douglas *et al.*, 2000). In Gracia *et al.* (2003) study, dietary supplementation of alpha-amylase increased nutrient digestion and growth performance of broiler chickens fed a corn-soybean meal diet. A study using a mixture of enzymes has shown that supplementation of a cocktail of xylanase, amylase, and protease individually or in combination with phytase in corn-soybean meal based diets increased ileal N and P digestibility of 21 days old broiler chickens (Olukosi *et al.*, 2007). In the study, growth performance of the birds was also increased, but the improvement in performance appears to be predominantly from phytase. Moreover, supplementation on bacterial enzymes might also increase the availability of digestive enzymes in the gut of broiler chickens fed cereal-based diets. In a study using Cobb 500 broiler chickens, Ondreci *et al.* (2006) showed that supplementation of α -amylase-producing bacterial culture in a corn-based diets increased nutrient digestibility, gut morphology, and growth performance of 21 days old broiler chickens. On the other hand, supplementation of protease and α -galactosidase in the diets improved the nutritive value of soybean meal in broiler cockerels and broiler chicks (Ghazi *et al.*, 2003). Therefore, it is possible that TM supplementation optimizes digestive process in the gut of broiler chickens. These effects may be responsible for why BA and TM increased energy utilization and stimulate digestive system in broiler chickens. Moreover,

the improvement of the energy metabolizability and AME due to combination of BA and TM in the current study was additive.

Results of the current study indicate that the combination of BA and TM at the rate of 1 and 5 g/kg was optimum because it resulted in a greater stimulation of the jejunal histomorphology and the growth performance of the birds, compared to those receiving individual supplementation of 2 g/kg BA or 10 g/kg TM or a combination of 2 g/kg BA and 10 g/kg TM. Combination of BA and TM at the rate of 1 and 5 g/kg respectively also reduced the digesta pH of broiler chickens.

It is concluded that BA and TM can be used alone or in combination to promote gut health and improve growth performance of broiler chickens. Combination of BA and TM at the rate of 1 and 5 g/kg respectively, was optimum for promoting growth performance, improving jejunal digestive cells growth, and enhancing energy utilization. Future studies evaluating the efficacy of a blend of herbal products would be useful to further elucidate the mode of action of herbal products in promoting health and growth of broiler chickens.

5. GROWTH PERFORMANCE, INTESTINAL pH, AND ENERGY AND NUTRIENT UTILIZATION OF BROILER RECEIVING DIETS SUPPLEMENTED WITH GARLIC AND TURMERIC MEAL INDIVIDUALLY OR IN COMBINATION

5.1. INTRODUCTION

Plant products (herbs, spices, and essential oils) have been widely used for many years as spices in human food culinary, as herbal remedies to prevent and cure various diseases (Nadkarni, 1976; Ammon *et al.*, 1992), and as feed additives in animal diets in many countries, such as in Indonesia (Kirana *et al.*, 2003; Murnigsih *et al.*, 2005), Pakistan (Mahmood *et al.*, 2009), Nepal (Eigner and Scholz, 1999), China and Japan (Cao *et al.*, 2001). Plant products are thought to have active compounds (phytochemicals) in root, stem, leaves, barks, or seed that possess important pharmacological properties. Various herbal products are found to have beneficial properties which might make them useful as alternatives to antimicrobial growth promoter (AGP) formerly routinely used in poultry diets. The primary mode of action of the phytobiotics as potential growth promoters is attributed to their ability to inhibit the growth of harmful intestinal microflora in the GIT (Benjilali *et al.*, 1984; Hammer *et al.*, 1999; Lopez *et al.*, 2005) and by stimulating the function of digestive organ (Jang *et al.*, 2004).

It has been shown *in vitro* that garlic (*Allium sativum*) extract has antibacterial, antifungal, and antiviral properties (Johnson and Vaughn, 1969; De Wit *et al.*, 1979; Ahsan *et al.*, 1996). Sarica *et al.* (2005) reported that garlic meal supplementation reduced the population of pathogenic coliform in the gut of broiler chickens. In addition, Gorinstein *et al.* (2005) and Kim *et al.* (2009) reported that garlic products have antioxidative properties in broiler chickens and layer hens.

The phytochemicals in turmeric (*Curcuma longa*) include curcumin, curcuminoids, demethoxycurcumin, zingiberene, curcumenol, eugenol, turmerin, turmerones, among others (Smith and Robinson, 1981; Aggarwal *et al.*, 2007) which are responsible to the efficacy of turmeric as a green additive. Curcumin is the main active compound that furnishes turmeric with its characteristic yellow colour and is recognized as being responsible for most of its therapeutic effects, including antibacterial, antifungal,

antiprotozoal, antiviral, antioxidant, antiinflammatory, and hypocholesteromic activities (Chattopadhyay *et al.*, 2004). Curcumin and curcuminoids possess anti-coccidial (Allen and Fetterer, 2002), anti-nematocidal (Kiuchi *et al.*, 1993), antioxidative (Ruby *et al.*, 1995; Sugiyama *et al.*, 1996), immunomodulatory (Yarru *et al.*, 2009), and anti-inflammatory (Mukhopadhyay *et al.*, 1982) properties. In poultry studies, turmeric meal stimulated growth performance (Gowda *et al.*, 2008; El-Hakim *et al.*, 2009) and the immunomodulatory system in broiler chickens (Kumari *et al.*, 2007) and laying hens (Sawale *et al.*, 2009). Feeding diets containing phytobiotics may result in inhibition of the growth, and colonization of entero-pathogenic microbes in the digestive tract, thus contributing to the balance of gut microflora (Harris *et al.*, 2001), and promoting the growth performance and health of birds (Adibmoradi *et al.*, 2006).

Because of the benefits of using turmeric and garlic meals in broilers when supplemented individually, there is the likelihood that a combination of both phytobiotics will confer additional benefits than the use of each individually. However, there is no information on the use of combination of garlic and turmeric meals on enteric health, growth performance and nutrient utilisation responses in broiler chickens. Therefore, the objective of the current study is to investigate the response of broiler chickens to diets supplemented with garlic and turmeric meals, using growth performance, intestinal pH, and energy and nutrient utilisation as response criteria. The possible associative effects and additivity between garlic and turmeric meal have also been investigated.

5.2. MATERIALS AND METHODS

5.2.1. Birds and Housing

A total of 300 male one-day old Ross 308 broiler chickens were used in this study that comprised two experiments. All animal experiment work was approved by the SAC Animal Experimentation Committee. The chicks were vaccinated at the hatchery against Infectious Bursal Disease and no additional vaccinations were administered during the study. At day old, the chicks were wing-tagged, weighed and allocated to 30 floor pens (110 cm x 150 cm) which were equipped with feeders and bell drinkers. The pens were situated in environment-controlled rooms with facilities to control temperature, light, and humidity. The room temperatures on day 0 to 7, day 7 to 14, and day 14 to 21 were kept at 35, 32, and 27 °C, respectively.

5.2.2. Birds Grouping, Diets, and Experimental Design

The chicks were allocated to 5 treatments in randomized complete block design with a 2×2 + 1 factorial arrangement. Each treatment had 6 replicates with 10 birds per replicate pen. Decisions on the number of replicates used in this study were based on power calculation during the planning of the study as well as experience from similar experiments. All of the 5 wheat-soybean meal based experimental diets were formulated to meet the Ross 308 specifications. The birds in treatment 1 (control) did not have garlic (GM) or turmeric meal (TM) in their diet. Birds in treatments 2 (T2) and 3 (T3) were fed control diet plus GM or TM added at the rates of 10 g/kg. Birds in treatment 4 (T4) were fed control diet plus a combination of GM and TM at the rates of 5 g/kg each, whereas birds in treatment 5 (T5) were fed control diet plus a combination GM and TM at the rates of 10 g/kg each. Titanium dioxide (0.3 %) was added to all diets as a digestibility marker. The birds received the experimental diets for the entire 21 days of the experiment. Birds had free access to feed and water, but no coccidiostat or enzymes were added to the diets. The ingredients and chemical compositions of the diets are presented in Table 5-29.

5.2.3. Birds Grouping

On day 14, 150 birds (5 randomly selected birds from each of the 30 pens) were transferred to 30 metabolism cages for digestibility trial (Expt. 2) and the remaining 150 birds remained in their pens to continue growth performance and intestinal health study (Expt. 1). The birds transferred to metabolism cages continued to receive the corresponding diets they were receiving previously.

5.2.3.1. Experiment 1: Growth Performance and Intestinal pH

The 150 birds that continued in the floor pens were used for this experiment that last for 21 days. The birds and feed were weighed both on day 1 and 21. On day 21, all of the birds were euthanized by cervical dislocation prior to the determination of digesta pH. pH of the digesta in the crop, proventriculus, jejunum, ileum, and caeca were measured using a sterile glass pH electrode (HI 99163, HANNA Instruments, Romania). Two independent pH readings were taken from each site.

Table 5-29. Ingredients composition and nutrient calculation of the experimental diet

Item	Dietary treatments ¹				
	T1	T2	T3	T4	T5
<i>Ingredients composition, g/kg</i>					
Wheat, White	611.00	611.00	611.00	611.00	611.00
Soybean meal -48%	259.50	259.50	259.50	259.50	259.50
Soybean oil	31.20	31.20	31.20	31.20	31.20
Gluten meal	14.50	14.50	14.50	14.50	14.50
Limestone (38% Ca)	16.00	16.00	16.00	16.00	16.00
Dicalcium phosphate ²	17.00	17.00	17.00	17.00	17.00
Common salt	3.50	3.50	3.50	3.50	3.50
Vitamin/mineral premix ³	4.00	4.00	4.00	4.00	4.00
DL-Methionine	3.00	3.00	3.00	3.00	3.00
L-Lysine HCl	5.00	5.00	5.00	5.00	5.00
Threonine	0.30	0.30	0.30	0.30	0.30
Titanium dioxide premix ⁴	15.00	15.00	15.00	15.00	15.00
Garlic meal	0.00	10.00	0.00	5.00	10.00
Turmeric meal	0.00	0.00	10.00	5.00	10.00
Gluten meal carrier	20.00	10.00	10.00	10.00	0.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00
<i>Calculated Nutrients and Energy</i>					
Protein, g/kg	220.98	216.02	222.22	219.12	217.26
ME, kcal/kg	3001.01	2971.25	3008.45	2989.85	2978.69
Ether Extract, g/kg	45.86	45.66	45.91	45.78	45.71
Ash, g/kg	25.34	25.23	25.36	25.30	25.26
Calcium, g/kg	10.72	10.72	10.72	10.72	10.72
Total Phosphorus, g/kg	6.89	6.85	6.90	6.88	6.86
Ca:P	1.55	1.56	1.55	1.56	1.56
Sodium, g/kg	1.71	1.71	1.71	1.71	1.71
Chloride, g/kg	3.72	3.72	3.72	3.72	3.72
Potassium, g/kg	7.87	7.84	7.87	7.86	7.85
Lysine, g/kg	13.72	13.64	13.74	13.69	13.66
Methionine, g/kg	6.41	6.25	6.45	6.35	6.29
Threonine, g/kg	10.65	10.49	10.68	10.59	10.53
Isoleucine, g/kg	9.04	8.86	9.09	8.97	8.90
Arginine, g/kg	12.29	12.14	12.33	12.24	12.18
Valine, g/kg	9.61	9.39	9.67	9.53	9.45

¹T1=control (C); T2=C+10g/kg garlic meal (GM); T3=C+10g/kg turmeric meal (TM); T4=C+5 g/kg GM+5g/kg TM; T5=C+10g/kg GM+10g/kg TM.

²Contain 21.3% Ca and 18.7% P.

³Vitamin/mineral premix supply per kilogram of diet: vitamin A, 16,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin B1, 3 mg; vitamin B2, 10 mg; vitamin B6, 3 mg; vitamin B12, 15 µg; hetra, 5 mg; nicotinic acid, 60 mg; pantothenic acid, 14.7 mg; folic acid, 1.5 mg; Biotin, 125 µg; choline chloride, 25 mg; iron, 20 mg; copper, 10 mg; manganese, 100 mg; cobalt, 1.0 mg; zinc, 82.222 mg; iodine, 1 mg; selenium, 0.2 mg; and molybdenum, 0.5 mg.

⁴Titanium dioxide (TiO₂) premix to be added as indigestible marker at a ratio of 1:4 of TiO₂:gluten meal.

5.2.3.3. Experiment 2: Metabolism Trial

The metabolism trial period consisted of five days of adaptation and two days of sample collection. The 150 birds transferred to the metabolism cages were used for this trial. The five days of adaptation enabled the birds to adapt to the environment of the metabolism cages. Following the adaptation period, total excreta voided were collected on days 20 and 21, excreta were pooled within a pen and stored in a freezer at -20°C prior to processing.

On d 21, all the birds were euthanized. The distal sections of ileum (portion of the small intestine from Meckel's diverticulum to approximately 1 cm anterior to the ileo-cecal junction) were removed and contents were gently flushed with distilled water into aluminium containers. Digesta from birds within a pen were pooled, and stored at -20°C prior to processing.

5.2.4. Calculations and Statistical Analyses

The dry matter (DM), organic matter (OM), ash, N, and gross energy (GE) values were determined in samples of diet, ileal digesta, and excreta. Dry matter was determined by drying the samples in the oven (Uniterm, Russel-Lindsey Engineering Ltd., Birmingham, England, UK) at 105°C for 24 hours (Method 934.01; AOAC, 2006). Organic matter was determined by ashing the samples in the muffle furnace (Carbolite Furnace, Bamford, Sheffield, England, UK) at 500°C overnight (Method 934.01; AOAC, 2006). Total nitrogen content was determined by the Leco combustion method (Method 968.06; AOAC, 2006). Gross energy was determined in an adiabatic oxygen bomb calorimeter using benzoic acid as an internal standard (Model 6200, Parr Instruments, Illinois, USA).

Ileal digestibility was calculated using the index method, with Ti as a digestibility marker. There are generally two similar methods for determining Ti content of the diet, except that one involves wet ashing (Short *et al.*, 1996) whereas the other involves dry ashing (Myers *et al.*, 2004). Ileal digestible and metabolizable energy in current study were calculated by multiplying ileal energy digestibility and energy metabolizability with feed gross energy. Ti concentration in samples of diets and ileal digesta were determined as per the method of Short *et al.* (1996) because the analysed Ti was very close to the expected dietary Ti. Therefore, the method is sufficient for calculation of digestibility.

The apparent total tract dry matter (DMR) or nitrogen (NR) retention was calculated using total collection method. Metabolisable energy was corrected for zero-nitrogen retention (AMEn) using the value of 8.22 kcal per g nitrogen retained in the body as described by Larbier and Leclercq (1992).

Growth performance, nutrient and energy utilization, and intestinal pH data were subjected to an Analyses of Variance employing Randomized Complete Block Design. Means were statistically separated by Tukey (Snedecor and Cochran, 1989). GenStat Release 11th edition (2008) Software was used for all statistical analysis. All statements of significance are based on a probability of less than 0.05.

5.3. RESULTS

5.3.1. *Growth Performance*

Growth performance responses of male broiler chickens to GM or TM supplementation on growth performance are summarized in Table 5-30. There were no treatment effects on feed intake. Supplementation of GM or TM individually added at the rate of 10 g/kg each in the diets had no effect on final weight, body weight gain, and gain to feed ratio of 21 days old broiler chickens relative to the control. However, combination of GM and TM at the rate of 10 g/kg each increased the body weight gain ($P < 0.005$), final body weight ($P < 0.01$), and gain to feed ratio relative to the control and the diet with GM supplementation alone. The effect of supplementation of TM alone or a combination of GM and TM at the rate of 5 g/kg each on growth performance was intermediate between the combination of the two additives at 10 g/kg each and the control as well as GM alone treatment.

5.3.2. *Digesta pH*

Table 5-31 shows the data for digesta pH in different sections of the digestive tract of the 21-day old male broiler chickens and the response to GM or TM supplementation. There were no treatment effects on digesta pH in the jejunum and ileum. Dietary supplementation of GM at the rate of 10 g/kg alone or in combination with TM at the rate of 5 g/kg each did not give any effects on the digesta pH in any section of intestine. However, supplementation of TM alone at the rate of 10 g/kg reduced ($P < 0.05$) the digesta pH in the crop and caeca. The digesta pH of the crop and proventriculus were reduced ($P < 0.05$) when the diets were supplemented with combination of GM and TM mixture at the rate of

Table 5-30. Growth performance responses of broiler chickens to diets containing different levels of garlic and turmeric meal on as-fed basis¹

Items	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
Final Weight, g	810.94 ^b	839.74 ^b	892.36 ^{ab}	891.37 ^{ab}	958.69 ^a	22.96	0.002
Body Weight Gain, g	762.96 ^b	792.06 ^b	845.07 ^{ab}	844.09 ^{ab}	911.39 ^a	22.87	0.002
Feed Intake, g	1088.5	1089.0	1092.7	1096.2	1110.6	15.92	0.859
Gain:Feed	700.94 ^b	726.63 ^b	772.69 ^{ab}	769.27 ^{ab}	820.91 ^a	18.86	0.002

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens of 10 birds each per treatment.

²Diet T1=control (C); T2=C+10g/kg GM; T3=C+10g/kg TM; T4=C+5g/kg GM+5g/kg TM; T5=C+10g/kg GM+10g/kg TM.

Table 5-31. Intestinal pH in different sections of digestive tract in response to diets containing different levels of garlic and turmeric meal supplementation¹

Intestinal part	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
Crop	5.84 ^a	5.46 ^{ab}	5.30 ^b	5.48 ^{ab}	5.29 ^b	0.126	0.035
Proventriculus	2.86 ^a	2.24 ^{ab}	1.97 ^{ab}	2.06 ^{ab}	1.89 ^b	0.215	0.031
Jejunum	5.95	5.98	5.89	5.93	5.87	0.119	0.964
Ileum	6.90	7.02	6.50	6.81	6.65	0.210	0.169
Caeca	6.53 ^a	6.14 ^{ab}	5.86 ^b	6.31 ^{ab}	6.09 ^{ab}	0.138	0.032

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens of 5 birds per treatment.

²Diet T1=control (C); T2=C+10g/kg garlic meal (GM); T3=C+10g/kg turmeric meal (TM); T4=C+5g/kg GM+5g/kg TM; T5=C+10g/kg GM+10g/kg TM.

10 g/kg each. The digesta pH values in the crop and caeca of the birds receiving diets with GM alone added at the rate of 10 g/kg or in combination with TM at the rate of 5 g/kg each were intermediate between the control and treatment 3. The digesta pH value in the proventriculus of the birds receiving diets with GM or TM alone or in combination with TM at the rate of 5 g/kg each was intermediate between the control and treatment 5.

5.3.3. Energy and Nutrient Utilization

The apparent ileal nutrient and energy digestibility as well as the ileal digestible energy data for the experimental birds are presented in Table 5-32. Supplementation of GM and TM alone or in combination had no effect on the apparent ileal DM, OM, Ash, or N digestibility. However, TM supplementation alone added at the rate of 10 g/kg increased ($P < 0.05$) the apparent ileal energy digestibility and ileal digestible relative to those of non-supplemented birds. The energy digestibility values for treatments with GM alone at 10 g/kg or GM combined with TM at 5 g/kg and 10g/kg for each additive were intermediate between the control and TM supplemented diet. Ileal digestible energy of the birds receiving diets containing combination of GM and TM at the rate of 5 g/kg each was greater ($P < 0.05$) than that of non-supplemented birds. The IDE value for diets with GM alone or combination of GM and TM at 10g/kg each was intermediate between the control and treatments 3 and 4.

The apparent total tract nutrient retention and energy metabolizability as well as the AME of broiler chickens fed diets containing GM or TM supplementation individually or in combination are presented in Table 5-33. There were no treatment effects on total tract DM, OM or N retention. However, supplementation of GM alone or in combination with TM also at 10 g/kg increased ($P < 0.05$) the apparent total tract nitrogen retention compared with the control whereas supplementation of TM alone or combination of TM and GM at 5 g/kg each were intermediate. Total tract ash retention was lower in the diet with GM alone at 10 g/kg compared with the control whereas the other treatments were intermediate. Supplementation of GM or TM individually or in combination at all supplementation levels increased ($P < 0.01$) both AME and AMEn relative to the control.

5.3.4. Additivity or Interaction between the Feed Additives

Improvements of the growth performance responses as well as nutrient and energy utilization of broiler chickens due to combination of GM and TM meal supplementation

Table 5-32. Apparent ileal nutrient digestibility and digestible energy of broiler chicken fed diet containing garlic or turmeric meal supplementation on DM basis¹

Item	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	P-value
DMD, %	67.12	68.70	71.03	70.05	69.66	1.313	0.315
OMD, %	70.73	72.42	74.49	73.58	73.22	1.261	0.319
Ash.D, %	44.13	37.64	45.32	40.47	39.11	2.841	0.293
ED, %	68.87 ^b	70.39 ^{ab}	74.02 ^a	71.68 ^{ab}	71.30 ^{ab}	0.987	0.025
IDE, kcal/kg	2690.4 ^b	2897.6 ^{ab}	2954.7 ^a	2945.3 ^a	2909.4 ^{ab}	53.195	0.014
ND, %	73.87	75.70	77.07	76.05	75.54	1.145	0.420

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates pens per treatment. DMD is dry matter digestibility; OMD is organic matter digestibility; Ash.D is ash digestibility; ED is energy digestibility; IDE is ileal digestible energy; ND is nitrogen digestibility.

²Diet T1=control (C); T2=C+10g/kg garlic meal (GM); T3=C+10g/kg turmeric meal (TM); T4=C+5g/kg GM+5g/kg TM; T5=C+10g/kg GM+10g/kg TM.

Table 5-33. Total tract nutrient retention and metabolizable energy of broiler chicken fed diet containing garlic or turmeric meal supplementation on DM basis¹

Item	Dietary treatments ²					Statistics	
	T1	T2	T3	T4	T5	SEM	<i>P</i> -value
DM, %	74.28	74.60	74.38	75.27	74.70	0.509	0.681
OM, %	76.60	77.19	76.78	77.65	77.15	0.489	0.611
Ash, %	44.53 ^a	40.07 ^b	43.72 ^{ab}	43.67 ^{ab}	43.34 ^{ab}	0.912	0.026
N, %	65.19 ^b	68.28 ^a	66.73 ^{ab}	66.65 ^{ab}	67.91 ^a	0.581	0.012
EM, %	75.97	77.36	77.02	76.81	77.45	0.578	0.410
AME, kcal/kg	3024.5 ^b	3184.4 ^a	3146.5 ^a	3156.2 ^a	3160.2 ^a	23.524	< 0.001
AMEn, kcal/kg	2913.4 ^b	3088.1 ^a	3040.5 ^a	3081.4 ^a	3065.3 ^a	23.284	< 0.001

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).

¹Data represent means from 6 replicates per treatment. DMR is dry matter retention; OMR is organic matter retention; Ash.R is ash retention; EM is energy metabolizability; AME is apparent metabolizable energy; AMEn is nitrogen-corrected AME; NR is nitrogen retention.

²Diet T1=control (C); T2=C+10g/kg garlic meal (GM); T3=C+10g/kg turmeric meal (TM); T4=C+5g/kg GM+5g/kg TM; T5=C+10g/kg GM+10g/kg TM.

Table 5-34. Improvement of the growth performance responses of broiler chickens due to garlic and turmeric meal supplementation¹

Item	FW	BWG	FI	Gain:Feed
<i>Diets</i>				
C + 10 g/kg GM (2)	28.80	29.10	0.54	25.70
C + 10 g/kg TM (3)	81.41	82.11	4.16	71.75
C + 10 g/kg GM + 10 g/kg TM (5)	147.74	148.43	22.12	119.97
SEM	22.364	22.235	16.473	12.984
<i>P</i> -values for main effect of diets	0.012	0.011	0.625	0.002
<i>P</i> -values for contrast				
2+3 vs. 5	0.007	0.007	0.350	0.001

¹Data represent means from 6 replicates pens of 5 birds per treatment. FW is final weight (g); BWG is body weight gain (g); FI is feed intake (g); Gain:Feed is ratio of gain to feed.

Table 5-35. Improvement of the energy and nutrient digestibility of broiler chicken fed diet containing garlic or turmeric meal supplementation in ileal part and total tract¹

Item	ED	IDE	ND	EM	AME	AMEn	NR
Diets							
C + 10 g/kg GM (2)	1.52	207.20	1.83	1.38	159.98	174.73	3.10
C + 10 g/kg TM (3)	4.78	239.87	2.89	1.05	122.04	127.10	1.54
C + 10 g/kg GM + 10 g/kg TM (5)	2.44	219.01	1.68	1.48	135.77	151.90	2.73
SEM	1.405	67.003	1.293	0.673	27.506	29.007	0.610
<i>P</i> -values for main effect of diets	0.288	0.941	0.776	0.896	0.628	0.531	0.219
<i>P</i> -values for contrast							
2+3 vs. 5	0.687	0.957	0.675	0.759	0.879	0.978	0.597

¹Data represent means from 6 replicates per treatment. ED is energy digestibility; IDE is ileal digestible energy; ND is nitrogen digestibility; EM is energy metabolizability (%); AME is apparent metabolizable energy (kcal/kg); AMEn is nitrogen-corrected AME (kcal/kg); NR is nitrogen retention (%).

are presented in Table 5-34 and Table 5-35, respectively. Result of the orthogonal contrasts showed that GM and TM were additive for the feed intake but associative in their effects on final weight ($P < 0.007$), body weight gain ($P < 0.007$), and gain to feed ratio ($P < 0.001$). In addition, orthogonal contrasts showed that GM and TM were additive for their effects on the nutrient and energy utilization at both the ileal and total tract levels. Additivity was indicated when the contrasts for the effects of GM and TM individually was not different from the effects of the combination of the two additives.

5.4. DISCUSSION

Improvement in enteric health and growth performance due to supplementation with herbs and spices has been attributed to the beneficial properties of some phytochemicals in these additives (Yoshida *et al.*, 1987; Kiuchi *et al.*, 1993; Ahsan *et al.*, 1996). Compared to the synthetic antibiotics or inorganic substances, these plant-derived products have been proven to be safe, natural, non toxic, and residue free, and thus are believed to be beneficial as alternative to synthetic antimicrobials in poultry nutrition (Ultee *et al.*, 2000). The aim of this study was to investigate the growth performance, intestinal pH, and energy and nutrient utilisation responses of broiler chickens in response to the dietary supplementation with GM and TM. Possible associative effects and additivity between GM and TM were used in combination was also investigated.

The findings in this study showed that when given separately, dietary supplementation of 10 g/kg GM or TM did not affect the growth performance of 21 days old broiler chickens. However, when given in combination at the rate of 10 g/kg each, supplementation of GM and TM improved the final weight, body weight gain, and gain to feed ratio relative to the control diet. Various studies have shown that broiler chickens did not respond to supplementation of GM in the diets added at the rate of 4 g/kg (Toghyani *et al.*, 2011), 5 g/kg (Onibi *et al.*, 2009), or 10 g/kg (Horton *et al.*, 1991; Pourali *et al.*, 2010). However, the body weight gain of broiler chickens were significantly improved and FCR were decreased when the diets were supplemented with 0.1 g/kg (Aji *et al.*, 2011), 8.23 g/kg (Lewis *et al.*, 2003), or 10 g/kg of GM (Mahmood *et al.*, 2009). In addition, the broilers did not respond to supplementation of TM at the rate of 1 g/kg (Rahmatnejad *et al.*, 2009), 2 g/kg (Mehala and Moorthy, 2008; El-Hakim *et al.*, 2009), 5 g/kg (Gowda *et al.*, 2008; Yarru *et al.*, 2009), or 10 g/kg (Al-Sultan, 2003; Durrani *et al.*, 2006; Abbas *et al.*, 2010). On the other hand, there are reports of beneficial effects of TM supplementation in some trials. Turmeric meal supplementation at 1 g/kg improved growth performance in

Kumari *et al.* (2007) study. Allen *et al.* (1998) and Lee *et al.* (2010) noted that TM supplementation alleviated growth-depression effect of *Eimeria* infection. Similarly, Yarru *et al.* (2009) reported positive effects of TM supplementation in birds exposed to aflatoxin. In recent study, Rajput *et al.* (2013) showed that dietary supplementation of pure curcumin, an active compound in turmeric, in corn-soybean based diets at the rate of 0.2 g/kg increase body weight gain and reduce FCR of 42 days old Arbor Acre broiler chickens. The difference in responses of broiler chickens reported in those studies was due to the difference of the basal diets, rearing periods, or breed of the broiler used in those studies. It is clear from the current study that a combination of TM and GM was more effective in promoting growth performance than the use of each additive by itself.

However, findings of this study have indicated that when given in combination with turmeric meal, combined action of the active substances in TM and GM likely exerted greater antimicrobial properties to create a better milieu in the digestive tract. As reported in some studies, phytochemicals in garlic have strong antibacterial properties to combat *Escherichia coli*, *Salmonella*, (Johnson and Vaughn, 1969), *Clostridium botulinum* (De Wit *et al.*, 1979), and other pathogenic species. The antimicrobial properties of garlic may be attributed to the alliin and allicin, the active compounds found in garlic (Singh *et al.*, 1998). On the other hand, turmeric contains phytochemicals that are known to have antimicrobial, antioxidant, and digestive stimulant effects in broiler chickens (Ruby *et al.*, 1995; Araújo and Leon, 2001). Reduction of pathogenic species in the digestive tract might reduce toxic substances, cause over-production of the mucin barrier by goblet cells, stimulate better growth of absorptive cells, and reduce competition for available nutrient with the host, thus resulting in a better intestinal milieu to support growth of the birds. Therefore, feeding diets containing the GM and TM mixture might produce double inhibition of the growth of entero-pathogenic bacteria, thus contributing to maintaining a healthy balance of microbial populations in the gut (Harris *et al.*, 2001), and resulting in a greater growth performance improvements (Lewis *et al.*, 2003; Adibmoradi *et al.*, 2006). Moreover, Kumari *et al.* (2007) and Yarru *et al.* (2009) reported that that curcumin and curcuminoids in the garlic has immunomodulatory properties that might beneficially accelerated digestive system.

Improvement in the growth performance in this study might also be related to the reduction of the digesta pH in some intestinal sections due to TM and GM supplementation. Digesta pH in the crop, jejunum, and caeca of 21 days old broiler

chickens in current study dropped when the diets were supplemented with TM alone or in combination with GM. Reduction of the digesta pH in current study might control the population of entero-pathogens in the gut. Reduction of the load, growth and colonization of entero-pathogenic microflora will not only result in a less competition for vital micro-nutrient between microbes and the host animals (Thomke and Elwinger, 1998), but also prevent excessive production of mucin, reduce the adverse effects of toxin production (Ferket, 2004), stimulate beneficial bacteria (Willis *et al.*, 2009), reduce the incidences of sub-clinical intestinal infections (Russell, 1992), stimulate pancreatic secretions (Rao *et al.*, 2003; Platel and Srinivasan, 2004), and stimulate the alteration of absorptive sites in the intestinal wall (Bravo *et al.*, 2011; Saki *et al.*, 2012). Therefore, a reduction in the digesta pH should provide a more optimal intestinal condition for an efficient nutrient utilisation.

Reduction of the digesta pH due to GM and TM supplementation in this study was in the line with the findings of Mikulski *et al.* (2008) who reported that 0.3 g/kg oregano essential oil and spice extracts of turmeric and capsicum blend reduced digesta pH in the crop and ileum. In Japanese quail, Denli *et al.* (2004b) reported that dietary supplementation of 60 mg thyme essential oil or 60 mg black seed essential oil per kg diet decreased the intestinal pH of 38 d old quails. The reduction in intestinal pH and the tendency of greater growth improvement due to TM supplementation in current study indicate that a reduction in pH is associated with enhanced growth performance and that TM is more efficacious than GM in promoting this effect. Moreover, combination of TM and GM at 10 g/kg each reduced the digesta pH more than individual supplementation of the additives.

Dietary supplementation of TM individually at 10 g/kg or in combination with GM at the rate of 5 g/kg each in current study increased energy digestibility and ileal digestible energy. Supplementation of GM or TM individually at 10 g/kg or in combination at 5 or 10 g/kg each in the diets also increased the apparent metabolizable energy (AME), nitrogen-corrected AME (AMEn), and total tract nitrogen retention. Improvement in the energy and nutrient utilization in current study was in the line with the findings of Sarica *et al.* (2005) who reported that dietary supplementation of GM reduced the concentrations of total aerobic bacteria and *E. coli* in the small intestine of broiler chickens.

Improvements in the nutrient and energy utilization in current study might be attributed to the antimicrobial properties of phytochemicals in garlic (Johnson and Vaughn, 1969) and turmeric (Araújo and Leon, 2001). Reduction of the pathogen load, growth, and

colonization in the gut due to the presence of both additives might help reduce the competition for available nutrients with the host, stimulate the growth of absorptive cells in the small-intestinal mucosa, and trigger the secretion of innate digestive enzymes. These enhancements should create a gut environment that is more suitable for optimal nutrient digestion and absorption, resulting in a greater nutrient and energy utilization efficiency. Apart from the antimicrobial properties of the phytochemicals in both additives, turmeric is also known to have digestive stimulant properties which may have the capacity to improve nutrient and energy metabolism in the birds. In rats, dietary supplementation of turmeric increased the bile acids production (Platel and Srinivasan, 2001; Platel and Srinivasan, 2004) and stimulated pancreatic secretion, such as amylase, lipase, proteases, and chymotrypsin (Platel and Srinivasan, 2000; Platel *et al.*, 2002; Rao *et al.*, 2003). Those enzymes play crucial roles in the digestion process of macro-nutrients. Phytochemicals in turmeric have also been reported to reduce the digesta passage time in the intestine (Platel and Srinivasan, 2001) thus facilitating more available time for the digestion process. In broiler chickens, dietary supplementation of herbal products in the diets resulted in a digestive acceleration through enhancement of the activities of pancreatic α -amylase (Lee *et al.*, 2003a), pancreatic trypsin, and intestinal maltase (Jang *et al.*, 2007).

Findings from the current study indicated that combination of GM and TM at the rates of 5 g/kg each had the same effect as supplementation of 10 g/kg of GM or TM alone. Although diets with combination of 10 g/kg each of GM and TM were mostly numerically greater in growth performance responses, it appears that a combination of 5 g/kg each of GM and TM will be sufficient to obtain optimum response.

It is concluded from the current study that GM and TM can be used alone or in combination to support intestinal health and stimulate growth performance of broiler chickens, as shown by the reduction of digesta pH in some intestinal sections, and improvement of the nutrient and energy utilization. Combination of GM and TM at the rate of 5 g/kg each was optimum for enhancing nutrient and energy utilization and promoting growth performance of broiler chickens.

6. SUMMARY AND GENERAL DISCUSSION

The objective of the studies reported in the thesis was to understand the efficacy of some alternative feed additives to enhance performance and enteric health in broilers in the absence of antibiotics. In order to be able to relate growth performance and nutrient utilisation with gut pH (an indication of gut health), the association between these responses was also investigated.

Evidence from literature showed that antibiotics have been used in non-therapeutic fashion as antimicrobial growth promoter (AGP) for about 50 years in many parts of the world to help improve the growth performance of broiler chickens. However, the use of these antimicrobials in animal feed is being curtailed in view of the threat to public health, occurring through microflora developing resistance to antibiotics. Casewell *et al.* (2003) observed that the removal of AGPs resulted in substantial increase in infections in poultry. As a consequence, the poultry industry needs to find alternatives to AGP in order to stem the spike in infection rates. Feed additives that have been evaluated include organic acids, herbs, spices and various plant extracts, probiotics or direct feed microbials, prebiotics, synbiotics, and dietary enzymes. Because many of these additives have different modes of action (many not well understood), they have been used either individually or in various combinations with the aim of maximizing the benefits from the additives.

The first study (Chapter 2) investigated the relationship between digesta pH, body weight and nutrient utilisation in broilers of the same breed but with different propensity for weight gain at different ages. Because the birds were of the same breed and received the same diets, but gained weight at different rates, they provided a model for studying the influence of the individual gut environment on growth performance and nutrient utilisation with the confounding influence of breed and diet differences. It was observed that during the starter (day 14) and grower phases (day 28), birds in group H (heavy) consumed more feed than those in group L (light) (Table 2-6). During the same periods, birds in group H had lower pH in the caeca whereas during the grower and finisher (day 42) phases, birds in group H had lower proventriculus pH (Table 2-7). The apparent ileal DM digestibility in grower phase was greater for group H than group L, whereas in finisher phase birds in group L had greater ileal digestibility of DM, N, energy, as well as ileal digestible energy (Table 2-8). Analyses showed that caecal pH in grower phase was correlated with the total tract retention of DM and energy utilisation at both the ileal and total tract levels whereas

during the finisher phase, there were correlations between crop pH and ileal nutrient utilisation and between jejunum pH and total tract energy utilisation (Table 2-11). The separation of the data into L and H groups and separate correlation analysis indicates that the main driver for correlation between caecal pH and energy utilization observed in the combined population was mainly driven by the stronger correlation between digesta pH and energy utilisation in the L group. It was concluded from the experiment that differences in body weight are also reflected in differences in gut pH, which is likely to be indicative of differences in intestinal condition between birds with heavier or lighter body weight. In addition, differences in the pH of GIT explained about half of the variations in total tract nutrient and energy utilisation. Lower gut pH is favourable for colonization of beneficial bacteria but unfavourable for colonization of pathogenic ones and hence it is likely that birds with the same genetic potential may have differences in growth performance based on the type of bacteria colonizing their gut.

Having established the relationship between gut health and gut pH, the second study (Chapter 3) investigated the response of broiler chickens to the supplementation of benzoic acid (BA) using growth performance, nutrient and energy utilization, intestinal acidity and histomorphology of the intestine as response criteria. Supplementation of BA at 0.53 g/kg (BA1) increased body weight gain and reduced FCR in the grower phase and overall growth phase of broiler chickens (Table 3-15). Supplementation of BA at 3.20 g/kg (BA2) reduced the feed intake without affecting the body weight gain and consequently resulted in a better FCR during the grower phase. In the starter phase, BA supplementation had no effect on energy intake but protein intake was greater in BA1 compared to the control whereas BA2 was intermediate (Table 3-16). As a proxy for nutrient digestibility, efficiency of utilisation of protein and energy for weight gain was also investigated in the study. Protein and energy efficiency ratios were greater in BA-supplemented diets than in the control diet in both the starter and grower phases. There were no effects of dietary treatments on protein or energy intakes or on protein and energy efficiency ratios in the finisher phase or the overall experimental period with the exception of energy efficiency ratio which tended to be greater in BA supplemented diets compared with the control. BA supplementation (BA2) reduced the digesta pH in the caecal content, but not in the crop or ileum (Table 3-17). The pH of caecal content was similar between BA1 and BA2. Supplementation of 3.20 g/kg BA stimulated the proliferation of the absorptive cells in the jejunum, as shown in the improvement of the villus length and width, as well as the crypt depth and width (Table 3-18). However, the thickness of the mucosal layer in the jejunum

was lower in the birds fed control diet compared with those receiving BA2 diet. The data from this study indicated that dietary supplementation of BA improved growth performance and beneficially modified the intestinal milieu of broiler chicks at 42 d of age.

It was pointed out earlier on that organic acids and phytochemicals have different modes of action but they have both been claimed to result in improved gut health and hence growth performance of birds. In addition, studies have shown the benefit of using these additives individually, very few or virtually no study has examined the benefits of combining the two. Consequently, in the third study, two experiments were conducted to examine the benefit of using BA and turmeric meal (TM) individually as well as in combination. The objective of the study reported in Chapter 4 was to determine the growth performance, nutrient utilization, and intestinal health responses of broilers to supplementation of BA and TM when supplemented alone or in combination. The study was also assessed the impact of the additives on the growth of digestive organs and thus determine how the additives affected whole-body energetics. The possible associative, additivity and interactivity of BA and TM were assessed as well. Supplementation of a combination of 1 g/kg BA and 5 g/kg TM improved ($P < 0.05$) body weight gain relative to the control (Table 4-20). Based on body weight gain, the equivalency of BA over TM individual supplementation was calculated to be 1.80 g/kg for 10 g/kg TM supplementation. Dietary supplementation of 2 g/kg BA reduced the pH in the crop and jejunum, whereas combination of BA and TM at the rate of 2 and 10 g/kg respectively reduced digesta pH in the crop, jejunum, and caeca (Table 4-21). Supplementation of BA at the rate of 2 g/kg alone or combination of BA and TM at the rates of 1 or 2 and 5 or 10 g/kg, respectively increased villus height, crypt depth and crypt width relative to the control (Table 4-23). Supplementation with 10 g/kg TM alone in the diet increased the crypt depth and width relative to the control. Crypts were wider in all the treatments relative to the control but none of the treatments had any effect on the villus height to crypt depth ratio.

Supplementation of BA alone at the rate of 2 g/kg improved the apparent ileal ash digestibility relative to the control whereas supplementation of 10 g/kg TM alone was intermediate (Table 4-24). TM supplementation alone at the rate of 10 g/kg increased energy digestibility and IDE relative to the control treatment. Energy metabolisability was greater in diets with combination of TM and BA relative to the control whereas the diets with individual supplementation of BA and TA were intermediate (Table 4-25).

Supplementation of BA and TM individually and in combination increased AME and AMEn relative to the control diet. Orthogonal contrasts showed that BA and TM were additive in their effects on the growth performance responses, digesta pH in the proventriculus, jejunum, and ileum, as well as on the energy utilization (Table 4-26, Table 4-27, Table 4-28). On the other hand, orthogonal contrasts showed that these additives were associative for the ileal digestibility of ash and energy digestibility, but negatively interacted for the crop and caecal pH. However, none of the dietary treatment altered the relative weight of the digestive tract of 21 days old broiler chickens (Table 4-22). The absolute length and relative length to the weight of the small intestine were also not affected by the dietary treatments.

In the final study, two experiments were conducted to investigate the response of broilers to two additives of plant sources (TM and garlic meal, GM) and study possible additivity in the effect of the two additives. Response criteria in the fourth study (Chapter 5) were growth performance, intestinal pH, and energy and nutrient utilisation. Results showed that combination of GM and TM at the rate of 10 g/kg each increased the body weight gain, final body weight, and gain to feed ratio relative to the control and the diet with GM supplementation alone (Table 5-30). Based on body weight gain, the equivalency of TM over GM individual supplementation was calculated to be 3.54 g/kg for 10 g/kg GM supplementation. Digesta pH in the crop and caeca were reduced when the diets were supplemented with TM alone at 10 g/kg (Table 5-31). The pH in the proventriculus also dropped when the diets were supplemented with combination of GM and TM mixture at the rate of 10 g/kg each. Supplementation of TM alone at 10 g/kg or in combination with GM at the rate of 5 g/kg each increased the apparent ileal energy digestibility and ileal digestible energy (Table 5-32). Supplementation of GM alone or in combination with TM at 10 g/kg increased the AMEn retention compared with the control whereas supplementation of TM alone or combinations of TM and GM at 5 g/kg each were intermediate (Table 5-33). Supplementation of GM or TM individually or in combination at all supplementation levels increased both AME and AMEn relative to the control (Table 5-34, Table 5-35). Orthogonal contrasts showed that GM and TM were additive for feed intake, nutrient and energy utilization at both the ileal and total tract levels, but associative in their effects on body weight gain and gain to feed ratio.

Taken together, these studies showed that organic acid and herbal products can be supplemented in the diet alone or in combination to improve the enteric health, nutrient

and energy utilization, and growth performance of broiler chickens. Improvement to the growth performance might be attributed to the reduction of the entero-pathogens in the gut, enhancement of the intestinal health, alteration of the absorptive cells in the intestinal wall, and an improvement in the nutrient and energy utilization.

6.1. DISCUSSION

The growth performance of broiler chickens is closely related to the growth, development, and health of the digestive tract as was also demonstrated in the first on the series of studies reported in this thesis. The balance of microflora population in the gut is the most important characteristic of a well-functioning gut. The growth and health of the gut may decline by surrounding growth depressing factors. There are several problems associated with the gut health which results in growth depression, such as a high rate of colonization by pathogenic microflora, which will result in greater incidences of bacterial infections, a poor growth and proliferation rates of absorptive cells, and diminished nutrients absorption and utilization rate (Van Immerseel *et al.*, 2006). To address the issues, antibiotics had been used for many years to protect poultry from pathogenic organisms, reduce overstimulation of intestinal mucus production, and facilitate better feed efficiency and growth performance. The growth enhancing properties of AGPs are closely related to their antibacterial properties by selectively modifying gut microflora, reducing pathogenic colonization, resulting in improved gut health.

Nevertheless, the increase of public outcry about possible development of resistant strains of pathogens in the host animal, which also poses potential public health hazard has made the government in many countries to choose to withdraw the authorisation of several antibiotics and growth promoter as feed additives in the European Union since 1997 (Dibner and Richards, 2005). It is therefore timely to explore alternate sources of AGPs in the diets. Considering the safety aspects, the alternatives should be environmentally friendly, easy to administer, and harmless for both animals and humans who consume animal products. Some studies have reported that organic acids, herbal products, probiotics, prebiotics, or exogenous enzymes can be used as alternatives to AGP. Dibner and Buttin (2002) reported that organic acid (OA) can be chosen as these weak acids indirectly control the intestinal microflora by reducing the digesta pH, which results in the reduction of the load and growth of entero-pathogens colonizing the gut. On the other hand, herbal products (phytobiotics) contain phytochemicals, the active compounds,

which has digestive stimulator (Jang *et al.*, 2004) and antimicrobial (Kamel, 2001) properties.

The relationship between birds having different rates of gain and their intestinal condition is an intriguing one. In order to investigate the relationship, birds of the same breed and sex and subjected to similar experiment condition but whose body weight were at the opposite sides of the normal curve were used as model. The heavy (H) birds had higher feed intake and the faster growth rate than the light (L) birds. It was speculated that the birds in H group had greater efficiency for digestion, absorption and utilisation of dietary nutrients to meet requirements for maintenance and growth. This supposition is supported by greater ileal DM digestibility in the H group during the grower phase. It would appear that the heavier birds were not necessarily more efficient at nutrient utilisation because the total tract retention was consistently lower for the birds in the H group from the grower phase onwards. However, further observation revealed that the birds in H group retained more nutrients when expressed in absolute terms, primarily because they had greater feed or nutrient intake, but when nutrient retention is expressed as a percentage of nutrient intake, the birds in H group were less efficient than the birds in the L group. Therefore greater digestibility and retention values of the birds in L group are likely a compensatory mechanism to account to counteract their lower level of feed or nutrient intake (Zubair and Leeson, 1996).

In general, heavier birds in this study had lower digesta pH value. This finding conforms the results in Angel *et al.* (2010) study where intestinal pH changed with GIT section and age. The faster growth rate in the H birds in this study might be related to the lower pH in the digestive tract that inhibited the growth and colonization of enteropathogens in the gut. Reduction of pathogenic species alongside the reduction of toxic metabolites should allow better nutrient utilization for growth (Lewis *et al.*, 2003). As reported in various studies, the lower pH in the digestive tract is beneficial by providing a more optimal intestinal milieu for the growth and colonization of beneficial microflora to the exclusion of the pathogenic organisms. Dibner and Buttin (2002) pointed out that reduction of these pathogens might beneficially reduce toxins produced by these organisms, which in turn reduce enteric problems and result in a more conducive gut structure. In addition, Xia *et al.* (2004) and Pelicano *et al.* (2005) noted that a reduction in the population of entero-pathogens in the intestine can indirectly optimize mitotic division of cells which have a responsibility for micronutrient absorption. Therefore, it can be

expected that birds with lower intestinal pH have a healthier intestinal environment that is more conducive for optimal performance.

A preliminary study was done to assess the response of broiler chickens to diets supplemented with organic acid using growth performance, intestinal pH, jejunal histomorphology, and nutrient and energy utilization as responses criterion. Results showed that supplementation of benzoic acid (BA) in this study reduced the digesta pH, improved nutrient and energy utilization, stimulated the growth of absorptive cell in the jejunal wall, and stimulated the growth performance of 42-d male broiler chickens. Improvement of the nutrient and energy utilization as well as the growth performance in current study might be related to the pH-reducing properties of BA which might reduce the load, growth, and colonization of entero-pathogenic microflora in the gut. Reduction of the pathogenic microflora should reduce the adverse effects of over colonization of harmful microflora in the gut and hence provide better intestinal milieu for nutrient absorption to support maintenance and production purposes.

Reduction in caecal pH due to BA supplementation observed in this study likely altered the intestinal ecosystems (Canibe *et al.*, 2001) by providing an unfavorable acidic environment to pathogenic microflora, which in turn reduced the toxic substances and other growth depressing microbial metabolites and stimulated the colonization of beneficial microflora (Partanen *et al.*, 1998) in the gut. The proposed mechanism of action of OA for controlling microbial growth involves depolarization of the bacterial membrane, an alteration in internal pH, and changes in the nutrient transport and synthesis within the bacterium (Eklund, 1983; Davidson *et al.*, 1997). Organic acids have the ability to penetrate the cellular membrane of bacteria. In the cell, OA releases protons in the alkaline cytoplasm, resulting in the reduction of intracellular pH. Such a reduction is unfavorable for intestinal colonization of entero-pathogenic bacteria which is sensitive to pH changes (Pelicano *et al.*, 2005), but at the same time could be favorable for stimulating the growth of beneficial bacteria (Eklund, 1985). Therefore, a reduction in the digesta pH facilitates better intestinal condition for greater nutrient and energy utilization.

The efficiency of energy and nutrient utilization for growth in this study was investigated using energy and protein efficiency ratios (EER and PER). Improvement of PER and EER in grower phase could be attributed to the pH-reduction properties of BA that could reduce the entero-pathogens colonization in the gut. A reduction in the pathogenic microflora might reduce the competition between pathogenic microbes and the

host for the digested nutrients (Dibner and Richards, 2004), enable utilization of more nutrients and energy for productive purposes and lower nutrient and energy requirements for maintenance. The findings in this study support the argument that the efficiency of utilization of dietary protein and energy for weight gain was greater in BA supplemented diets, especially during starter and grower phases.

Intestinal histomorphology examination showed that dietary BA supplementation helped the development of the absorptive cells of the intestinal mucosa by increasing intestinal villi and crypts size, resulted in longer and wider villi as well as deeper and wider crypts. The observed longer villi and greater ratio of villus height to crypt depth is an indication of capacity for greater nutrient absorption. The longer villi provide greater absorptive surface area and deeper crypt indicate a more enhanced capacity for replenishing the enterocytes (Geyra *et al.*, 2001; Mathlouthi *et al.*, 2002). The longer villi in this study may have resulted from the BA contributing to a pH reduction in the intestine and, consequently, reducing colonization of the intestine by entero-pathogenic bacteria (García *et al.*, 2007). As described by Sklan (2004), explosive growth of entero-pathogens causes the intestine to secrete excessive mucus quantity to protect the absorptive surface of the intestine from luminal irritants and bacteria. Therefore, minimization of the load, growth and population of pathogenic microflora due to BA supplementation will lead to the reduction of toxic bacterial metabolites which in turn will maximize digestive efficiency by facilitating greater proliferation of the absorptive cells (Iji and Tivey, 1998).

In order to determine the response of broiler chickens to the diets supplemented with organic acids and herbal products, consequent studies have been done to examine the response of broiler chickens to benzoic acid (BA) and turmeric meal (TM) supplementation using growth performance, intestinal pH, and nutrient and energy utilization as response criteria. The impact of the additives on the growth (length and weight) of digestive organs as a proxy of the whole-body energetics and possible associative, additivity and interactivity of BA and TM when used in combination were also been assessed.

Supplementations of dietary additives in this study improved the growth performance of 21-d broiler chickens, although the improvement in the body weight gain was more pronounced with BA than with TM. Improvement in the growth performance responses in this study may be attributed to the pH reduction properties of BA (Dibner and Buttin, 2002). As shown in the study, the digesta pH was lower in birds receiving diets

containing BA compared to the diet containing only TM. Canibe *et al.* (2001) reported that the antimicrobial effect of organic acids in broilers takes place mostly in the upper part of the gut, such as the crop where the acidity is more relevant for the action of the acid. Reduction of the digesta pH might also stimulate the pancreatic secretion which will enhance lipid and starch digestion (Dibner and Buttin, 2002). Kamel (2001) also noted that herbal product might stimulate pepsin activity by reducing pH of the gut to the optimum level for pepsin activity. Improvement in the growth performance responses due to the combination of BA and TM in this study was likely due to the combined effects of BA and TM as antimicrobial and antioxidant agents (Canibe *et al.*, 2001) which may result in stimulation of protein synthesis (Osawa *et al.*, 1995).

Dietary supplementation of BA alone or in combination with TM in this study stimulated the growth of absorptive cells in the jejunal cell wall. The increase in the length and width of the villus as well the depth and width of the crypts of jejunum in this study might be an indirect effect of a reduction in the population of entero-pathogens in the gut (Araújo and Leon, 2001; Dibner and Buttin, 2002). These improvements in villi growth may be the explanation for why BA and TM supplementation in this study improved the nutrient and energy utilization. But in addition to the effect of the additives on gut morphology, as an organic acid, BA may weaken the structure of crude fibre of the feed, enabling gastric juices to penetrate more easily through the cell wall (Atapattu *et al.*, 2005), which in turn will make the macro-nutrients in the diets more available to enzymatic digestion. On the other hand, TM contains curcumin which enhances the activities of terminal digestive enzymes of small intestinal mucosa through the stimulation of pancreatic lipase, amylase, trypsin, and chymotrypsin secretion (Khan *et al.*, 2012) that play a crucial role in maximizing macronutrient digestion. Moreover, BA and TM was appeared to be additive on their effects to improve of the energy metabolizability and AME.

However, the dietary treatments did not affect the length or weight of the digestive tract of the broiler chickens similar to reports of Gunal *et al.* (2006) and Gowda *et al.* (2008). Profile of digestive tract can be as an indicator of the health status of the gut as it is affected by the shift in and formation of the microflora that develop a synergistic relationship with the host (Torok *et al.*, 2009). Sporadic growth of intestinal microflora will trigger excessive stimulation of the intestinal epithelial barriers (van der Klis and Jansman, 2002; Ferket, 2004), which in turn will physically increase the relative weight of

the intestine. On the other hand, a reduction in the relative weight and length of the gut are believed to be beneficial for the whole body energetics. Spratt *et al.* (1990) indicated that the gut of broiler chickens only represents around 1.5% of the total body weight, but consumes approximately 6-8% of the energy derived from the diet. Therefore, the shorter the length of the intestine, the less energy and nutrient required to maintain the section.

The responses of the birds to the diets supplemented with garlic meal (GM) or turmeric meal (TM) alone or in combination were investigated in a subsequent study in order to investigate how two additives of plant sources but with different modes of action work in tandem to stimulate growth performance of birds. Results showed that a combination of GM and TM improved the final weight, body weight gain, and gain to feed ratio of 21-d broiler chickens. These findings indicated that the combined action of the active substances in TM and GM likely exerted greater antimicrobial properties to create a better milieu in the digestive tract. Garlic meal contains alliin and allicin, the two phytochemicals that have strong antibacterial properties to combat various enteropathogens (De Wit *et al.*, 1979). On the other hand, TM contains curcumin and curcuminoids that have antimicrobial, antioxidant, and digestive stimulant effects in broiler chickens (Yarru *et al.*, 2009). Feeding diets containing GM and TM mixture likely produced double inhibition of the growth of entero-pathogenic bacteria, thus contributing to maintaining a healthy balance of microbial populations in the gut (Harris *et al.*, 2001), and resulting in a greater growth performance improvements than obtained using only one additive (Lewis *et al.*, 2003; Adibmoradi *et al.*, 2006).

Improvement in the growth performance in this study might also be related to the reduction of the digesta pH in the crop, jejunum, and caeca that might control the population of pathogens in those intestinal sections. The observation in the current study that GM and TM reduced digesta pH indicate that pH reduction in the previous study was not simply due to supplementation of benzoic acid but also that the additives were able to increase intestinal acidity consequently encouraging the growth of more beneficial microflora. The effect of the additives on intestinal microflora should create a gut environment that is more suitable for optimum nutrient digestion and absorption, resulting in a greater nutrient and energy utilization efficiency. The results from the current study suggest that combination of GM and TM at 5 g/kg each is the optimum for enhance the nutrient and energy utilization and promoting growth performance of broiler chickens. It was also shown that GM and TM were additive in their effects on the feed intake and

nutrient and energy utilization at both ileal and total tract levels, but associative for the final weight, body weight gain, and gain to feed ratio.

6.2. RECOMMENDATIONS AND FUTURE DIRECTION

Results of the experiment reported in this thesis showed that organic acid and herbal products can be used as alternatives to AGPs in the post antibiotic era. However, nutrition is not only about the impacts of the dietary supplementations on the enteric health and growth performance, but also the other factors which are related to the physiology of nutrition and the meat quality. Therefore further studies are needed to explore the efficacy of feed additives in the following area:

- a. The impacts of the additives on the growth and population of specific beneficial microflora as response criterion. This may help improve understanding the mechanism of action of both additives in improving intestinal health and growth performance.
- b. The benefits of using the additives on animal products (e.g. meat and egg) with focus on structure and biochemistry. These may result in a better understanding of the efficacy of both additives to improve meat quality.
- c. Exploration of the best dose of organic acids and herbal products cocktail in broiler chickens.

7. APPENDIX

Photo 1. Muffle furnace for ashing samples (Carbolite Furnaces)



Photo 2. Bomb Calorimeter for energy determination (Parr Instrument Model 6200)



Photo 3. pH meter for measuring digesta pH (HANNA Instrument HI99163)



Photo 4. Microtome for micro-slicing tissue sections (Leitz-1512 Microtome)



Photo 5. Jejunal samples ready to read under the microscope



Photo 6. Laboratory microscope for histomorphology examination (Olympus BX41)



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