The Role of the Gut Microbiota in Inflammatory Diseases of Childhood

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Summary

The bacteria located within the human gastrointestinal tract (the gut microbiota) perform numerous protective, immunological and metabolic functions for the host. They are increasingly implicated in the pathogenesis of acquired inflammatory diseases of the gut in childhood, namely: necrotising enterocolitis (NEC) and inflammatory bowel disease (Crohn's disease (CD) and ulcerative colitis (UC)). Study of the role that the microbiota may play in the development of such diseases may lead to new therapies to modulate or even cure them. However many current techniques depend on the ability to study such bacteria outwith their natural ecosystem. Although molecular techniques can identify species independent of standard cultures they can enlighten little on the metabolic activity of identified bacterial species, which may be important in the propagation of inflammatory responses. Little is known about the potential of novel therapeutic strategies, such as probiotics, to modulate diseases such as NEC. In addition inadequate scientific rigour has been applied to the science of probiotics. The aims of the study described in this dissertation were to test the following hypotheses.

Hypotheses:

1. Probiotics prevent NEC in at risk infants of very low birth weight (VLBW).

2. The human gut microbiota can be labelled by stable isotope probing (SIP) to measure metabolic activity.

3. Quantitative measurement of the metabolic activity of the unculturable gut microbiota is a useful way of studying changes in the microbiota, compared with measures of bacterial diversity, and may enlighten our understanding of bacterially mediated inflammatory stimuli in inflammatory gut diseases of childhood.

Aims:

1. To evaluate the evidence for the use of prophylactic oral probiotics for the prevention of NEC by systematic review of the published literature.

2. To develop stable isotope probing as a novel tool to study the metabolic activity of the gut microbiota in children.

Methods:

1. Systematic review of randomised controlled trials (RCT) and quasi-RCTs of the administration of oral probiotic preparations to preterm infants, looking for outcome measures including; incidence, severity, need for surgery and mortality in NEC. Electronic searches were performed on MEDLINE and CINAHL using keyword and subject heading (MeSH) using combinations of the terms infant, preterm; infant, VLBW; enterocolitis, necrotising; probiotic. In addition, all potential studies had citation searches performed.

2. Stable isotope probing methodology was developed to examine bacterial RNA extracted from human faecal samples. The potential to enrich bacterial RNA with ^{13}C from candidate tracers 16s rRNA was then to be isolated by adapting oligonucleotide probe capture technology to measure eubacterial and group specific 16s rRNA ^{13}C enrichment as a proxy for bacterial metabolic activity.

Results:

1. Systematic review of more than 45000 citations identified five randomised controlled trials of sufficient quality. 640 infants were in the treatment group in comparison to 627 controls. Statistical analyses demonstrated a cumulative reduction of NEC in the treatment group. However major heterogeneity in terms of type, dosage and administration of probiotics, precluded further interpretation of meta-analysis. No studies reported on long-term safety or observations of unintended outcomes.

2. Using a non-radioactive isotope of carbon (^{13}C), enrichment of faecal bacterial RNA was reliably achieved and detected by liquid chromatograph isotope ratio mass spectrometry (IRMS) and enrichments were related to stimuli for bacterial activity and correlated with faecal short chain fatty acid profiles. A protocol for the isolation of ^{13}C enriched bacterial 16s rRNA was developed and ^{13}C urea was identified as a reliable tracer to produce enrichment in 16s rRNA. 16s rRNA enrichments were shown to correlate with total RNA enrichment across varying stimuli for bacterial activity. Phylogenetic group specific probes also suggested some specificity when probing the faecal bacterial consortia. However sufficient

specificity of ^{13}C labelled sequence specific RNA was not reliably demonstrated, despite examination of many steps in the protocol using a highly ^{13}C enriched *E. coli* model.

Conclusions:

1. The data appear to support the use of oral probiotics for the prevention of NEC in preterm VLBW infants. However the data are insufficient to comment on their short and long-term safety. Type of probiotics as well as timing and dosage are still to be optimised. The gut microbiota undoubtedly play a pivotal but, as yet, not clearly defined, role in the pathogeneses of inflammatory gut diseases of childhood. However before the development of targeted therapies to modulate these diseases can be successfully implemented expansion of the evidence base is required. Future work should aim to improve definitions and understanding of mechanisms of action and safety of probiotic preparations and to enhance the methods to determine the effects of unculturable bacteria on the ecology of the gastrointestinal tract.

2. RNA SIP-IRMS methodology can be applied to study changes in the metabolic activity of the unculturable bacterial in human faecal samples. The use of oligonucleotide probe capture techniques potentially allows the study of the metabolic activity of individual bacterial species within such complex consortia. Studies of unculturable bacteria should focus on both metabolic activity and diversity because of functional redundancy within the microbiota. Optimisation of the existing SIP methodology could lead to a powerful new way of investigating the metabolic activity and diversity of the gut microbiota.

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List of Accompanying Material

Publications and presentations arising directly from this work

Full peer review papers

Probiotics for the prevention of necrotising enterocolitis; A Systematic Review. AR Barclay, B Stenson, JH Simpson, LT Weaver DC Wilson. *J Paediatr Gastroenterol Nutr* 2007;45:569-76

What is the role of the metabolic activity of the gut microbiota in inflammatory bowel disease: Probing for answers with stable isotopes. A R Barclay, D J Morrison, LT Weaver. *J Pediatr Gastroenterol Nutr* 2008:46;486-95

Abstracts

Probing the metabolic activity of the human gut microbiota with different stable istopes. A R Barclay, L T Weaver, D J Morrison. *Gut* 2008;57 (suppl II): A332

Stable isotope incorporation into faecal bacterial RNA reflects predictable changes in short chain fatty acids. A R Barclay, K Gerasimidis, C A Edwards, L T Weaver, D J Morrison *Gut* 2008;57 (suppl II); A332

Probiotics for the prevention of necrotising enterocolitis in preterm and VLBW infants: A Systematic Review. A R Barclay, B Stenson, J H Simpson, L T Weaver, D C Wilson. *Gut* 2007;56 (Suppl III) A297

Stable Isotope probing of the human gut microbiota: A novel method to link activity with diversity. A R Barclay, L T Weaver, D J Morrison. *Gut* 2007;56 (Suppl III) A114

Probiotics for NEC?: A Systematic Review. A R Barclay, B Stenson, J H Simpson, L T Weaver, D C Wilson. *Paediatrics and Child Health* 2007;17:411

Stable Isotope probing of the human gut microbiota: A novel method to link activity with diversity. A R Barclay, L T Weaver, D J Morrison, was awarded the Alex Mowatt Award for best scientific presentation at the British Society for Paediatric Gastroenterology, Hepatology and Nutrition Annual Winter meeting. Southampton January 2008

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Finally to my wife and family, without whom, this would be a pointless exercise

This thesis is dedicated to Robert Peter Clarkson Barclay, an inspirational father, teacher and paediatrician.

Author's Declaration

The original idea for this thesis was conceived in two parts. The need for further review of the subject of probiotics for NEC was my own. The development of this into a systematic review was originally suggested by Dr David Wilson. The study was designed by myself after consultation with Marschall Dozier at the University of Edinburgh Medical Library. Other contributors to this work were required to comply with approved systematic review methodology namely; David Wilson agreed on study design of literature; my own opinions of the available literature had to be validated by two reviewers, Dr Ben Stenson an Dr Judith Simpson, who graded the quality of studies.

The original idea for stable isotope probing of the gut microbiota was conceived by Dr Douglas Morrison and Professor Lawrence Weaver following work performed in the Department of Child Health, University of Glasgow using 16s rDNA probe technology. Individual experiments were designed by myself and Douglas Morrison and I collected all stool samples prepared all laboratory conditions and isolated all nucleic acids. Analysis of the ^{13}C incorporations were performed at the Scottish Universities Environmental Research Centre (SUERC) with the aid of Dr Douglas Morrison.

Collation and analysis of data was performed by myself on Excel for Windows, or in conjunction with Dr Douglas Morrison when using SPSS.

1 Introduction

1.1 The Human Gut Microbiota

The mucosal surface of the gastrointestinal tract represents the largest and most active interface between the host and its external environment (1). A single monolayer of cuboidal cells separates the sub-epithelium from the luminal contents. Many species of bacteria have evolved and adapted to live in the human intestinal tract. In the adult human, microbial cells contained in the gut out-number the total eukaryotic cells of the body by a factor of 10. These several hundred grams of bacteria play many important roles in physiological homeostasis and metabolism for health. However most cannot be easily studied outwith the environmental niches they inhabit.

The 'gut microbiota' can be defined as all the species within the ecosystem (gastrointestinal tract) which are involved in symbiotic relationships with the host (2). Many bacteria are potential pathogens, causing infections and sepsis in the susceptible host when barrier function or immunity is compromised. The gut microbiota have been long known to have this duality and recently they are being increasingly linked to the pathogenesis of complex acquired diseases (3). The nature of the gut microbiota is complex and many factors influence it. As the intraluminal environment of the gut changes along its course, so does the microbial composition. Many bacteria cannot be directly studied outwith the gut because of the specific nutrient requirements and conditions that they are provided with *in vivo*. All this makes the study of the gut microbiota's effects on preventing or propagating acquired diseases difficult.

The aim of this chapter is to review current knowledge of the gut microbiota, the factors influencing its formation and composition, its role in the maintenance of health, methods of analysis of its composition and how it is implicated in acquired diseases of childhood, highlighting in particular necrotising enterocolitis (NEC) and inflammatory bowel disease (IBD).

1.1.1 Development of gut microbiota from birth

The human gut is sterile at birth but rapidly colonised thereafter (4). Although protozoa and fungi come to inhabit the gut, by far the most numerous micro-organisms are bacteria. Many environmental factors, including maternal colonisation, mode of delivery, length of gestation, composition of first enteral feed and antibiotic exposure, affect the rate of bacterial colonisation and species diversity of the newborn (1;5;6). This process continues until adult type diversity and numbers are established by around two years, by which time around 10^{14} bacteria are resident in the gut, outnumbering human eukaryotic cells by an order of magnitude (7).

The composition and number of bacteria increase cranio-caudally along the gastrointestinal tract and also vary in number radially from lumen to mucosa. In the upper gut only relatively small numbers, of few species, are found, which have adapted to survive the acidic pH of the stomach. Many bacteria in this part of the digestive system are commonly carried by food particles in the diet and generally do not establish themselves permanently on the mucosa or in the lumen and are thereby considered 'transient'. The relative number of bacteria rises in the small bowel until in the colon 10^{11}-10^{12} bacteria per g of faecal material are present (8). Bacteria in the colon are relatively stable and colonies will establish themselves permanently there, both in the lumen and on the mucosal surface, where they exist within biofilms on the mucous layer overlying the epithelium.

In the lower gastrointestinal tract of healthy individuals the majority of bacteria are strict anaerobes, outnumbering the facultative anaerobes 10-100 fold (8). The biofilm microbiota differ in composition from the luminal bacteria (9). Within individuals species numbers and diversity are stable over time (10) and these bacteria are therefore considered 'resident'. Luminal bacteria in health include a mixture of resident and transient bacteria. Mucosal biofilms tend to be more static in healthy individuals and comprise mainly resident bacteria. Inter-individual variations in the microbiota, however, are marked (10), giving rise to difficulty in characterising a 'normal' healthy microbiota. Nevertheless, in spite of such variability the gut microbiota undoubtedly perform important protective, immunological and metabolic functions for the host in health.

1.1.2 Influences on gut microbiota composition in infants and young children

Colonisation of the gut begins with organisms derived form the maternal vaginal, intestinal and skin microbiota. Aerobic and facultative anaerobes such as *Staphylococci* and

Enterococci colonise within the first week. Oxygen consumption by such bacteria results in a drop in the redox potential within the gut allowing strict anaerobes such as *Bifidobacteria, Bacteroides* and *Clostridia* to colonise (11). However many exogenous factors have been shown to affect colonisation patterns. Infants born by caesarean section are less likely to acquire *Bifidobacteria* by ten days of age in comparison to vaginally delivered infants (4). The effect of early diet on colonization patterns has been studied extensively. Breast-fed infants have less bacterial diversity than formula-fed infants with a predominance of *Bifidobacteria* species. Such differences disappear when mixed diets are introduced (12).

Length of gestation at time of delivery also appears to be of importance. Infants born prematurely are likely to have slower rates of colonisation with fewer overall numbers of bacteria present, with less species diversity and a relative lower incidence of *Bifidobacteria* in comparison to healthy term controls. It is difficult, however, to separate the relative roles of immature gut immune function, neonatal care (nursing in incubators, strict hygiene, and antibiotic usage) and feeding (tube feeding and formula feeds) in the genesis of this pattern (4-6). Infants delivered in Westernised countries have more delayed colonization of bacteria and fewer *Bifidobacteria* than those delivered in developing countries, suggesting that environmental factors such as hygiene are of importance to the term infant also (6). The effects of such differences have not been studied but the hypothesis relating environmental changes to the development of acquired disease (hygiene hypothesis) discussed in section 1.3 may be relevant.

1.2 Functions of the Gut Microbiota in Health

1.2.1 Protective functions

Resident bacteria within the gut provide resistance against enteropathogens (colonisation resistance). Mucosal bacteria compete for binding sites on the epithelial surfaces of the gut, thus preventing attachment and subsequent invasion of cells by pathogenic bacteria (13). Luminal bacteria can inhibit the growth of enteropathogens by competing for common substrates (14), by reducing intraluminal pH and by producing specific bacterocidins against such species (15).

1.2.2 Immunological functions

Microbiota affect the development of the gut associated lymphoid system (GALT). The intestine contains 70% of the body's circulating lymphocytes, many of which are found

within the epithelium (16). In the lamina propria there are several lines of immune cells, key to the host response to microflora, such as macrophages, dendritic cells and myofibroblasts (17-19). Animals bred in sterile conditions have low densities of gut lymphoid cells and are particularly susceptible to infection (20;21). Lymphoid tissue, and surface and circulating immunoglobulin concentrations rise substantially with the introduction of bacteria to the gut of such animals (20;22). In early human life, pioneering species in the gut interact through surface cell receptors on gut immune cells, such as CARD15 and toll-like receptors (TLRs), to promote the expression of host genes that generate an intraluminal and mucosal environment that further favours their colonisation (23).

Host bacteria and the immune system are constantly engaged in a process of sensing and feedback with one another (24). Pioneer bacteria within the human gut can regulate host responses to maintain an intraluminal environment which favours their continued establishment, meaning that early colonisation contributes to life-long gut microbial patterns and host immune responses (23). Immune stimulation by the microbiota in early life can also affect differentiation of immune memory and response to antigens. This interactive modelling of gut microbial composition and host immune response is referred to as 'crosstalk' (23). The exposure of the host to a numerous and diverse microbiome programmes the primed immune system to react appropriately to perceived gut commensals and eneteropathogens (oral tolerance) (1;25). Such mucosal immune responses are lessened with exposure to heat treated bacteria, as opposed to live organisms, suggesting that such mechanisms involve the metabolic products of bacterial activity as well as bacterial cell-receptor mediated sensing (26).

1.2.3 Metabolic functions

Colonic bacterial diversity equips the host with a range of enzymes and biochemical pathways not present in the human repertoire (27). This allows the metabolism of otherwise indigestible food products, such as carbohydrates, peptides and proteins to generate substrates for colonocyte function and energy salvage for use by the body (28;29). Of high functional importance is the fermentation of non-digestible carbohydrates (NDC) to short chain fatty acids (SCFA) which can regulate colonocyte metabolism and other host metabolic functions (figure 1).

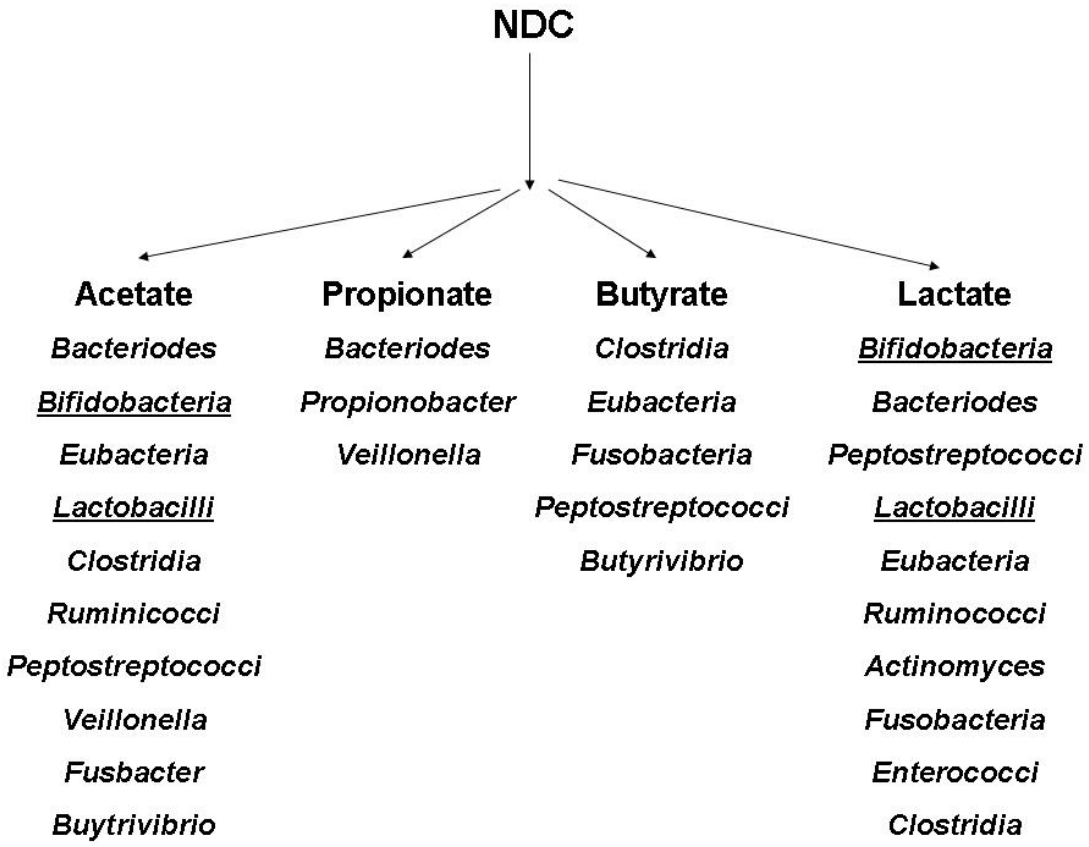

Figure 1: SCFA producing species of bacteria within the human gut (probiotic species underlined)
NDC= non digestible carbohydrates

SCFA produced within the gut lumen can have trophic effects on colonocyte differentiation and cell turnover (8;30). Such products of bacterial activity may also play an important role in bacteria-gut crosstalk in health (16;26) and therefore may be involved in regulation of the inflammatory response in disease.

1.3 Gut Microbiota in Disease - The Hygiene Hypothesis

The recent progressive decline in morbidity and mortality from infectious diseases in developed countries has been mirrored by a concurrent rise in allergic and immune mediated illness (21). Asthma, hay fever and allergic dermatitis have increased in incidence in the last 20 years (31). Migrant families moving to Westernised countries appear to develop such diseases after a generation (3). Gastroenterologists have also noted dramatic rises in the incidence of inflammatory bowel disease (IBD) (32;33), particularly in children (see 1.6.2). Epidemiological studies initially related increases in allergic disease to decreasing household size and increasing levels of hygiene (34). An immunological hypothesis for this phenomenon is that increasing hygiene alters the programming of the developing acquired immune system (hygiene hypothesis).

T helper (TH) lymphocytes can develop into two distinct categories: TH_1 and TH_2. When activated these cells secrete different profiles of immunomediators (35;36). Allergic diseases are noted for their predominance of TH_2 response (37). The hygiene hypothesis suggests that Westernized lifestyles lead to a skewing of the TH axis to TH_2 resulting in increased allergic disease. An extension of this hypothesis is that the TH_2 predominance results in a decline in illnesses characterized by a TH_1 response. However TH_1 characterised illnesses, including Crohn's disease (CD), coeliac disease, multiple sclerosis and autoimmune diabetes, have also shown dramatic increases in incidence (38-42) over the last few decades. Observations on the effects of improved hygiene on populations are flawed because poor hygiene is associated with other confounding environmental variables such as poor dietary intake, lifestyle and limited access to medical care (42). The pathogeneses of such illnesses are now thought to be subtler and more complex than accounted for by this simple hygiene hypothesis alone, and are not fully understood (8;21;43). A prevailing theory for acquired illnesses is that they are the product of interactions between human and environmental factors in genetically susceptible individuals, resulting in immune dysregulation and clinical disease (44-46). The rising prevalence of IBD may be explained this way. Moreover Crohn's disease, because of its growing incidence, and NEC, because of its increasing burden on the ex-preterm infant, are of great interest to the paediatric gastroenterologist (47).

1.4 Probiotics

Probiotic are defined as live microbial organisms that, when consumed in adequate quantities confer health benefits to the host (48). The possibility of bacteria having positive as well deleterious effects to the host was first postulated by Ilya Metchnikoff in 1913 after he observed the longevity of Bulgarian peasants who consumed fermented milk products (49). However it was Lilly and Stillwell who coined the phrase 'probiotic' (defining them as ingested bacteria with benefits to the host) in 1965 (50) leading to a revival of interest in this area of science. Commercial manufacture of probiotics products, in particular yogurt based products, has increased dramatically over recent years. The scientific evidence to support the health claims of such products remains limited and in the 1990s attempts were made to standardise criteria and definitions for probiotic bacteria (51).

The Lactic Acid Bacteria Industrial Platform (LABIP) workshop of 1998 summarised criteria for probiotic bacteria to include: human origin, non-pathogenic, resistant to gastric acid and bile, adherent to gut epithelial tissue, persistent within the gut epithelial tract,

production of antimicrobial substances, modulation of immune responses and metabolic activities (52). Other authors have argued that these bacteria should be studied in isolation and that data from closely related strains cannot be extrapolated to other strains of bacteria. Where possible evidence of beneficial effects should be demonstrated in well-designed placebo randomised double blind controlled trials in humans (53;54). Species commonly identified under these criteria as 'probiotic' belong to the genus*: Lactobacillus, Bifidobacterium, Bacillus, Streptococcus, Enterococcus* and the yeast *Saccharomyces*. However the FAO/WHO have reported that the *Enterococcus* species should not be considered probiotic (55). The species *Lactobacillus, Bifidobacterium* and *Streptococcus* have been most commonly studied to date because they are common and 'natural' members of the intestinal microflora (53;56). However authors have noted that differences between the behaviour of such bacteria *in vitro* and *in vivo* limit our ability to study their mechanisms of action (51). In addition these bacteria do not necessarily need to be alive, as products of the bacteria, such as cell wall and bacterial DNA, can modulate the profile of the gut microbiota and immune responses (55;57).

1.4.1 Prebiotics and synbiotics

'Prebiotics' are defined as non-digestible food components that confer a health benefit to the host by the selective stimulation of one or more species of probiotics (58). The majority of prebiotics are non–digestible carbohydrates (NDC) which require colonic bacteria for their degradation. NDCs such as galacto-oligo saccharides (GOS) and fructo-oligo saccharides (FOS) have been included within artificial milk formula for infants (59). GOS and FOS have been shown to increase faecal *Bifidobacteria* counts and soften stools in term and preterm infants (60). Some observations of decreased numbers of enteropathogens have also been made (61). Some NDCs may also directly affect gut barrier function in addition to modulating the gut microbiota. A 'synbiotic', as the name implies, is an ingestible combination of prebiotics with probiotic species with the purpose of assisting the establishment of probiotic bacteria in the host gut (58).

1.4.2 Evidence of probiotic mechanisms of action

Production of antimicrobial compounds by lactic bacteria has been well demonstrated. These compounds can include fatty acids, hydrogen peroxide and diacetyl (51). Peptides with direct bacterial inhibitory properties have been termed 'bacteriocidins', of which there are now purified products available for human consumption (62). The gut epithelium is a selective barrier to epitopes and increased intestinal permeability has been implicated in the aberrant immune response. *Lactobacillus* has been demonstrated to enhance intestinal

barrier function in IBD animal models and in patients with IBD (63;64). Localised immune responses have been demonstrated with up-regulation of non specific mucosal IgA and IgE production (65;66). Increased antibody response has also been demonstrated in patients receiving vaccinations and concomitant probiotics (67). Enhanced activity of natural killer cells and phagocytosis by peripheral monocytes has been reported (68). Monocyte activity and cytokine profiles can be altered by exposure to *Lactobacillus* GG and VSL#3 (69;70). Probiotic induced increased cytokine (IFN-γ INF-α IL-2) production has also been demonstrated (71). Inhibitory immunomediators can also be induced by exposure to VSL#3 (72). T-cell function and redistribution of T-cells within the gastrointestinal tract has been demonstrated with *Saccaromyces boullardii* (73).

Table 1: Mechanisms of action of probiotics

Mechanism	Biological effect
Microbial compounds	Bacterial inhibition
Intestinal barrier function	Enhanced barrier function
Immunoglobulin	Increased surface and circulating immunoglobulin levels
Antigen presentation	Enhanced natural killer cell activity, increased phagocytosis
Cytokine production	Both inhibition and up-regulation of cytokines
T-cells	Redistribution of T-cells within gastrointestinal tract

1.4.3 Evidence of clinical effects of probiotics in adults

The first studies of the prevention and treatment of acute infectious diarrhoea with probiotics were conducted on children (74). A Cochrane review of probiotics to treat infectious diarrhoea, collating data from 1917 patients, demonstrated that probiotics were associated with less diarrhoea at three days and a shorter duration of diarrhoea (75). It was unable to conclude what was the most effective type of probiotic or probiotic effects on reducing mortality. Meta-analysis of the use of oral probiotics for the prevention of antibiotic associated diarrhoea in adults (AAD) suggests that they can reduce the frequency of AAD when administered concomitantly with oral antibiotics for nosocomial infections (76). Probiotics have been demonstrated to moderately reduce *Helicobacter pylori* infection rates in adults patients with gastritis and increase eradication rates and reduce side effects when used as adjunct therapy (77).

Probiotics have also been used to modulate complex acquired disease. In a Finnish study 132 expectant mothers, who either had atopic dermatitis (AD) or first degree relatives with AD, were randomised to receive either *Lactobacillus* GG or placebo for two months antenatal and four months post-nasally (78). The frequency of allergy of the offspring in

the probiotic group was found to be half that of the placebo group at two years of age. Four year follow-up of this study replicated this finding (79). Several small studies have been performed to investigate the use of probiotics in alleviating the symptoms of irritable bowel syndromes, some with positive results (77). In contrast to this, a recent study of acute pancreatitis suggested an adverse effect of probiotic administration (80).

1.4.4 Evidence of clinical effects of probiotics in children

Many clinical studies using *Lactobacilli*, *Bifidobacterial* and the yeast *Saccaromyces bouldaardi* have been performed, both for prevention and treatment of infectious diarrhoea in children. A Cochrane review in 2003 pooled results from 1449 children (75). Conclusions for children alone were that probiotics reduced diarrhoea rates at three days and overall diarrhoeal duration. Meta-analysis for the use of probiotics for prevention of diarrhoea are not as conclusive (81). Studies in Finland suggested that probiotics could reduce the likelihood of AAD in children receiving antibiotics for respiratory or urinary tract infection (82;83). Meta-analysis from ten paediatric studies demonstrated a pooled reduction in the per protocol incidence of AAD by probiotics. However the intention-to-treat analysis did not reach statistical significance (84). More recent studies have supported this indication. Of note, is a French study administering fermented milk products with inactivated bacteria or control formula to 971 infants which demonstrated a significant reduction in rates of consultation with a physician and for prescription of oral rehydration solution (57). A Finnish study in which *Lactobaccillus* GG was supplemented to 971 nursery day-care children found a significant reduction in number of days of non-attendance for respiratory or gastrointestinal infection when compared to the treatment group (85).

Following publication of the data from Finnish mothers, probiotics have been trialled for the treatment of AD. Weston et al (86) used as an adjunct to conventional therapy, 16 weeks of treatment with a *Lactobacillus* in children with moderate and severe exacerbations of AD. They demonstrated a modest reduction in patient's subjective symptoms of AD in the treatment group. In contrast to this Taylor et al (87) found that the administration of *Lactobacillus GG* to newborns at risk of allergy was associated with an increased risk of having AD and positive skin prick tests at 12 months of age. Several trials, with conflicting results for the treatment of allergy, have been published and the evidence remains inconclusive (88). Other conditions where limited evidence for positive effects exists for children include irritable bowel syndrome, constipation and the treatment of *Helicobacter pylori* (77).

1.4.5 Conclusions: probiotics

The data supporting the clinical use of probiotics for both adults and children is growing. However promising therapeutic results in the areas of diarrhoea treatment and prevention of infection have not been consistently replicated. In particular the lack of standardisation of probiotic preparations in terms of bacterial species, dose and timing, limits the generalisability of many studies. As the pathogenesis of different diseases is diverse, the mechanisms by which bacteria impact on diseases processes are unique. Therefore the application of species beneficial in one illness will not necessarily hold for another. The use of such preparations for the treatment of childhood inflammatory diseases therefore requires further study of both disease pathogenesis and mechanisms of action of probiotics.

1.5 Necrotising Enterocolitis (NEC)

Necrotising enterocolitis (NEC) is the commonest acquired abdominal emergency of the newborn (89). It is characterised by mural intestinal ischaemia which can result in necrosis, perforation and death (90-92). Perforation of the bowel may occur in up to one third of affected infants (93). Clinical manifestations include abdominal distension, bloody stools, acute shock and systemic collapse (92). Changes on abdominal x-ray of pneumatises intestinalis (intramural air) are pathognomic (94). Perforations can be managed either with placement of peritoneal drains or laparotomy and resection of non-viable gut (95;96). Resected intestinal tissue demonstrates oedema, necrosis, intramural air and mucosal ulceration (97).

1.5.1 Epidemiology

The overall incidence of NEC has been estimated to vary between 1 and 3 per thousand live births (98). The principal risk factor is prematurity with 90% of cases of <33wks and of very low birth weight (VLBW) <1500g (90;93). NEC rates in this population have been calculated to be between 3 and 7% (99-101). This figure rises to 10% in the extremely low birthweight (ELBW) (<1000gm) (102). There is wide variability in rates between individual centres and sporadic incidence with clustering of cases has been reported (103).

Minor risk factors have been noted with a slight male predominance and ethnicity, and a greater risk for Black and Hispanic populations noted in North America (104;105). The association of increased risk with lower socio-economic status is most likely a reflection of poorer infant nutritional status and lower birthweight (106). NEC is an acquired disease; it is not observed in stillbirths and very rarely occurs before the commencement of milk

feeds (91). There is a lower incidence of NEC in infants fed maternal breast milk as opposed the formula milk (107;108). The use of donor expressed breast milk may also reduce risk (109). The antenatal administration of corticosteroids to mothers in preterm labour has also been shown to consistently decrease NEC incidence (105).

1.5.2 Diagnosis

Suspicion of NEC is aroused by clinical signs, symptoms, biochemical and inflammatory indicators. Abdominal distension, bloody aspirates and stools are often accompanied by non-specific signs such as apnoea, poor skin capillary perfusion and sudden circulatory collapse (92). Abdominal tenderness is often difficult to detect clinically. Bell's criteria classify NEC into three stages (94): Stage 1 suspected, Stage 2 confirmed by x-ray and Stage 3 perforated or 'surgical'. Stage 1 and 2 are usually managed conservatively (intravenous antibiotics and nil by mouth) while stage 3 requires surgical intervention. Meta-analysis of biochemical indicators prior to development of NEC has demonstrated acidosis, hypoglycaemia and thrombocytopenia to be potent predictors (110). Prolonged and severe thrombocytopenia also correlates with mortality. However, none of these indicators is specific to NEC and are present in other causes of neonatal morbidity. This has led investigators to seek specific non-invasive markers of NEC.

Calprotectin, a calcium-binding protein found in neutrophils, has been shown to be a reliable indirect marker of gut inflammation in adults and children with IBD (111). A study of over a hundred preterm infants demonstrated that elevated faecal calprotectin can be detected in cases with NEC. However identification of elevated calprotectin in the stool was detected before NEC was clinically suspected only in the minority of cases (112). Salivary epidermal growth factor (S-EGF) is detectable from the second trimester onwards and contributes to gut maturation. Attempts to correlate low serial levels of S-EGF infants with the onset of NEC have generated some promising preliminary results (113). Fatty acid binding proteins (FABs) are a class of organ specific cytoplasmic small mass proteins. Elevations of intestinal and hepatic FABs have been demonstrated in the serum of preterm infants with NEC suggesting their potential as early biomarkers (114). Paradoxically rises in faecal butyrate levels have also been correlated with intestinal injury in preterm injury (115) whereas a sizeable body of evidence supports its protective role post weaning and into adulthood (116;117). Whether butyrate itself is pathogenic or simply a biomarker for colonising pathogenic bacteria which may elevate the risk of developing NEC remains to be elucidated.

1.5.3 Management and current outcomes in NEC

Clinical management of NEC is usually informed by a sliding scale according to Bell's classification (94). Stage 1 NEC is managed with broad spectrum antibiotics and a period of observation with gut rest (118). Confirmed NEC requires patients to receive nothing by mouth for a prescribed period of time (89). Supportive management, including blood volume expansion, inotropic drugs, ventilation and blood transfusion may be necessary to maintain perfusion and oxygenation to vital tissues. Reintroduction of feeds should be cautious, as stricture formation and reoccurrence complicate recovery from stage 2 NEC (95). Stage 3 NEC is managed in conjunction with surgeons. Options include laparotomy and resection of tissue or the placement of a drain and deferral of surgery unless or until complications such as stricture formation require it. Although many clinicians suspect that the removal of an inflammatory mass by resection will be of benefit to patients, careful analysis of treatment options has failed to demonstrate significantly better results (95;96). Overall mortality for infants with stage 2 and 3 NEC remains high with little significant reduction in peri-operative mortality over the last decade (98). However other major contributors to morbidity and mortality in VLBW infants, such as bronchopulmonary dysplasia and intraventricular haemorrhage, have declined (98;104). This means that the relative contribution that NEC has made to infant mortality and survival with morbidity has increased. Survivors of NEC have an overall uncertain prognosis with higher risk of neurodevelopmental delay and cerebral palsy in comparison to other preterm populations (119;120). Infants surviving massive intestinal resection face inadequate gut function and intestinal failure (121). Although long-term management with parenteral nutrition (PN) can permit intestinal adaptation, this treatment carries significant morbidity and mortality through recurrent line sepsis and intestinal failure associated liver disease (IFALD) (121;122). Although long term survival for intestinal failure is improving, data from centres with high NEC rates suggest that mortality is still significant even after extended PN (123). This significant long term morbidity and mortality continues to focus attention on efforts to better understand the pathogenesis of NEC and strategies for its primary prevention.

1.5.4 Pathogenesis of NEC

The 'Santulli hypothesis' is now over 30 years old (92). Santulli argued that NEC required the presence of excessive luminal substrate, abnormal bacterial colonisation and intestinal ischaemia (92) (figure 2). The exact pathogenesis of NEC remains unclear. However the combination of several factors resulting from, or affecting, the immature gut appear to be responsible (118). The intestinal migrating motor complex does not fully develop until

33wks of post-conceptual age resulting in inadequate digestion of food substrates which can exert direct antigenic stress on the gut (124). Immature circulatory function has been postulated as a mechanism for enterocyte damage and inflammation, as suggested by animal models in which hypoxic ischaemic injury can induce NEC (125). However more recently this has been called into question as hypoxia and ischaemia have been found to be less potent predictors of NEC than inflammatory cytokine levels (126;127).

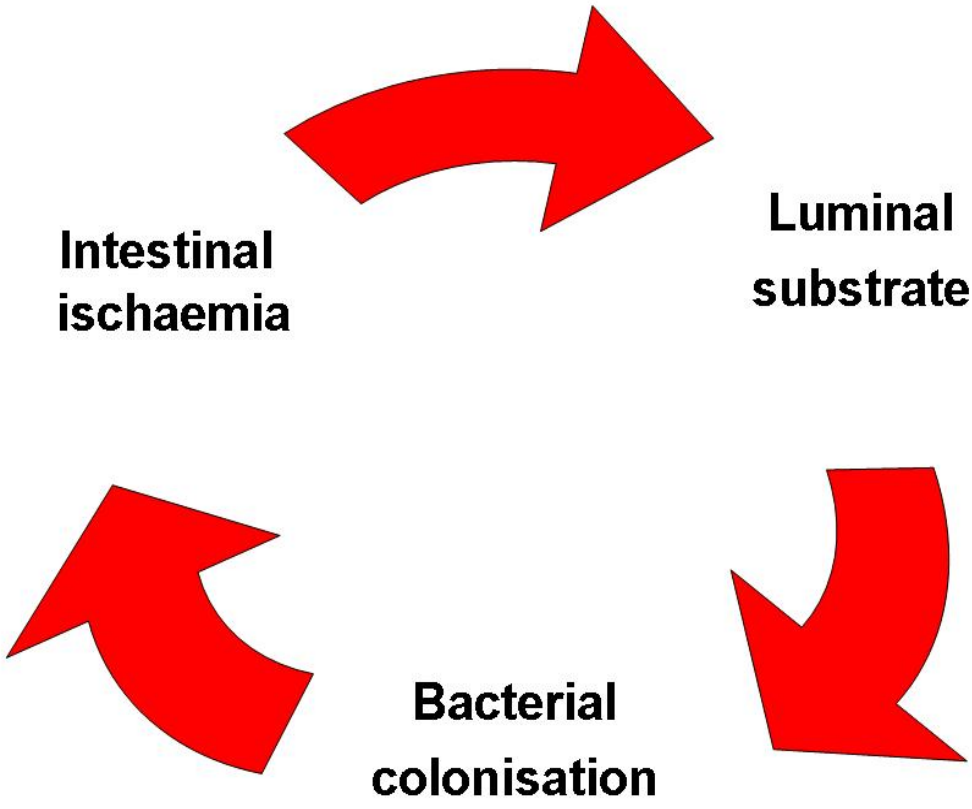

Figure 2 :The inflammatory cycle of NEC

Microcirculatory abnormalities may lead to local nitric oxide deficiencies. Nitric oxide synthase deficiency is implicated in other diseases of the preterm infant, including bronchopulmonary dysplasia and retinopathy of prematurity (128). Inadequate intestinal barrier functions has also been demonstrated in preterm infants with reduced levels of mucin, alpha-defensins and prostaglandins, which may all regulate intestinal barrier function (129). Intestinal permeability can also be compromised (130).

1.5.5 Gut microbiota in NEC

Several lines of evidence point to the involvement of the gut microbiota in the pathogenesis of NEC. NEC does not occur in the sterile environment *in utero* (92). As discussed earlier, the bacterial colonisation of preterm infants is often delayed and less diverse than that of the healthy term infant (see 1.1). Faecal samples from infants' exhibit colonisation with *Clostridia spp, Enterobacteria* and coagulase negative *Staphlycocci* prior to the development of NEC (129;131). Abnormal colonisation with *Salmonella sp*. and *E. coli* can lead to inflammatory responses in immature intestinal tissue (132). Alterations in the metabolic profile of the gut microbiota also have been observed prior to NEC development (115). Epidemiological studies of NEC demonstrate temporal and geographical clustering that can be reduced by preventative measures (118) (see 1.5.7). Gas taken from intestinal pneumatosis is derived from the metabolic activity of pathogenic bacteria. Perceived enteropathogens have been cultured from the blood of patients with NEC (118). NEC most commonly affects the terminal ileum and colon, the region of the gut most densely colonized with bacteria (90).

However several different organisms are associated with NEC rather than a single identifiable agent (133). In addition, many preterm infants develop NEC despite being on intravenous antibiotics (105;106). In animal models, NEC rates induced by cold stress are reduced but not eradicated by the absence of bacteria (134). This suggests that rather than inducing infection directly, bacteria are an important co-factor in propagating the inflammatory response seen in NEC. Whether the inflammatory response in NEC is an appropriate response to clinical infection by pathogenic micro-organisms or it represents an inappropriate inflammatory response to bacterial colonisation is a topic of debate (118). The introduction of substrates into the lumen of the gut (most often milk feeds) appears to predispose to abnormal patterns of gut microbiota that are a factor in the development of inflammation and ischaemia. However it may be that these substrates merely provide for abnormal bacterial colonisation rather than being pro-inflammatory themselves.

1.5.6 Inappropriate immune response

Jillings et al (134) elegantly demonstrated the interaction of the innate immune system and gut microbiota in the pathogenesis of NEC in an animal model. They induced NEC by cold and hypoxic stress in neonatal rats. Pups were randomised to feeding with formula delivered via a sterilised feeding catheter, or one that was freely colonised by environmental bacteria. The sterile fed pups had significantly lower NEC rates than the standard feeding pups (20% vs.53%) demonstrating the proinflammatory role of bacteria.

However in a second experiment toll-like receptor 4 (TLR-4) knockout mice were bred. TLR-4 is a key component for bacterial sensing of the innate immune system. These mice were compared to wild type mice under cold hypoxic stress and the TLR-4 knockout mice had significantly less NEC in comparison to the wild type mice (12% vs. 62%). This demonstrated that the innate immune response to bacteria is important in propagating inflammatory responses in NEC. The inflammatory response is now well characterised in NEC with demonstration of the key roles of cytokines including platelet aggregating factor 1, TNFα, IL-6 and IL-8 (90;135).

1.5.7 Novel preventative strategies for NEC

Other than reducing prematurity, the single best modifiable factor for reducing NEC to date is the administration of human milk, as opposed to preterm formulae (136;137). Components of human breast milk, such as amino-acids, nucleotides and long chain fatty acids, have been found to have direct positive effects on the immune response. Infants fed on formula milk have a different profile of bacterial colonisation from breast fed infants, the latter with greater numbers of *Bifidobacteria, Lactocbacilli* and fewer perceived enteropathogens such as *Clostridia, Bacteroides* and *Pseudomonas* (138-141). Preterm infants display a delay in bacterial colonisation with less species diversity and greater numbers of perceived eneteropathogens (4). Recent attempts to identify potential protective strategies for NEC have focused on mimicking or augmenting the effects of human breast milk, in terms of immune response or manipulation of the gut microbiota (142).

1.5.7.1 Altering the immune response

A simply conceived method to alter the preterm immune response is to supplement formula fed infants with immunoglobulin, because human-milk may exert some of its protective effects through the presence of IgA in colostrum and by human milk's ability to stimulate IgA production in the preterm neonate (143). However trials of the administration of both intravenous IgA and IgG have demonstrated only a non-significant trend towards NEC reduction (144;145).

Initial research into the therapeutic use of the amino-acids glutamine and arginine showed promise. Glutamine is the most abundant amino-acid in muscle and plasma and is a preferential substrate for rapidly dividing cells such as enterocytes (146). It has been suggested that glutamine becomes 'essential' during periods of stress and therefore deficiency in the newborn exposes enterocytes to the risk of oxidative damage. A study of

enteral supplementation of glutamine in 436 preterm infants demonstrated a reduction in NEC-related morbidity (147), but a larger study examining parenteral administration of glutamine did not show any significant reduction (148). The recently updated Cochrane review on the use of enteral glutamine demonstrates no clear effects on NEC rates in the preterm infant (149). Arginine is an essential precursor of nitric oxide synthesis via nitric oxide synthase, deficiency of which is implicated in several diseases of the newborn (127). Again arginine appears to be conditionally essential for the newborn. A single controlled study of arginine supplementation to preterm formula fed infants demonstrated a significant reduction in the incidence of NEC in the treatment group (6.7 vs. 23% $p<0.01$) (150). However this small study (154 infants) has not been replicated. Down-regulating the immature inflammatory response by enhancing gut maturation is another measure and correlations of gut maturation with growth factors such as EGF have been demonstrated (113). However, to date, only one study using insulin-like growth factor 1 (IGF-1) (151) in which preterm VLBW infants were randomised to receive either IGF-1 supplement or placebo in their milk, has been conducted. No effect on NEC rates was observed in this small study, although the treatment group did establish full feeds over a significantly shorter duration than controls. Reduction in NEC rates has been demonstrated with other feed components in NEC models. Lu et al (152) demonstrated significant reductions in NEC rates in a rat model by supplementing formulae with differing long chain fatty acids, However, as yet, no uses of this type of 'immunonutrition' have advanced to clinical trial.

1.5.7.2 Manipulating the gut microbiota

Direct manipulation of the gut microbiota can be achieved by the administration of enteral antibiotics. This practice has found little favour amongst neonatalogists, due to concerns about the development of antibiotic resistance. However it was first trialled thirty years ago (153). Cochrane meta-analysis has identified five studies which used either enteral gentamicin or vancomycin for the prevention of NEC (154). Cumulative results suggest a reduction in the incidence of NEC and NEC-related mortality. It also demonstrated an increased incidence in colonisation with resistant bacteria in the treatment group. Despite these promising results concerns remain about a therapy that may make such drastic and potentially long-term changes to the gut microbiota in these vulnerable infants (103;118;154). Clinicians are inclined to seek safer methods to change the composition of the microbiota.

Prebiotic-supplemented infant milk formulations now exist containing either GOS, FOS or a combination thereof. Several studies have demonstrated that these prebiotics are well

tolerated and can enhance faecal *Bifidobacterial* populations in term and preterm infants (59;61) and reduce faecal enteropathogen numbers (61;155). They have been shown to result in softer stool consistency (60;156) and increased levels of secretory IgA (157). However, no large clinical trials of their effects on NEC rates have been performed. In the light of data on the benefits of mother's breast milk, and now the effects of donor human milk (109) on NEC rates, it is more likely that the formula fed preterm infant will become increasingly rare. Therefore to most effectively reduce NEC rates in the VLBW population, strategies which will confer benefits over and above those of human milk feeding must be adopted. The most pertinent strategy therefore might be the administration of prophylactic oral probiotics.

1.5.8 Probiotics for NEC?

Interest in the use of oral probiotics for the prevention of NEC began to develop in the late 1980s as the understanding of NEC pathogenesis evolved. Enthusiasm for the perceived benefits were tempered by a lack of knowledge as to whether oral probiotics could establish themselves in the immature preterm gut, safety concerns over probiotic sepsis in the immunocompromised infant and their long-term effects on nutrition and immune function (132). Nevertheless by the early 1990s a neonatal rat model had demonstrated a reduction in NEC rates with the supplementation with *Bifidobacteria* (158) and a pioneer infant trial demonstrated changes in microflora composition in infants receiving oral *Bifidobacteria* (159). In a large retrospective case control study Hoyos (160) studied the administration of *Lactobacillus acidophilus* and *Bifiodbacteria infantis* to 1237 infants and compared it to 1282 infants from the previous year. The study results showed a significant reduction in the NEC rates and NEC-related mortality in the treatment group. These developments led to attempts to examine the evidence for the benefits of oral probiotics for the prevention of NEC in VLBW infant. This question forms the basis of experimental work in chapter 2.

1.6 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory disease of the bowel, which can affect the whole gut (Crohn's disease CD), be localised to the large bowel (ulcerative colitis - UC) or exhibit features of both (indeterminate colitis) (161). IBD is characterised by bloody diarrhoea, abdominal pain and weight loss (161). Extraintestinal manifestations of IBD include specific rash (eythema nodosum, pyostomatitis vegetans), arthropathy, posterior uvietis and primary sclerosing cholangitis. Diagnosis is made by

macroscopic examination of the bowel mucosa either directly (endoscopically) or indirectly (capsule biopsy) and with histological tissue examination (162).

Crohn's disease is characterised by discontinuous disease that can affect any region of the gastrointestinal tract from mouth to anus (163). In children the most commonly affected regions are the distal small bowel, colon, peri-anal region and mouth. Rectal sparing is common in CD. Affected areas demonstrate transmural thickening, oedema, chronic inflammation causing lumen narrowing which can lead to intestinal obstruction (164). The mucosal surface may show apthous ulceraction which can coalesce into extensive linear serpiginous ulceration (cobblestoning). Microscopic appearances are dependant on the duration and severity of inflammation, ranging from superficial apthoid ulcerations to confluent depressed ulcer beds, sub-mucosal obliteration, crypt abscesses and epitheloid granuloma formation (164).

Ulcerative colitis most often affects a continuous segment of large bowel ranging from a limited short segment (proctitis) to affecting the entire large bowel (pancolitis) (165). Inflammation is characteristically confined to the mucosal surface. It varies from generalized eythema with loss of normal mucosal vascularity, to surface ulceration with exudative flecks and deep ulcers and pseudo-polyps. Neutrophillic infiltrate of crypts with crypt abscesses and chronic inflammation in the lamina propria are typically seen on histological examination (165).

In around 10% of cases of IBD in which the inflammation is limited to the large bowel it may be impossible, at least at presentation, to differentiate between CD and UC. These cases are referred to as indeterminate colitis (166).

1.6.1 IBD in children

Inflammatory bowel disease is often characterised by bloody diarrhoea, abdominal pain and weight loss (163), but only around 25% of children present with this classic triad (167). Younger age of onset is associated with delay in diagnosis which can lead to malnutrition, growth failure (168-171), inadequate bone mineralisation (172-174) and pubertal delay (175). There is a higher prevalence of abdominal pain in children with CD (33). Children with UC have a greater incidence of pancolitis (33;176). Children with IBD are more likely to have a positive family history for IBD, suggesting that genetic factors are more important in these patients than in adults (177). The Scottish Childhood IBD population is characterised by extensive intestinal involvement with rapid progression of disease, in particular an early requirement for surgery in UC (178).

1.6.2 IBD epidemiology

The incidence and prevalence of all age on-set IBD has risen dramatically world-wide in the last four decades. Both CD and UC have risen in Europe (179;180), the UK (33;181-183) and North America (184) where their incidences have always been relatively high. Childhood onset IBD has also seen a dramatic increase over the same time period although the proportion of patients diagnosed with IBD in childhood and adulthood has remained relatively equal (184). There is a slight female predominance of CD and a male predominance of UC (185). Within North America, African Americans and Hispanics are less likely to develop IBD than the Caucasian population (186). However in the UK ethnic minorities appear to have a similar risk of IBD to Caucasian populations (33;187). The single greatest risk factor for IBD is a family history of IBD, with 5-35% of newly diagnosed IBD patients having a first degree relative with established IBD (188). Disease concordance rises to 66% in monozygotic twins (188). Appendicectomy and cigarette smoking are the only firmly established environmental factors: smoking protects against the development of UC, as does appendicectomy for proven appendicitis, whereas, in contrast, smoking is a risk factor for the development of CD (189;190). Other potential environmental modifiers include childhood immunisations, unrefined dietary sugars and breast-feeding (191;192). Within the UK cases of CD in children appears to have increased three-fold between 1960-1990 whilst UC rates remained relatively static (193). A prospective study of the UK population for 1998-1999 calculated that the incidence of childhood onset IBD has risen again to 5.2/100 000 child years (CD 3.1 and UC 1.4/100 000 child years) (33). The Scottish population has a yet higher incidence, with 6.5/100 000 child years of IBD (CD 4.2 and UC 1.8/100 000). Better disease recognition may in part account for this rapid rise, although elsewhere in Europe and North America similar rises have been recorded (180;184).

1.6.3 IBD genetics

Epidemiological studies have long supported a genetic basis for IBD (183;194;195). There is a greater risk of IBD amongst first degree relatives, with higher concordance between monozygotic twins than dizygotic twins (188). Early onset disease may confer a higher risk of IBD, especially CD, to first-degree relatives. There are also marked differences in incidence with ethnicity (196). There is a north to south cline across Europe, with the highest prevalence of childhood onset IBD found in Scandinavia and Scotland (33;180). Several gene loci have been associated with an increased susceptibility to IBD, and the strongest evidence for genetic linkage in IBD susceptibility is located on chromosome 16 and is designated IBD1 (197). Within the IBD1 locus the causal gene has been identified

as the caspase activating recruitment domain gene (CARD15), otherwise known as the nucleotide oligomerisation domain (NOD2) with allelic variations in the CARD15 protein conferring an increased risk for the development of CD (198;199). The CARD15 protein is an intracellular receptor that identifies pathogen associated molecular pathways from bacterial muramyl dipeptide (MDP) (200). MDP is a common component of most Gram-positive and Gram-negative bacteria. CARD 15 protein mutations also appear to interplay with a family of equivalent cell surface receptors known as toll-like receptors (TLRs) (196), numerously present on the enterocytes of the gut mucosa (45). Specific allelic variations have been characterised in the Scottish childhood onset IBD population (201-203) and these other IBD candidate genes also appear to encode for components involved in immuno-surveillance (196). These recent observations have focused interest on the interface between the immune system of the gut and its microbiota (figure 3).

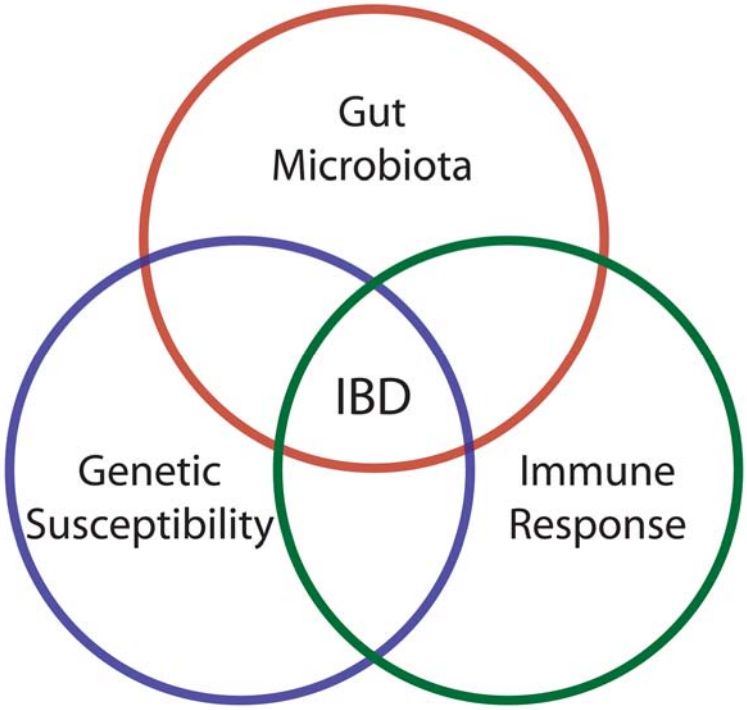

Figure 3: Interrelationship of factors associated with the pathogenesis of IBD

1.6.4 Immunology in IBD

The involvement of the immune system in the pathogenesis of IBD, via inflammatory cytokines, has been demonstrated in animal experimental models (204). A self-perpetuating inflammatory cascade occurs due to an imbalance between pro- and anti-inflammatory mediators (44). Cytokines implicated in the initiation and perpetuation of inflammation includes; interleukin-1 (IL-1), IL-6, IL-8 IL-12, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNFα) (23;44;204-206).

The pro-inflammatory effects of TNFα in both local inflammation and peripheral organ involvement in IBD is elegantly demonstrated by the effectiveness of specific TNFα antibodies to reduce intestinal inflammation (207;208) and extra-intestinal manifestations of IBD such as pyoderma gangrenosum (209). TNFα may exert its effect upstream through regulation of transcription factors (210). Nuclear factor κB (NFκB) appears to be one such important factor in the propagation of intestinal inflammation. Activated NFκB up-regulates the expression of pro-inflammatory molecules in IBD (211). The precise regulation of NFκB in the inflammatory response is unclear, but is known to involve the CARD15 protein, TLRs, TNFα and bacterial lipopolysaccharide directly (212;212-215). This implicates the bacterial microbiota in triggering and propagating immune dysfunction, and thus inflammation, in IBD.

1.6.5 Gut microbiota in IBD

The intestinal T-cells of patients with CD are hyper-reactive to bacterial antigens. CD can be characterized by a T_h1 skewing of T-cell response (216;217), whereas UC exhibits a T_h2 response (44). The initiating and perpetuating stimuli for such immune dysregulation are not fully explained by genetic predisposition, as the number of known IBD promoting gene mutations, such as CARD15, greatly exceeds the clinical prevalence of disease (196). Mouse models also demonstrate that clinically identical mucosal inflammation can be initiated by different genetic immune defects (44). This indicates that other modifying factors must be present and suggests that it is changes in the luminal environment that are crucial to the pathogenesis of IBD. The fact that experimental colitis does not develop in germ-free animals (205), that the introduction of single bacterial species can induce mucosal inflammation in animal models (218) and that diversion of the faecal stream is an effective treatment of active CD (219), further implicates gut bacteria in the pathogenesis of IBD.

1.6.5.1 Exaggerated or altered immune response?

There are two prevailing hypotheses which might explain these findings:

- IBD is the result of a normal (if prolonged and aggressive) immune response to a single pathological agent.

- IBD is the result of an altered immune response to a ‘normal’ microbiota (loss of tolerance).

Certain mycobacteria have been detected in the gut wall of patients with CD (220;221). However the lower incidence of CD in rural areas, the protective effects of poor sanitation in the development of CD and the effectiveness of immunosuppressive agents in the treatment of intestinal inflammation argue against a single infective agent as the cause of CD (222). Moreover anti-tuberculosis therapy does not cure IBD. Such observations may also be a consequence of loss of intestinal mucosal integrity due to the inflammatory process of IBD, rather than to an aetiological factor. Other agents implicated have been various bacteria, Coxsackie, Reovirus, Norwalk virus, Influenze B, Herpes, Paramyxovirus and measles. However more stringent analysis of such observations suggest these are no more than co-factors (223).

Another working model for the pathogenesis of IBD is that changes in the gut flora disturb the balance of pro- and anti-inflammatory responses (44;224). Rather than implicating a single species, it is hypothesised that such disturbance of immunoregulation involves changes across many of the bacteria present. This concept of an imbalance between 'harmful' and 'protective' bacteria has been termed dysbiosis (225;226). Probiotics, have been shown to suppress local inflammation in experimental colitis, supporting the hypothesis that bacteria are involved in both promoting and down-regulating the inflammatory responses in IBD (227;228).

Changes in the luminal bowel flora, including loss of population diversity leading to a dominance of pathogenic species such as *Clostridia difficle, Bacteriodes vulgates* and *E. coli,* have been observed before relapse in IBD (229;230). Reduced bacterial diversity has been demonstrated in mucosal biopsies of patients with active IBD, with loss of commensals such as *Clostridium leptum*, *Eubacteria* and *Bifidobacteria* (231;232). Such changes in bacterial diversity have been shown to be independent of CARD15 mutations (233). In addition, modifications of the composition of the bowel flora have been observed in patients with CD between relapse and remission (234;235).

Although bacterial sensing has been implicated in the pathogenesis of IBD, recent research has demonstrated that the products of bacterial activity have a regulatory effect on inflammation in IBD (116;117). The SCFA, butyric acid, is a product of the bacterial fermentation of non-digestible carbohydrate and in the acidic pH of the colon exists predominantly in its anionic form; butyrate. Butyrate is the preferential energy substrate for human colonocytes (236). It has a regulatory effect on colonocyte metabolism, cell division and apoptosis (237). Butyrate down-regulates mucosal inflammatory responses *in vitro* by inhibiting the activation of NFκB (117;216;238). Butyrate has been used as a

proxy measure of bacterial metabolic activity when comparing stool samples of healthy controls and patients with IBD (239). Loss of butyrate-producing species has been demonstrated in faecal samples of patients with CD (230;239). Moreover butyrate has been shown to be an effective local topical treatment of UC (240-242) and oral therapy has been shown to positively affect mild to moderate CD (240;241;243). This suggests that changes in the metabolic activity, as well as diversity of bacteria, play a part in the pathogenesis of IBD.

Luminal and mucosal bacteria may play differing roles in the pathogenesis of IBD as luminal bacteria only interact indirectly with the gut epithelium through production of metabolites and bacteriocidins, whereas mucosal bacteria lying on the epithelium are more likely to interact with cell surface receptors. In the healthy gut, direct bacteria-mucosa interaction is inhibited by the mucin layer which keeps the biofilm bacteria away from direct contact with the mucosal surface. However in active IBD the mucin layer thickness can be decreased or absent resulting in disturbances in the mucosal biofilm (244). This loss of barrier function leads to increased numbers of bacteria adhering to cell surfaces and increased permeability allowing more of these bacteria to injure or invade the epithelium. This suggests that in active IBD bacteria normally present in the lumen, as opposed to the mucosa can also play a role in direct host-bacteria crosstalk.

These findings pose two key questions concerning the role of the microbiota in the pathogenesis of IBD:

- How are changes in bacterial diversity involved in the pathogenesis of IBD?
- Are there changes in metabolic activity that instigate or propagate IBD?

Whilst these two questions are inextricably linked, the tools with which each can be investigated are not equally well developed. The colonic microbiota, whilst diverse, is also complex and the activity of groups of bacteria within it varies temporally and spatially within the gut lumen, regulated by nutrient supply, host immunological response and inter-microbe interaction and competition for survival and growth (see 1.1).

1.6.5.2 Bacterial diversity versus activity?

The bacterial genomic reservoir of the collective human gut is very diverse (>100 000 species) but within each human individual the number of species may be much smaller (around 1000 species) (245). This has lead to the concept of a ‘personalised microbiome’.

Different bacterial strains may be performing the same protective, immunological and metabolic activities in different individuals, which could be either preventing or stimulating the inflammatory response in IBD. This is termed 'functional redundancy'. However, molecular bacterial species diversity assays alone may be poor indicators of functional activity because they rely on the amplification of short sequences of DNA/RNA, which can be derived from active, inactive or dead bacteria. Around 80% of the gut microbiota can be characterised only by culture-independent methods and these do not assess the complete genome of bacteria, far less which genes are being expressed, nor information about their metabolic activity (246). Assumptions cannot be made about the functions of even highly phylogenetically similar species as other factors in the microbial genome can affect the expression of genes encoding for metabolic function.

To understand the complex changes in the gut microbiota that may predispose to the genesis and/or relapse in IBD, techniques are needed which can assay and link metabolic activity and diversity of the unculturable bacteria (figure 4). Bacterial activity *in vivo* has been assessed, by proxy, through analysis of metabolites such as SCFAs, ammonia, hydrogen-sulphide, phenols and bile acids (247;248). SCFA analysis has been used to study gut bacterial metabolic activity in culture models and human faecal samples (12;249;250). However such measures are affected by confounding factors within the colonic environment including intestinal motility and transit time, absorption and colonocyte metabolism. A more direct measure linking the microbiota's functional activity with diversity is required (see 1.9-1.12).

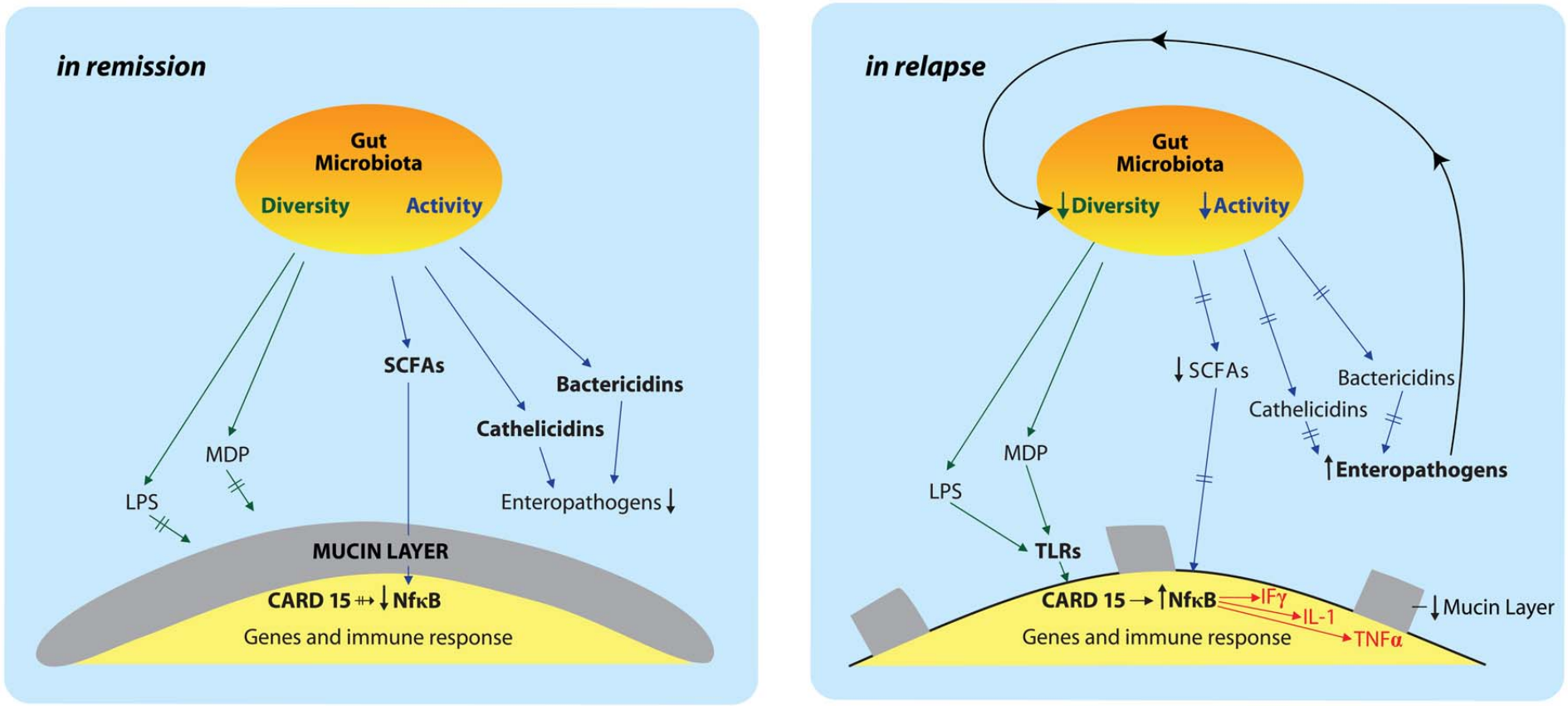

Figure 4: Hypothesised interrelationship between bacterial diversity and activity in relapsing IBD

LPS= lipopollysaccharide, MDP= muramyl dipeptide, NfκB= nuclear factor kappa B, SCFA= short chain fatty acids, IFγ= intereferon gamma, IL-1= interleukin 1, TNFα= tumour necrosis factor alpha, TLRs= toll like receptors

1.7 Medical Therapies in IBD

Effective medical therapies for the treatment of IBD began with the use of 5-aminosalicylates (5-ASA) and oral corticosteroids in the 1940s and 1950s respectively (251). These therapies work primarily by targeting the inflammatory cascade at various levels to reduce mucosal and peripheral inflammation. Modern medical therapies are now categorised into corticosteroids, 5-ASA, immunomodulators and biological therapies (164).

1.7.1 Corticosteroids

Corticosteroids bind to specific intracellular receptors to effect the transcription of steroid responsive genes (252). It is also recognised that activated steroids receptors interact with NF-κβ in regulating the inflammatory response (252). Pro-inflammatory proteins suppressed by corticosteroids include IL-1, IL-2, IL-8, IL-6, γ-interferon, and TNF-α. These in turn result in impaired inflammatory signalling in terms of antigen presentation, and inflammatory cells activation (253).

Oral corticosteroids (primarily prednisolone) have long been used to induce remission in CD (254) and they have been proven effective in the treatment of adults and children for more than four decades. A Cochrane systematic review has demonstrated that they are effective in achieving clinical remission in CD in comparison to placebo (255). Corticosteroids are also effective in inducing remission in patients with active UC (256).

However the adverse effects of oral corticosteroids are high with Cushingoid appearance, hypertension and increased infections being commonly encountered in the short-term and osteoporosis, adrenal suppression and growth retardation as long-term concerns (253). Oral budesonide is a topically-active glucocorticoid that has a much lower systemic bioavailability due to first pass metabolism and evidence suggests that it can be effective in treating active CD with fewer systemic adverse effect (257;258). 20% of patients encounter recurrence of symptoms on tapering of oral corticosteroids (259) and a Cochrane review suggests that there are no benefits in using steroids to maintain long-term remission in patients with quiescent CD (260). There is also little evidence to support their use in the maintenance of remission in UC (256).

1.7.2 5-Aminosalicylates (5-ASA)

Sulphalasazine was the first 5-ASA used in clinical practice for the treatment of IBD (261). Sulphasalazine consists of a sulphapyridine linked to a 5-aminosalicylate, which is the active moiety, released as a result of bacterial cleavage in the colon (262). Further 5-ASAs have been developed, including mesalazine and olsalazine. Aminosalicylates down-regulate inflammatory responses through inhibition of IL-1, IL-2 and IL-6, lymphocyte proliferation and epithelial HLA-DR expression and enhancement of barrier function (263). High level evidence supports the use of 5-ASAs for induction of remission in UC (264) and for a moderate benefit in maintenance of remission in UC (264). Modest benefits have been demonstrated with 5-ASAs in inducing remission in patients with mild to moderate colonic CD, but, the evidence does not support their use in maintaining remission in CD (265). 5-ASA's also have a well documented adverse effect profile, including rash, headache and pruritus and, more rarely, toxic hepatitis, leucopenia, pancreatitis and neurotoxicity (265). Because of the limitations of both steroids and 5-ASA in maintaining remission in many IBD patients often require additional therapy with immunomodulating agents is required.

1.7.3 Immunomodulators

The imidazole derivative 6-mercaptopurine (6-MP) and its precursor, azathioprine, have been used in clinical practice for the treatment of IBD for over four decades. The majority of clinical experience, in the UK, is with azathioprine, which was developed in 1957 as a slower release formulation of 6-mercaptopurine (266). Azathioprine is rapidly metabolised to 6-MP in the body which exerts its effect by inhibiting purine synthesis in DNA and RNA and thus reducing cell proliferation and thereby causing immunosuppression (266). Azathioprine has been shown to be effective in both inducing remission and for maintenance therapy for CD, with significant steroid-sparing effects. (267). Azathioprine may reduce the requirements for steroids in inducing remission in UC (268) and appears to reduce maintenance relapse rates long term (269). The related compound, cyclosporine, has been used as a rescue therapy in patients with severe UC before establishment on azathioprine (270).

Methotrexate also causes immunosuppression by inhibiting purine synthesis in DNA and reducing T-cell proliferation. Methotrexate at higher dose (25mg weekly) appears to induce remission in CD (271). Methotrexate has also been shown to be superior to placebo for the maintenance of remission in CD and is now commonly used as an adjunct to therapy in patients who relapse on or are intolerant to purine metabolites (272).

Adverse events with immunosuppressants are significant with nausea and vomiting both common. Over-immunosuppression leading to leucopenia is a potential risk with both azathioprine and methotrexate, which requires frequent white cell level monitoring (268). In addition methotrexate is also associated with the development of hepatitis and hypersensitivity pneumonitis (272). Fatal sepsis and malignancy risk are low (273). A significant proportion of patients are intolerant of, or do not achieve long-term remission of symptoms, with immunosuppressive agents.

1.7.4 Biological therapies

Because of the limited success of immunosuppressive agents to treat moderate and severe IBD, and because of their adverse effect profile, newer drug therapies have been developed to target more specific aspects of the inflammatory cascade. Infliximab, a chimeric monoclonal antibody to TNF-α, was the first such 'biologic' agent employed for the treatment of IBD (207). Infliximab binds to both trans-membrane and circulating TNFα preventing it binding to receptors on target cells, thus down regulating the inflammatory cascade (274). Infliximab appears effective in inducing remission in active CD (275) and has been used extensively in adults and children for this purpose (208). Clinical trials suggest that repeated infusion can help maintain remission in infliximab-sensitive patients (276;277). Infliximab, as adjunct therapy, also appears to promote mucosal healing and reduce short-term need for surgery in UC (278). Limited evidence also suggests a role for infliximab as maintenance therapy for UC (279). 'Human' anti-TNF (adulibumab, certolizumab) therapies are now in use and appear to have similar effects (280). Enthusiasm for the biological agents, particularly in North America, has led authors to promote their early use in CD (280). Other specific biological agents of promise include Il-1 antibodies, TNF-α converting enzyme inhibitor, CD cell antibodies and B-cell directed therapies (281;282).

Adverse events reported with biological agents include acute infusion reactions, delayed hypersensitivity reaction and auto-immune phenomena (258;283). Immunosuppression related infections have been reported, with concerns over fatal sepsis and the development of tuberculosis. Case reports of malignancy have been described as infusion numbers have increased, with most recently reports of invariably fatal hepato-splenic T-cell lymphoma (284;285).

1.7.5 Conclusions: medical therapies

Medical therapies have evolved over the last fifty years to meet the increasing complexity of the natural history of IBD. Although mortality remains consistently low in IBD, despite maximal medical therapies, a significant minority of patients do not achieve long-term remission or experience significant complications of primary disease or treatment (need for surgery, malnutrition, poor bone mineral density). In children and adolescents growth failure and pubertal delay remain significant clinical problems despite the availability and use of more complex therapies (174). Medical therapies target the inflammatory cascade rather than the stimuli for inflammation, such as the gut environment. They also do not address malnutrition as a factor contributing to the inflammatory process nor its effect on the individual patient. The adverse events of medical therapies contribute to the long-term morbidity of patients with IBD, and now appear to pose a significant mortality risk. With these issues in mind, a separate approach of 'nutritional therapies' for IBD has been developed over the last two decades.

1.8 Nutritional Therapies in IBD

Theories linking the aetiology of IBD to dietary factors have existed for some time. These have implicated Westernised diet, in particular refined sugars (286), 'fast food' (287), margarine (288) and dairy produce (289). However critical examination of these hypotheses are inconclusive and specific dietary recommendations cannot go beyond general advice about healthy eating (290).

Disease related malnutrition (undernutrition) is common in both CD and UC, with decreased adipose tissue, lean body mass, and muscle bulk, linear growth and bone mineral density all described (290). Gut rest and PN were previously used as a primary therapy for active CD, but they have now been shown to have little impact on clinical remission rates and they are limited to patients with severe active disease and undernutrition (290).

The possibility that enteral nutrition had a direct positive effect on the natural history of IBD was speculated in the 1970s when it was noted that patients with IBD receiving exclusive elemental feeds for preoperative supplementation appeared to have an improvement in their clinical condition (291). A broad experience in the use of nutritional therapy now exists for IBD. In adults, nutritional therapy has been shown to be effective in achieving clinical remission in CD and to a limited extent in UC. However meta-analysis has consistently shown that corticosteroids are more effective than enteral nutrition in

achieving remission in adults and their role in adult practice remains controversial (292;293). However, in children with IBD, enteral nutrition appears to be at least equipotent at achieving remission in CD (294) and has additional benefits on nutritional status and linear growth (295). Adult data suggesting that disease location is important in predicting clinical responsiveness to nutritional therapy in CD have not been replicated in paediatric studies, where response has been good even in patients with disease limited to the large bowel. Nutritional therapy is regarded as the first line treatment of choice for paediatric CD in the UK and Europe (296). Some data also suggest that long term supplementation with enteral nutrition reduces relapse rates and improves linear growth (297;298).

1.8.1 Mechanisms of action of enteral nutrition?

The first clinical trials of enteral nutrition focused on elemental formulae (in which nitrogen is delivered in the form of amino acids). It was postulated from *in vitro* and *in vivo* observations that the mechanism by which gut rest and enteral nutrition stimulated mucosal healing was through a reduction of antigenic stimuli (299;300). However recent analysis has shown no difference between elemental, semi-elemental (oligopeptides) or polymeric (whole protein) feeds in terms of efficacy (292;301). Micronutrient deficiency has been shown to be commonly present in IBD and nutritional therapies (302;303) have been shown to increase enterocyte levels of micronutrients prior to mucosal healing. The anti-inflammatory properties of enteral nutrition have been demonstrated by the reduction in inflammatory markers, including serum I-6 preceding nutritional restoration in polymeric liquid fed CD patients (304;305). This has in particular led to interest in the lipid content of feeds, especially the relative composition of long chain fats and ω-3 fatty acids. Changes in dietary lipids can alter cell membrane phospholipid profiles, which in turn can modulate cellular inflammatory responses. In addition ω-3 fatty acids may also directly interact with surface receptors of the GALT (306). Preliminary data support the hypothesis that the lipid profile of enteral nutrition may play a role in its therapeutic effects in IBD (307).

1.8.2 Mechanism of action of enteral nutrition: modulation of the gut microbiota?

As previously discussed in section 1.5.6, evidence exists implicating the gut microbiota in the pathogenesis of IBD. Modulation of the gut microbiota to ameliorate the inflammatory process has been demonstrated with diversion of the faecal stream in colitis and the use of antibiotics in perianal CD (205;219). It has been speculated that the primary anti-

inflammatory mechanisms of nutritional therapy may lie in its restoration of a 'healthy' gut microbiota. This prebiotic effect may result in the restoration of metabolic functions and products (such as butyrate) to promote mucosal healing. However the effects of enteral nutrition on the metabolic function of the flora in the small bowel are not well characterised (308). One study has demonstrated changes in the faecal flora during treatment with enteral nutrition for active IBD. Lionetti et al (234) studied the faecal flora of nine children with active CD who were all treated for eight weeks with an excusive polymeric feed. Faecal samples were obtained every 2-3 weeks and the bacterial consortia typed by TGGE of 16s rRNA PCR products. All children showed changes in bacterial banding patterns during treatment and although species stabilisation was maintained after treatment the band diversity was not as marked as during the treatment period. The direct effects of nutritional therapy on the gut microbiota require more study, and very little is known about their effects on the small intestine.

1.8.3 Probiotics for IBD?

With our evolving knowledge of the role that the gut microbiota play in the pathogenesis of IBD there has been increasing interest in the possibility of using probiotics to modify the natural history of the disease. Post-operative pouchitis, a common complication of ileal pouch anal anastamosis in UC, has been studied by Giochetti et al (309) who demonstrated a benefit in the maintenance of antibiotic induced remission in twenty patients treated with a cocktail of *Bifidobacteria, Lactobacilli* and a non-pathogenic *Streptococci* (VSL#3) in comparison to placebo. The findings that VSL#3 extends time to pouchitis relapse has been confirmed by other independent researchers and demonstrated with *Lactobacillus GG* (310-312). The successes with pouchitis led to great enthusiasm for the possibility of a new class of treatment for IBD. However the treatment of IBD in general has been met with more conflicting results.

Initial small studies into inducing remission and for maintenance for CD reported two positive outcomes (313;314) and three negative outcomes (315-317). Larger trials have since been conducted. Marteau et al (318) recruited 98 patients with CD in clinical remission and randomised them to six months of *Lactobacillus johnsoni* or placebo. They demonstrated no significant difference in the relapse rate between the groups at the end of the study. These findings were replicated by another group (319). The only paediatric study of probiotic usage in IBD to date was performed by Bousvarous et al (320), who randomised children with CD in remission to receive either *Lactobacillus GG* or placebo

over two years. There were no significant differences in time to relapse between the groups by the end of the study.

Early studies of probiotic use for the treatment of UC were more positive. Four small studies demonstrated the benefits of *Bifidobacteria, Sarccharomyces boulardii*, *E. coli Nissle* 1917 and VSL#3 over placebo in inducing remission (321-324). Rembacken et al (325) demonstrated, in a larger study, that oral *E. coli Nissle 1917* was as effective as standard therapy (mesalazine) in inducing remission. One study has shown *E. coli Nissle* to be effective in maintaining remission (326) whereas two studies have shown no significant treatment effect (325;327). Two studies have also failed to demonstrate any beneficial effects in the use of *Lactobacillus salivarus, Bifidobacterium infantis* and *Lactobacillus GG* (328) respectively.

Non digestible carbohydrates have been used for their 'prebiotic' effects in small trials in IBD. A double-blind cross over study examined the effect of inulin in patients with chronic pouchitis (329) and demonstrated a significant reduction in clinical and endoscopic markers of ileal inflammation. A small randomised study of oligofructose and inulin and *Bifidobacteria* significantly reduced mucosal TNFα levels in 18 patients with UC; however clinical parameters were not statistically significantly altered possibly due to small study numbers (330). Oligofructose has also been given to adults with CD in an open label non-controlled study with reduction in disease activity as an adjunct to conventional therapies (331). The current limited data need to be extended with larger well designed placebo controlled studies.

1.8.4 Conclusions: nutritional therapies

The recent developments in enteral nutritional therapies have provided benefits to many patients with IBD, particularly children, in terms of reduction of symptoms, and adverse events. However much is still poorly understood about nutritional therapies. The exact mechanisms for the mode of action of exclusive enteral diets remains obscure and a significant proportion of patients remains recalcitrant to nutritional therapy (both predictably and unpredictably). Potential benefits of probiotics have been seen in the maintenance of remission of pouchitis and in the treatment of active UC, but in other forms of IBD results have been disappointing. This has led authors to speculate that the effects of enteral nutrition and probiotics may be site-specific (location in the GI tract) and patient-specific (differing genetic susceptibilities) (226;332;333). To understand the potential benefits of such nutritional therapies, and therefore to design new tailored treatments, it is

firstly necessary to understand the activity of the full microbial population of the gastrointestinal tract and secondly to understand the impact of nutritional therapies on their metabolic activities *in vivo*. The development of innovative culture-independent techniques will be necessary produce objective measurements which can be used to determine the effects of therapies on the metabolic activity of the gut microbiota. The evolution of the techniques used to determine the nature and function of bacteria will therefore be discussed next.

1.9 Studying the Gut Microbiota

1.9.1 Traditional methods: microbiological culture techniques

There is a variety of methods available to characterise the microbiota of the large bowel including those that may be involved in the aetiology of IBD and NEC. Traditional bacterial culture techniques allow the activity of species to be studied under varying conditions, albeit in isolation from their natural ecosystem. However they have limitations because the vast majority of species found in the colon are anaerobic and difficult to culture. It has been suggested that less than 20% of the gut microbiota have been cultivated and identified in this manner (246).

1.9.2 Molecular techniques

The development of culture-independent methods of identifying micro-organisms has rapidly expanded since their first description in the mid 1980s (334;335). These utilised the key discovery of the polymerase chain reaction (PCR) to facilitate dot-blot analysis and fluorescene *in situ* hybridization (FISH) (336). Isolation of bacterial DNA and gene sequencing has led to a rapid expansion of gene libraries of bacterial species. The most commonly used DNA sequences now encode 16S rRNA due to its combination of hyper-conserved and variable regions, allowing phylogenetic typing. Over 120 000 sequences have been identified (334;337;338). A specific library of the microbiota of the human intestine identified using molecular techniques has been established through a unique European collaboration (245). This has demonstrated that the human bacterial genome is vast (>100 000 species). However within each individual the number of species is far lower (around 1000 species).

The use of phylogenetically-specific RNA probes and temporal temperature gradient gel electrophoresis (TTGE) can resolve complex flora into the dominant phylogenic groups (10). This produces a picture loosely related to the dominance of each group, known as a

'community profile'. TTGE has been used to characterise the microbiota of healthy individuals and of patients with IBD. Seksik et al (235) used such methods to demonstrate that 30% of bacteria identified in stool samples from patients with CD were not usually found as dominant phylotypes in the microbiota of healthy individuals. These differences were seen in patients whether in remission or with active CD. In addition two major groups of bacteria were identified in patients in disease remission but were lost when these patients relapsed. Other groups identified during relapse were not present when patients achieved remission. These floral changes are confusing and appear paradoxical. It is worth noting that PCR techniques 'identify' bacteria through a single copy of DNA which may be derived from active, inactive or dead bacteria. Quantitative and real time PCR experiments have been performed for single organism studies (339), but as yet this technique is unable to detect quantitative changes in diversity in complex consortia with sufficient sensitivity (340).

PCR and DNA phylotyping has expanded our knowledge of bacterial diversity. Of the 52 characterised major bacterial phyla, 26 have been identified by culture-independent methods alone (334). In the human gut microbiota, molecular techniques have revealed that 80% of the species identified had never been cultured and thus no information on their metabolic activity is known (246). Assumptions cannot be made about the functions of even phylogenetically similar bacteria as many factors influence the microbial genome and the expression of genes encoding for metabolism. The interactions of these bacterial species may be better understood by assessing their relative activities between relapse and remission rather than by simple species identification. This dilemma has led to the development, over the last ten years, of techniques that combine culture-independent identification with assays of metabolic function.

1.10 Techniques to Assay Bacterial Metabolic Activity

1.10.1 Metabolite assays

Bacterial activity *in vivo* has been assessed, by proxy, through analysis of metabolites such as SCFAs, ammonia, hydrogen-sulphide, phenols and bile acids (247;248). SCFA analysis has been used to study gut bacterial metabolic activity in culture models and human faecal samples (12;249;250). Faecal SCFA measurements have also been measured in VLBW infant and correlated with gut inflammation (115). However addition these markers cannot be used to construct phylogenetic relationships since bacteria that are phylogenitcally distant may possess similar genes which encode for saccharolytic enzymes which produce

similar SCFA profiles; the concept of 'functional redundancy'. A more direct measure linking the microbiota's functional activity with diversity is required.

1.10.2 Fluorescence in situ hybridisation-microautoradiography

The combination of fluorescence *in situ* hybridisation and microautoradiagraphy (FISH-MAR) was the first such technique to measure metabolic activity, described in 1999 (341;342). Bacterial communities are incubated in environments enriched with a radioactively labelled substrate. Fluorescene probe labelled bacteria were identified microscopically, and assessed as to whether they have sequestrated the labelled substrate, using a microradiograph (343). FISH-MAR has been used to extend our knowledge of the activity of single species within microbial consortia in the environment and to give quantitative functional data on microbial activity (344). FISH-MARs application in studying bowel flora is however limited by the number fluorescent probes that can be used (usually a maximum of seven) meaning that not all functionally active phyla of bacteria can be studied together.

1.10.3 Isotope array

The isotope array allows identification of all bacteria involved in the degradation of a radio-labelled substrate by using rRNA oligonucleotide probes which are covalently linked to fluorescent dye (345). The multiple RNA probe technique allows the identification of many bacteria with a specific metabolic activity (such as ^{14}C-glucose consumption). Combining analysis of radioactivity and fluorescence of a bacterial population can also give quantitative data on relative substrate degradation. However the use of radioactive substances in metabolic studies may not be readily applicable to the study of patients *in vivo*. An alternative to this is the use of non-radioactive (stable) isotopes.

1.10.4 Stable isotopes

Stable isotopes (SIs) are non-radioactive isotopes of elements (such as ^{15}N, ^{18}O, ^{2}H, ^{13}C). They differ in their atomic mass from the most commonly occurring forms of that element in the environment, by an additional neutron in their nucleus. There are three natural isotopes of carbon: ^{12}C ^{13}C and ^{14}C. Due to the natural abundance of these isotopes the relative atomic mass of carbon is 12.011 (figure 5).

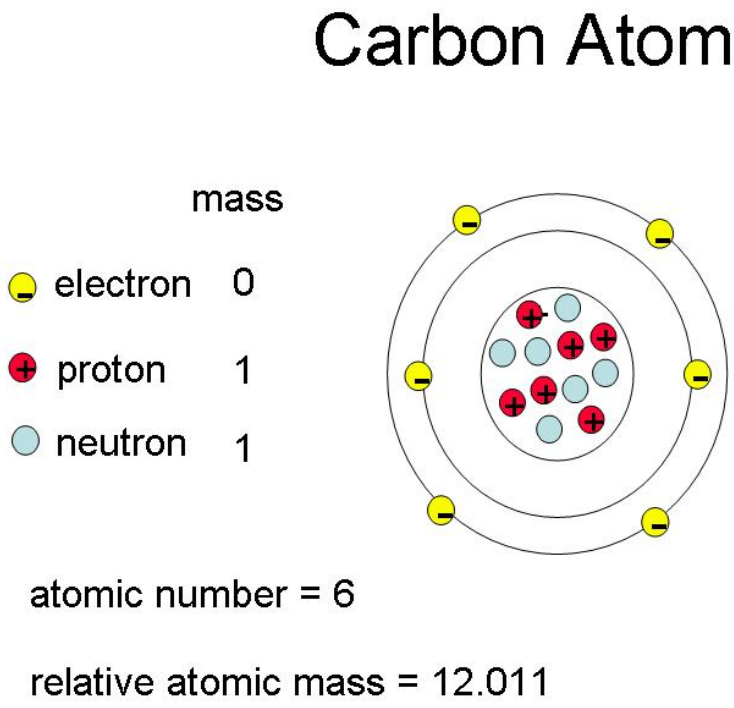

Figure 5: Atomic structure of carbon (reproduced with permission of Tom Preston)

Compounds containing stable isotopes are distinguishable by any methods that can resolve mass, such as mass spectrometry. This principle allows SIs to be used as tracers of metabolic and gastrointestinal function *in vivo* (29;346-348). SIs have been used to indirectly measure intestinal bacterial function *in vivo* by studying their effects on substrate metabolism. This has included investigation of SCFA production and SCFA interconversion using SIs (349;350). ^{13}C containing urea (lactose ureide) has shown excellent potential as a marker of intestinal function as ingested, it remains intact until hydrolysed by bacteria in the large bowel (351). Carbon flow from ^{13}C urea can be followed into the large intestine and systemically by breath testing and urinary monitoring of the isotope (348). Urea can potentially be used as a precursor of bacterial *de novo* RNA and DNA synthesis since it is readily hydrolysable by bacteria, produces inorganic C (HCO_3^-/CO^2) and therefore can potentially label the building blocks of RNA. Urea and lactose ureide, as colon specific urea delivery systems, therefore have potential as *in vivo* tracers of bacterial activity.

1.11 Stable Isotope Probing (SIP)

Stable isotope probing (SIP) is a term used to describe a technique of exposing environmental samples containing bacteria to stable isotope enriched substrates and

subsequently analysing labelled biomarkers such as RNA or DNA(338;352). Current SIP experiments have used mainly ^{13}C and ^{15}N labelled substrates (353;354). SIP works on the principle of being able to separate 'heavy' labelled DNA from 'light' unlabelled DNA. SIP experiments conducted in environmental and human ecology studies to date have isolated labelled DNA via density gradient centrifugation. DNA obtained containing ^{13}C represents that of the collective genomes of the metabolically active micro-organisms that have incorporated the labelled substrate into their nucleic acids. The greater the incorporation of SIs, the more metabolically active the bacteria. SIP is being used increasingly to link the identity of micro-organisms with their function within their natural environment (338;352). The central premise of SIP studies has been to determine which bacterial groups are involved in substrate specific metabolism. Clearly in human studies, even the use of simple primary substrates for bacteria would introduce bias since not all bacteria, for example, would be primary glucose utilisers. An alternative approach is to use generic tracers of *de novo* purine and pyrimidine synthesis which, at least theoretically, are less prone to substrate specific bias. The technique of using SIs of commonly occurring molecules to study bacterial turnover was used long before the term SIP was coined. ^{13}C labelling through *de novo* nucleic acid synthesis was the basis of the first demonstration of the semi-conservative replication of DNA in *E. coli* in 1958 (355).

1.11.1 DNA SIP

SIP experiments on DNA have been used to isolate methane-degrading bacteria in soil and biodegradation of multi-carbon pollutants (356;357). Isolated DNA can be amplified via PCR and species identified using primers targeting the 16S rRNA gene. ^{13}C labelled DNA SIP also provides a potentially novel method of bacterial identification and classification via their genetic sequence (metagenomics). A potential advantage of DNA SIP taxonomy over 16S rRNA is that it can be used to classify bacterial phyla according to common metabolic function (338). Such techniques offer a new way of studying the activity of the colonic microbiota. However there are potential limitations in these approaches, particularly when trying to measure bacterial activity in the stool samples of patients with IBD. To achieve adequate incorporation of stable isotopes into bacterial DNA requires relatively lengthy incubation periods, owing to the time taken for DNA replication. Moreover faecal samples do not fully resemble the colonic environment and laboratory analysis of the former will not necessarily reflect the latter.

1.11.2 RNA SIP

RNA synthesis occurs more rapidly than DNA turnover. This makes RNA SIP a potentially more attractive technique for analysing bacterial activity in humans over short time-frames which may retain a more representative picture of *in vivo* activity (358;359). RNA based SIP experiments have now been carried out successfully to examine bacterial degradation of phenol in an industrial bioreactor (358;359). Such techniques can potentially be scaled down to study bacterial metabolism in the smaller bioreactor of the human colon. It has been proposed that time-based SIP, using a combination of both RNA and DNA methods over time, could track the flow of ^{13}C through micro-organisms in the environment (360).

1.12 Application of SIP to Assay Gut Microbiota in IBD

SIP experiments on DNA and RNA have been used within bioreactors and in their natural ecosystem to identify unculturable bacteria which carry out specific degradative functions (356). Isolated DNA can be amplified via PCR and species identified using primers targeting the 16S rRNA gene. ^{13}C labelled DNA SIP also provides a potentially novel method of bacterial identification and classification according to their genetic sequence (metagenomics). Combining DNA and RNA SIP experiments over time has enabled temporal tracking of labelled substrates through ecosystems. This can include the flow of a labelled substrate into secondary degradators (cross-feeding) (361). Such methods lend themselves to the measurement of the metabolic activity of the gut microbiota *in vitro* (340) and *in vivo*. However there are a number of obstacles that must be overcome before SIP will elucidate our understanding the possible central role of the gut microbiota in the pathogenesis of IBD. Obtaining stool samples from patients with IBD, both in remission and relapse, and performing SIP experiments on them is a logical starting point to gain an insight into changes in bacterial metabolic activities that precede (or lead to) changes in microbial diversity. However the application of SIP *in vivo* demands adequate appropriate bacterial genetic material and sufficient enrichment of target nucleic acids. This involves;

- Enrichment and isolation of RNA/DNA
- High sensitivity of analytic techniques of isotopic enrichment, and
- Validation of an appropriate substrate for bacterial metabolic activity.

1.12.1 Isolation and enrichment of nucleic acids

There are potential limitations to centrifugal methods of isolation of RNA/DNA that rely on the separation of light and heavy bacterial nucleic bands, particularly when working with faecal samples. To achieve sufficient incorporation of SIs into bacterial DNA for adequate band separation by centrifugation requires relatively lengthy incubation periods, owing to the time taken for DNA replication and cell turnover. Moreover faecal samples do not fully resemble the colonic environment and laboratory analysis of the former does not reflect the latter. This can result in changes in the metabolic activity of the flora over time that could lead to a misrepresentation of bacterial activity. RNA, with its more rapid turnover, would therefore seem to be a better candidate nucleic acid for such studies (359) and coupling isotopic enrichment with taxonomic profiling afforded by 16s rRNA could overcome some of the limitations of DNA SIP.

SIP has proved useful in environmental microbiology through the exploitation of density ultra-centrifugation to separate isotopically heavy nucleic acids from unlabelled light nucleic acids. This has the potential advantage of limiting bias and removing the requirement for *a priori* knowledge of species diversity. However, there are some disadvantages of applying this methodology, particularly to SIP studies of the human microbiota. Effectively separating heavy nucleic acid bands from light bands requires significant enrichment in the heavy nucleic acid fraction (362;363). Achieving this enrichment in the nucleic acid fraction often requires extended incubation times. Maximising time for nucleic acid enrichment must be balanced against the changes in the faecal bacterial community which occurs once a stool is voided. Exposure to oxygen, type of culture media, loss of immunological stimuli and secondary degradation of substrates (cross-feeding), whether in batch or continuous culture, can effect bacterial diversity and metabolic activity (350;364;365).

The current achievable resolution afforded by centrifugal separation (i.e. band separation of heavy and light nucleic acids) can only be improved by increasing isotopic incorporation into studied bacterial nucleic acids. Bands of uniform enrichment will travel together, but bands of lower enrichment will migrate a shorter distance within the gradient owing to their buoyant density, resulting in a band profile reflecting isotopic enrichment. Across a spectrum of bacteria this may result in overlapping bands from bacteria with low isotopic enrichment and bacteria with a high guanine and cytosine content of their nucleic acids, due to their similar buoyant densities within the gradient. This effect becomes more problematic when trying to study RNA at lower enrichments, such as after short incubation

times (i.e. those most closely reflecting the colonic environment). 16s RNA SIP experiments performed *in vitro* on bacteria in conditions simulating the human intestine have shown that high concentrations of labelled tracer are needed to separate labelled and unlabelled RNA (366). Investigators have suggested that lower limits of enrichment required would be 20atom% for ^{13}C and 40atom% for ^{15}N (362;367). Trying to establish this level of enrichment in an *in vivo* ecosystem, such the human colon, is problematical due the systemic dilution of tracer within the whole host.

1.12.2 High resolution, high precision SIP

Whilst the advantages of not requiring *a priori* knowledge of bacterial diversity and the potential sensitivity of using centrifugal methods and PCR amplification of isolated heavy nucleic acids are acknowledged, alternative approaches are possible when studying the activity of the human gut microbiota. As discussed in section 1.1 the gut microbiota, particularly in the large intestine, are part of a densely populated ecosystem containing in excess of 200g of faecal material and 10^{10}-10^{12} viable organisms per g (8;28). This represents a significant pool of microbial nucleic acids, of which RNA may account for 20% of dry weight (68). This is many orders of magnitude greater in cell density than is often observed in extra-intestinal microbial ecology and alternative low-sensitivity but high-resolution approaches become possible.

Gene capture technology has used biotin labelled oligonucleotide probes to capture DNA targets (*Helicobacter pylori*) and streptavidin coated magnetic bead capture technology to extract and isolate the target DNA (368). This method can be modified to target 16s rRNA. Magnetic bead capture of 16s rRNA coupled with isotopic analysis has been pioneered in single species and environmental samples (369). An extension of this technique to capture 16s rRNA from complex consortia by using oligo-dt coated paramagnetic beads has been described (370).

1.12.3 Universal substrates for gut microbiota activity

SIP experiments have previously focussed on bacteria according to their metabolic functions by exploiting their preferential metabolism and sequestration of isotope from specific ^{13}C labelled substrates. Probing all metabolically active bacteria in the faecal flora requires the adoption and validation of a ‘universal’ substrate to obtain results which are not subject to substrate bias. The requirement for an isotope labelled substrate that avoids phylogenic selection bias upon incubation is paramount if the functional activity of the whole microbiota is to be assessed. The highly complex and diverse nature of the colonic

microbiota means that the metabolism of many substrates, particularly carbohydrates, will vary within and between different bacterial phyla, resulting in selection bias for bacteria that can utilise the substrate. The addition of even relatively simple labelled substrates such as ^{13}C-glucose will result in selective changes in metabolic activity. ^{13}C-glucose has been shown to inhibit lactic acid producing bacterial activity in an *in vitro* model of the human gut as measured by RNA-SIP and nuclear magnetic resonance spectroscopy (371). The ideal substrate therefore should lead to efficient enrichment of all active bacterial RNA over the shortest timescale without bias toward any phylogenetic group. Such candidate substrates are isotope-labelled precursors of *de novo* bacterial RNA synthesis (figures 6, 7). Simple substrates that are inherent to these pathways (rapidly producing HCO^{3-}/CO^{2} in bacterial systems), such as urea, aspartate, glycine and uracil, have optimal potential for SIP experiments.

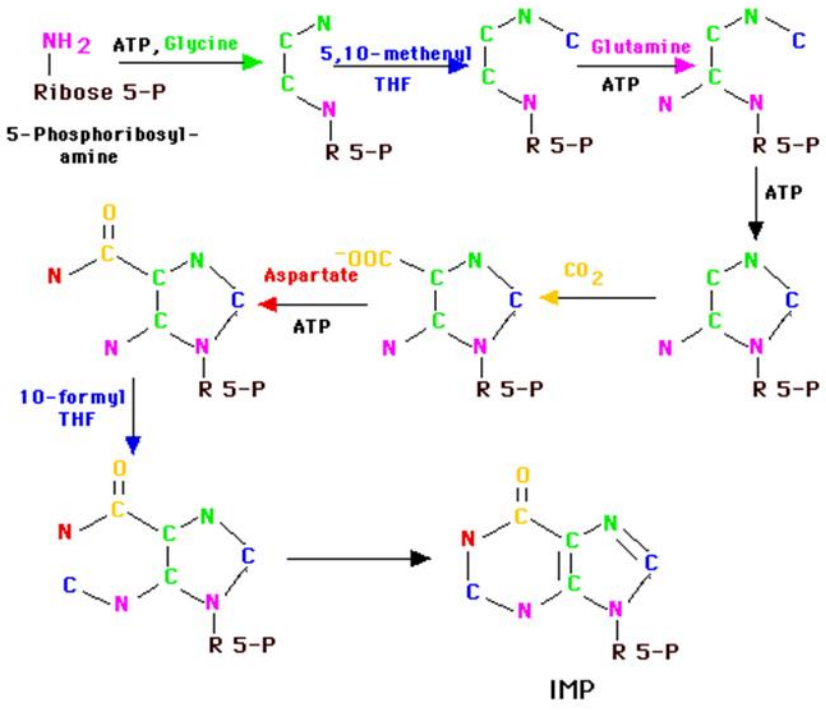

Figure 6: Purine synthesis

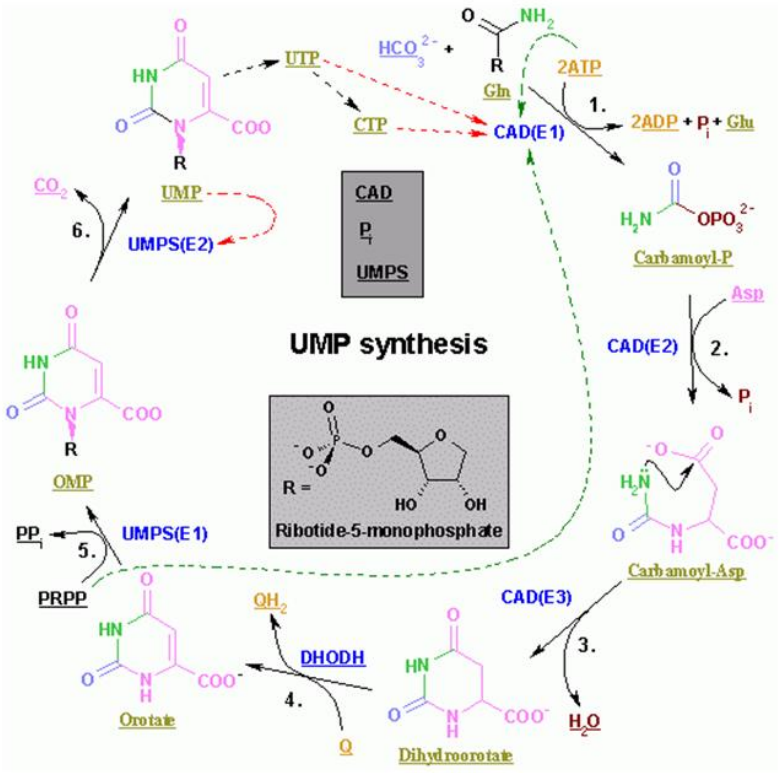

Figure 7: Pyrimidine synthesis

1.13 Mass Spectrometry

Mass spectrometry is a method of measuring the molecular and sometimes elemental composition of substances and is considered the gold standard method for the identification and quantitification of complex organic compounds. Mass spectrometry is the tool of choice with which to determine isotopic natural abundance and enrichment at low levels.

1.13.1 Isotope ratio mass spectrometry

For measurements of the isotopic ratios of light elements such as C, N, and S, very high precision is required (372). The concept of isotope ratio mass spectrometry (IRMS) was first demonstrated in 1947 by the seminal work of Neir and colleagues (373). A critical advance in modern IRMS experiments on solid material was that analytes are combusted prior to passing through the ionisation field. C, N, O, S and H, are combusted to CO_2, N_2, CO, SO_2 and H_2. After passing through the electron beam which results in a positive charge of particles they are deflected by magnetic field and collected by Faraday cups (372). It is the static nature of this mass spectrometer design that leads to the high precision analysis necessary for isotope ratio measurements. For the measurement of ^{13}C three collectors are used to measure the ion beams at m/z 44, 45 and 46 to collect isotopes of carbon in the form of carbon dioxide; $^{12}C^{16}O_2$, $^{13}C^{16}O_2$, $^{12}C\ ^{18}O^{16}O$ (46 is measured to correct for the contribution of the isotopomer $^{12}C^{17}O^{16}O$ at m/z 45 based on the $^{18}O/^{17}O$ ratio; the Craig correction (374)).

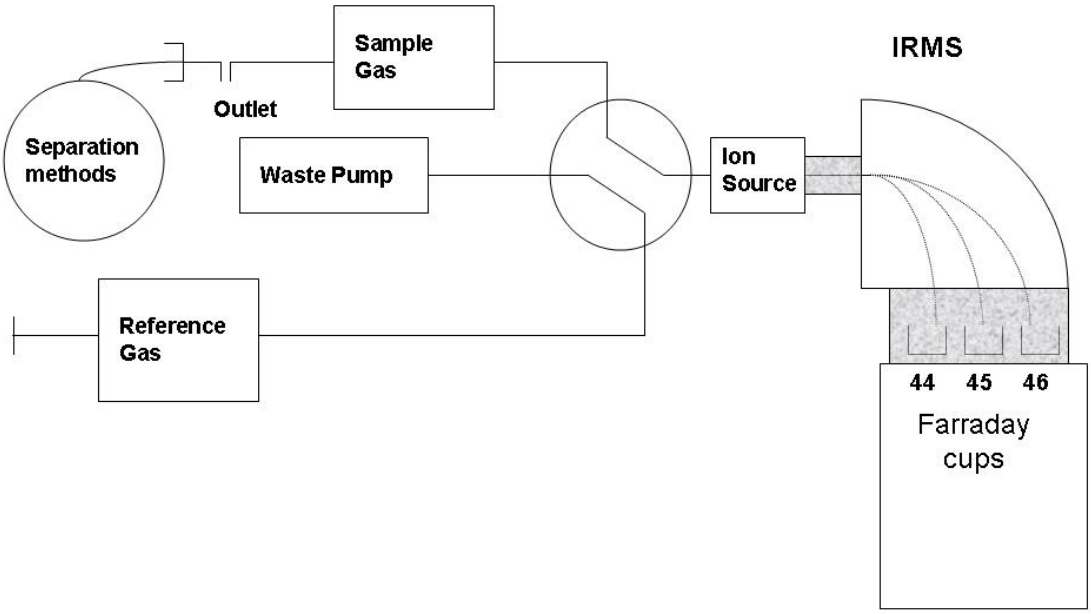

Figure 8: IRMS methodology

1.13.2 Elemental analysis IRMS

The analysis of ^{12}C:^{13}C ratios can be performed on relatively small samples by coupling IRMS with an elemental analyser (EA). A compound is loaded into a combustion chamber and all organic compounds are transformed to simple gases by hot-oxidation followed by reduction. These simple gases are easily directed into the ionisation field (figure 8). The use of EA-IRMS allows the examination of compounds with much smaller enrichments (down to natural isotopic abundances) of SIs than standard molecular mass spectrometry (1 atom% to 0.00001atom%) (375).

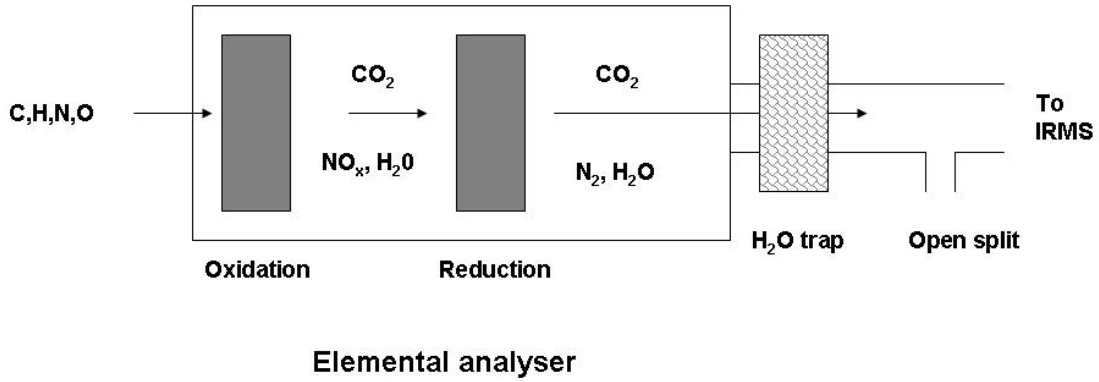

Figure 9: Elemental analyser method

However the need to maintain high carrier gas flow of material through the EA system before entry into the ionisation chamber (100ml/min) can limit the sensitivity of the system when analysing very small amounts of compounds (<1mg C). Even this level of sensitivity may be inappropriate for small amounts of 16s rRNA in SIP analysis. The analyses of low ^{13}C enrichment in very small quantities by combining IRMS liquid chromatography (LC) has been described; so called LC-IRMS (375).

1.13.3 Liquid chromatography (LC) IRMS

In LC-IRMS the transportation of soluble organic compounds in a liquid phase prior to oxidation enables reduction in He carrier flow rate into the ionisation field, thus increasing sensitivity of analysis (375). Carbonaceous compounds are oxidised to CO_2 and thereafter the dissolved gases are stripped from the liquid flow into a helium (He) counter flow by means of a microporous PTFE membrane (figure 10). The use of low-flow He prior to introduction to the ionisation chamber (1mL/min) enables the analysis of very small amounts of organic compounds (<1μg).

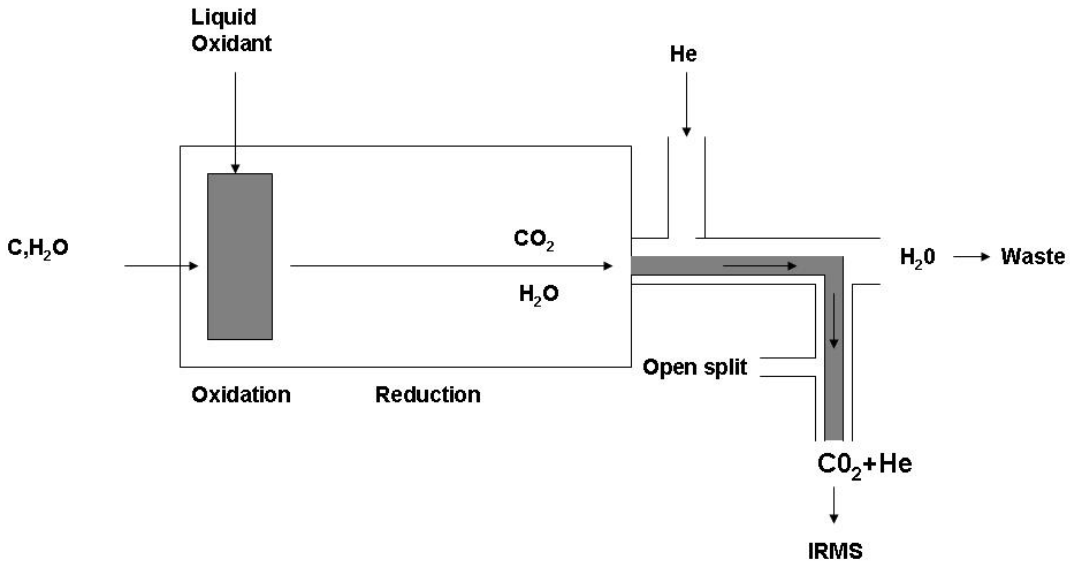

Figure 10: LC-IRMS schematic

1.13.4 Delta notation

As such small variances in $^{13}C/^{12}C$ ratios occur between stable isotopes at natural abundance a unit of expression is required. The δ notation termed in units of parts per mil has been adopted to express deviations from an internationally accepted standard (arbitrarily set at 0):

$$\delta^{13}C\ (^{0}/_{00}) = [(R_{sample}/R_{standard})\text{-}1]\ x10^{3}$$

Where $R=^{13}C/^{12}C$ ratio of the sample or the standard

$$\Delta^{13}C = \delta^{13}C \text{ sample-} \delta^{13}C \text{ baseline}$$

A change in $\delta^{13}C$ of $1\ ^{0}/_{00}$ is equivalent to a change of 0.001099 atom%. This nomenclature for expressing isotope ratios have been standardised and accepted by the isotope community (375).

1.14 16s rRNA-IRMS-SIP

As discussed in section 1.10 the development of culture-independent methods to determine the metabolic activity of unculturable gut microbiota may afford the opportunity to gain new insights into human intestinal microbial ecology and disease pathogenesis. The investigation of faecal microbiota with SIP can be conceptualised (figure 11). This can be conceived as either utilising ultracentrifugation methods or, as preferred and discussed above, 16s rRNA probe technology with IRMS.

The exposure of a faecal sample to a ^{13}C labelled precursor of *de novo* nucleic acid synthesis would result in the metabolically active bacteria utilising ^{13}C and incorporating it into its cell structure and nucleic acids. After isolation of nucleic acids, analysis can be performed by either buoyant density ultracentrifugation or by oligonucleotide probe extraction and IRMS. This latter approach was identified as the most suitable due to its applicability to short incubation times and RNA/DNA with low levels of enrichment not amenable to centrifugal separation.

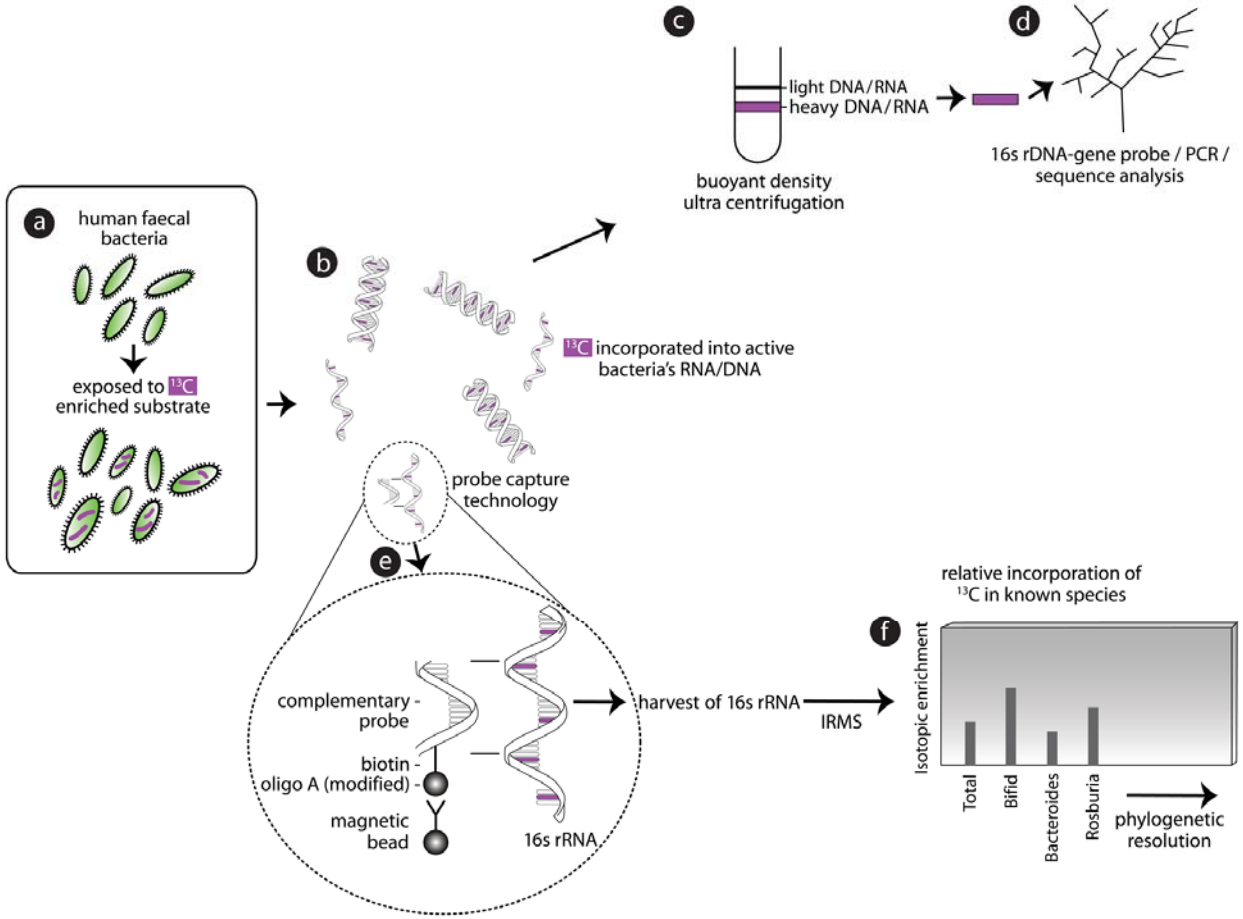

Figure 11: Conceptualisation of SIP experiments using human faecal material

a) Faecal material exposed to a ^{13}C rich substrate the metabolically active bacteria incorporate ^{13}C into cell structures during cells replication. b) nucleic acids can then be attained by centrifugal methods c) light and heavy labelled DNA can separated by buoyant densities and then phylotyped by d) PCR and phylotyping or alternatively e) 16s rRNA of previously identified bacteria can be captured by biotin labelled probes. Relative incorporation of ^{13}C can then be analysed by f) isotope ratio mass spectrometry

1.15 Hypotheses and Aims of Research

Chapter one outlined the background to the subject of this thesis: an investigation into the role of the gut microbiota in inflammatory gut diseases of childhood; NEC and IBD. The gut microbiota plays a critical but, as yet, poorly defined role in the aetiology of these diseases. This suggests that manipulation of the gut microbiota might have positive therapeutic effects in such diseases. As the science of intestinal ecology is evolving there are many hypotheses that might be tested to expand knowledge in this field. The aim of this thesis was to test the three hypotheses:

1. Probiotics prevent NEC in at risk infants of very low birth weight (VLBW).

2. The human gut microbiota can be labelled by stable isotope probing (SIP) to measure metabolic activity.

3. Quantitative measurement of the metabolic activity of the unculturable gut microbiota is a useful way of studying changes in the microbiota, compared with measures of bacterial diversity, and may enlighten our understanding of bacterially mediated inflammatory stimuli in inflammatory gut diseases of childhood.

To test the first hypotheses a systematic review of the published literature was undertaken, designed to evaluate the current data concerning the routine administration of oral probiotics to VLBW and <33wk infants with particular interest in their safety and efficacy.

To test the second two hypotheses a series of experiments was undertaken to develop a new methodology, stable isotope probing (SIP) for use in the field of human gut microecology. These experiments were designed to develop a working protocol for 16s rRNA SIP using human faecal samples.

2 Prospective Study of the Evidence for Use of Oral Probiotics for the Prevention of NEC

As discussed in 1.5, NEC results in significant morbidity and mortality of the preterm and VLBW infant. The introduction of feeds or other nutritional substrates to the lumen of the gut appears to be factor in initiating and propagating inflammation and such substrates can modify this response as evidenced by the effects of human milk feeds as apposed to milk formulae. Modulation of the gut microbiota is hypothesised as a contributor to this effect. Rather than manipulating the composition of feeds, a more direct method of modifying the colonising microbiota might be the administration of enteral probiotics to 'at risk' infants. A large number of studies have been undertaken in which probiotics have been given to newborn infants in an attempt to modulate the gastrointestinal environment and thereby change biochemical, physiological and clinical outcomes. However the current level of evidence of the efficacy and particularly the safety of such preparations are not well documented.

2.1.1 Background: Why a systematic review?

When institutions or individuals are formulating policy decisions on whether to implement new treatments for patient populations, much emphasis is now placed on the use of evidence based medicine (EBM) (376). This has informed many of the recent changes in the structure and nature of health services in the UK. In brief, EBM is the conscientious use of the current best available data in combination with individual clinical experience to make valid treatment decisions for patients. Reviews of rapidly advancing areas of medicine are of vital importance to clinical decision-makers as they can synthesize the available data and interpret the results of many studies, which often takes far more time than is expected or available. Moreover narrative reviews, commissioned from experts in the field, have been shown to exhibit the cultural and professional biases of individual reviewers. In a landmark article in 1987 (377) Mulrow reviewed 50 review articles in four leading medical journals and demonstrated that, according to published guidelines on narrative syntheses, only one article defined methods of selecting articles and only three articles used a quantitative method of synthesis. Mulrow stated that the review article fell below the scientific standards in appraising available evidence. Since Muldrow's article the focus of many reviews has been to ascertain and evaluate all the available evidence on a topic before drawing conclusions on that subject. These 'systematic reviews' require the same level of methodology as that of original research and should be considered as of

equal potential value. Systematic review is now considered to be an integral part of the practice of EBM (378).

The systematic review's power to influence policy has been shown to be greater than that of the narrative review. In 1993 the Cochrane Collaboration (http://www.cochrane.org/), an international not-for profit organisation, was formed to create an international library of systematic reviews and meta-analyses, in order to help individuals and institutions practice EBM. Cochrane methodology is highly structured to minimise bias in a review. Expert committees on subject areas have been assembled to reach consensus for search strategies for specific questions. National health policies on therapeutic interventions are now often determined by the examination of current evidence under such methodology. The National Institute of Clinical Excellence (NICE; www.nice.org.uk) in England and Wales bases its decisions as to which specific medications will be funded by the national health service on from the results of commissioned systematic reviews of the evidence. Within Scotland a specific commission has been assembled to create guidelines for clinical management based on systematic review the Scottish Intercollegiate Guideline Network (SIGN; www.SIGN.ac.uk). As a result of these and other initiatives the science of systematic review has grown with rapid expansion of the number of guidelines on appraisal and meta-analysis, as well as an exponential increase in the number of published systematic reviews in the last two decades. However the methodology of committee-based reviews such as SIGN or Cochrane has been replicated so that individuals can answer their own clinical questions using systematic review. The methodologies of systematic reviews are now the subject of consensus from experts in this field (379-381).

Table 2: The QUORUM reporting of meta-analysis (Adapted from Moher et al (382))

Heading	Subheading	Descriptor
Title		Identify the report as systematic review or meta-analysis
Abstract		Uses structured format
	Objectives	Explicit clinical question
	Review Methods	Selection criteria, methods for validity assessments, data abstraction, study characteristics and quantitative data synthesis in sufficient detail to permit replication
	Results	Characteristics of the RCTs included and excluded; qualitative and quantitative findings and subgroup analysis
	Conclusions	The main results Describe
Introduction		The explicit clinical problem, biological rationale for the intervention, and rationale for review
Methods	Searching	The information sources, in detail (e.g. databases, registers, personal files, expert informants, agencies, hand-searching) and any restrictions (years considered, publication, status, language of publication)
	Selection	Inclusion and exclusion criteria (defining population, intervention, principal outcomes and study design)
	Validity assessment	The criteria process or processes
	Review Methods	Selection criteria, methods for validity assessments, data abstraction, study characteristics and quantitative data synthesis in sufficient detail to permit replication
	Data abstraction	The process or processes used
	Study Characteristics	The type, study design, participants, characteristics, details of intervention, outcome, definitions and how clinical heterogeneity were assessed
	Quantitative data synthesis	Principle measures of effect, method of combining results, handling of missing data, how heterogeneity was assessed, a rationale for any a-priori sensitivity, subgroup analysis and publication bias
Results	Trial flow	Provide a meta-analysis profile summarising trial flow
	Study characteristics	Present descriptive data for each trial
	Quantitative data synthesis	Report agreement on selection and validity; present simple summary results; present data required to calculate effect size and confidence intervals in intention to treat analysis
Discussion		Summarise key findings; discuss clinical inferences based in internal and external validity; interpret the results in light of the available evidence; describe biases in review process and future research agenda

When combining the results of studies using variable methods and treatments but studying the same outcomes, inadequate attention has often been given to reporting individual study quality which should include: study design, combinability, control of bias, statistical analyses, sensitivity analyses and problems of applicability (383). In particular not only should authors report the methods of their own review, but the methods used in each individual study included in their review. In 1999 a consensus based guideline for the reporting of studies in meta-analysis was devised at the Quality of Reporting of Meta-Analysis Conference (QUORUM) (382). A QUORUM checklist and flowchart (figure 12) was devised which summarised the key points of meta-analysis methodology (table 2). The QUORUM methodology increases clarity for the reviewer permitting him or her to assess the quality and appropriateness of meta-analysis in any systematic review. The use of the QUORUM methodology has been incorporated into the requirements for systematic of several high quality peer review publications. The success of these guidelines has also led to the development of methodology guides for the reporting of non-randomised (TREND) (379) and observational studies (STROBE and MOOSE) (380;381).

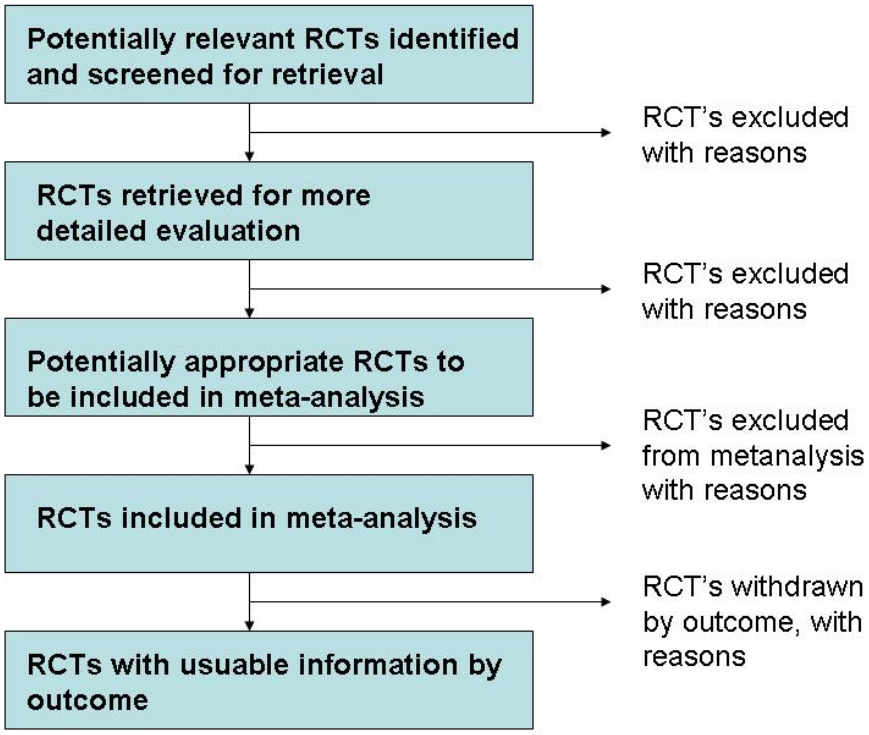

Figure 12: Progress through the stages of meta-analysis for RCTs QUORUM flow chart (adapted from Moher et al (382))

2.2 Aims of Study

In light of the large body of literature that exists concerning the use of probiotics in the newborn a systematic review requires to be undertaken to evaluate their quality. The objectives of this systematic review were: 1. to assess published documentary evidence concerning the safety and the effectiveness of the oral administration of probiotics to VLBW in the prevention of NEC and 2. To suggest how deficiencies in the existing data may be redressed in order to develop evidence to justify the rational use of probiotics for VLBW infants. This was formulated into the clinical question 'Should VLBW infants be routinely administered oral probiotics to prevent necrotising enterocolitis'.

2.3 Methods: Systematic review

2.3.1 Setting the clinical question

A clear focused question is required to set the clinical context for the systematic review and will influence the search terms and areas searched. A framework for clinical question setting is PICOS (384). The question should include the problem or population (P), the intervention (I) the comparator (C) and the dependant variable of interest or outcome (O), study limitations (S) can also be applied such as only using higher level evidence (RCTs). The clinical question 'Should VLBW infants (P) be routinely administered (C) oral probiotics (I) to prevent necrotising enterocolitis (O)' was selected.

2.3.2 Assembling a systematic review team

Although this study was primarily designed as an independent piece of work the systematic review requires a minimum number of individuals to independently validate steps of the review process in to order reduce bias in terms of selection and interpretation of studies. To maintain a broad range of clinical and research experience the invited team consisted of Paediatric Gastroenterologists and Neonatologists from two tertiary paediatric units.

- David Wilson, Reader in Paediatric Gastroenterology, University of Edinburgh
- Judith Simpson, Consultant Neonatologist, Queen Mother Maternity Hospital, Glasgow
- Ben Stenson, Consultant Neonatologist, Simpson Centre for Reproductive health Edinburgh

- Lawrence Weaver, Professor of Child Health, University of Glasgow

These colleagues agreed the framework for systematic review and assisted in independently validating i) the search strategy ii) the inclusion and exclusion of studies and iii) the means of synthesis (if any) of appropriate studies.

2.3.3 Search framework and search strategy

Once the decision to appraise the clinical question 'Should VLBW infants be routinely administered oral probiotics to prevent necrotising enterocolitis' was made an appraisal of the current available literature was undertaken in order to formulate the methods for systematic review. Due to perceived lack of scientific rigour involved in much of the published data in the area of probiotics there were specific concerns over the combinability of data from probiotic trials in VLBW infants. In view of this the decision was made to observe the QUORUM statement on methodology and reporting of systematic reviews as this method was most applicable.

A search was made for publications reporting randomised placebo controlled and quasi-randomised controlled trials of infants at high risk of NEC (born <33wk or with birth weight <1500g) that involved the oral administration of probiotics, defined as bacteria that will modify gut microflora to give a predominance of non-pathogenic organisms. Within these studies outcome measures were sought that included incidence of NEC diagnosed by Bell's Classification or a modified Bell's Classification (385;386) (table 3), severity of NEC defined by Bell's stage 2 and 3 of NEC, requirement for surgery, mortality attributed to NEC, overall mortality, and adverse reactions attributed to probiotic; defined as episodes of sepsis with positive blood culture containing probiotic strain or delay in tolerance of enteral feeds.

Table 3: The Bell's Classification of NEC (Adapted from Bell et al (385))

Stage I (Suspected)

a) Any one or more historical factors producing perinatal stress
b) Systemic manifestations- temperature instability, lethargy, apnoea, bradycardia
c) Gastrointestinal manifestations- poor feeding, increasing pre-gavage residuals, emesis (may be bilious or test positive for occult blood)mild abdominal distension, occult blood may be present in stool (no fissure)
d) Abdominal radiographs show distension with mild ileus

Stage II (Definite)

a) Any one or more historical factors
b) Above signs and symptoms plus persistent occult or gross gastrointestinal bleeding: marked abdominal distension.
c) Abdominal radiographs show significant distension with ileus; small bowel separation (oedema in bowel wall or peritoneal fluid), unchanging or persistent "rigid" bowel loops, pneumatosis intestinalis, portal vein gas.

Stage III (Advanced)

a) Any one or more historical factors
b) Above signs and symptoms plus deterioration of vital signs evidence of septic shock or marked gastrointestinal haemorrhage.
c) Abdominal radiographs may show pneumoperitoneum in addition to those listed in IIc

Electronic searches of MEDLINE (January 1966 – December 2006) and CINAHL (January 1982-December 2006) databases were performed using all combinations of the following subject heading and keyword searches: infant, preterm; infant very low birth weight; enterocolitis necrotising and probiotic. Adaptations for UK and US English were made to the word necrotising (necrotizing) for all searches and no language filters were applied. The abbreviation probiot$ was also used to obtain all word stems. All abstracts from the combination searches were reviewed. For publications which involved trials of probiotics administered to preterm or VLBW infants, the full paper was reviewed to determine whether NEC was a primary or secondary outcome measure. To identify any relevant further papers, citation searches were performed on potential studies using MEDLINE, CINAHL and Web of Science databases, and the references of all reviewed papers were also checked for further studies. Additional hand searches were performed from the proceedings of the Society for Pediatric Research (SPR) and European Society for Paediatric Research (ESPR) from the last ten years to identify any unpublished trials. Two colleagues (ARB and DW) constructed the search strategy and identified trials for consideration. Two colleagues (BS and JS) were masked to outcome of trials and independently assessed the methodological quality of potential studies using a previously validated three-point methodology rating scale (387) (appendix 1). A score of three or greater defined inclusion in our review.

2.4 Results

The aforementioned subject headings and keywords generated 45860 hits (citations). Limiting citations to those that had two or more of all combinations of subject headings and keywords in their title or keyword list reduced the potential citations to 745 hits. The abstracts of all 745 hits were reviewed to identify all potential clinical trials and review articles of importance. Eighteen studies involving administration of probiotics to preterm and VLBW infants were identified (table 4). Two of the studies were in foreign languages, with the abstract only available in English (388;389). Both studies were read by a native speaker with a science degree to ascertain whether NEC outcomes were described. This was not the case for either study. Only six randomised controlled trials described the incidence of NEC as a primary or secondary outcome (figure 13). Of these, one study (160) was identified as ineligible as it was assessed as <3 on the rating score (387). This left five eligible studies (390-394) which are discussed in section 2.4.1 and summarised in table 5.

Table 4: 18 identified studies that administer probiotics to infants

Name	Preparation	Dose	Numbers	Outcomes reported
Reumann et al 1986 (395)	*Lactobacillus acidophilus*	$9x10^6$/ml formula	15 infants 15 controls	*L. acidophilus* in rectal swab
Bennett et al 1992 (396)	*Bifidobacterium breve* *Bifidobacteium longum* *Lactobacillus acidophillus*	$3x10^9$ tds Breast, 1 formula	11 infants	*Bifidobacteria* and *Lactobacillus* in stool
Akiyama et al 1993 (388)	*Bifidobacterium longum biovar* *Bifidobacterium longum*	$5x10^8$ daily	5 infants 5 controls	*Bifidobacteria* in flora at 2wk
Stansbridge 1993 (397)	*Lactobacillus* GG	10^8 cfu bd	25 infants	Changes in urinary SCFA profiles
Millar et al 1993 (398)	*Lactobacillus* GG EBM /DEBM formula	10^8 cfu bd	20	Colonisation with *LactobacillusGG*, reduction in pathogens in stool
Kitajima et al 1997 (159)	*Bifidobacterium breve*	$0.5x10^9$	46infants 46 controls	Changes colonization, better feed tolerance
Gronlunnd et al 1997 (399)	*Lactobacillus casei Rhamonosus* (GG)	$2.5x10^8$ bd	30 infants	GM neg bacteria in stool after supplementation
Uhlemann et al 1999 (389)	*Bifidobacterium tophfer bifidus*	$1.25x10^8$ tds	50 infants 50 controls	*Bifidobacteria* dominance in stool, reduction in sepsis
Hoyos 1999 (160)	*Bifidobacterium infantis,* *Lactobacillus acidophilus*	$2.5x10^8$ od	1237 infants	NEC reduction
Dani et al 2002 (392)	*Lactobacillus GG*	$6x10^9$ od	295 infants 290 controls	NEC and sepsis rates
Marini et al 2003 (400)	*Sarcaromyces Boullardii,* *Bifidobacterium subtilis,* *Lactobacillus GG*	Not specified	30 infants 10 controls	Faecal colonisation with probiotic species
Costalos et al 2003 (391)	*Sarrcaromyces Boullardii*	10^9 bd	51 infants 36 controls	Faecal bacterial diversity

Table 4b: 18 identified studies that administer probiotics to infants continued

Name	Preparation	Dose	Numbers	Outcome
Mihatsz 2004 (60)	*Bifidobacterium lactis*	$6x10^9$ Mixed feed	65 infant 63 controls	Sepsis rates
Lin 2005 (393)	*Lactobaccillus acidophilus* *Bifidobacterium infantis*	$1x10^7$ od	180 infants 187 controls	NEC
Bin-Nun 2005 (390)	*Bifidobacterium infantis* *Strep thermophilus* *Bifidobacterial bifid us*	10^9 do	72 infants 73 controls	NEC
Manzoni et al 2006 (394)	*Lactobacillus GG*	$6x10^9$/day breast	80 infants	Candida in gastric asp, stool, rectal swab
Mohan 2006 (401)	*Bifidobacterium lactis*	$4.8x10^9$ day	37 infants 32 controls	Molecular stool stechnique to measure enterobacters.
Argarwal et al 2003 (402)	*Lactobacillus GG*	10^9od	24 infants 15 control	*Lactobacillus* in stool flora

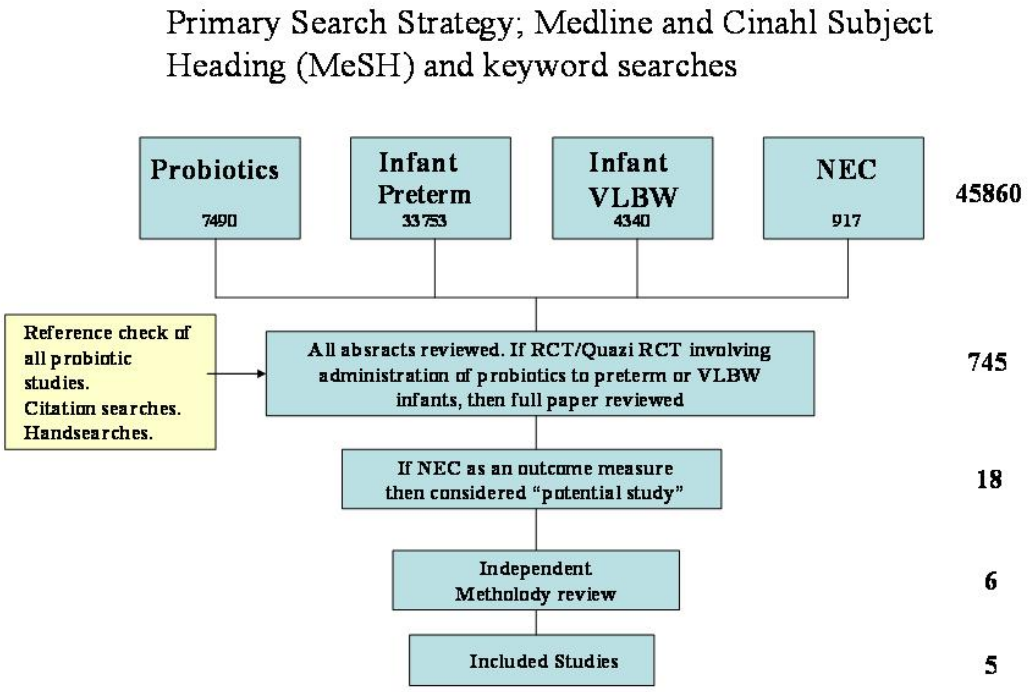

Figure 13: QUORUM flowchart for search strategy for included studies

Table 5: Characteristics of five studies reviewed in detail

Study	Methods	Participants	Intervention	Outcomes	Limitations
Dani et al (392)	Randomisation by sealed envelope. Blinding of care giver: yes. Blinding of assessors: yes. All or almost all subjects analysed: yes.	All infants of gestational age <33wk or birth weight <1500g admitted to 12 NICU's. Exclusion included, congenital malformation, death within 2wk of life and lack of parental consent.	Daily administration of *Lactobacillus GG* 6x 10^9 cfu until discharge (295) or given placebo (290).	Reported outcomes: incidence of UTI, incidence of NEC and incidence of bacterial sepsis.	No initial power calculation. Low background incidence of NEC which fell during study.
Costalos et al (391)	Randomisation by sealed envelope. Blinding of caregivers: yes. All or almost all subjects analysed: yes.	All infants between 28-32wk some >1500g admitted in two NICU's. Exclusion criteria included, breast feeding, antibiotic administration, congenital malformation.	12 hourly administration of 10^9 cfu/kg of *sarccharomyces boulardii* until discharge (51) or placebo (36).	Reported outcomes: weight gain, enteral tolerance, sepsis and incidence of NEC ≥2.	Not powered to detect changes in NEC rates. Non significant trend toward less NEC in treatment group. Any NEC rather than ≥2 reported.
Lin et al (393)	Randomisation by sealed envelope. Blinding of caregiver: yes. Blinding of assessors: yes. All or almost all subjects analysed: yes.	All VLBW infants admitted to NICU. Exclusion criteria, Death <7d and NEC <7d. All received maternal or banked breast milk.	After day 7 all enterally fed infants received *L acidophilus* 1 million units and *Bifidobacter infantis* (180) or placebo (187).	Reported outcomes: incidence of NEC, severe NEC, sepsis and death.	Any NEC primary outcome, although NEC≥2 also reported. NEC or death reported as secondary outcome although overall mortality reported.
Bin-Nun et al (390)	Randomisation by computer. Blinding of caregiver: yes Blinding of assessors: yes. Almost all subjects analysed: yes.	Infants <1500g admitted to single neonatal unit (143).	Daily administration of 10^9 cfu *Bifidobacteria bifidus, Streptococcus thermoplhillus* and *Bifidobacter infantis* (71) or placebo (72).	Reported outcomes: incidence of NEC, severity of NEC and mortality in relation to NEC.	Combined outcome of NEC or death or sepsis measured rather than overall mortality.
Manzoni et al (394)	Randomisation by computer generated table. Blinding of care givers: yes. All or almost all subjects analysed: yes.	Infants <1500g admitted to single neonatal unit.	Daily administration of 6x10^9 colony forming units/day of *Lactobacillus GG* (39) or placebo (41) until 6wks age or discharge.	Reported outcomes. Bacterial colonization rates, NEC 2 and 3, overall mortality.	Not powered to detect changes in NEC rates. Non-significant trend towards less NEC and mortality in the treatment group.

2.4.1 Description of selected studies

The five selected studies summarised in table 5 were carried out in a variety of locations (three European, one Asian and one from the Middle-East). All of the studies were set in what would be described in the UK as level three neonatal care units (those managing extremely preterm infants). Two of the studies were multi-centred whereas three were on single sites. The details of neonatal practice including antenatal steroid policy, administration of surfactant to VLBW infants, antibiotic policy and postnatal feeding strategies are not described. However the individual feeding practice and probiotic administrations are described for each individual study.

The study by Dani et al (392) was carried out in twelve Italian NICUs. The authors enrolled 585 infants with birth weight <1500g or gestation <33 wk. They were randomly assigned to receive milk supplemented with *Lactobacillus GG* or a placebo (maltodextrins), from first feed until time of discharge. Infants received mother's milk, donor expressed breast milk or preterm formula. *Lactobacillus GG* was stopped after NEC but not after UTI or sepsis. Incidence of NEC ≥2 was a primary outcome (defined by Bell's staging (385)). UTI, sepsis and adverse events also measured. Continuous data were expressed as mean +/-SD. Mean birth weight was <1300g in both groups with some of the population >1500g (but <33 weeks gestation).

The study by Costalos et al (391) was performed in two NICUs in Athens, Greece. The authors enrolled 87 infants between 28-32wk. Patients were randomised to receive either preterm formula with additional polyamines and *Saccaromyces boulardii,* or standard preterm formulae to which placebo (maltodextrins) were added. Patients discontinued supplementation if they developed NEC, sepsis or vomiting. Clinical data gathered included weight changes, enteral tolerance of feeds and sepsis. Incidence of NEC (defined by a modified Bell's criteria (386)) was a secondary outcome measure. Continuous data were expressed as median with interquartile ranges.

The study by Lin et al (393) involved 367 infants of <1500g birth weight admitted to a single NICU in China. Infants were randomised to receive either human milk (mother's or donor) supplemented with *Lactobacillus acidophilus* and *Bifidobacterium infantis* twice daily, or unsupplemented human milk, from seven days of life to discharge. Demographic data included risk factors for NEC. Primary measures were incidence of NEC ≥1 (defined by a modified Bell's criteria (386)), severe NEC (≥2), sepsis, death attributable to NEC

and adverse effects attributable to probiotics. Continuous data were expressed as mean +/- SD.

The study by Bin-Nun et al (390) from Israel included 143 preterm infants weighing <1500g who were randomised to receive milk supplemented with *Bifidobacterium infantis, Streptococcus thermophilus* and *Bifidobacterium bifidus* or unsupplemented milk. Infants received mother's milk or preterm formula. Clinical parameters relating to NEC were collected. Continuous data were expressed as mean +/-SD. Primary outcomes included incidence of NEC (defined by Bell's criteria (385)), severe NEC and death. No adverse events attributable to probiotics were noted.

The study by Manzoni et al (394) was carried out in a single Italian NICU. The authors enrolled 80 infants with BW<1500g. Patients were randomly assigned to receive human milk (mothers or donor) supplemented with *Lactobacillus GG* or unsupplemented human milk. Clinical data gathered included bacterial colonisation rates, invasive fungal infections, enteral milk tolerance. Stage 2 and stage 3 NEC (defined by Bell's criteria (385)) were secondary measures. Continuous data were expressed as a mean +/- SD.

2.4.2 Methodological quality

Randomisation of patient groups by sealed envelope was reported by Dani et al (26), Costalos et al (27) and Lin et al (29), and caregivers and assessors were blinded. Manzoni et al (32) described randomisation by computer generated table. Bin Nun et al (31) patients were randomised by computer assignment (personal communication, Hammerman C, Jan 2007). Demographic data in all studies on the two groups showed them to be well-matched.

2.4.3 Incidence and severity of NEC

Dani et al (392) found a lower incidence of NEC≥2 in the probiotic group (1.4% vs. 2.8%) but this did not reach statistical significance. Patients were not stratified by severity. Costalos et al (391) reported a non-significant trend towards less NEC of any severity in the treatment group (9.8% vs. 16% p= 0.5). Lin et al (393) found a lower incidence of all NEC in the probiotic group (1.1% vs. 5.3%, p=0.04). There were six cases of severe NEC (≥2) in the control group versus none in the probiotic group (p=0.03). Bin-Nun et al (390) found a significantly lower incidence of all cases of NEC in the probiotic group (4% vs. 16.6%, p=0.031) and severe NEC (≥2) (1% vs. 14% p=0.013). Manzoni et al (394)

reported a non-significant trend towards less severe NEC in the treatment group (2.6% vs. 4.9% p=0.51).

2.4.4 Requirement for surgical intervention

In the study of Dani et al (392) two patients from the control group underwent surgery as opposed to none in the probiotic group (no statistics included). Manzoni et al (394) reported one surgical case of NEC in the control group versus none in the treatment arm. Costalos et al (391), Lin et al (393) and Bin-Nun et al (390) did not record need for surgery.

2.4.5 Mortality attributable to NEC and overall mortality

Dani et al (392) recorded two deaths attributable to NEC, both in the control group; all deaths before two weeks of life were excluded from analysis. Costalos et al (391) gave no figures on mortality. Lin et al (393) reported a significantly lower mortality rate in the probiotic group (3.9% vs. 10.7%, p=0.009) but did not differentiate between death attributed to NEC or other causes. Infants that died before seven days of life were not enrolled in the study. Bin-Nun et al (390) described a non-significant trend to less NEC related mortality in the probiotic group (0/3 vs. 3/12). Overall mortality was significantly lower in the probiotic group (6/73 vs. 17/72, p=0.025). Manzoni et al (394) described a non-significant trend towards lower mortality in the treatment group (12.8% vs. 14.3%) but did not differentiate between those attributed to NEC or other causes (table 6). All of the systematic review team concluded that the heterogeneity of probiotic preparations and the timing and methods of interventions made further synthesis and meta-analysis of data inappropriate.

Table 6: Cumulative results of five included studies

Study	Study group	Control	Any NEC	NEC ≥ 2	NEC 3	NEC Mortality	Overall Mortality
Dani et al (26)	298	290	4 vs. 8	4 vs. 8	-	0 vs. 2	-
Costalos et al (27)	51	36	5 vs. 6	-	-	-	-
Lin et al (29)	180	187	2 vs. 10	2 vs. 10	0 vs. 6	-	7 vs. 20
Bin Nun et al (31)	72	73	3 vs. 12	1 vs. 10	0 vs. 3	0 vs. 3	3 vs. 8
Manzoni et al (32)	39	41	1 vs. 2	1 vs. 2	0 vs. 1	-	5 vs. 6
Cumulative	**640**	**627**	**15 vs. 45**	**8 vs. 30**	**0 vs. 10**	**0 vs. 5**	**15 vs. 34**

2.4.6 Adverse reactions attributed to probiotic

Dani et al (392) recorded no episodes of *Lactobacillus* sepsis. Time to full enteral feeds was also not significantly different between groups. Costalos et al (391) recorded no episodes of *Sarccaromyces boulardii* sepsis and time to full enteral milk feeds and weight gain were similar in the two groups. Lin et al (393) also recorded no episodes of *Lactobacillus* or *Bifidobacteria* sepsis. Time to full enteral milk tolerance was also noted to be similar in the two study groups. Bin-Nun et al (390) reported similar enteral tolerance between study groups and reported no adverse events. Manzoni et al (394) reported no episodes of *Lactobacillus* GG sepsis and did not comment on time to full enteral feeds.

2.5 Discussion

Five eligible randomized controlled studies of the use of probiotics to prevent NEC in preterm and VLBW infants were identified which, between them, enrolled 1267 infants. Heterogeneities of study design, in the opinion of the reviewers preclude meta-analysis, but the studies broadly suggest that probiotics are likely to be useful in preventing and reducing the severity of NEC. However there are limitations to the existing data and the degree to which interpretation can lead to firm conclusions that probiotics prevent NEC. Mortality rates are related to local attitudes to NEC and the management of VLBW infants, but it is of note that overall mortality also appeared to fall in all of the probiotic groups (table 6). In the identified studies the authors used a wide variety of dosages and types of probiotics. These dosages in terms of cfu/kg often bore little relation to those used in other clinical trials of probiotics in children and adults (74;310). This massive variability in dosage may reflect a lack of targeted specificity in the desired effects of probiotics in preventing NEC.

Despite strict definitions of NEC and staging, individual management and indications for surgical intervention, and thus mortality, may vary from neonatal unit to unit. The outcomes measured in individual studies also varied, with some authors reporting suspected NEC (Bell's grade 1) and others confirmed cases (Bell's grade ≥2). Mortality directly arising from NEC and overall mortality are the most important outcome measures, but these were not consistently reported by all authors. Studies demonstrating positive outcomes for the administration of probiotics to prevent NEC to date have reported high background rates of NEC. It is hypothesised that such rates can be attributed to local environmental factors, such as antenatal antibiotic policy, surgical delivery rates and breast feeding rates, which affect rates of infant bacterial colonisation (see section 1.5). The only

study performed in neonatal units with low background rates of NEC (392) generated results that failed to reach statistical significance, this may have been due to an inadequate number of infants. The existing studies also have variable entry criteria, feeding practices and insufficient statistical power. Breast milk feeding, which may affect NEC rates (107;108), was universal in two studies (393;394), not in two (390;392) and preterm formula was exclusively used in one study (391). The practice of human milk donation is not universal in neonatal intensive care units (NICUs) and donor rates within Europe are low. This effects the generalisability of these data to units within the UK No adverse events attributable to probiotics were recorded in any of the studies. However insufficient information was given on observation for perceived adverse outcomes or for other morbidities suffered by the VLBW infants at follow-up. In addition none of the studies had sufficient power to attribute safety of administration of oral probiotics to this immunocompromised population of VLBW and preterm infants. Insufficient numbers of infants were included in these trials to reach statistical significance for overall mortality.

The exact pathogenesis of NEC remains unclear. However the triad (figure 2) of intestinal ischaemia, substrate (often feeds) and microbial colonisation with pathogenic bacteria all play a part in the inflammatory cycle (92;118). Colonisation of the gut is significantly delayed in preterm neonates and differs from healthy term infants with a greater proportion of perceived enteropathogens (4-6;403) (see section 1.1.2). However host-bacterial interactions (cross-talk) through the products of bacterial metabolic activity, or by sensing of bacterial components, may be important in both stimulating and inhibiting the inflammatory response in NEC (23;134;404). Genetic variations in components of the gut immune system involved in bacterial sensing, such as toll-like receptors, result in reduction in NEC rates in experimental models (as discussed in 1.5.6.)(134). This suggests that predisposed immune responses to enteropathogens may also affect the development of NEC.

The mechanisms by which probiotics may ameliorate NEC are not fully understood. Probiotics compete for intraluminal substrates and binding sites on the mucosal surface of the gut, and produce an array of antimicrobial compounds, thus reducing enteropathogen numbers and ability to attach and invade the gut epithelium (13-15;405). Pioneer bacteria in the gut have been shown to up-regulate the expression of host genes which favour the continuance of an intraluminal environment that will promote their own proliferation and dominance. This again encourages commensal bacteria rather than pathogenic species (23). Bacteria are required for the development of a normal healthy mucosal and systemic immunity (20), and probiotics have been shown to up-regulate markers of immune system

function in human studies (58). Commensal bacteria can also down-regulate programmed inflammatory responses to bacterial sensing (oral tolerance) (25).

The reduction of NEC rates from probiotics may be accounted for by any of the above mechanisms rather than simply through reduction of numbers of, or invasion by, enteropathogens. The reduction in overall mortality rates identified, independent of NEC mortality, suggests that probiotics may have other beneficial effects on the preterm neonate, such as immune stimulation or reduction of bacterial sepsis rates. A greater understanding of probiotic functions including the development of methods to better analyse bacterial metabolic activity may help to refine probiotic administration through better definition of type, dose and timing of interventions for future studies. The development of such methods to measure the pathophysiological and clinical effects of the gastrointestinal microbiota is the subject of sections 1.9-1.12.

2.6 Conclusions

The conclusions arising out of this systematic review illustrate the difficulties involved in designing intervention studies to evaluate dietary products for infants and children. Apparently ‘similar’ therapeutic strategies can reveal major heterogeneity in terms of species, viability, dose and timing of interventions when pharmaceutical standards are applied. Quite apart from those alluded to in section 2.5, infant feeds affect not simply growth and nutritional status, but also the development of gastrointestinal, respiratory and allergic disease in childhood and metabolism and health in later life (406-409). None of the studies reviewed involved long-term follow-up of infants. The ESPGHAN committee on nutrition has been critical of previous research into the use of probiotics in infants and children (55). It found limited high quality data on the safety and efficacy of probiotics added to infant formulae, follow-on formulae and special foods, and no information on the long-term benefits or adverse effects of such supplementation. The ESPGHAN committee has published recommendations on the design of trials into dietetic products for infants and children (410), which are applicable here (table 7).

Table 7: Summary of recommendations on future research on the use of infant milk formulae or dietary products (adapted from Aggett et al (410))

- Nutritional adequacy should be determined by outcome studies at 4-6 months.
- Appropriate clinical studies of safety should be performed on all new components and combination of components in infant formulae and dietary products.
- New modifications to formulae or dietary products should be informed by the systematic review of relevant existing information.
- Formulation changes for reasons other than novel nutritional or clinical benefit should be at least subjected to trials of acceptability and nutritional equivalence.
- All infant trials should be characterised with regard to factors that may affect outcome. Adequate blinding and good clinical practice is important.
- Ethical approval and informed parental consent and required and should be declared on publication.
- The possibility of unexpected adverse clinical outcome be addressed.
- Study design should consider short-term and long term effects. It should include statistical power of the study and confidence limits of study should be reported.
- Pilot study should be used to anticipate outcomes and enable the views of caregivers to be taken into account.
- Manufacturers, scientific, academic and professional groups should collaborate to agree an essential portfolio of data and out-comes which will be recorded in all nutritional studies.
- A central register of current trials should be established and information shared by manufacturers and clinical researchers
- Confidentiality of original participants should be preserved
- The results of abandoned studies should be made available publicly.

The field of systematic review continues to grow at an exponential rate. In 2007 2500 new systematic reviews were listed on Medline, an increase of over 100 fold from the rate in 1991 (411;412). Over 100 groups conduct reviews and commission systematic reviews world wide (413). Reproduction of Muldrow's analysis, even soon after its first publication in 1987, demonstrated a paradigm shift towards greater scientific method and reproducibility in terms of reviewing medical subject matter (414). The ESPGHAN committee on nutrition illustrates this evolution in review with successive improvements in the methodology and reporting and therefore the clinical importance of its position statements over time (410;415;416).

Systematic reviews undoubtedly give 'added value' to the available evidence. However there are specific limitations to systematic review in the area of nutrition, particularly variables that are independent of the intervention and likely to affect outcome, such as food intake, conversion of food intake into nutrient values, methods and timing of intakes. Often the value of systematic reviews is contingent on the quality of reporting of individual studies, as inadequate description of 'methods' can exaggerate the effectiveness of an intervention by ≥ 30% (417). The variability in results of systematic reviews has demonstrated that even subtle differences in clinical question, search strategy and synthesis can result in marked differences in study results and recommendations (413;418-420). It is of note that a systematic review conducted in parallel to the one reported here generated similar results in terms of studies and reported outcomes. However the authors agreed that synthesis of these data was appropriate and generated significant outcomes for NEC prevention (421). This illustrates that systematic reviews are still open to cultural bias at the level of data interpretation.

The need for improvements in systematic reviews has informed recommendations on the reporting of clinical trails. The findings of the QUORUM group and its recommendations have improved the reporting of individual randomised controlled trials by modelling the Consolidated Standards of Reporting of Trials (CONSORT) statement (422). Increasingly adherence to guidelines for reporting RCTs and systematic reviews are a requirement for publication. The outlined difficulties in performing randomised interventions in nutrition may make it infeasible to definitively answer clinical questions with RCTs alone. It is estimated that this reduces enquiry to around only 10% of original published research (423). The specific nature of individual areas of medicine, such as nutrition, may require individual guidelines for methods and reporting of studies. The CONSORT statement has a version specifically for holistic interventions. A future framework for nutritional systematic reviews could include the development of a specific capacity and training

programme in nutritional systematic review using existing reporting guidelines, and the development of nutrition specific guidelines and a central repository for data, which reviews the content and quality of current reviews and develops a list of research priorities (413).

It is clear that to develop the field of probiotic therapies further for the prevention and treatment of both NEC and IBD greater rigour must be taken to describe the administration of such therapies in terms of definition and standardisation of live bacteria with gene-typing of strains. Treating such therapies as pharmaceutical agents (rather than food additives) requires greater understanding of their mode of action. A greater understanding of probiotic functions, including the development of tools to analyse bacterial metabolic activity, may refine our understanding of the value of probiotic administration, through better definition of type, dose and timing of interventions for future studies. Thus whilst new nutritional therapies herald a promising era in treatment and prevention, it is the development of new tools to study changes in bacterial metabolic activity which are required to advance this. This will form the subject of the remainder of this thesis.

3 Development of SIP in human Faecal Microbiota: Experiments with Total RNA

As discussed in the previous chapter the potential for nutritional therapies in inflammatory gut diseases of childhood is illustrated by the positive evidence for the prophylactic use of probiotics for the prevention of NEC. However, as discussed in the introduction, the role that the gut microbiota plays in the pathogenesis of IBD is not clear. Nutritional therapies have been shown to be effective in the treatment of IBD since exclusive enteral feeding, and to a limited extent probiotics, are effective in treating active inflammation. However the mechanisms of action of such therapies are poorly understood, in particular their effects on the metabolic activity of the gut microbiota.

To better understand disease pathogenesis in IBD, experiments were designed to extend the current methodology of SIP to study human faecal microbiota. This programme of research was aimed to utilise SIP methodology in anticipation of the measurement of metabolic activity of faecal bacterial samples. This involved experiments to determine

- Reliable extraction of bacterial total nucleic acids from human faecal samples;
- Reliable enrichment of total RNA with ^{13}C from labelled tracers;
- Validation of total RNA ^{13}C enrichment against established markers of bacterial metabolic activity;
- The development of 16s rRNA capture techniques to isolate target RNA sequences, both at a group and a species specific level.

The sequences of experiments were based on a number of considerations. The overall plan was that candidate tracers would be selected from compounds known to be involved in *de novo* synthesis of RNA and DNA as nucleic acid replication is a generic bacterial function. Ideally incorporation of ^{13}C would then be dependant on the metabolic activity of the bacteria rather than from stimulus by tracer (as seen with ^{13}C-glucose (371)). The purine and pyrimidine bases would therefore be the target for incorporation with ^{13}C labelled substrates known to be involved in the synthesis pathways; urea, glycine, aspartate and uracil (figures 14, 15); (glutamine was discounted because it only donates nitrogen to RNA/DNA synthesis). Urea is an attractive substrate because its carbon enters into one-

carbon pool (after rapid hydrolysis by bacterial urease) and it is potentially less susceptible to substrate bias. Moreover its existing potential for use *in vivo* has been demonstrated with the use of lactose ^{13}C-ureides (348). Provisional data would be gathered using ^{13}C urea. However, the suitability of urea as a universal tracer would be tested against the other candidate tracers in later experiments. The reproducibility of SIP as a marker of bacterial activity would also be tested against a previous validated marker of bacterial metabolic activity such as SCFA production.

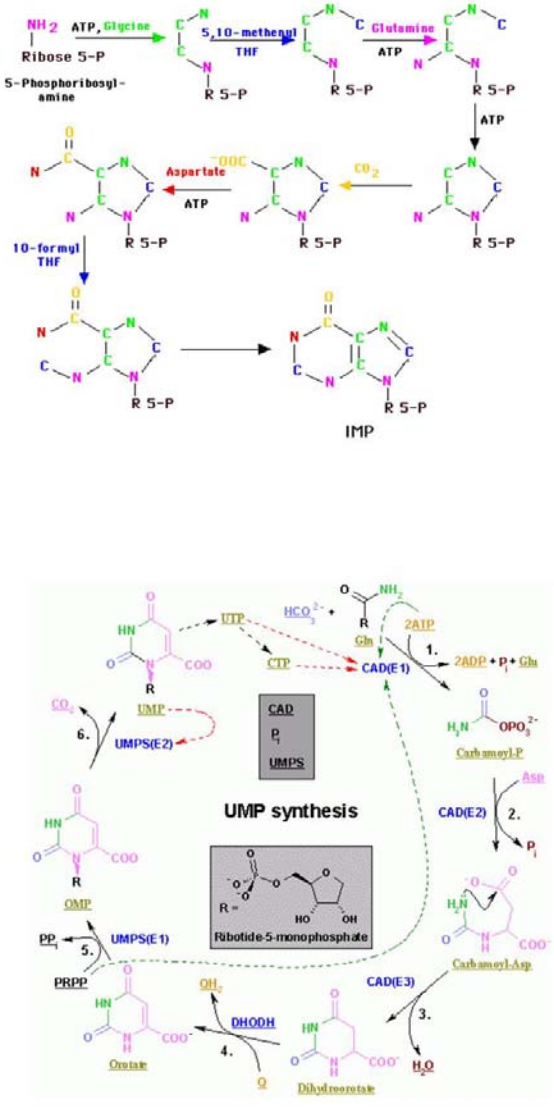

Figure 14: Purine and Pyrimidine synthesis

In terms of IRMS analysis, where materials could be reduced to dry weight with a predicted carbon content of ≥500μg the EA-IRMS would be deemed sufficiently sensitive for analysis. Where samples were likely to have a smaller carbon content or where the ^{13}C signature would be particularly low, then samples were kept in solution and analysed via the LC-IRMS. ^{13}C incorporation was expressed as $\Delta^{13}C$ ($\Delta^{13}C = \delta^{13}C$ sample – $\delta^{13}C$ baseline) above baseline.

Unless stated otherwise, comparisons between sets of data were made by Student's t-test (2 tailed) in Microsoft Excel (Windows XP) and ANOVA using SPSS (SPSS Inc, Chicago, USA). A p value of ≤0.05 was taken as statistically significant.

The first series of experiments focused on the extraction of total RNA from faeces, enriching total RNA with ^{13}C and the comparison of ^{13}C enrichment of total RNA with SCFA profiles.

3.1 Experiment 1: Nucleic Acid and RNA Extraction

3.1.1 Aims

The aim of this experiment was to determine that adequate yields of RNA could be reliably obtained from 6g stool samples to perform 16s rRNA isolation experiments. A nominal yield of 10mg of RNA was calculated to be adequate to perform multiple probe experiments.

3.1.2 Materials and methods

Human stool samples were used for all experiments; these were obtained from healthy volunteers from within the Division of Developmental Medicine, University of Glasgow. All samples were voided between 0800-1100 on the day of use and were used within 2hr of voiding. Nucleic acids were extracted from the faecal slurry as previously reported (368). 6g of fresh stool were homogenized in a stomacher 400 (Seward Medical, London, UK) for 2min as 20% faecal slurries in a phosphate buffered solution (PBS) (0.1mM phosphate buffer, 0.0027mM potassium chloride, 0.317mMsodium chloride pH 7.4 (Sigma-Aldrich, London, UK) before use for nucleic acid extraction (for initial studies an aerobic buffer was permitted). Faecal slurries were then centrifuged at 15,000g for 30min and bacterial cells were obtained by pipetting out 1ml of the creamy layer of the pellet (containing high concentrations of bacterial cells). Bacterial cells were lysed by the addition of Prepmann Ultra (Applied Biosystems, Foster City, USA) and then boiled for 10min. After mixing

with equal volumes of phenol:chloroform:isoamyl alcohol (24:25:1) the resulting emulsion was centrifuged at 20 000g for 10min the aqueous phase was then removed. This was repeated a further two times before being repeated with chloroform alone. Nucleic acids were then precipitated by placing at -20^{0}C with 0.1 volume of 3mM sodium acetate and two volumes of ethanol for 30min, followed by centrifugation at 20 000g for 15min. The pellet of nucleic acids were dried at room temp for 20min and then re-suspended in 2ml eppendorf tubes in 266µl of tris-phosphate EDTA buffer. To isolate purified RNA, DNA was removed by the addition of 40µls per eppendorf of RNAase free DNAase (1U/µl) (Promega, Southampton, UK) after addition of 30µls of the accompanying reaction buffer (Promega, Southampton, UK) and incubation at 37^{0}C for one hour. Enzymes were removed by sodium acetate and alcohol precipitation as previously described.

3.1.3 Results

Following the standard protocol, extractions were on average 10-20mg of bacterial RNA per 6g of faecal material. Table 8 shows the yield of total nucleic acids from the first eight samples processed with the protocol.

Table 8: Yield of bacterial RNA from first 8 extractions from 6g of faecal material

Experiment	Weight (mg)
1	13.1
2	17.2
3	14.8
4	14.8
5	10.4
6	12.6
7	13.7
8	19.5
Mean	**14.5**
SEM	**2.8**

3.1.4 Conclusions

This protocol provided a reliable method of extracting adequate total RNA for 16s rRNA isolation experiments, working on the assumption that 20% of total RNA is 16s rRNA. A nominal figure of 1mg 16s rRNA would be sufficient for standard elemental analysis IRMS (EA-IRMS). Therefore the next logical step was to develop a model for enrichment of total RNA with ^{13}C tracers before proceeding to 16s rRNA experiments. A set of experiments was designed to test candidate ^{13}C tracers, and then to examine the validity of changes in ^{13}C incorporations in total RNA as a marker of metabolic activity.

3.2 RNA Labelling Experiment: Multiple ^{13}C Tracer Incorporation

3.2.1 Aims

To test the suitability of various ^{13}C labelled tracers in the labelling of total nulciec acids and total RNA in human faecal samples.

3.2.2 Materials and methods

Two separate freshly voided faecal samples (from the same individual on two occasions) were divided into 26 x3g aliquots as 10% w/v faecal slurries in two (13 in medium 1, 13 in medium 2) separate culture media. To these culture media supportive electrolytes, to a Sorenson's phosphate buffer solution pH 6.6, were added. These had been boiled for 20min and then purged with nitrogen for 10min to achieve anaerobic conditions. Culture media differed in amounts of supportive carbohydrate (see table 9).

Table 9: Culture media used in ^{13}C tracer incorporation experiments

Culture Medium	Contents
1	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, *1000mg NH4Cl* 300mg Pectin, 1660mg cornstarch
2	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, *1000mg NH4Cl* 2000mg Raftilose
3	250ml Sorensons buffer pH 6.6 2000mg Raftilose
4	250ml Sorensons buffer pH 6.6

All specimens were again equilibrated in a waterbath at 37^0C for 30min prior to the start. At time zero two bottles, one of each media, were snap frozen as before and labelled T_0. The remaining 24 samples were inoculated with one of the following ^{13}C tracers (6 samples, 3 for each media): 37.5mg ^{13}C urea (99 atom%), 5mg [U-$^{13}C_4$ ^{15}N] aspartate (99 atom%), 35mg [4-5$^{13}C_2$] uracil (99 atom%), 24mg [U-$^{13}C_3$] glycine (99 atom%). These tracers were all normalised for total weight of ^{13}C, except for aspartate which was given at one seventh of weight due to its cost. The tracers were selected as they are precursors in the de novo synthesis of purines and pyrimidines, but enter the pathways at different points. They all therefore have potential as generic tracers of metabolic activity. Samples were sequentially removed at 2, 6 and 24hr and snap frozen and stored as before (one sample in each medium with each tracer at every time point). Samples were then defrosted

and total nucleic acids and total RNA were obtained as described in 3.1.2. Total RNA was predicted to be small so was analysed by LC-IRMS for total carbon content and ^{13}C incorporation.

3.2.3 Results

In both media (medium 1 and medium 2) significant enrichments above natural abundance (T_0 samples) were seen in RNA over time (figures 15, 16). $\Delta^{13}C$ enrichments were consistently higher with the ^{13}C-urea tracer and highest enrichments were seen over shorter time-points (2 and 6hr) but these enrichments were not statistically significant.

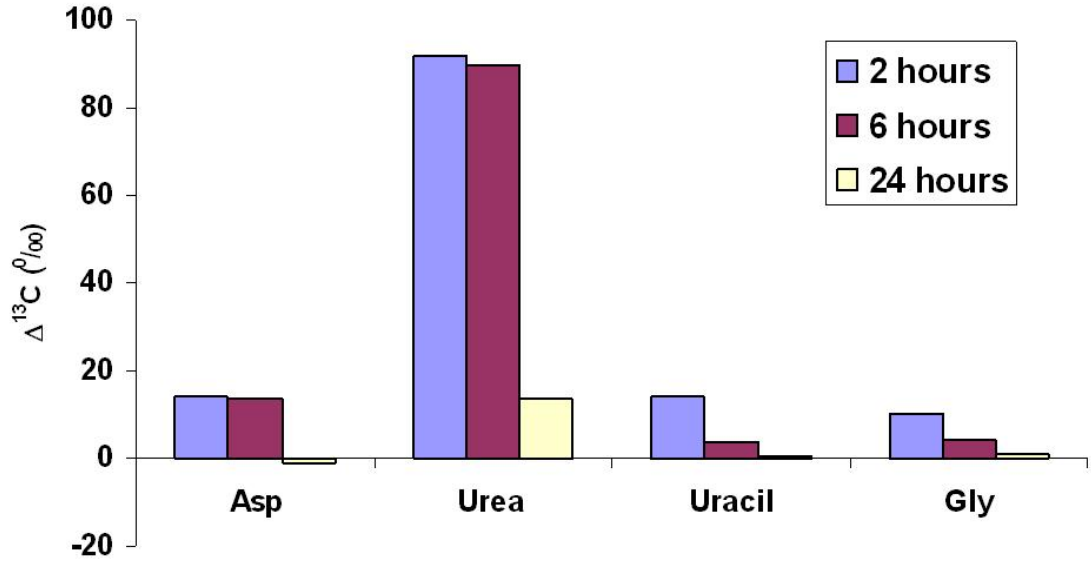

Figure 15: ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in total RNA over three time points using four tracers in culture Medium 1

Asp= aspartate, Gly= glycine

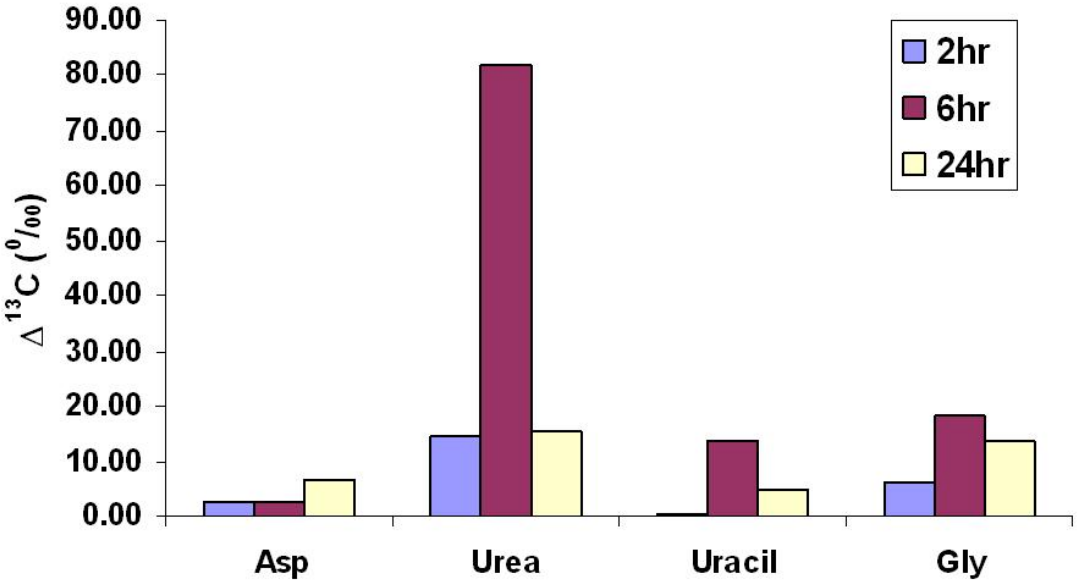

Figure 16: ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in total RNA and Eubacterial 16s rRNA over varying time points using multiple tracers in culture Medium 2

Asp= aspartate, Gly= glycine

When examining the mean incorporations of ^{13}C for all four tracers there was significantly greater ^{13}C enrichments from ^{13}C urea in comparison to all other tracers (figure 17). When correcting for initial concentration of tracers (scaling up $^{13}C_4$, ^{15}N aspartate), the mean incorporations across both culture media showed most consistent ^{13}C incorporation for $^{13}C_4$, $^{15}N_2$ aspartate and ^{13}C urea in RNA. Again only ^{13}C urea had significantly greater incorporations than $^{13}C_2$ uracil and $^{13}C_3$ glycine (figure 18).

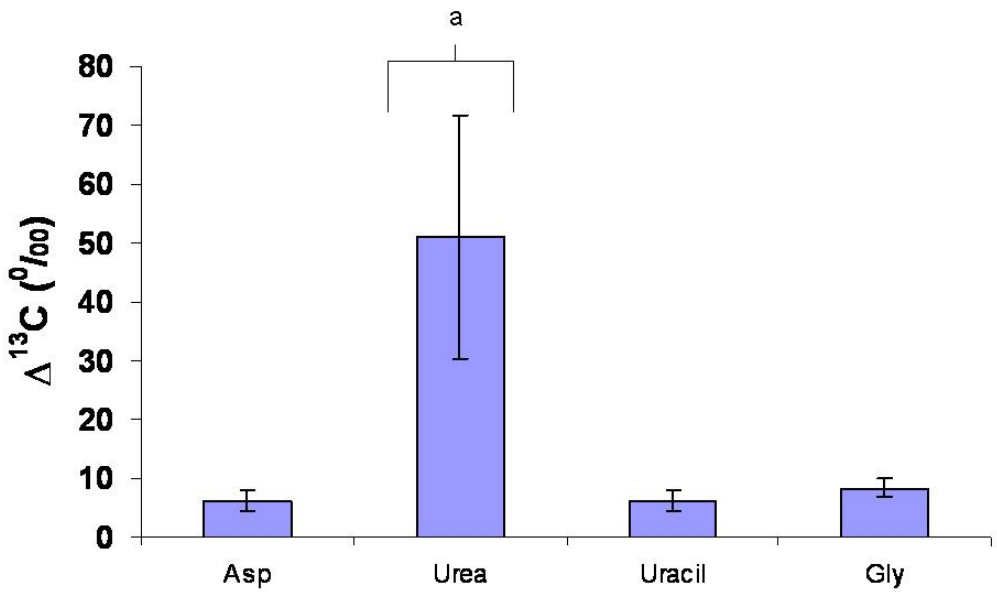

Figure 17: Mean ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) (SEM) of four tracers in both media

Asp= aspartate, Gly= glycine, a= significantly greater incorporation than aspartate, uracil and glycine $p<0.05$

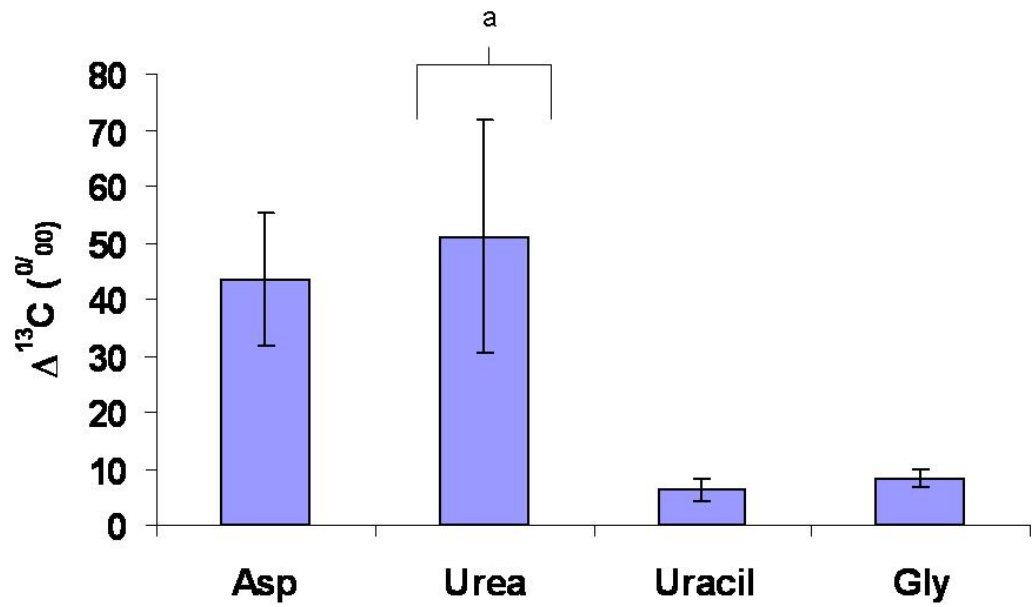

Figure 18: Mean ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) (SEM) across all tracers in both media correcting $^{13}C_4$ $^{15}N_2$ aspartate for concentration

Asp= aspartate, Gly= glycine, a= greater incorporation than uracil and glycine $p<0.05$

3.2.4 Conclusion

^{13}C urea showed consistently greatest enrichments in comparison to other SI-labelled tracers in this culture model for faecal bacterial metabolic activity. The ability of urea to be incorporated most readily into RNA fitted with our understanding of where ^{13}C, from ^{13}C urea hydrolysis, enters the one-carbon pool. Although [$^{13}C_{4,}$ $^{15}N_2$] aspartate also showed promise, its metabolism may be less generic and the costs of obtaining tracer preclude large scale experiments. It was therefore concluded that using ^{13}C urea tracers was the next logical step, until such time as these tracers could be retested using 16s rRNA. The first step was to determine whether such perturbations in ^{13}C incorporation were a reliable proxy for bacterial metabolic activity by comparing ^{13}C urea enrichment with a validated marker.

3.3 Experiment 2: ^{13}C Enrichment of Bacterial Total RNA and comparison with SCFA

3.3.1 Aims

The following experiments were designed to develop a method for the reliable incorporation of ^{13}C tracer into the nucleic acids of faecal bacteria, and to demonstrate that tracer incorporation can be predictably changed by manipulation of selected substrates for metabolic activity. The validation of ^{13}C incorporation into nucleic acids was to be done in comparison with alternative markers of metabolic activity. SCFAs were chosen as previous data exist on SCFA changes upon fermentation of specific substrates in these types of culture model experiments, knowledge of the relevance of SCFA changes to disease pathogenesis, and the potential use of butyric acid as therapeutic agent (115-117;240;241;424).

3.3.2 Subjects, materials and methods

Early morning stool samples were obtained from four healthy adults. Subjects reported themselves as ‘well’ with no specific dietary regimens and had not received oral antibiotics within eight weeks of participating in the study. Stool specimens were used within 2hr of voiding. A minimum of 36g stool was required for each experiment.

Aliquots of stool were homogenised into multiple 20% slurries for 1min using a high speed domestic blender. As each protocol variation was carried out in triplicate, homogenisation was carried out in 18g or 9g batches (3g or 6g depending on size of initial stool sample).

Sorenson's phosphate buffer (SPB) pH 6.6 was used as the base for the slurries. The phosphate buffer had been prepared the previous 24hr, deoxygenated by boiling for 5min followed by purging with nitrogen for 20min and then sealed and stored at room temperature overnight. Three additional variations were made to the SPB with the addition of electrolytes and a carbohydrate source; either Raftilose®, (oligofructose, Beneo-Orafti, Tienen, Belgium), pectin or L-rhamnose (table 10). The fourth solution acted as a control with Sorenson's phosphate solution only.

Table 10: Culture media 1 to 4 for RNA incubation experiment

Medium	Contents
1	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg NH_4Cl 1000mg Oligofructose
2	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg NH_4Cl 1000mg Pectin
3	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg *NH_4Cl* 1000mg L-Rhamnose
4	250ml Sorensons buffer pH 6.6

Specimens of 30ml or 15ml slurries were decanted into 50ml crimped topped glass vials and each flask was deoxygenated by flushing with nitrogen for 30sec. All specimens were placed in a 37^0C waterbath agitating at 40 cycles per min and allowed to equilibrate for 20min. At time zero all vials were spiked with weight equivalents of 99% ^{13}C urea (either 50mg in 500µls or 25mg in 250µl) and shaken for 1min. One control vial (T_0) was immediately snap frozen by placing in ethanol which had been chilled to <-20^0C with dry ice. The other twelve samples were replaced in the water bath and left to incubate for 4hr. Samples were then removed from the waterbath, 2.5ml of slurry was removed from each sample and mixed with 2.5ml of 1mol/L sodium hydroxide to fix SCFA profile, these samples were then stored at -20^0C for future SCFA analysis. The slurries were then all snap frozen with chilled ethanol and stored at -20^0C for future nucleic acid extraction. Nucleic acids were extracted from the faecal slurry by the method previously described. The resultant RNA pellets were weighed and resuspended in ultrapure water at a concentration of 2.5mg/50µls. Purity of RNA was demonstrated by spot check with ultraviolet spectrophotometric analysis and agarose gel elctrophoresis (figure 19). The concentration of total RNA was determined by measuring the ratio of the absorbance of total RNA to protein. Typically 2µl of RNA extract were dissolved to a final volume of 50µl with distilled water and its absorbance was measured in UV at 260 (nucleic acids) and

280 (proteins) nm corrected for the background absorbance at 320 nm. A ratio of 260:280nm greater than 1.8 represented RNA fraction of high purity.

The amount and integrity of RNA were estimated visually by electrophoresis on agarose gel (figure 20). Typically 2µl of RNA template were mixed with 3µl of loading buffer (Bromophenol 6 X (B0126, Sigma)) and loaded onto the wells of an agarose gel (0.8% containing ethidium bromide 5µl for 50ml of agarose). The electrophoresis was run in 1 x TBE (Tris Borate EDTA) for 30 minutes at 100V.

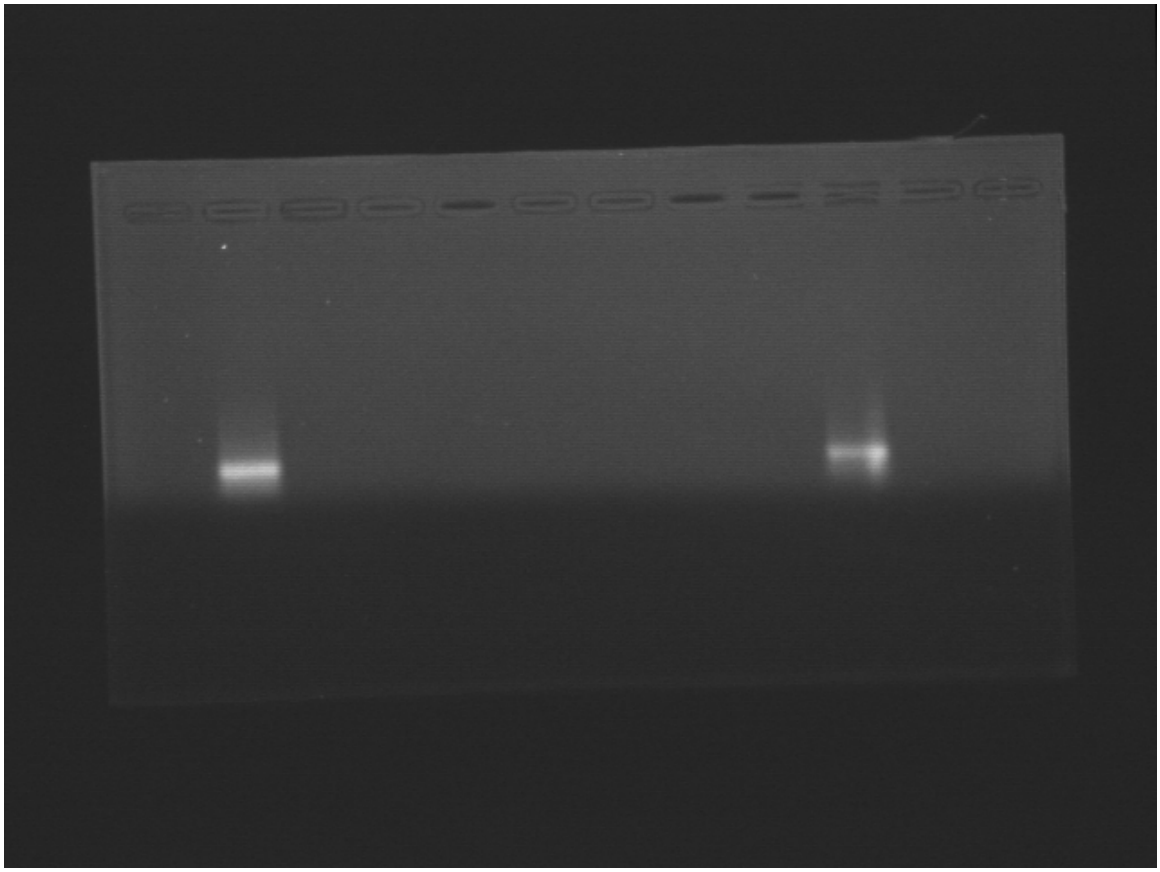

Figure 19: Agarose gel elctropheresis confirming purity of RNA in bacterial specimen

3.3.2.1 Analysis of ^{13}C incorporation in total nucleic acids and RNA

Samples were diluted to 1 in 100 concentration with ultrapure water were and analysed in 25µl aliquots by LC-IRMS. Total C in sample and relative ^{13}C:^{12}C were analysed. ^{13}C incorporation was expressed as $\delta^{13}C$ parts per mille above ^{13}C natural abundance ($\Delta^{13}C$).

3.3.2.2 SCFA extraction and analysis

SCFAs were extracted using methods based on the previously described departmental protocol (425). 100µl of internal standard solution (86.1mmol/L 2-ethylbutyric acid) and 100µl of orthophosphoric acid were added to 800µls of ultrapure water which had 50µl of

the faecal slurry/sodium hydroxide solution added to it. After homogenisation 3ml of diethyl ether was added to each sample which was vortexed for 1min, and the pooled ether phase was collected as a supernatant. This step was repeated twice.

SCFA analysis of the pooled extract was performed using a TRACE 2000 gas chromatographer (ThermoQuest Ltd, Manchester, UK) using nitrogen (30ml/min) as a carrier. Calibration was carried out using an external standard which was run five times prior to analytical run and the response factor for each SCFA calculated from the average. A coefficient of covariance was calculated as a quality control check for the performance of the instrument. An external standard was also run after every twelfth sample. For measurement 1µl of pooled extract was automatically injected at 230^0C onto the column. The column was a FFAP column (30m × 0.32mm; 0.22um film thinkness; Promochem, UK). The column temperature was held at 80^0C for 1min and increased by 15^0C per min until it reached a final temperature of 210^0C. The total concentrations of acetic, propionic, and butyric acid were calculated automatically by the software by peak area ratio analysis against the internal standard and corrected for the response factor of each SCFA determined by the external standard. The relative proportions of SCFAs were measured in each assay sample.

3.3.2.3 Statistical comparison of ^{13}C RNA incorporation vs. SCFA profiles

Comparisons were made between total SCFAs and culture media, relative SCFA profiles and culture media and SCFA profiles and ^{13}C incorporations were analysed using ANOVA model in SPSS. Correlations between all measured parameters (total SCFAs, total C2, C3, C4, relative C2:C3:C4 proportions and ^{13}C incorporations versus media support) were analysed using the Pearson Correlation Coefficient.

3.3.3 Results

^{13}C enrichments were recorded in all four samples from all four individuals, indicating bacterial sequestration of labelled tracer (figures 20-23). Intra- and inter-individual variations were apparent. However mean ^{13}C incorporations for the group across all four individuals showed reasonable reproducibility as expressed by standard error of mean (SEM). Significant differences were seen in mean ^{13}C incorporation between groups. Incorporation was significantly higher in medium 2 (pectin) and medium 3 (L-rhamnose) (figure 24).

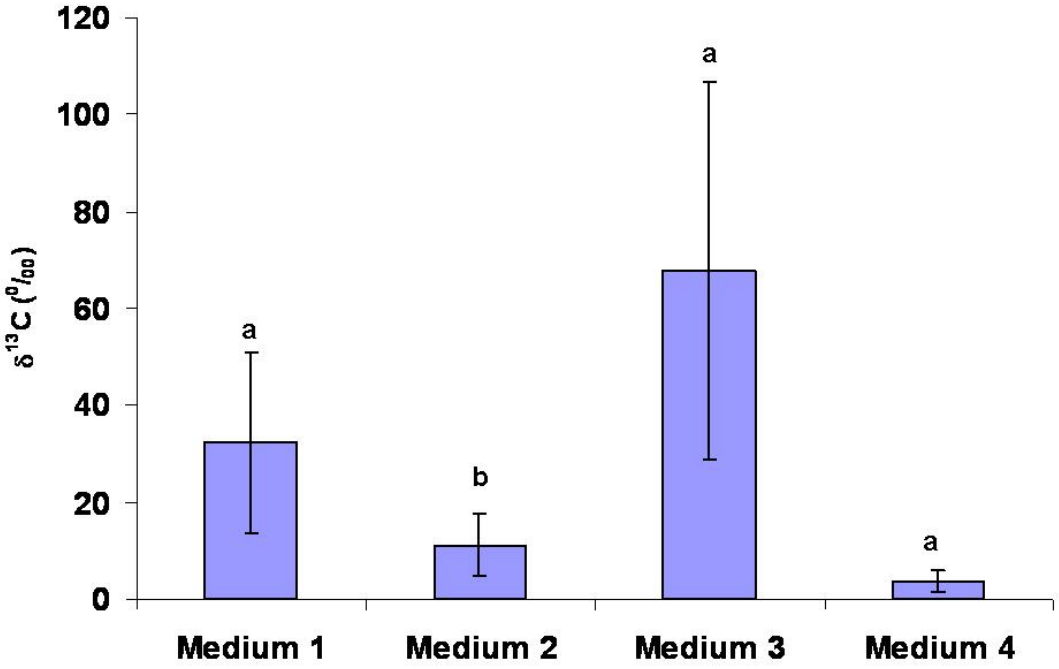

Figure 20: Mean (SEM) ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial RNA in four culture media in subject 1

a=ns, b=significantly greater incorporation than medium 4 p<0.05)

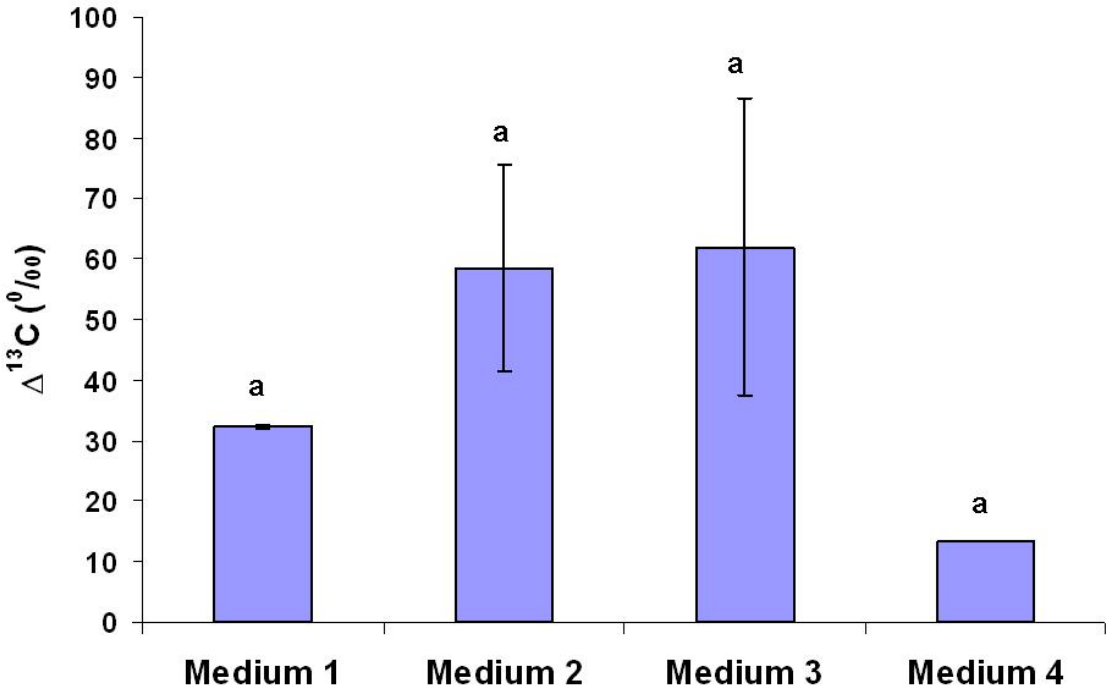

Figure 21: Mean (SEM) ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial RNA in four culture media in subject 2

a=ns

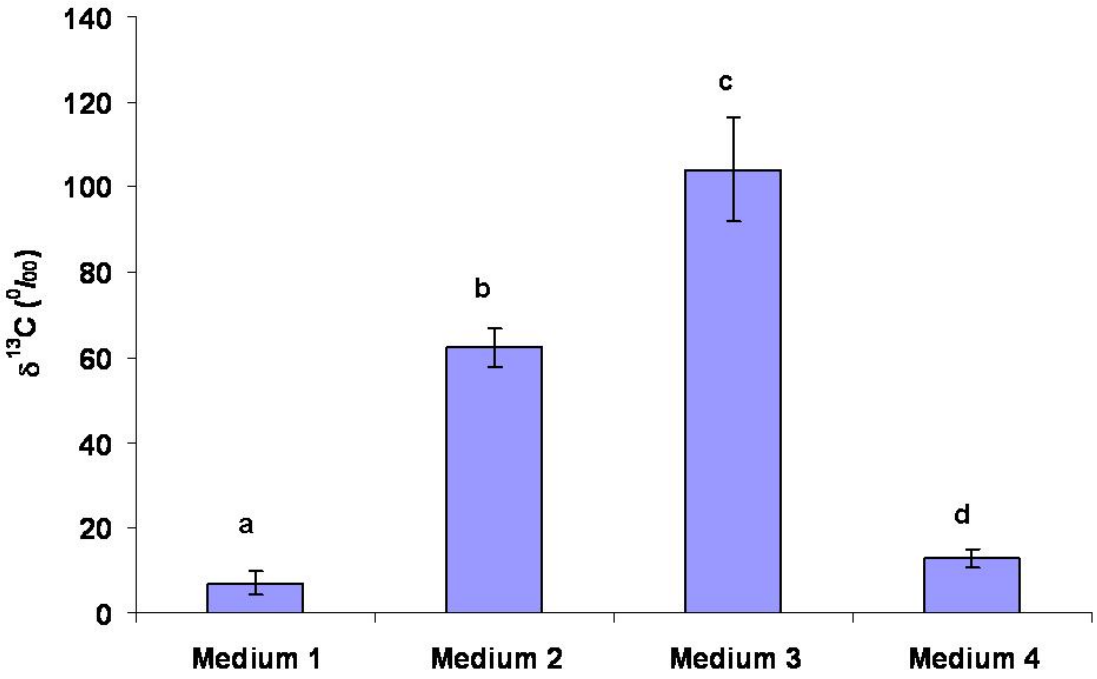

Figure 22: Mean (SEM) ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial RNA using four culture media in subject 3

a= less incorporation than media 2, 3, 4 ($p<0.05$), b=significantly greater incorporation than medium 1 and 4 and less than medium 3 ($p<0.05$), c= significantly greater incorporation than media 1, 2 and 4, d= significantly greater incorporation than medium 1 and less than media 2 and 3 ($p<0.05$)

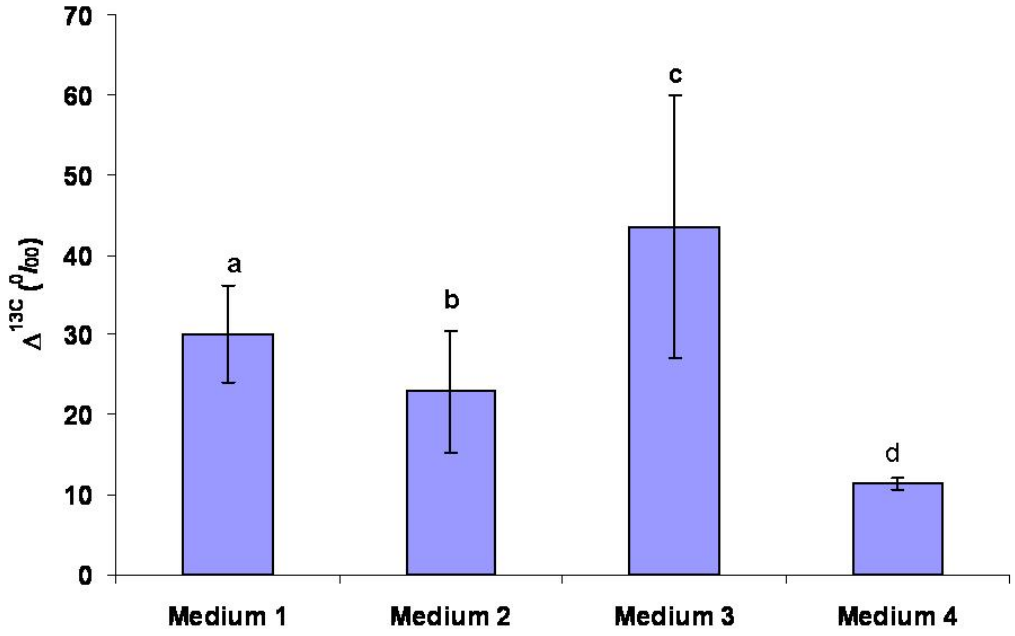

Figure 23: Mean (SEM) ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial RNA in four culture media in subject 4

a= significantly greater than medium 3 ($p<0.05$), b=significantly less than medium 3 and greater than medium 4 ($p<0.05$)

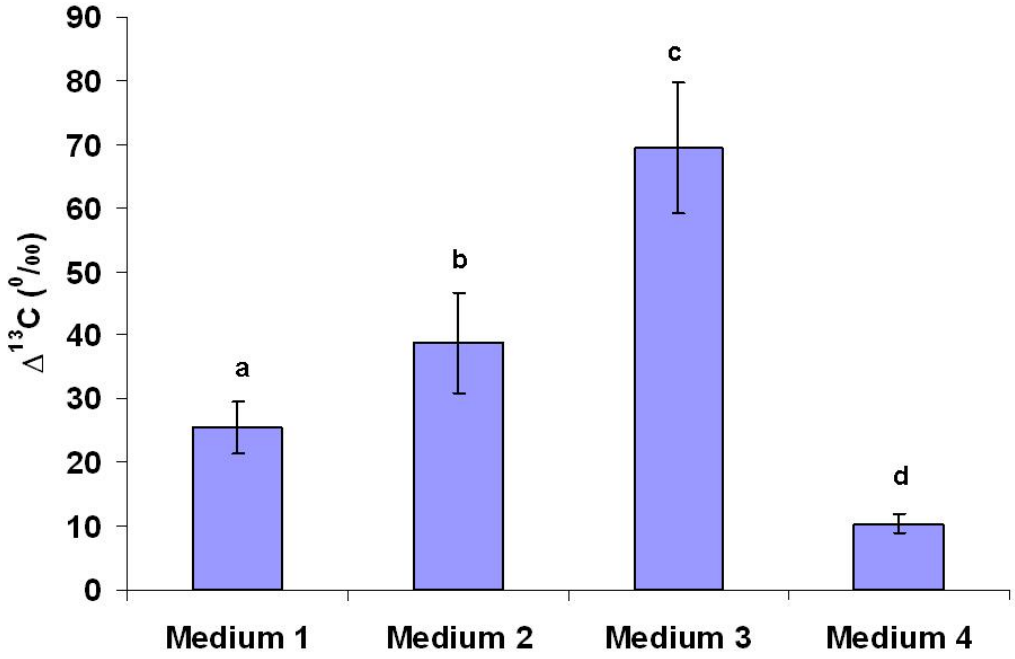

Figure 24: Mean (SEM) ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) into bacteria RNA in all four subjects

a= significantly less incorporation than medium 3 and greater than medium 4 ($p<0.01$), b= significantly less than medium 3 ($p<0.05$) and greater than medium 4 ($p<0.01$), c= significantly greater incorporation than medium 2 ($p<0.05$) and media 1 and 4 ($p<0.01$), d= significantly less incorporation than media 1, 2 and 3 ($p<0.01$)

SCFA analysis showed intra-individual reliability between triplicate samples. Total SCFAs were increased in all subjects in media 1-3 in comparison to medium 4 (figure 25). Addition of all three carbohydrates (oligofructose, pectin and L-rhamnose) was associated with significantly increased production of acetate, propionate, and butyrate, ($p<0.05$ vs. no carbohydrate) except that pectin was not associated with increased propionate nor was L-rhamnose associated with increased butyrate production. SCFA profiles (% of total SCFA) showed significant changes in the relative proportion of C2-C4 depending on carbohydrate support. L-rhamnose was associated with significantly more propionate production and significantly less acetate and butyrate (figure 26). When comparing SCFA profiles with ^{13}C incorporation there was a significant correlation between propionate production and RNA enrichment and an inverse relationship between RNA incorporation and butyrate production ($R^2=0.44$ $p<0.017$) (figure 27).

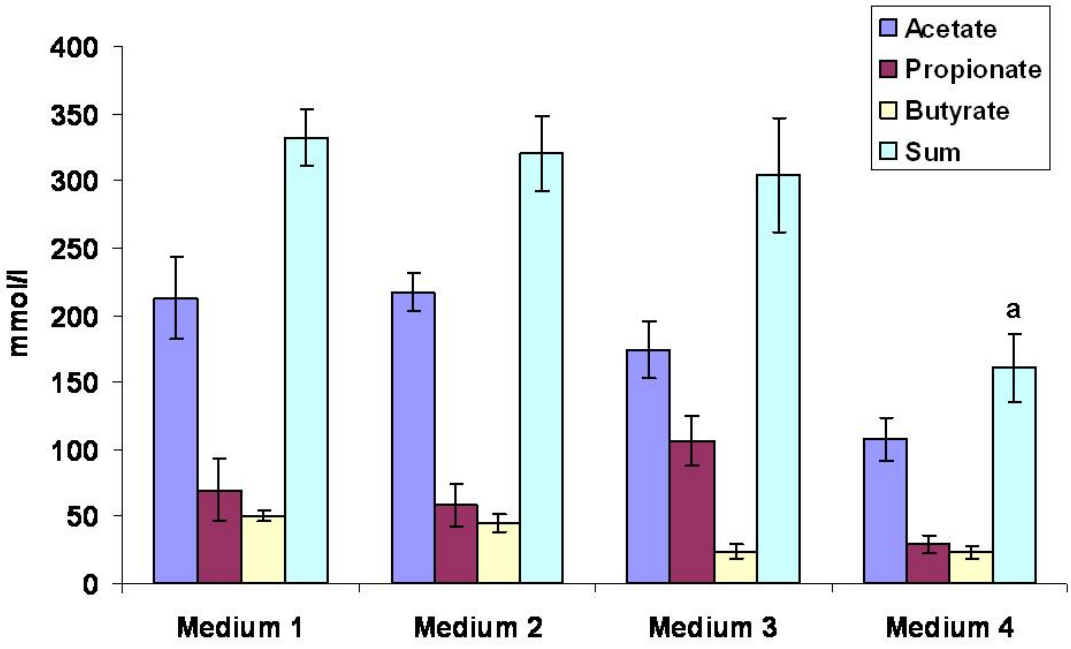

Figure 25: Mean total SCFAs (µmol/ml) in all four subjects in four different media
a= significantly less total SCFA in comparison to media 1, 2 and 3 ($p<0.05$)

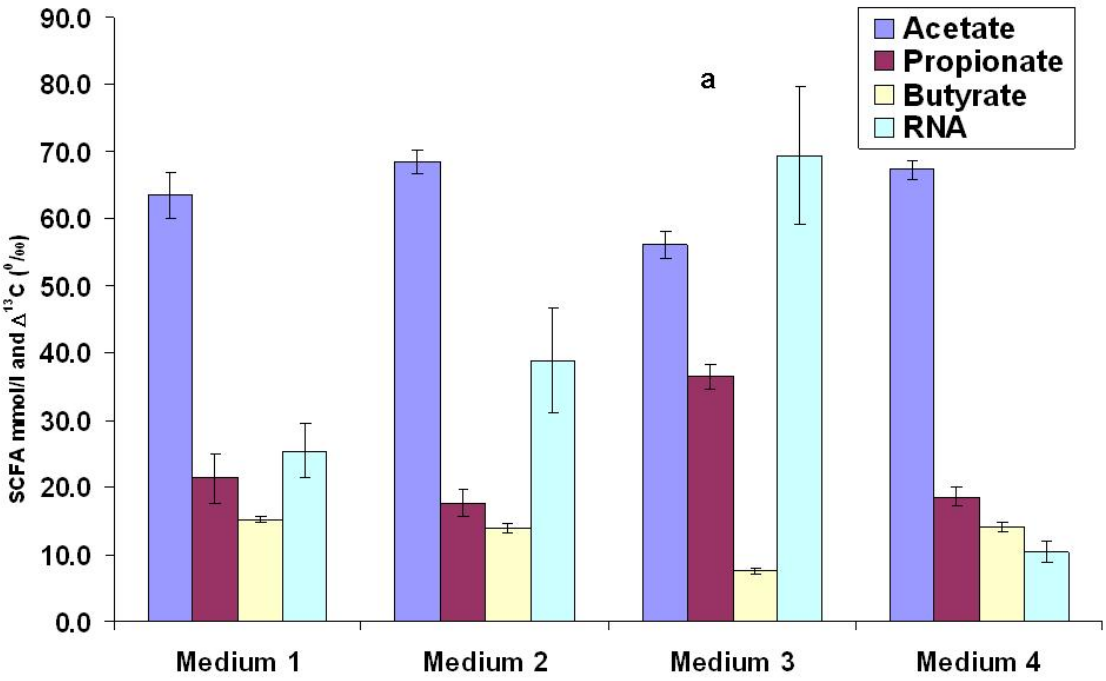

Figure 26: Mean SCFA proportions (%) and ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial RNA in all four subjects in four media

a= significantly greater production of propionate and less acetate and butyrate than media 1, 2 and 4 ($p<0.05$)

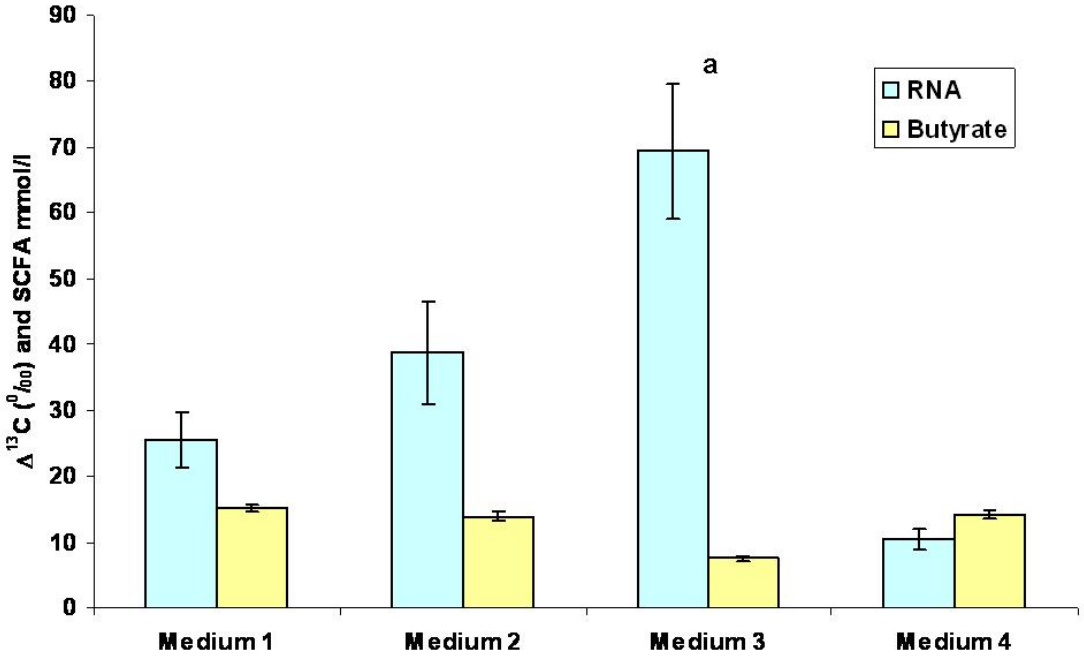

Figure 27: $\Delta^{13}C$ ($^{0}/_{00}$) and butyrate concentrations across 4 media in all subjects

a= negative association between RNA incorporation and butyrate production $R^2=0.44$ $p<0.017$

3.4 Discussion

This set of experiments demonstrated adequate isotopic enrichments of bacterial RNA to achieve sufficient ^{13}C incorporation for detection by LC-IRMS. These enrichments showed reliable and predictable differentiation under stimuli for metabolic activity. There was marked inter-individual variation in enrichments. However the microbiome, and therefore metabolic signatures, of the gut microbiota show great variation from individual to individual. More importantly intra-individual variation in terms of ^{13}C enrichment according to media was less marked. Carbohydrate substrate support is known to relate to SCFA production by bacteria within the gut (426;427). Fermentation of SCFAs *in vivo* is regulated by total fibre content of a complex diet and the relative resistance to digestion of NDCs, colonic transit time and the existing microbial pool (428). Faecal concentrations of SCFAs are however kept relatively stable by absorption and utilisation of SCFAs making faecal SCFA profiling relatively uninformative in the *in vivo* profile (429).

Human *in vivo* studies have shown a wide variety in individuals' ability to excrete SCFA in stool samples (428). Pectin supplementation of the diet has been demonstrated to increase total SCFAs in human faecal samples, although ratios between them are most often unchanged. *In vitro* studies have shown clearer correlations with substrate support and SCFA profiles. Pectin has been correlated with increased propionate production in a rat colonic fermentation model (430). L-rhamnose has been correlated with marked increases in propionate production in *in vitro* models of human faecal samples (431). The current *in vitro* model experiment conforms to previous work that suggests bacterial fermentation has been increased by the addition of carbohydrates as indicated by increased total SCFAs in comparison to controls (430). In this experiment the mean proportion of propionate was increased by the addition of L-rhamnose, in keeping with previous observations (431). Changes in mean RNA ^{13}C enrichment correlated with increased total SCFA production and changes in SCFA profiles between different media. This suggests that ^{13}C incorporation is also a marker of bacterial activity. ^{13}C enrichment of RNA represents the utilisation of substrate into cell structure in a replicating organism. Because RNA turnover is more rapid than that of DNA it may be a better marker of rapid cell division or metabolism. This idea is strengthened by the observation that RNA enrichment is greater than total nucleic acid enrichment in some of the samples (data not shown).

However significant changes in SCFAs and ^{13}C incorporation according to culture media were not observed for each individual. Data relating phylogeny of bacteria present in faecal

microbiota and fermentative capacity are limited. It has been shown however that SCFA ratios can be affected *in vitro* by the presence of significant numbers of methane producing species (431). These observations, in terms of the variable fermentative capacity of microflora, may be a reflection of markedly different bacterial consortia between individuals. Such differences may limit the validity of combining inter-individual results to create aggregated results. The ability to link phylogenic diversity, and activity, to different metabolic profiles such as faecal SCFA concentration, would greatly improve our ability to study the metabolic capacity of such faecal samples.

The development of the ability to probe individual species 16s rRNA for relative ^{13}C incorporation should enable validation of SIP as a measure of metabolic activity. For example, increased ^{13}C incorporation, relative to the total bacterial species, in identified propionate producing species (such as *Bacteriodes* and *Propionobacter)* could be linked to SCFA profiles. ^{13}C enrichments appear to be unaffected by scaling down of the weight of the samples (from 6g to 3g) and, assuming 20% of total RNA is 16s rRNA, provides adequate material for the development of a probe capture technique to analyse species incorporation. The scaled down protocol may be suitable for the study of stool specimens from children or those from patients with IBD in relapse, where the concentration and total numbers of bacteria are likely to be significantly lower.

4 Development of 16s rRNA SIP for Assay of Human Gut Microbiota: Preliminary Experiments

The experiments described in chapter 3 demonstrated that bacterial RNA could be reliably obtained from faecal samples and ^{13}C enrichment of bacterial total RNA is a valid proxy of bacterial metabolic activity. However to understand the pathophysiology of faecal bacteria in IBD, metabolic changes at the species level, as well as the whole consortia, must be studied. Capture and recovery of 16s rRNA, as previously discussed, is the first step to be examined before the study of metabolic changes at a species level. In this series of experiments the development a protocol for the isolation of 16s rRNA from human faecal samples is described.

4.1 16s rRNA Isolation Experiment 1: Initial Protocol

4.1.1 Aims

16s rRNA probes designed for the molecular characterisation of human faecal microbiota have been previously described (432). In initial experiments the aim was to obtain adequate and reliable amounts of 16s rRNA by adapting previously described technique of magnetic bead capture of 16s rRNA (368).

4.1.2 Materials and methods

A starting experimental protocol was devised based on previous observations of DNA probe capture work within the Department (368) and using data from the manufacturers. Two eppendorfs with 50µls of faecal bacterial RNA (obtained as described in section 3.1) at a concentration of 10mg/50µl and 50µl of hybridisation buffer (5ml, SSC buffer, 20µl of N-lauroyl sarcosine, 20mg of NaCl, 4mg of SDS, 4ml of formamide made up to 10ml with ultrapure water) were mixed in a microeppendorf and then hybridised at 70^0C in an incubating oven for 10min followed by 30min at room temperature (18^0C). 5µl (1.78nmol) of a specifically designed 16s rRNA capture probe (100µmolar solution) (Sigma-Geonosis, Suffolk, UK) with oligonucleotide sequences corresponding to a Eubacterial 16s rRNA (bact 338 5'-GCTGCCTCCCGTAGGAGT-3') and left to incubate under gentle mixing overnight at 25^0C. The RNA solution was then added to 890µl (8.9mg) of M-280 streptavidin coated paramagnetic beads (Dynal Biotech, Wirral, UK) which had been washed three times (with magnetic capture of beads on each occasion) and re-suspended in the same volume of hybridisation buffer (here the formamide was substituted for ultrapure

water). The solution was incubated for 2hr under gentle mixing for 2hr at 25^0C. The streptavidin coated beads were then recaptured with a magnet and washed three times in ultrapure water and re-suspended in 100µl of ultrapure water. The streptavidin coated beads with bound 16s rRNA were then harvested with the PTC-200 Peltier thermocycler (MJ research, GMI, Minnesota, USA) in a 300µl microeppendorf tube by heating to 90^0C for 3min. The resultant supernatant was removed and pipetted into a preweighted 200µl tin capsule (Elemental Microanalysis, Southampton, UK) and left to dry at 50^0C until only the precipitate is left.

4.1.3 Results

There was no appreciable increase in weight after complete drying of both eppendorfs.

4.1.4 Conclusions

This experiment demonstrated inadequate yield of material. A rate limiting step for the 16s rRNA isolation could occur at several steps of the protocol. Theoretically the concentration of total RNA was adequate, but hybridisation conditions, in terms of time, temperature concentrations of solvents and formamide appeared to affect RNA hybridisation for binding to capture probes.

Capture probe volume (1.78nmols) had been calculated, on the assumption of 90% binding efficiency (on the basis of product information) and 16s rRNA length of 1542 base pairs in length, to be saturated for the total amount of RNA and binding capacity of streptavidin coated particles. However binding efficiency of these and the binding capacity of streptavidin coated beads may have limited capture of 16s rRNA. A series of experiments was required to identify which factors could be optimised.

4.2 16s rRNA Isolation Experiment 2: Hybridisation Conditions

4.2.1 Aims

The aim of this set of experiments was to identify if variations in the hybridisation conditions which would lead to an increase in measurable nucleic acid yield.

4.2.2 Materials and methods

Six microeppendorfs containing 50µl of faecal bacterial RNA (10mg/50µls) were prepared as described in section 4.1. The following variations in the protocol were performed; microeppendorfs 1 and 2 had 50µl of buffer solution added and the samples were hybridised at 70^0C for 10min and at room temperature for 30min (per-protocol). Micro eppendorfs 3 and 4 had no additional buffer added and were then incubated at 70^0C for 10min followed by 30min at room temperature. Microeppendorfs 5 and 6 had 50µl of buffer solution added to them but were not incubated at 70^0C for 10min.

Following hybridisation the following variations in probe capture were performed: micoreppendorfs 1, 3 and 5 had 1µl (0.36nmol) of bact-338 probe added whilst 2, 4 and 6 had 5µl (1.78nmol) of bact-338 added to each. All microeppendorfs had added 890µls (8.9mg) of M-280 paramagnetic streptavidin coated beads which had been prewashed as previously described. They were all left for overnight mixing and harvested per protocol. The supernatants (or bands of 16s rRNA) were pipetted into preweighted tin boats and dried at 50^0C. All boats were reweighed at the end of drying (figure 28).

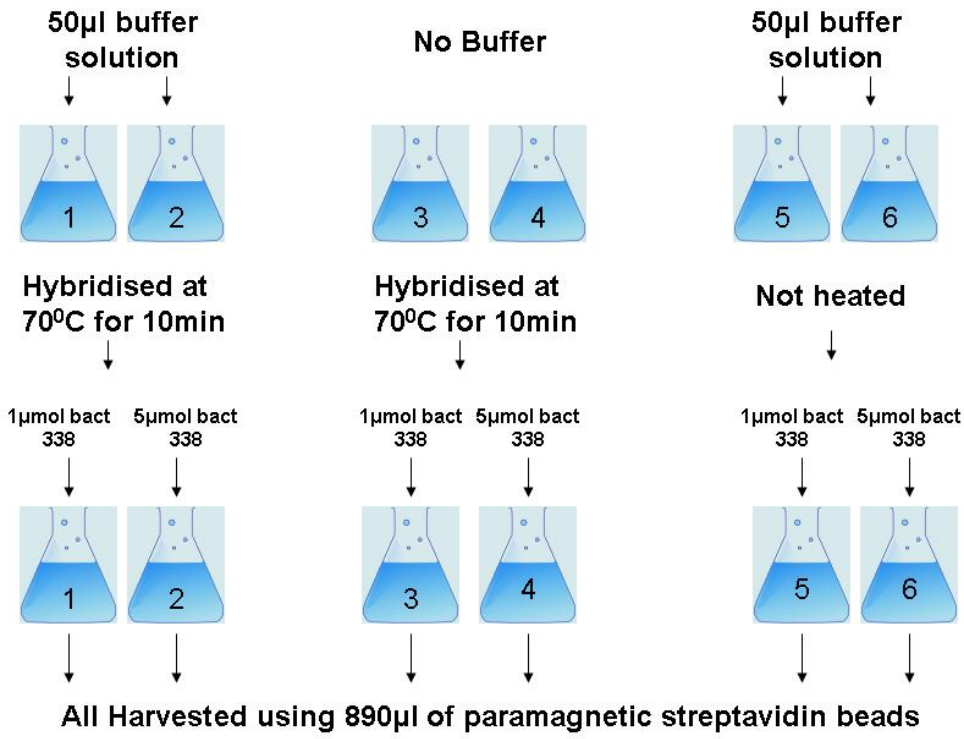

Figure 28: Schematic conditions of methods 4.2.2

4.2.3 Results

The initial experiment resulted in tin boats yielded between 0µg and 300µg of residue post drying (table 11). This yield was unsatisfactory with two blank cycles. It was also on a scale too small for accurate analysis by EA-IRMS.

Table 11: Dry-weight of precipitates under varying hybridisation conditions

Eppendorf	Experimental Conditions	Precipitate µg
1	50µl RNA, standard buffer, hybridised at 70^0C, 1µl capture probe	300
2	50µl RNA, standard buffer, hybridised at 70^0C, 5µl capture probe	100
3	50µl RNA, no buffer, hybridised at 70^0C, 1µl capture probe	0
4	50µl RNA, no buffer, hybridised at 70^0C, 5µl capture probe	100
5	50µl RNA, standard buffer, not heated, 1µl capture probe	300
6	50µl RNA, standard buffer, not heated, 5µl capture probe	100

4.2.4 Conclusions

Variations in the hybridisation conditions resulted in an appreciable precipitate in tin boats. However this was inadequate for analysis by EA-IRMS and no condition produced an order of magnitude increase in amount. A further variation on capture probe concentrations (1µ, 5µl and 50µl, (0.36nmol, 1.78nmol, 17.8nmol)) resulted in no improvements in yield (data not shown). Therefore exploration of other steps in the protocol was required to see if yield could be increased. Binding capacity of streptavidin coated beads and concentrations of buffer solution were other potential rate limiting steps in the protocol and these were examined next.

4.3 16s rRNA isolation Experiment 3: Binding Capacity

4.3.1 Aims

The aim of this experiment was to examine the role of magnetic capture bead binding capacity and formamide concentrations in relation to dry weight of RNA obtained at the end of the protocol.

4.3.2 Materials and methods

Four microeppendorfs of 100µl of RNA solution (10mg/50µl) were prepared as before. To microeppendorfs 1 and 2, 50µl of standard buffer solution were added and hybridised at 70^0C for 10min followed by 30min at room temperature. To microeppendorfs 3 and 4, 50µls of a modified hybridisation solution with lower concentration of solutes and formamide (3ml, SSC buffer, 10µl of N-Lauroyl sarcosine, 10 mg of NaCl, 2mg of SDS, 3mls of formamide made up to 10ml with ultrapure water) was added and hybridised as above. Following hybridisation all microeppendorfs had 5µl (1.78nmol) of bact-338 capture probe added and were incubated overnight at 37^0C as before. The following morning to eppendorfs 1 and 4 were added to 9.0mg of prewashed streptavidin coated beads in 900µls of buffer. To microeppendorfs 2 and 3 were added 9.0mg of MagnaCell-streptavidin cellulose/iron oxide particles (Bio-Nobile, Turku, Finland), which have a greater surface area to weight ratio and therefore potentially a greater binding capacity, in 1800µl of buffer solution. Mixtures were hybridised at 25^0C for 2hr and then harvested as before in the thermocycler at 90^0C for 3min with the washed supernatant being placed in preweighted tin boats and then dried at 50^0C (figure 29).

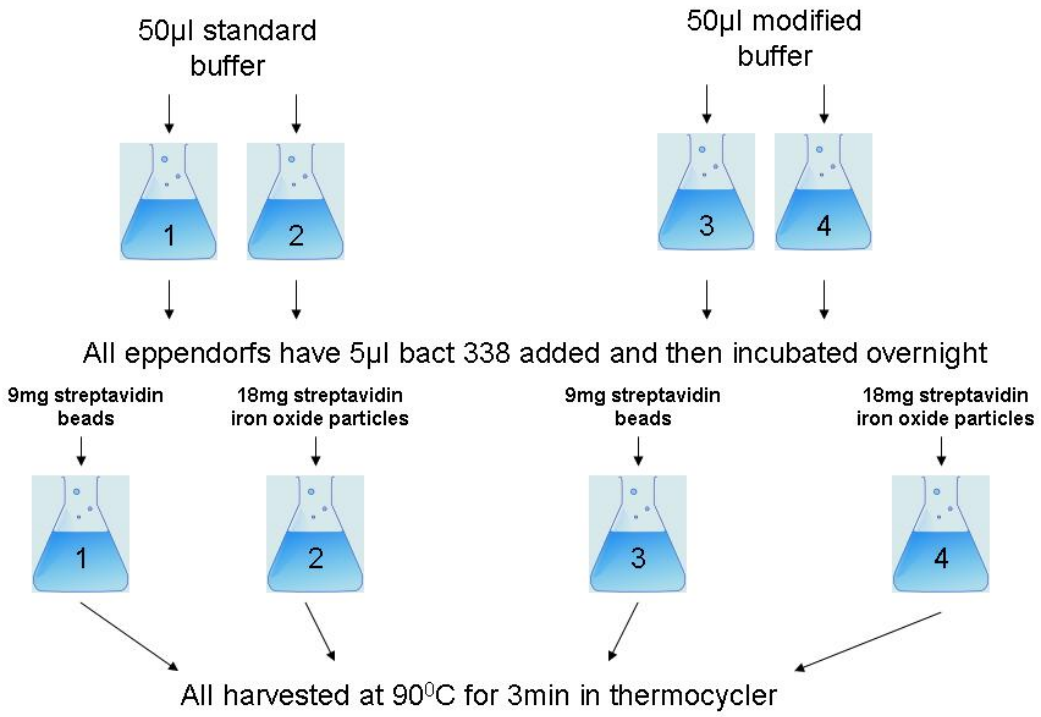

Figure 29: Schematic of methods for 4.3.2

4.3.3 Results

Weighted precipitates were greater in boats 2 and 3 (iron oxide particles) than in boats 1 and 4. Table 12 and figure 30 demonstrate the effectiveness of the iron oxide particles.

Table 12: Dry-weight precipitates via streptavidin beads and streptavidin iron oxide coated particles

Microeppendorf	Experimental conditions	Dry-weight µg
1	Standard buffer, Streptavidin beads	150
2	Standard buffer, Streptavidin particles	400
3	Modified buffer, Streptavidin particles	500
4	Modified buffer, Streptavidin beads	10

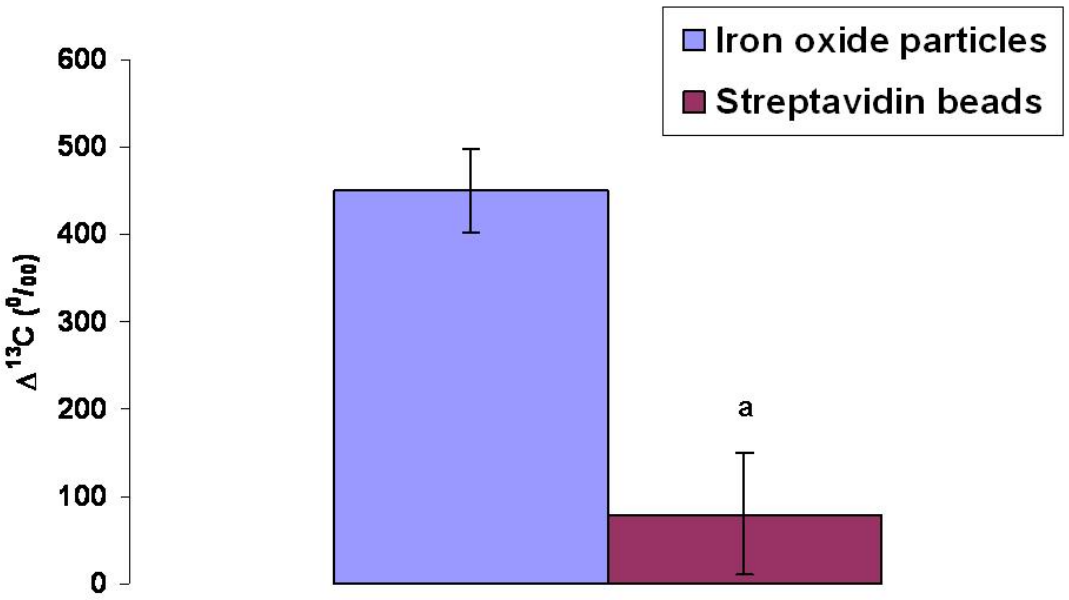

Figure 30: Comparison of mean dry-weight (SEM) between iron oxide particles and streptavidin beads
a= significantly less dry weight than with iron oxide particles ($p<0.05$)

4.3.4 Conclusions

The binding capacity of streptavidin coated beads appears to play a role in limiting isolation of 16s rRNA with the possibility of streptavidin capture particles increasing yield. However optimisation of other elements of the protocol remained to be demonstrated. Hybridisation conditions can only be optimised when a reliable baseline yield of material

can be obtained. In addition measurements of samples by EA-IRMS should confirm carbon signature and purity of precipitants. The concentration of total RNA may be important for binding potential as it may affect both hybridisation of 16s rRNA making it available for binding and stearic inhibition of binding by competing fragments of RNA. The investigation of RNA concentrations appeared to be the next logical step to examine.

4.4 16s rRNA isolation Experiment 4: RNA Concentration

4.4.1 Aims

The aim of this experiment was to determine the effect of concentration of RNA and iron oxide particles on the yield of dry weight precipitate of 16s rRNA at the end point of the protocol.

4.4.2 Materials and methods

Six microeppendorfs of faecal bacterial RNA were prepared in the following manner: 1 and 2 had 50µl (10mg) of RNA added, 3 and 4 for had 25µl (5mg) added and 25µl of ultrapure water, 5 and 6 had 12.5µl (2.5mg) of RNA added and 37.5µl ultrapure water. To all microppendorfs 50µl of standard hybridisation buffer was then added and they were hybridised at 70^0C for 10min followed by room temperature for 30min. Microeppendorfs 1,3 and 5 then had 5µl (1.78nmol) of bact-338 probe added whilst 2, 4 and 6 had 10µl (3.56nmol) of bact-338 added. All mixtures were then incubated at room temperature under gentle mixing overnight. The following day to all were added 8.9mg of streptavidin iron oxide particles and incubated at 25^0C for 2hr and then harvested in the thermocycler using a longer harvesting cycle of 90^0C for 6min (as suggested by manufacturer). Supernatants from each mixture were dried in preweighted tin boats as before and then reweighed when dried out (figure 31).

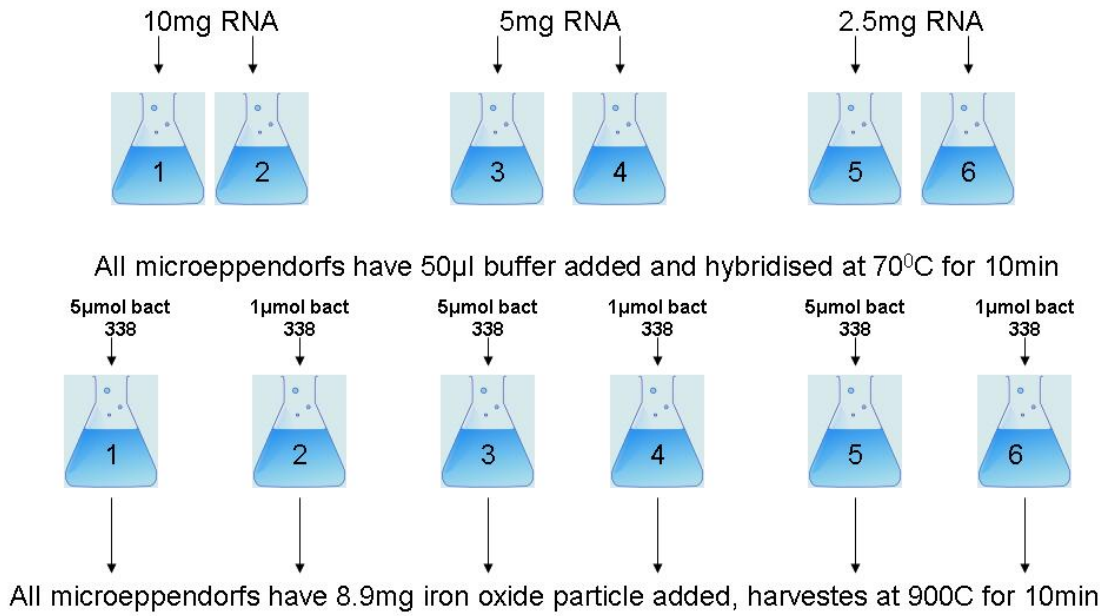

Figure 31: Schematic of methods for 4.4.2

4.4.3 Results

Total yields of precipitate demonstrated a clear trend towards increasing yield with increasing concentrations of RNA in the hybridisation mixture. The binding capacity also appeared related to total ammount of iron oxide particles for the reaction (figure 32). However there was no consistent trend in variation in capture probe concentration suggesting that this was not a saturated step in the protocol.

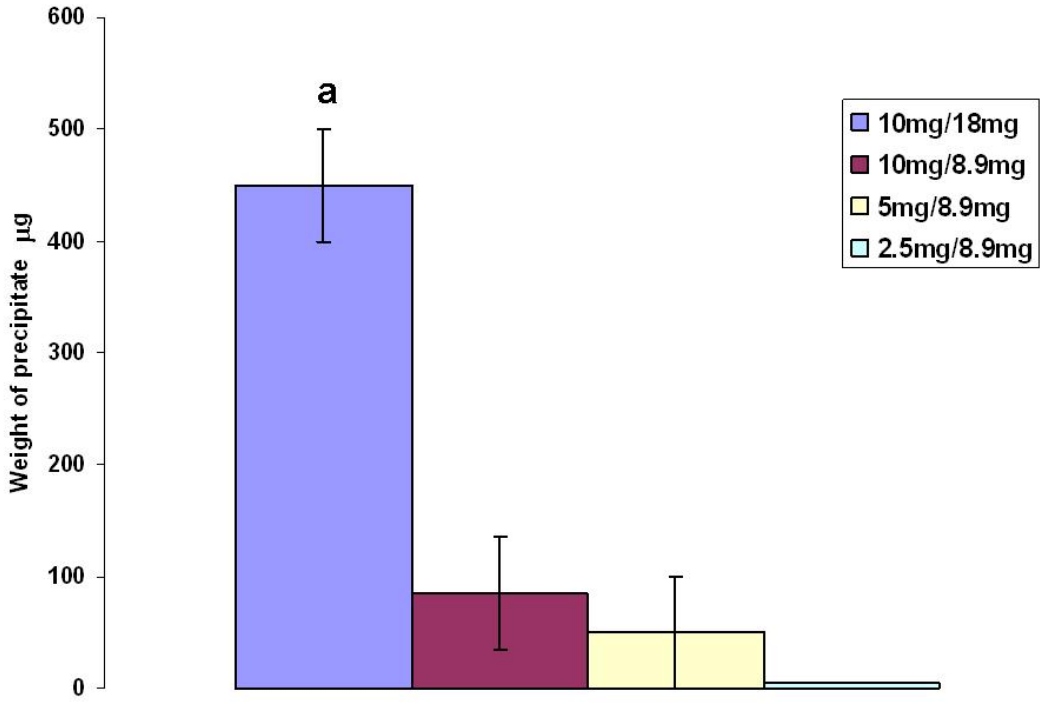

Figure 32: Mean dry-weight of precipitate (SEM) under differing concentrations of total RNA and capture particles

a= significantly greater weight than 10mg/8.9mg methods $p<0.05$

4.4.4 Conclusions

These experiments suggested that the protocol would now generate measurable and reproducible yields of precipitate. Although detectable by EA-IRMS, these yields were still relatively small to enable precise relative abundance ^{13}C to be determined. The protocol was repeated with open drying of tin boats substituted with freeze drying tin boats after snap freezing them in liquid nitrogen to reduce loss of 16s rRNA by capillary action over the sides of the tin capsule during the evaporation process. However this resulted in no greater yield of precipitate (data not shown).

The quantity of RNA proved limiting when trying to make multiple observations of bacterial enrichment on single specimens because high quantities of RNA are required for single experiments, which could not be yielded from dilute or small faecal specimens. In addition the quantity of capture particles required to yield sufficient material for EA-IRMS analysis would be prohibitively expensive for multiple observations. As stearic inhibition of competing strands of RNA was likely to affect binding, due to 16s rRNA being a long RNA strand (~1500 base pairs) it is proposed that a method that could reduce stearic inhibition for binding would improve the protocol. In addition the potential for scaling

down experiments with the use of highly sensitive LC-IRMS needed to be explored to maintain the feasibility of multiple SIP observations from single specimens.

A double probe method previously described by Pearson et al (370) appeared to be a promising approach that increased yield and reliability when probing for 16s rRNA. Here the oligonucleotide probe is adapted with the addition of a complementary sequence for another probe (CA- clamp 5' CCCACCCACCCACCCACCCCCCCCAAAAAAAAAAAAAAAAAAAA-3'), which has a poly-A tail, which can be hybridised to Poly-T sequences attached to a capture particle (figure 33).

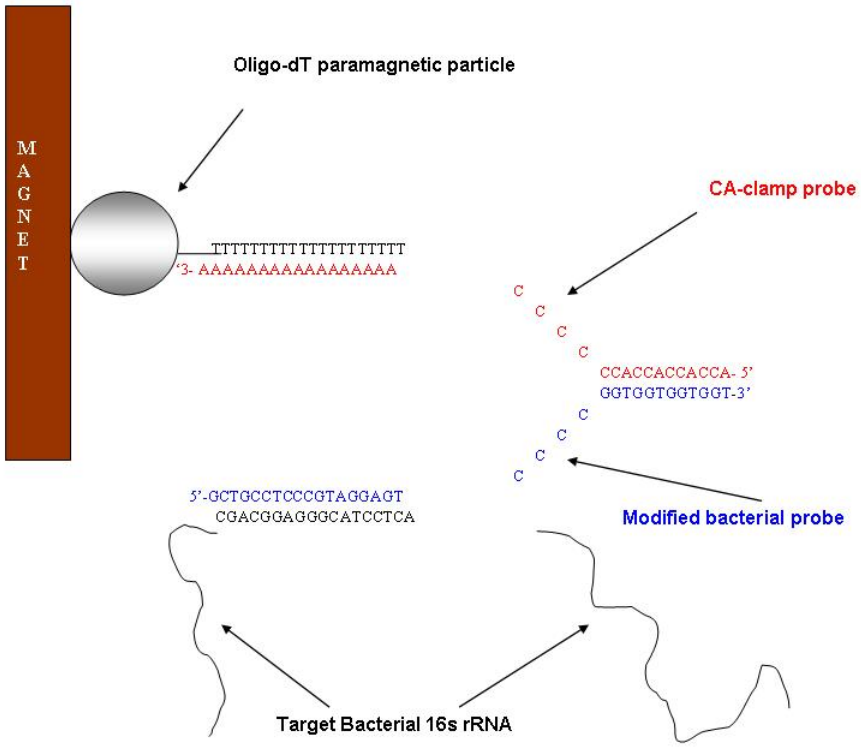

Figure 33: Modified "double probe" capture method for 16s rRNA

It was therefore decided to compare the optimum streptavidin capture protocol with a protocol based on modification of this double probe capture technique. An initial comparison was devised before proceeding to a definitive experiment using measurements of ^{13}C enriched material by LC-IRMS.

4.5 Streptavidin Double Probe Capture Comparison 1: Initial Comparison

4.5.1 Aims

The aim of the first experiment was to determine whether a double probe capture technique could be applied to faecal microbiota samples for the isolation of 16s rRNA. For this first experiment a larger volume and higher concentration of RNA were used to test the potential of this method.

4.5.2 Materials and methods

100µls (20mg) of RNA solution and 100µl of hybridisation buffer were hybridised as before. To the RNA solution 20µl (8.9nmol) of modified oligonucleotide probe; modified Bact 338- (5'-GCTGCCTCCCGTAGGAGTCCCCCGGGTGGGTGGGTGGGTGGG-3') (which has an additional sequence complementary to the CA-Clamp sequence) was added and again incubated overnight. In parallel 400µl (4mg) of Magnetite oligo dT capture particles (Novagen, Munich, Germany) were washed three times in ultrapure water and recaptured in the eppendorf each time with a magnet. After the final wash, the particles were suspended in 800uls of the hybridization buffer (formamide substituted with water) to keep the final volumes and concentrations the same as the streptavidin particle experiments. 5µl (1.78nmol) of CA-clamp probe were then added to the particle mixture and all eppendorfs were left to incubate overnight at 25^0C under gentle mixing. After overnight incubation each microeppendorf of RNA solution was added to a microeppendorf of particles and CA-clamp mixture and incubated for 2hr as before. Particles were then washed as before three times in ultrapure water before being re-suspended in 100µl water. Based on product information and previous data on using these particles (370), a lower temperature setting of 75^0C for 6min was used for 16s rRNA harvest, again in the PTC 200 Pelteir thermocycler. In parallel, a second 100µl of RNA (20mg) with 100µl of buffer solution was prepared and hybridised as before, before having 20µl of Bact-338 probe added and incubated overnight as before. This solution was then added to 1600µl (16mg) of streptavidin iron oxide particle and incubated for 2hr. After triple washing the solution was harvested at 75^0C for 6min. Both samples' supernatant was then dried in preweighted tin capsules and weighed after drying. Both tin capsules were analysed by EA-IRMS (figure 34).

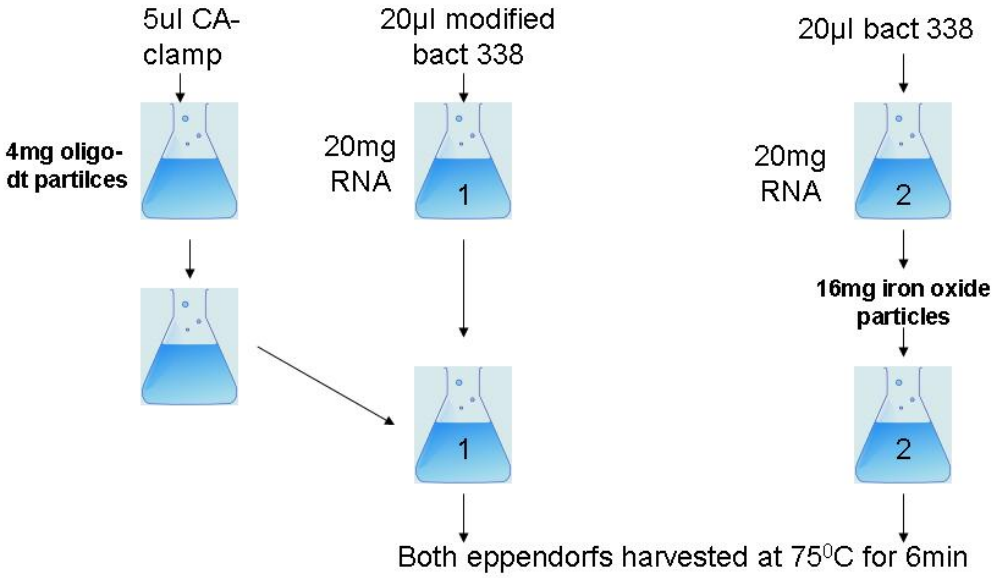

Figure 34: Schematic for methods 4.5.2

4.5.3 Results

Dry weight of tin capsules revealed around 200µg of precipitate from the oligo-dT experiment with no appreciable weight in the streptavidin boat. However analysis of the precipitate by EA-IRMS confirmed the presence of carbon with a signature in keeping with organic matter, but still of insufficient weight to accurately assess ^{13}C abundance of the material.

4.5.4 Conclusions

The double probe capture technique showed promise in reliably yielding greater amounts of 16s rRNA in that it generated a greater amount of precipitate despite using one quarter of the volume of capture particles, suggesting that binding efficiency was improved by the use of the CA-clamp. However the scale was still insufficient for determination of ^{13}C abundance by EA-IRMS. The ability of the protocol to yield ^{13}C enriched material was also required to validate the capture of 16s rRNA. This would most likely to best achieved by final analysis using LC-IRMS which can increase the sensitivity of the protocol by several orders of magnitude. An experiment to examine these factors was designed.

4.6 Streptavidin versus Double Probe Comparison 2: Definitive Experiment

4.6.1 Aims

To compare to the optimal streptavidin 16s rRNA capture technique with the double probe technique in terms of reliability of yield of carbon material and yield of ^{13}C enriched material via LC-IRMS.

4.6.2 Material and methods

A fresh 6g faecal sample was homogenized to 20% w/v slurry in Sorenson's phosphate buffer solution pH 6.6, which had been boiled for 20min and then purged with nitrogen for 10min to achieve anaerobic conditions. The sample was placed in a sterile crimped topped flask and allowed to equilibrate in a 37^0C waterbath for 30min under gentle mixing. At time 0 the bottle was spiked with 37.5mg of 99% ^{13}C urea in 75µls of water. The sample was then incubated for 6hr to allow bacterial activity to sequestrate ^{13}C urea before being removed and snap frozen by being placed in ethanol chilled to -30^0C by dry ice, to arrest bacterial activity. Sample were then stored in a -20^0C freezer. Total nucleic acids and total RNA were then extracted using the standard protocol. Six eppendorfs of the resultant RNA solution were prepared in parallel, (2 with 10mg/50µls, 2 with 5mg/50µls and 2 with 2.5mg/50µls). One microeppendorf of each concentration was prepared by the standard streptavidin protocol with 5µls (1.78nmols) of Bact 338 probe and 8.9mg/890µls streptavidin particles. The second set of three eppendorfs were prepared with the oligo-dT methods with 5µls (1.78nmols) of a modified Bact 338 probe (5'-GCTGCCTCCCGTAGGAGTCCCCCGGGTGGGTGGGTGGGTGGG-3') and 4mg/800µls oligo-dt particles. Samples were washed three times, resuspended in 100µls of ultrapure water and then harvested in the thermocycler at 75^0C for 6min. After harvest all samples supernatants were kept in a solution for analysis by LC-IRMS. The particles were washed once more with 100µl of ultrapure water and this was added to the solution to give a final volume of 200µl for analysis. The solutions were then analysed for total C and ^{13}C content in 25µl aliquots by LC-IRMS (Dionex ICS 3000, Sunnyvale CA, USA, coupled to Liquiface interface and Isoprime isotope ratio mass spectrometer (Isoprime, Cheedle, UK)).

4.6.3 Results

Consistently higher yields of carbon were obtained from the double probe samples (figure 35). The weight obtained appeared to be independent of RNA concentration via the double probe method. These yields were on a sufficient scale for analysis by the LC-IRMS to detect total carbon and relative changes in ^{13}C incorporation.

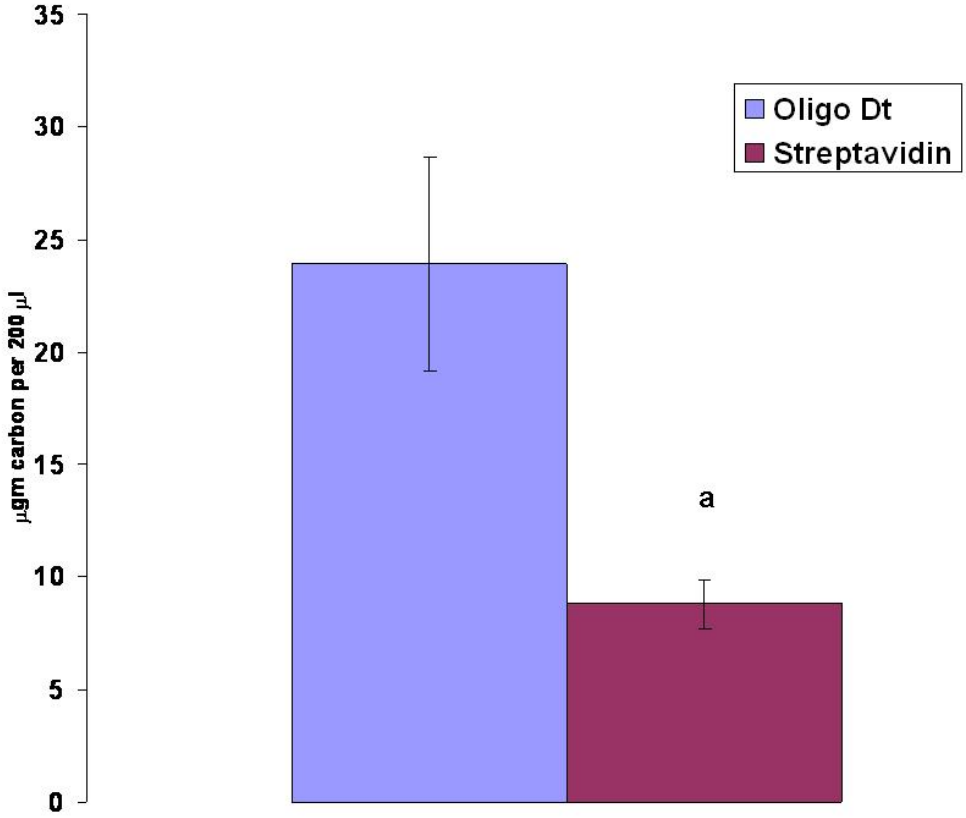

Figure 35: Comparison mean weight of carbon (SEM) in oligo-dT and streptavidin capture methods a= p=0.06

The presence of a $\Delta^{13}C$ above natural abundance in analysed samples confirmed the sequestration of labelled substrate by target bacteria. Yields of ^{13}C were reliable with the oligo-dT method with a reasonable standard error, whereas the streptavidin yielded only natural abundance in the 5mg and 2.5mg RNA eppendorfs with a wide standard error (figure 36).

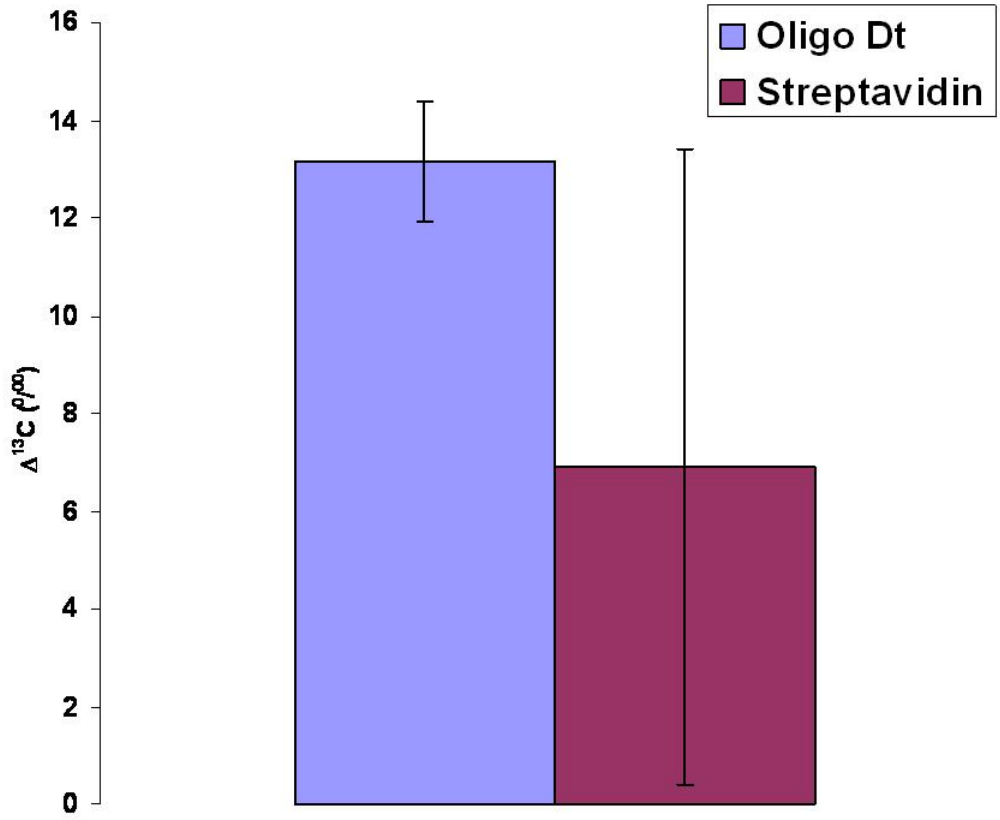

Figure 36: Comparison of mean ^{13}C enrichments $\Delta^{13}C$ ($^{0}/_{00}$) (SEM) of 16s rRNA via the oligo-dt double probe capture technique and streptavidin coated particles

4.6.4 Conclusions

The double probe capture technique showed greater potential for SIP experimentation in terms of yield of organic carbon after harvest and reliability of material yielded, demonstrated by consistency in ^{13}C enrichments in multiple specimens. Consistently, capture particles have shown a greater yield of total ^{13}C in comparison to capture beads. This makes sense in terms of our understanding of the protocol binding kinetics and the greater surface area afforded by capture technique. The presence of increased quantities of non-RNA C in the double-probe capture technique may potentially invalidate observations of greater carbon yield being related to improved binding efficiency. However this observation, in parallel with an apparent reliability in ^{13}C enrichment of capture material, suggests that the double-probe technique is more proficient. As 16s rRNA is a large molecule (~1500bp) it follows logic that a technique which can overcome the effects of competitive inhibition between RNA strands could lead to improved binding efficiency. Optimisation of this protocol was required, and it was felt that this could be performed in conjunction with examination of the SIP protocols ability to reflect varying bacterial metabolic activity using 16s rRNA as a proxy for total nucleic acid and generic RNA incorporation, at both eubacterial and group specific levels (phylogenetic level). For these

studies it was proposed to identify ^{13}C incorporations into 16s rRNA using all four of the original candidate tracers, to test the relative reliability of these tracers in 16s rRNA in comparison to total RNA. The use of group specific oligonucleotide probes would enable the study of relative incorporation of tracers across different phylogentic groups to assess whether tracers exhibit substrate bias.

5 16s rRNA SIP Experiments on Faecal Microbiota

To improve the SIP protocol and optimise the yield of target 16s rRNA the following studies on ^{13}C enriched faecal microbiota were undertaken. The aims of this section of work were to validate SIP for the study of ^{13}C enrichments of bacterial 16s rRNA as a proxy for total RNA incorporation and to assess whether these ^{13}C incorporations could be related to bacterial metabolic activity. This was to be achieved by validating a ^{13}C tracer at the 16s rRNA level, demonstrating that 16s rRNA yield would be consistently on a scale appropriate for LC-IRMS analysis and that appreciable ^{13}C incorporation can be detected with predictable differentials across different tracers, stimuli and bacterial species.

5.1 Faecal SIP Experiment 1: Multiple ^{13}C Tracer Incorporation at 16s rRNA level

5.1.1 Aims

To test the suitability of various ^{13}C labelled tracers in the SIP protocol applied to human faecal samples when probing at the 16s rRNA level.

5.1.2 Materials and Methods

Samples of frozen total RNA from the experiment described in chapter 3.2, from all four tracers ([U-$^{13}C_4$, ^{15}N] aspartate, ^{13}C urea, [4,5-$^{13}C_2$] uracil, [U-$^{13}C_3$] glycine), from both media (1 and 2) and all three time points (2, 6, 24hr) were defrosted and prepared as previously described in 2.5mg/50µl aliquots. 16s rRNA was obtained from all samples using 5µls (1.78nmol) of modified eubacterial probe (modified Bact 338) using the oligo-dT protocol described in section 4.6. Total RNA and 16s rRNA were analysed by LC-IRMS for total carbon content and ^{13}C incorporation.

5.1.3 Results

In both media (medium 1 and medium 2) significant enrichments above natural abundance (T_0 samples) were seen in RNA and 16s rRNA over time (figures 37, 38). ^{13}C enrichments were an order of magnitude greater in the total RNA in comparison to 16s rRNA. However relative changes in ^{13}C enrichment were in proportion over time-points. ^{13}C enrichments were consistently highest with the ^{13}C-urea tracer and best enrichments were seen over shorter time-points (2 and 6hr).

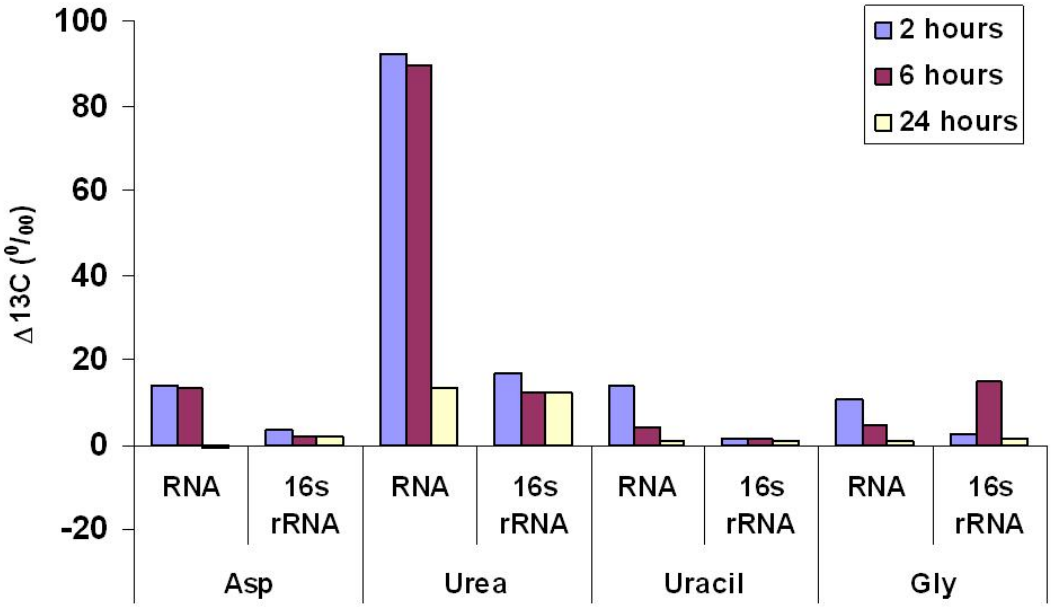

Figure 37: ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in total RNA and Eubacterial 16s rRNA at three time points using multiple tracers in culture Medium 1

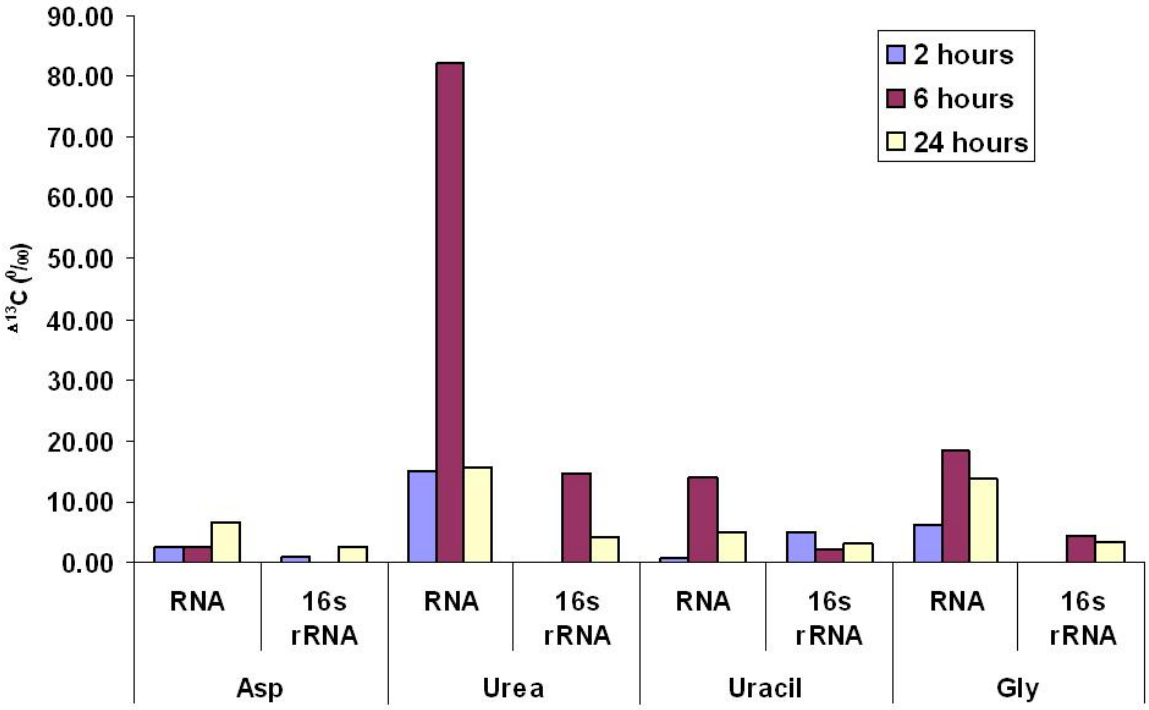

Figure 38: ^{13}C incorporation $\Delta^{13}C$ ($^{0}/_{00}$) in total RNA and Eubacterial 16s rRNA at three time points using multiple tracers in culture Medium 2

At the 16s rRNA level ^{13}C urea there was significantly greater ^{13}C incorporation than from all other tracers (figure 39). When correcting for initial concentration of tracers (scaling up $^{13}C_4$ N_2 aspartate), the mean incorporations across both culture media showed most consistent ^{13}C incorporation for [U-$^{13}C_4$, ^{15}N] aspartate and ^{13}C urea in both RNA and eubacterial 16s rRNA (figure 40). However only ^{13}C urea demonstrated significantly greater ^{13}C incorporation than $^{13}C_2$ uracil and $^{13}C_3$ glycine at the 16s rRNA level (figure 41).

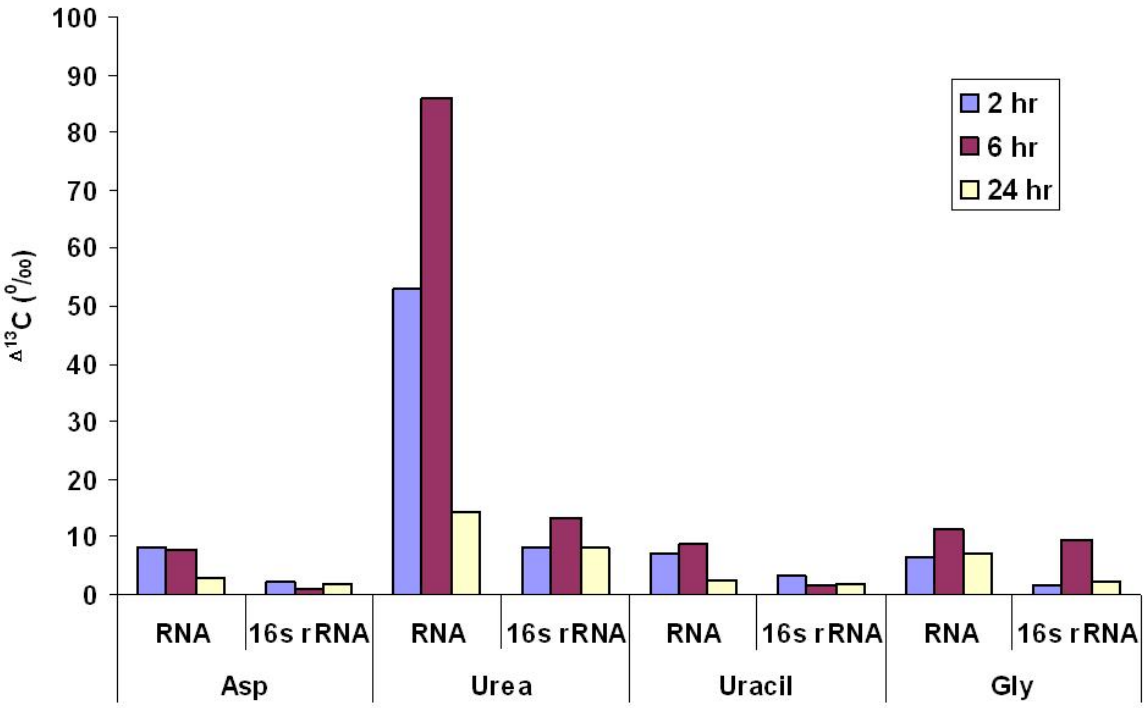

Figure 39: Mean ^{13}C incorporation $\Delta^{13}C$ ($^0/_{00}$) across all tracers in both media

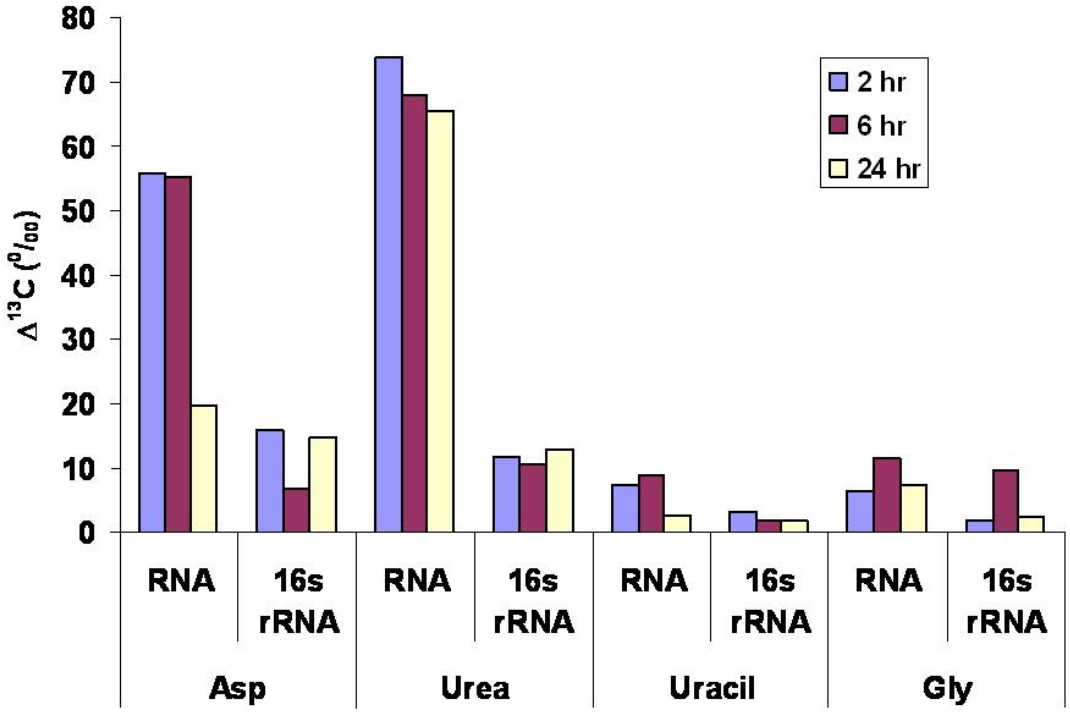

Figure 40: Mean ^{13}C incorporations $\Delta^{13}C$ ($^{0}/_{00}$) across all tracers in both media correcting [U-$^{13}C_4$ ^{15}N] aspartate for concentration

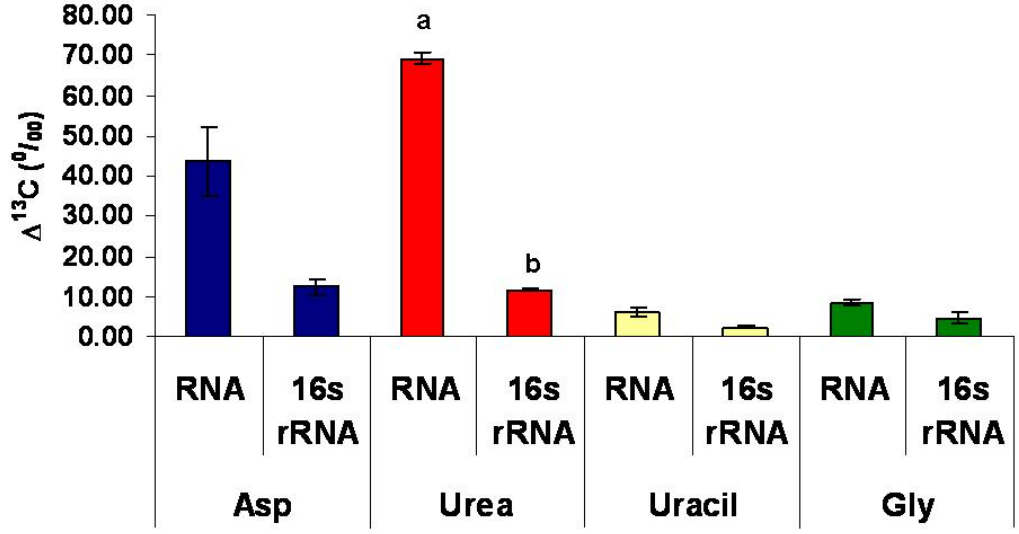

Figure 41: Mean ^{13}C incorporations for all time points in both media for all for tracers, correcting for [U-$^{13}C_4$ ^{15}N] aspartate concentrations

a+b= significantly greater incorporation to uracil and glycine $p<0.05$

5.1.4 Conclusion

^{13}C urea generated consistently greatest enrichments of nucleic acids in comparison to other SI-labelled tracers. However the relationship between total RNA and 16s rRNA was not linear, but appeared in proportion for the majority of samples. The ability of urea to be incorporated most readily into RNA fitted with our understanding of where ^{13}C urea enters into the one-carbon pool (after hydrolysis to HCO_3^-/CO_2) making it a potential 'universal tracer' i.e. by entering via the most basic substrates would be less prone to sequestration bias. The experiments showed that the solubility of [4,5-$^{13}C_2$] uracil was poor in solutions which reduced its availability for incorporation into bacterial nucleic acids. Aspartate showed reasonable levels of incorporation, but it was less reliable with inconsistent incorporation and, as a result, failed to reach statistically significant better incorporation than uracil or glcyine. Glycine demonstrated good solubility, yet resulted in low levels of tracer incorporation. The reasons for this are unclear as glycine enters purine synthesis at an early stage and is essential for nucleic acid synthesis. One explanation may be that the demands on glycine for bacterial protein synthesis reduce its availability for nucleic acid synthesis.

The ^{13}C signal was again diluted between observations of total RNA and 16s rRNA, the dilutional factors were consistent across all the tracers, suggesting that these were not a factor in this process. Dilution of the ^{13}C enriched 16s rRNA therefore must be an artefact of the 16s rRNA isolation and harvesting process. Contamination of the ^{13}C signal by unenriched C could be due to several steps of the protocol where carbon is added, such as from the oligonucleotide probe, residual CA-clamp or from capture particles. More detailed examination of the protocol was required to fully understand this.

It was therefore concluded that using ^{13}C urea tracers would be the next logical step to examine a working model of SIP as a potential tool to explore the stimuli for bacterial metabolic activity and species specific incorporations. However the limited number of samples and variation of isotope incorporation was beyond the ability of the chosen method to demonstrate differences in ^{13}C incorporation according to stimuli. Nor did it examine group specific activity. A set of experiments was then designed to examine these questions.

5.2 Faecal SIP Experiment 2: ^{13}C Urea Incorporation Under Varying Stimuli and Time Frames

5.2.1 Aims

To assess the potential for the current SIP protocol to detect changes in ^{13}C incorporation in bacterial 16s rRNA under varying stimuli for metabolic activity.

5.2.2 Materials and methods

Two freshly voided stool samples were each prepared in 13 separate crimp topped serum vials. Four separate culture media were prepared by the addition of supportive electrolytes to a volume of Sorenson's phosphate buffer solution pH 6.6, which had been boiled for 20min and then purged with nitrogen for 10min. Culture media differed in level of supportive electrolytes and supportive carbohydrate (table 13). 6g of stool was diluted to make 20% slurry with culture media and crimped topped tube was sealed before being flushed again with oxygen free nitrogen for 30sec. All specimens were then placed in a gentle agitating waterbath at 37^0C and left to equilibrate for 30min. At time zero each crimped topped vial was inoculated with 37.5mg of 99% ^{13}C urea in 75µls of water. Samples from each culture medium were sequentially removed at 2hr, 6hr and 24hr and snap frozen. Frozen samples were immediately stored at -20^0C until being defrosted for analysis. Total nucleic acids and total RNA were isolated according to the standard protocol. Samples of total RNA with highest enrichments (medium 1 to 3, time-points 2hr and 6hr) were then probed for 16s rRNA with a modified eubacterial probe (modified Bact 338) using the oligo-dT method from section 1.2.8.2 using aliquots of 50µls/2.5mg of RNA.

Table 13 Culture media used in 5.2.2

Medium	Contents
1	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg NH_4Cl 1000mg Oligofructose
2	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg NH_4Cl 1000mg Pectin
3	250ml Sorensons buffer pH 6.6 625mg NaCl, 60.15mg $MgSO_4$, 2.9mg $CaCl_2$, 1000mg *NH_4Cl* 1000mg L-rhamnose
4	250ml Sorensons buffer pH 6.6

5.2.3 Results

Total nucleic acids from all four media over all three time-points were analysed by LC-IRMS and shifts in ^{13}C enrichment above a nominal baseline from a T_0 sample were expressed as $\Delta^{13}C$ above natural abundance. Total nucleic acids enrichments were significantly higher in media 1 and 2 over the timeframe (figure 42, 43). Medium 3 had moderate enrichments with medium 4 having the lowest enrichment over all time-points. These findings correspond with the analysis of total RNA from all media and time points. Media 1 and 2 had all the significantly higher enrichments (figure 44, 45). Tracer incorporations were not as high in total RNA as in the total nucleic acids.

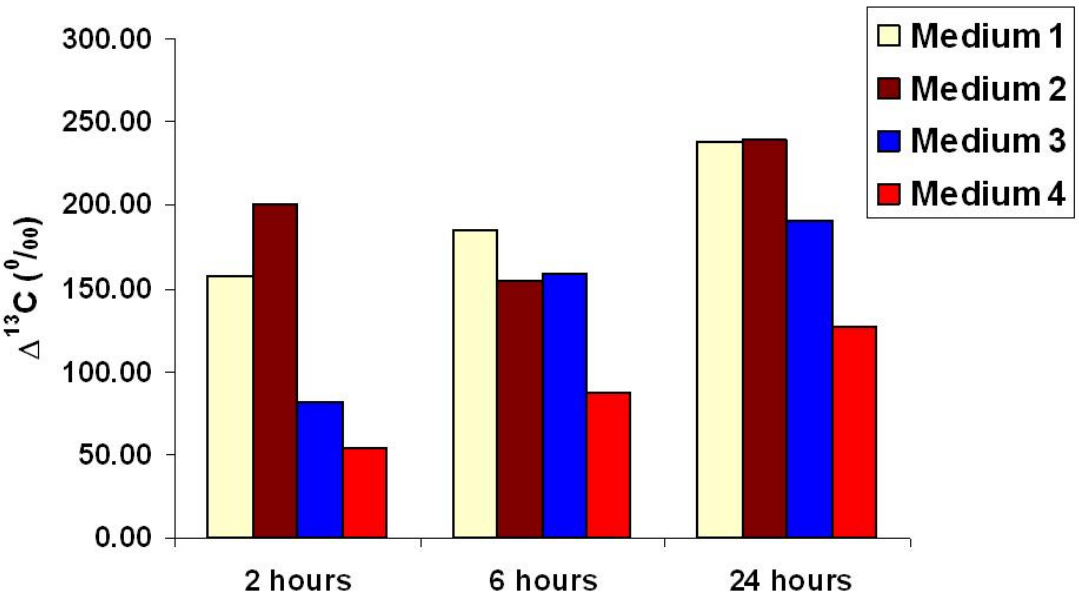

Figure 42: ^{13}C enrichments $\Delta^{13}C$ ($^0/_{00}$) in bacterial total nucleic acids from 4 culture media at three time points

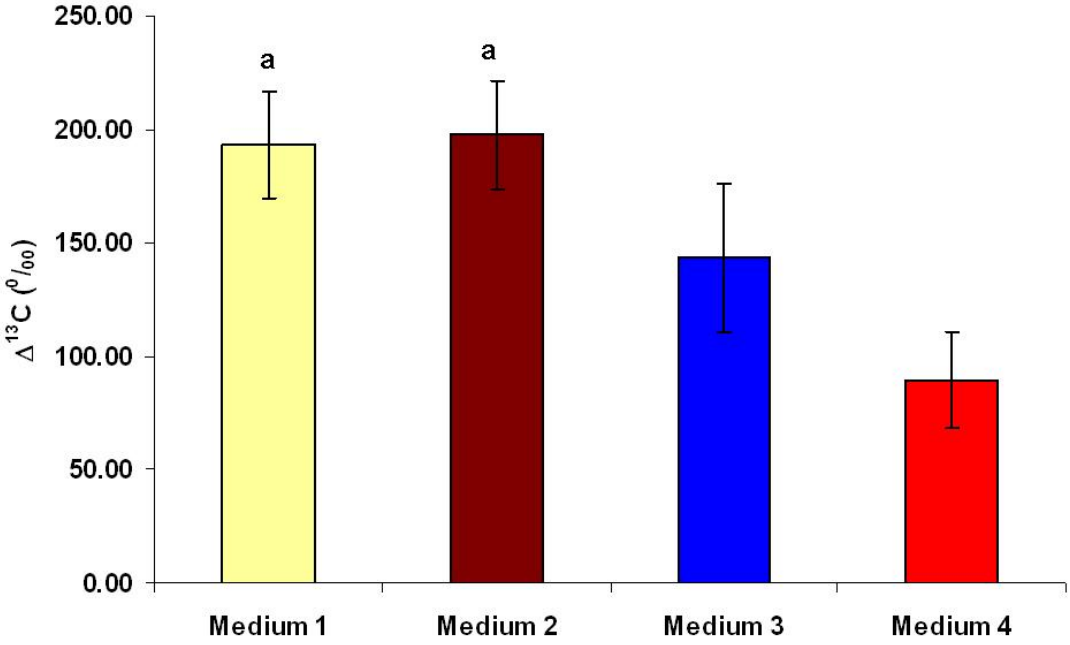

Figure 43: Mean ^{13}C incorporations $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial total nucleic acids from 4 culture media at the three time points

a=significantly greater incorporation than media 4 p<0.05

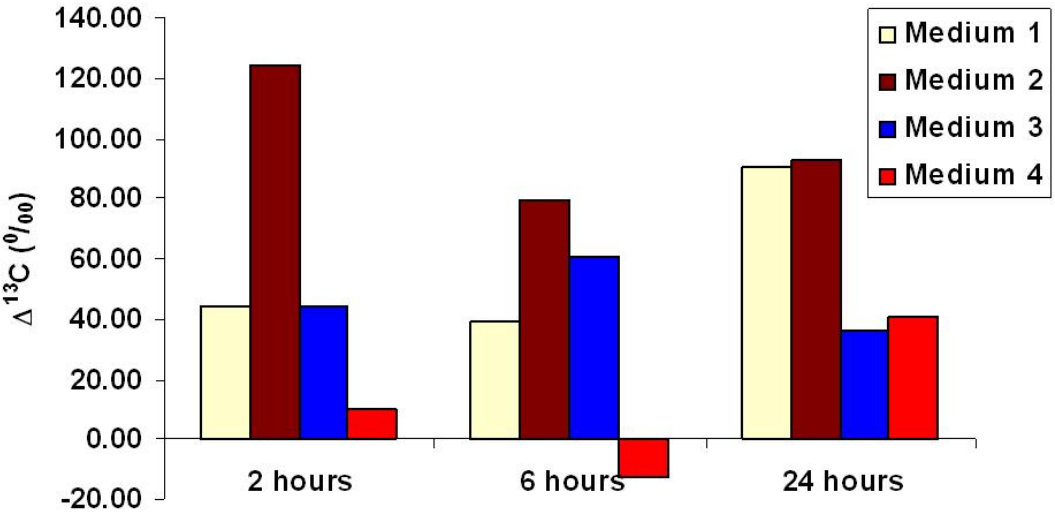

Figure 44: ^{13}C enrichments $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial total RNA from 4 culture media at the three time points

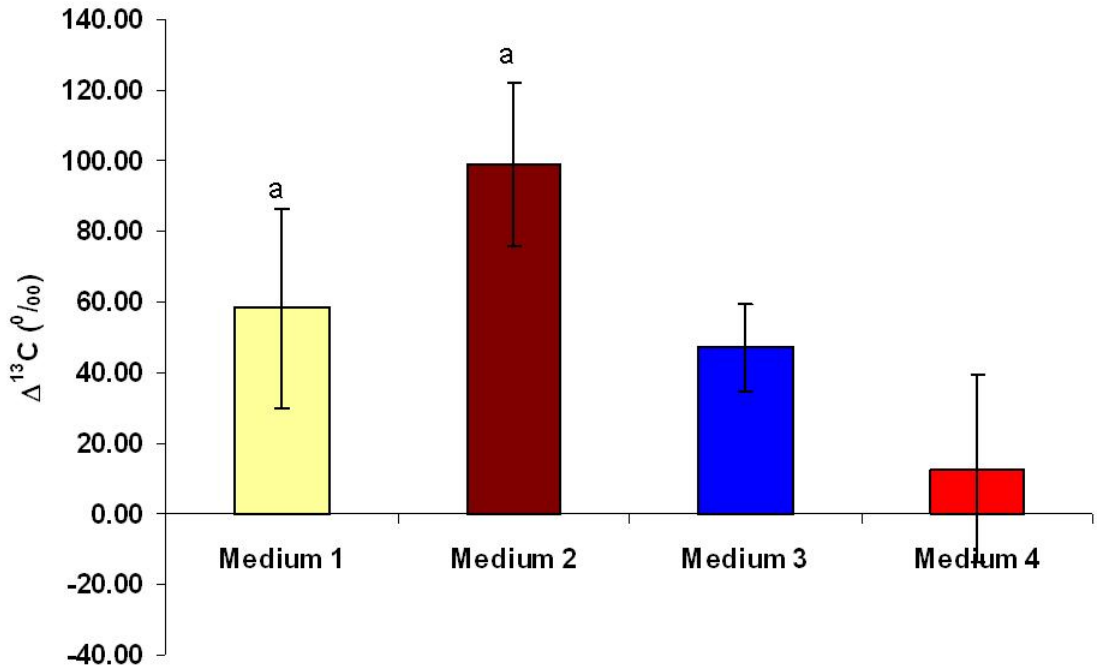

Figure 45: Mean ^{13}C incorporations $\Delta^{13}C$ ($^{0}/_{00}$) in bacterial total nucleic acids from 4 culture media at the three time points

a= significantly greater incorporation than 4 $p<0.05$

Isolated 16s rRNA again demonstrated lower enrichments of ^{13}C in comparison with total RNA; however yields were again in proportion with total RNA across the three media and both time points (figure 46).

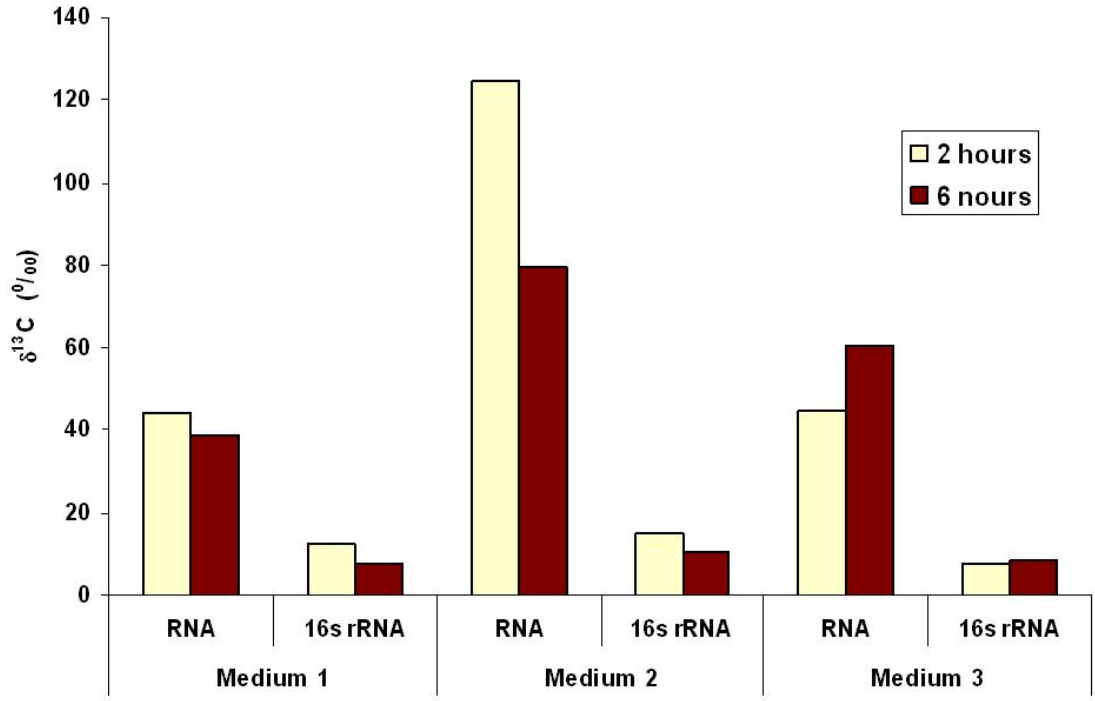

Figure 46: ^{13}C incorporations $\delta^{13}C$ ($^{0}/_{00}$) in bacterial total RNA and eubacterial 16s rRNA in culture media 1-3 at 2hr and 6hr

5.2.4 Conclusions

The 16s rRNA enrichments were in proportion to those in the paired total RNA samples. These enrichments also were significantly greater in the culture media that would have provided most support for bacterial activity. The protocol therefore offered opportunity to examine activity-related changes in the bacterial pool. The next logical step was to investigate the potential for detecting individual species specific changes in ^{13}C incorporation in comparison to the whole bacterial consortia.

5.3 Varying ^{13}C incorporations across Different Bacterial Species

5.3.1 Aims

To assess the potential for the current SIP protocol to detect species specific changes in ^{13}C incorporation into 16s rRNA according to metabolic stimulus.

5.3.2 Materials and methods

Residual total RNA yielded from the multiple culture media (section 5.2.2) was prepared in 2.5mg/50ul aliquots as before with a paired eppendorf of oligo-dT particles modified with 5µls (1.78nmol) of CA-clamp. The RNA/buffer mixture was then probed using 5µl (1.78nmol) of a species specific *Bifidobacterial* probe (modified Bif 164 5'CATCCGGC ATTACCACCCCCCCCGGGTGGGTGGGTGGGTGGG-3') to isolate Bifidobacterial 16s rRNA. This was done for limited highly enriched samples (medium 1 and at 2 time-points 2hr and 6 hr). Isolated material was analysed by LC-IRMS and compared with eubacterial ^{13}C incorporation for the same samples from the previously displayed data.

5.3.3 Results

Similar total weight of carbon was extracted using the species specific *Bifidobacterial* probe as from the eubacterial probe. Figure 47 displays the ^{13}C enrichments of *Bifidobacteria* relative to total bacteria. The *Bifidobacterial* enrichment continued to rise at the 6hr time-point, converse to total RNA and eubacterial 16s rRNA, this trend in rising enrichment was more pronounced in the oligofructose medium (medium 2).

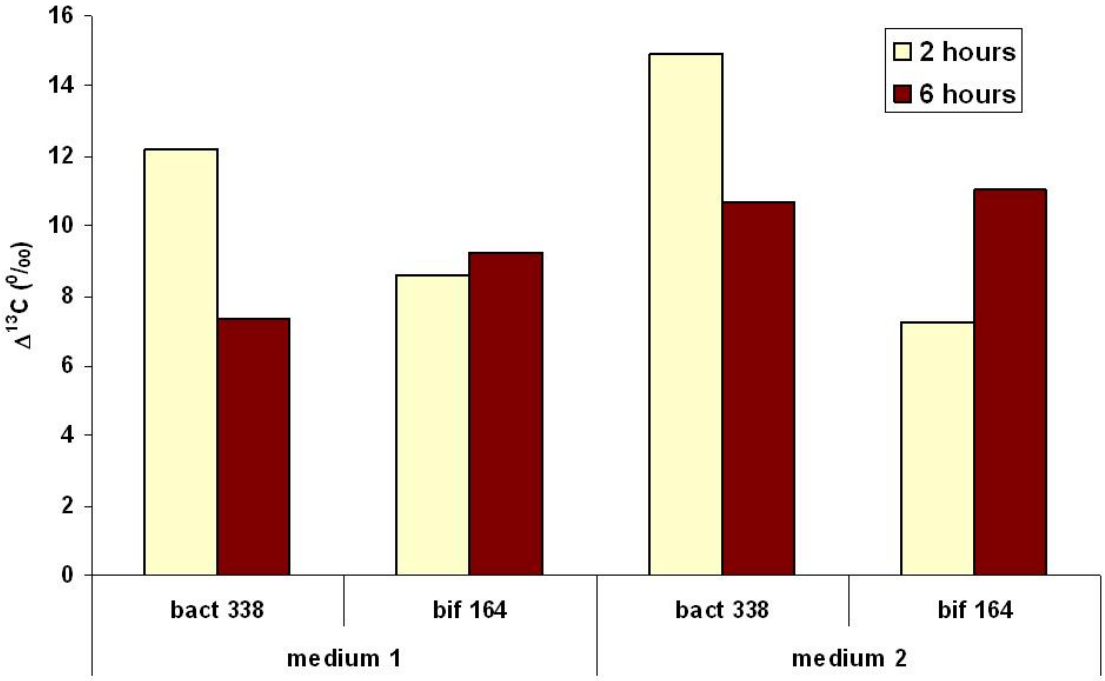

Figure 47: ^{13}C encorporations $\Delta^{13}C$ ($^{0}/_{00}$) in eubacterial 16s rRNA and *Bifidobacterial* 16s rRNA in media 1 and 2 at 2hr and 6hr

5.4 Conclusions of Faecal Microbiota SIP Experiments

The faecal bacterial experiments yielded encouraging results. The SIP protocol consistently detected a measurable and reproducible weight of carbon from each probe capture experiment and consistently detected ^{13}C enrichments above natural abundance. ^{13}C urea appeared to be the most reliable tracer at RNA and 16s rRNA levels with significantly greater incorporation than other candidate tracers. The enrichments of total RNA were related to support for bacterial activity, supported by changes relating to SCFA profile changes. These changes were also mirrored in the 16s rRNA from a eubacterial probe. The basic media chosen were designed to be as universally supportive of bacterial replication, at a pH that suited most gut microbiota (pH 6.6) and the electrolytes chosen were important co-enzymes for cellular function. The addition of a carbon source consistently increased bacterial metabolic activity as evidenced by a greater enrichment in total nucleic acids, total RNA and 16s rRNA in the samples to which a carbohydrate source was added. Cornstarch, as a complex carbohydrate, is a relatively generic carbon source for bacterial metabolic activity across species, whereas oligofructose is a preferential substrate for *Bifidobacteria*. Differences in the activity profile of *Bifidobacterial* probed 16s rRNA were detected and these appeared to be most marked in the oligofructose media although

these were only single observations. Urea still appeared to be a good potential tracer as incorporations were seen in all experiments and levels were related to metabolic activity.

RNA and 16s rRNA enrichments were different with a marked dilution of ^{13}C signal between the former and the latter in all experiments. The initial experiments with the single probe capture technique had inexplicable 'blank cycles' where no appreciable carbon was detected. This problem appeared to have been overcome by the use of the double-probe capture technique but there remained variation in the dilution from total RNA to 16s rRNA in apparently identically conditioned experiments. Although differential incorporations were observed from the eubacterial group and the *Bifidobacteria*, these differences were minimal and did not demonstrate clearly better incorporation by the *Bifidobacteria* when stimulated by the oligofructose medium.

The observations from these experiments appeared to lack specificity, the 16s rRNA signal was again diluted and possibly contaminated by 'background' carbon. This dilution could possibly involve non RNA material within the supernatant or non specific binding of other cell material less likely to be ^{13}C enriched over shorter time periods (DNA, cell proteins). The most likely explanation was that the blank carbon term results from recovery of probe nucleic acid sequences that were released into the supernatant in the high temperature melting of the probe-16s rRNA complex. The capacity of the protocols to isolate RNA without contamination with processing materials from protocol such as oligonucleotide probe, CA-clamp and capture particles is also important as these again will be sources of unenriched carbon. Although not necessarily a major problem if the amount of background carbon is consistent and reproducible, it does require greater ^{13}C incorporation into 16s rRNA to be able to detect differences against a background of natural abundance carbon. The specificity of probes for binding when examining differences in species activity is the limiting factor in observing significant differences between species. Although encouraging, the data from section 5 suggested that optimisation of binding specificity was still required.

Validation of the purity of specimens and a greater degree of reproducibility of the 16s rRNA results was sought in order to optimise the potential of SIP for the study of human faecal bacterial physiology and pathophysiology. It was decided that investigation of this would be best achieved by testing the experimental protocol further using a highly enriched model to amplify any potential differences in enrichment of target materials and thus to test the specificity of individual steps of the capture protocol.

6 16s rRNA SIP Experiments Using a ^{13}C Enriched Pure Culture Model

In chapter 5, experiments designed to determine that the SIP protocol could yield reliable quantities of ^{13}C enriched 16s rRNA were outlined. In addition, changes in ^{13}C incorporation predictable by stimuli for bacterial metabolic activity were demonstrated. However ^{13}C incorporation in 16s rRNA is greatly diluted in comparison to that in total RNA. This dilution is also likely to affect the accuracy of any observation made on incorporation into 16s rRNA, both at a consortia and a species level. The aims of the following experiments were to investigate the faecal microbiota protocol further, focusing on effects on yield, purity and specificity of the probe hybridisation for enriched 16s rRNA. This was to be done by utilising a ^{13}C enriched pure culture model to produce a sample of ^{13}C enriched RNA of a known nucleic acid sequence that a specific complementary oligo-nucleotide probe could be designed to target.

6.1 Preparation of ^{13}C Enriched *E. coli* RNA for Pure Culture SIP Experiments

An inactivated lyophilised specimen of a genetically characterised non-pathogenic *E. coli* (ATCC 25922; sourced from the Department of Microbiology, Royal Hospital for Sick Children Glasgow) was taken from a single transport swab and plated onto multiple agar gel plates with Maconkeys standard No. 3 gel under aseptic conditions. Culture plates were then incubated at 37^0C for 24hr to allow growth of pure colonies to form. Colonies were then removed by sterile ring and placed in a 2ml sterile flask containing 2ml of liquid culture medium. Bacteria were added until a standardised optical density of solution (80-88%) was achieved which correlated with10^6 cfu/ml. 1ml aliquots of this sub-culture medium were used to inoculate a 50ml sterile flask of culture medium. The flasks were then placed in a 37^0C waterbath under vigorous agitation (40 cycles/min) for 48hr.

As with previous experience within the laboratory, a relatively complex medium and a second sub-culturing of culture broth for a further 48hr were both required to yield optimum shifts in optical density of the culturing media. Sub-cultures were diluted to 10^{-2}, 10^{-4} and 10^{-6} density and plated on Mackonkeys number 3 and Columbian blood agar plates at each phase to ascertain the identity and purity of culture growths. Even with meticulous aseptic techniques *E. coli* were likely to be contaminated with at least one other

bacterial species after more than two sub-culture steps. Experiments were therefore limited to two sub-cultures.

After a second sub-culture phase the sterile flasks were decanted into two 30ml centrifuge tubes and centrifuged at 15,000g for 20min. A bacterial pellet was then identified at the bottom of the centrifuge tube. This was removed with 1ml of culture solution and pipetted into a 2ml eppendorf. Bacteria were then processed to total nucleic acids and total RNA by the standard protocol and frozen at a concentration of 2.5mg/50µls.

For ^{13}C enrichment of *E. coli* the only carbon source present in the medium was glucose (10atom% ^{13}C). This enriched glucose was added to both subculture media so that the bacteria were exposed to 10 atom% ^{13}C as the sole carbon source for at least 96hr. After extraction analysis of total RNA confirmed ^{13}C enrichment of around 3atom%. These were deemed suitably enriched to study the SIP protocol.

6.2 Pure Culture Experiment 1: Faecal Microbiota Comparator Experiment

6.2.1 Aims

To determine the ability of the current experimental protocol to demonstrate species specificity by utilising highly ^{13}C enriched pure cultures of *E. coli* as a positive control with unenriched faecal microbiota.

6.2.2 Materials and Methods

Six eppendorfs were prepared with 2.5mg/50µls of ^{13}C enriched *E. coli* total RNA and 50µls of hybridisation buffer, these were hybridised at 70^0C for 10min followed by 25^0C for 30min. To the first three samples (1, 2, 3) 5µl (1.78nmol) of modified eubacterial probe was added (Bact 338) and to the second three (4, 5, 6) 5µl (1.78nmol) of modified *Bifidobacteria*l probe was added (Bif 164). In parallel 6 eppendorfs were prepared with 400µls (4mg) of oligo-dT paramagnetic particles, which were washed three times in ultrapure water before resuspension in 800µls of hybridisation buffer. Each of these then had 5µls (1.78nmol) of CA-clamp probe added. All the eppendorfs were incubated overnight at 25^0C under gentle mixing. The RNA/probe solution were then each added to a particle eppendorf and then incubated for a further 2hr. The particles were removed by magnetic capture, washed three times with ultrapure water before resuspension in 100µl of ultrapure water. Particles were harvested in the peltier thermocycler at 75^0C for 6min. The

supernatant was removed and the particles were washed with a further 100µl of water to leave a 200µl sample. All samples were analysed in 25µl aliquots by the LC-IRMS.

This experiment was repeated again with the alteration that each 2.5mg/50uls of RNA as a composite of 20% highly enriched *E. coli* and 80% RNA from non enriched faecal bacteria.

6.2.3 Results

In the pure *E. coli* experiment, supernatant material demonstrated consistent enrichment with ^{13}C well above baseline in all six samples. Although 16s RNA enrichments were on a smaller scale than *E. coli* total RNA they were in proportion (roughly 4:1) and the pure *E. coli* arm, specimens 1, 2 and 3, were on a higher scale than in the previous faecal culture experiments. The 20%/80% mixed specimens, 4, 5 and 6, showed a proportional reduction in enrichments (figure 48). Neither of the experiments showed any specificity towards the Bact-338 probe and in the 20%/80% diluted sample enrichment appeared greater in the Bif-164 samples.

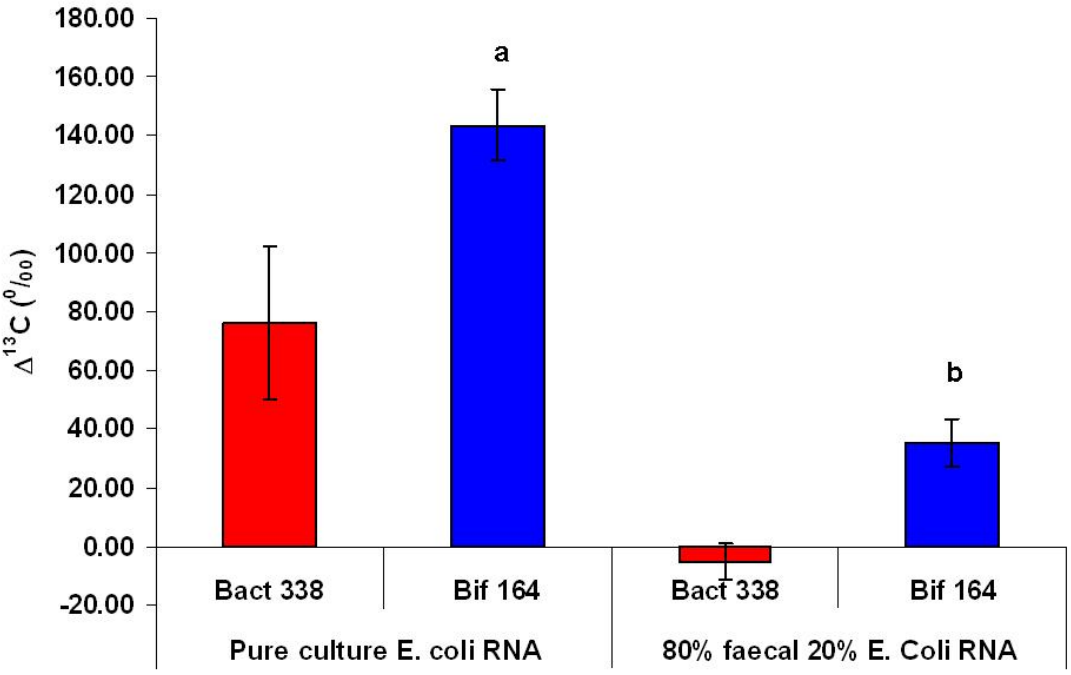

Figure 48: ^{13}C enrichments $\Delta^{13}C$ ($^{0}/_{00}$) of 16s rRNA from pure *E. coli* culture and an 80% faecal microbiota 20% *E. coli* solution, probed with eubacterial (bact 338) and *Bifidobacterial* (Bif 164) probes

a= significantly greater incorporation than the 80% faecal microbiota 20% *E.coli* in both bact 338 and Bif 164 probes, b= significantly greater incorporation than the 80% faecal microbiota 20% *E.coli* with the bact 338 probe, both $p<0.05$

6.2.4 Conclusions

This experiment demonstrated reproducibility in the capacity to obtain enriched material in proportion to the total RNA from which it was obtained. A higher enrichment might also help demonstrate significant differences in bacterial activity. However the protocol failed to achieve species or group selective binding suggested by the previous faecal *Bifidobacterial* experiments. In fact enrichments trended towards greater enrichment in the Bif-164 samples. A possible explanation for this may be non-specific binding of RNA to available sites on the poly-T tail of the particles. In particular, messenger RNA (mRNA) in some bacterial groups is terminated by poly-A sequences (433). If mRNA enrichment was greater than 16s rRNA and non specific binding of mRNA was greater in the Bif-164 probe arm this could generate the results seen previously. The logical next step was to investigate non-specific binding of the protocol.

6.3 Pure Culture Experiment 2: No Probe vs. Open Clamp Experiment

6.3.1 Aims

To evaluate the role that ^{13}C enriched non 16s rRNA material and non specific binding of mRNA might play in contaminating the ^{13}C signal in harvested material from the final step in SIP protocol. To test the hypothesis that contamination involves non-specific binding to the oligo-dT particles (i.e. open Poly-T sites) or to the bacterial complementary sequence on the oligo-nucleotide probe the following experiments were performed.

6.3.2 Materials and methods

Stored highly ^{13}C enriched *E. coli* RNA was defrosted and prepared in 12 x10µl aliquots (500µg) with 10µl of standard hybridisation buffer (40% formamide). RNA was hybridised at 70^0C for 10 min then allowed to rest at room temperature.

In parallel, six eppendorfs were also prepared with 1.2mg of oligo-dT particles in 200µls of buffer having been washed three times in ultrapure water (‘no probe’). A further 6 eppendorfs were prepared with 1.2mg of oligo-dT particles in 200µls of buffer which then had 1µl of CA-clamp added to each eppendorf (‘open clamp’). Oligonucleotide probes were not added to any of the eppendorfs. All RNA mixtures and particles were then incubated in parallel overnight at 25^0C.

The following day the contents of the 12 RNA eppendorfs were added sequentially to the particle solutions. This gave six pots with a 'no probe' set up for binding (figure 49) and six pots with an 'open clamp' set up (see figure 50). RNA particle mixtures were incubated together at 25^0C for 2hr before being removed. The particles were then captured by magnet and washed three in ultrapure water before being resuspended in 50µls ultrapure water. Three eppendorfs from each arm of the experiment were placed in tin boats and particles dried in an oven at 40^0C for 2hr. A further three sets of particles were placed in the peltier theromocycler and harvested at 75^0C for 6min. The resultant particles captured with a magnet and the 50µl supernatant kept for analysis. The particles were washed once more with 50µls ultrapure water to give a total of 100µls liquid sample for analysis. The post harvested particles were resuspended in 50µls of ultrapure water and dried in tin boats as before (figure 51). All particle samples were then analysed by EA-IRMS and the liquid samples were analysed by LC-IRMS.

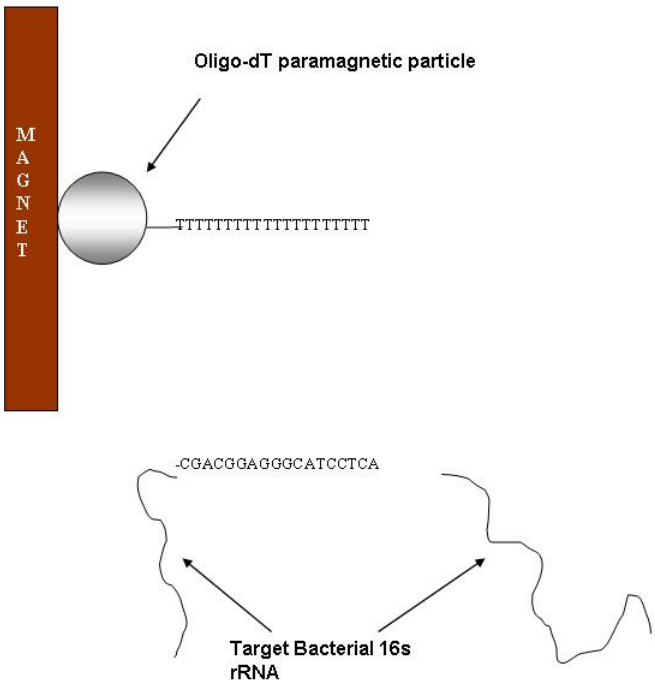

Figure 49: Schematic representation of the 'no probe' experiment

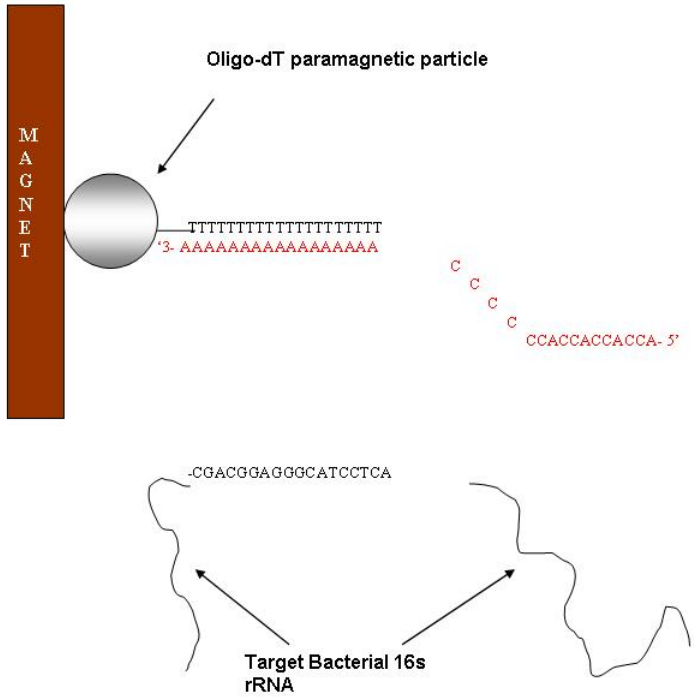

Figure 50: Schematic representation of the 'open clamp' experiment

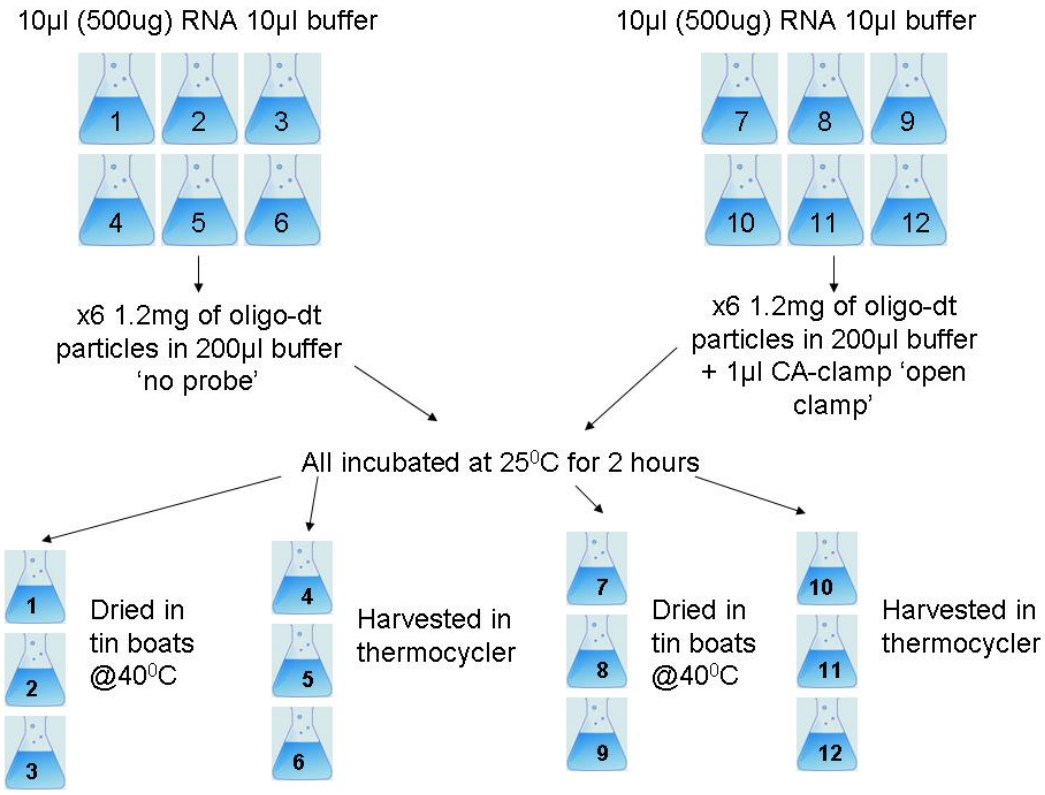

Figure 51: Schematic of 'no probe' versus 'open clamp' experiment

6.3.3 Results

Both the 'no probe' and 'open clamp' arms of the experiment yielded similar amounts of total carbon. Enrichment above a nominal baseline was detected, but these yields were very low in comparison to the high enrichment of total nucleic acids and in comparison to the per protocol yields from the previous experiment (figure 52). No significant difference was detected in enrichments between the two arms of the experiment. Analysis of the particles did not detect any significant differences in enrichment between particles that had been dried immediately and those that had been harvested via the thermocycler (not shown).

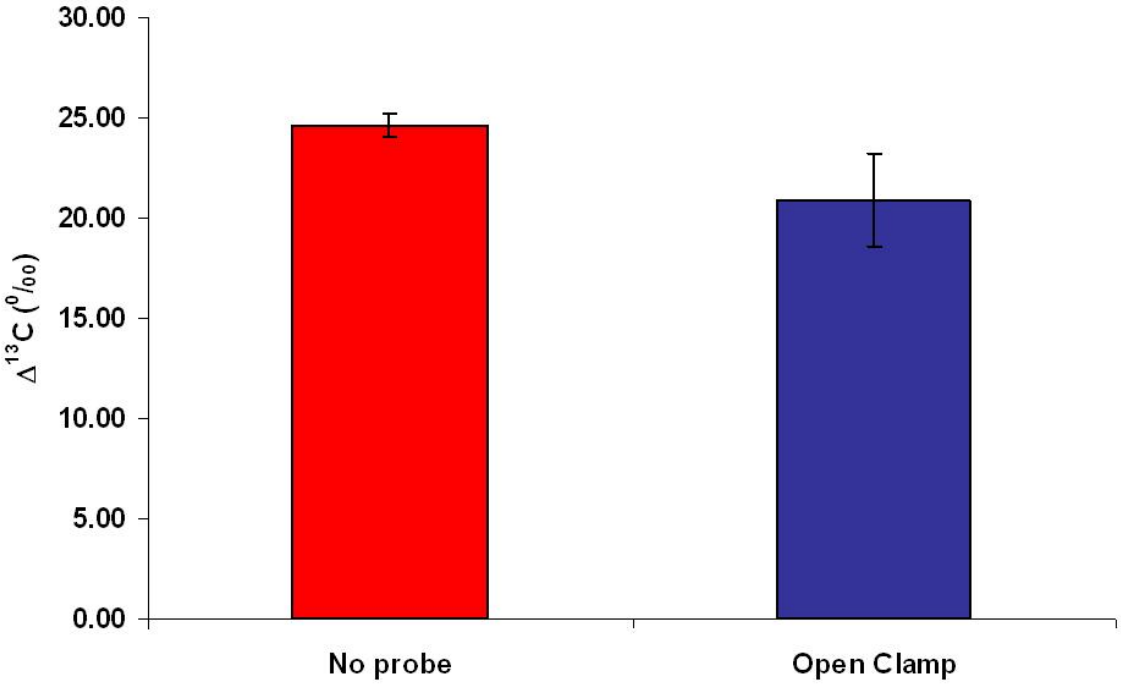

Figure 52: ^{13}C enrichments $\Delta^{13}C$ ($^{0}/_{00}$) for 16s rRNA yielded from the 'no probe' vs. 'open clamp' experiment (NS)

6.3.4 Conclusions

Enrichments of nucleic acids in this experiment were consistently lower than total RNA enrichments in comparison to the previous experiments. This suggested that if non specific binding occurred then in it might in part require the bacterial probe to facilitate binding. In order to determine that 'per protocol' methods yielded greatest enriched material further experiments were performed.

6.4 Pure Culture Experiment 3: No Clamp vs. per Protocol Comparator

6.4.1 Aims

To analyse the potential for non specific binding of probe and other RNA to Poly-T sites and to test the 'per protocol' approach as the optimal method to yield the highest enrichment of ^{13}C from total RNA.

6.4.2 Materials and Methods

Stored highly ^{13}C enriched *E. coli* total RNA was defrosted, prepared into six 50µl (2.5mg) aliquots and then hybridised at 70^0C for 10min with 50µl of hybridisation buffer (40% formamide). After equilibrating at 25^0C for 30min 5µls of modified *E. coli* probe was added to each eppendorf and then incubated at 25^0C under gentle mixing.

In parallel six eppendorfs were prepared with 400µl (4mg) of oligo-dT paramagnetic particles. Three eppendorfs were simply washed three times in ultrapure water, resuspended in 800µls of hybridisation buffer and then incubated over night at 25^0C ('no clamp') (figure 53). The second set were modified per protocol; after washing three times in ultrapure water they were resuspened in 800µls of hybridisation buffer and 5µl of CA-Clamp probe were added to each eppendorf and incubated overnight at 25^0C. The particles were washed three times with ultrapure water to remove any unbound CA-Clamp and then resuspended again in 800µl of fresh hybridisation buffer. RNA solutions were then sequentially added to the particles eppendorfs and incubated together for 2hr. All particles were then captured by a magnet removed from solution and washed three times in ultrapure water before being resuspended in 200µls of ultrapure water. The particles were then all divided into two 100µl aliquots, the first half being dried in tin boats at 40^0C for 2hr, the second half for harvest in the thermocycler. The particles for thermocycling were harvested at 75^0C for 6min and the 100µl supernatant was removed and the particles were washed with a further 100µl of ultrapure water which was also kept (giving a total of 200µls for analysis by LC-IRMS. The particles post thermocycling were also resuspended in 100µls ultrapure water and then dried in tin boats as before (figure 54). All stored particles were then analysed by EA-IRMS.

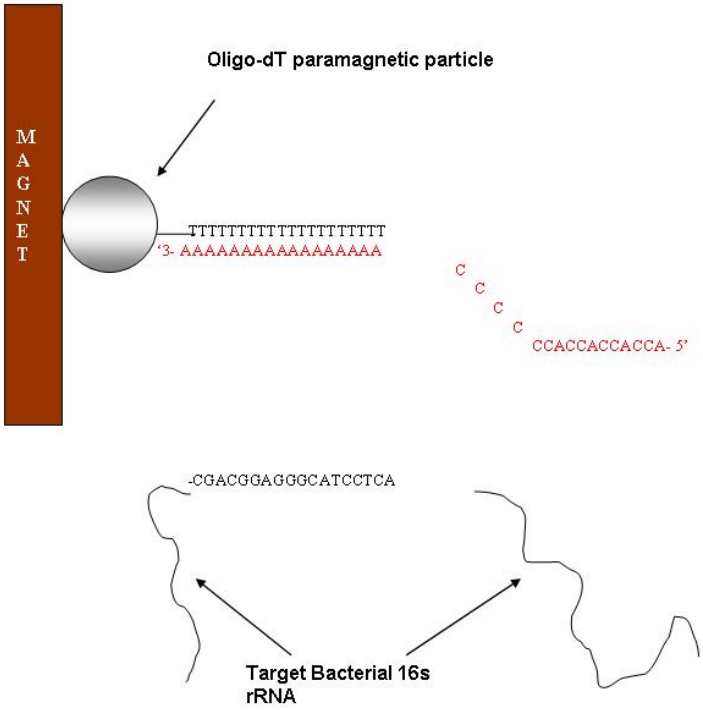

Figure 53: Schematic representation of the 'no clamp' experiment

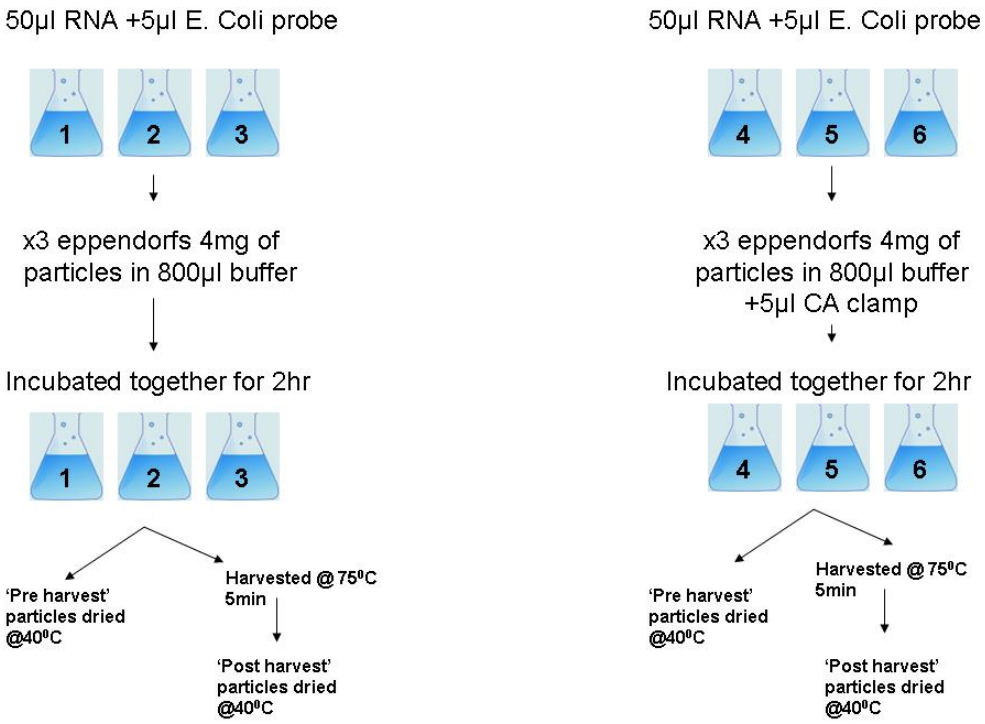

Figure 54: Schematic for 'no clamp' versus 'per protocol' experiment

6.4.3 Results

Both arms of the experiment yielded similar amounts of carbon. Significant enrichments of ^{13}C were seen above baseline in both experiment arms and these were significantly higher in the 'per protocol' arm (figure 55). Analysis of the oligo-dt particles revealed very modest enrichments of ^{13}C (in comparison to total C of the particles). These were greater in the pre harvest samples and the highest enrichments were in the 'per protocol' samples (figure 56).

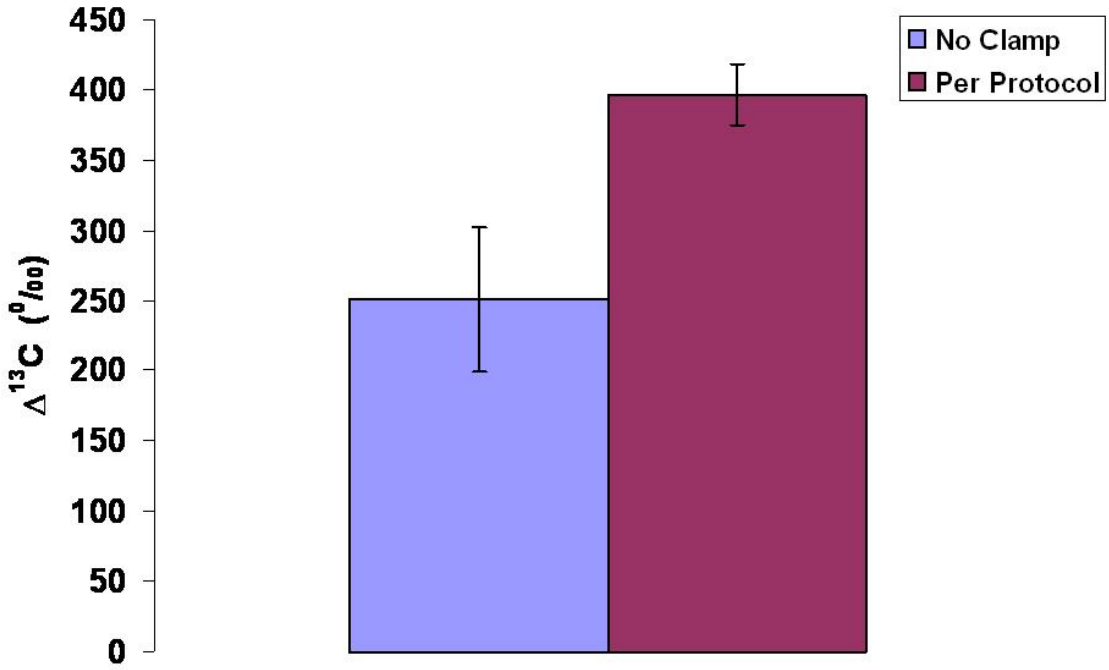

Figure 55: Mean (SEM) ^{13}C enrichments (parts/mille) of 16s rRNA samples from 'no clamp' and 'per protocol' experiments

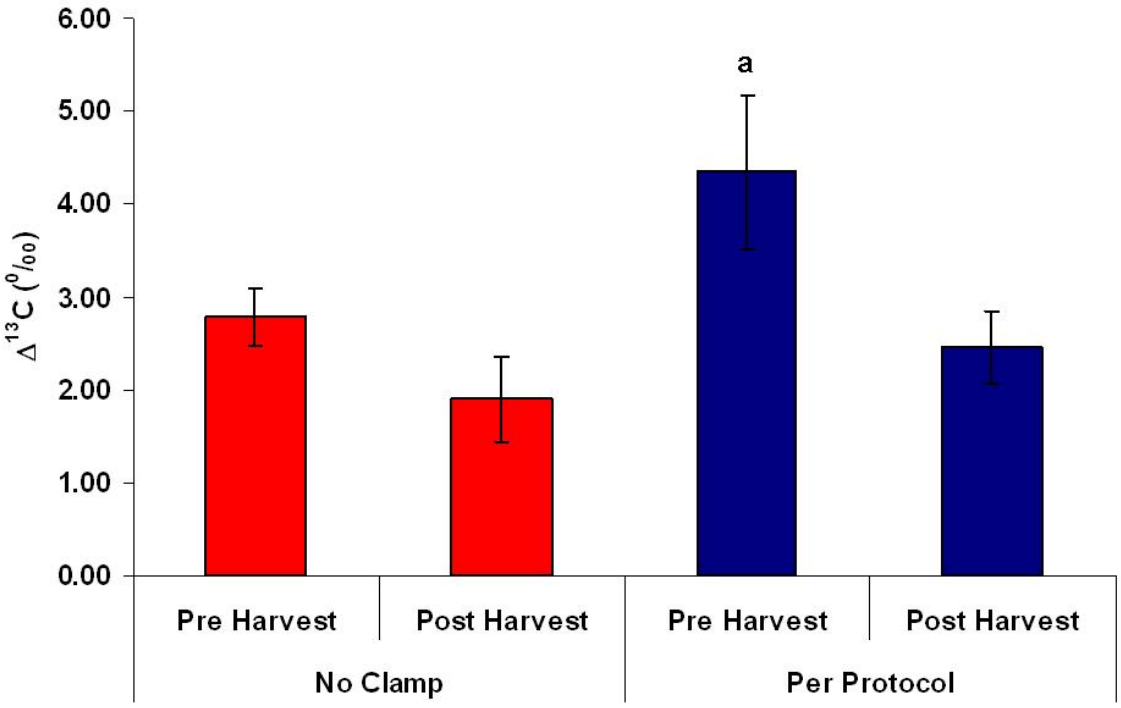

Figure 56: ^{13}C enrichments $\Delta^{13}C$ ($^{0}/_{00}$) of oligo-dt particles from the 'no clamp' and 'per protocol' experiments, before and after harvesting in the Peltier thermocycler

a= significantly greater incorporation than post harvest $p<0.05$

There was a significant difference in the pre harvest ^{13}C incorporation between the 'no clamp' and the 'per protocol' experiment with it being highest in the per protocol arm (figure 57). There was also a significant reduction in the particle enrichments between pre and post harvest in the 'per protocol' arm. Comparisons of all four recently used variations of the protocol revealed that the 'per protocol' arm yielded significantly ^{13}C incorporations than all other methods (figure 58).

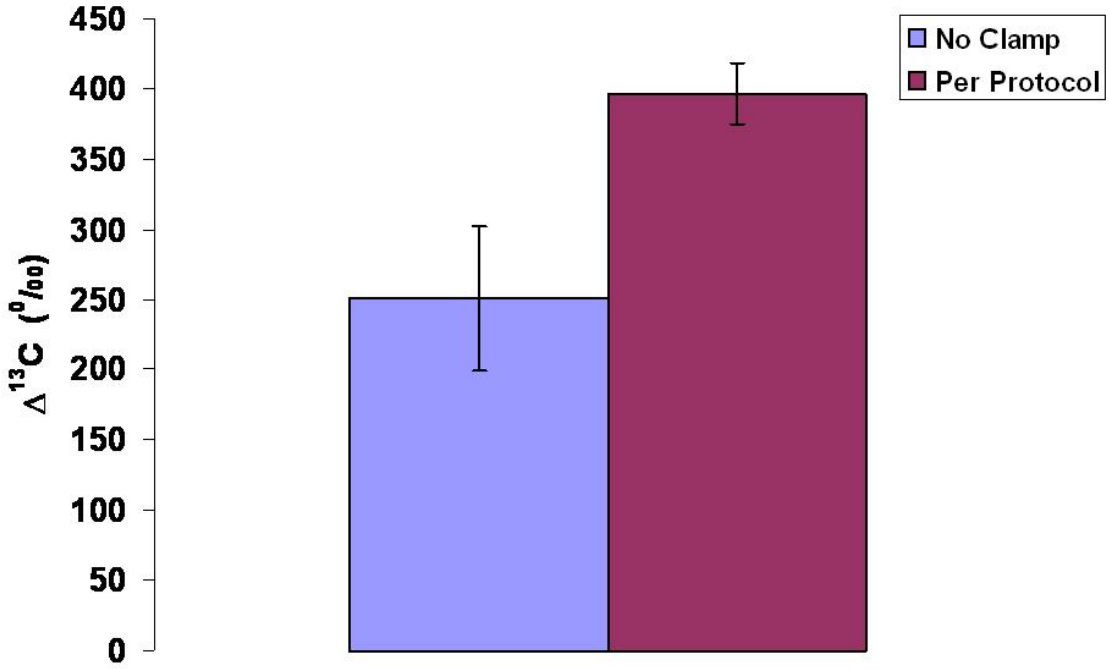

Figure 57: Mean (SEM) ^{13}C incorporations $\Delta^{13}C$ ($^{0}/_{00}$) in pre harvest particles in 'no clamp' and 'per protocol' arms

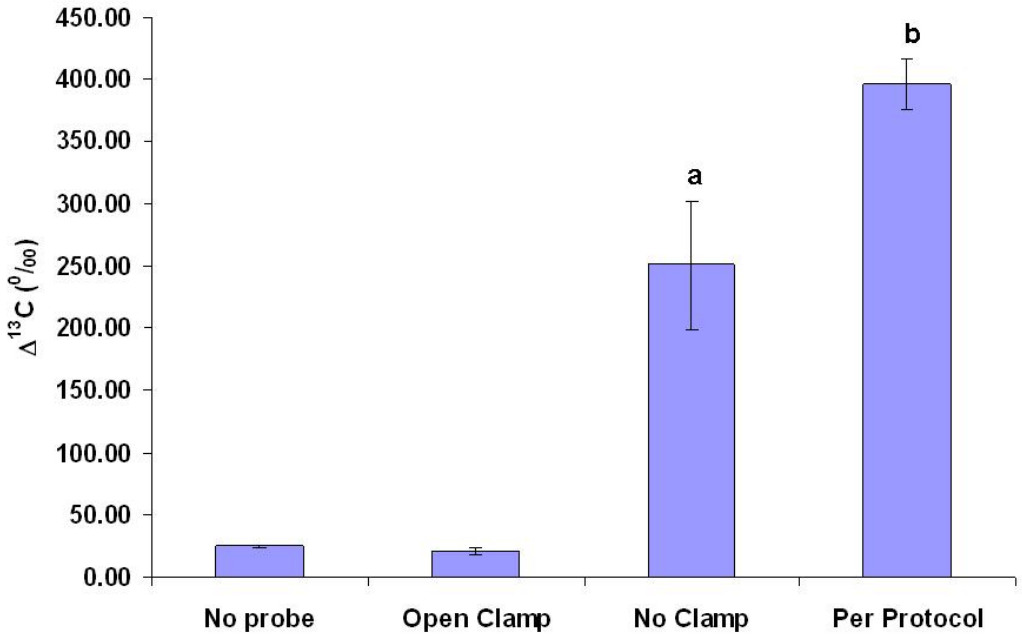

Figure 58: ^{13}C incorporations $\Delta^{13}C$ ($^{0}/_{00}$) across 'no probe', 'open clamp', 'no clamp' and 'per protocol' experiments

a= significantly greater incorporation than 'no probe' and 'open clamp'p<0.05, b= significantly greater incorporation than 'no probe' and 'open clamp' p<0.01

6.4.4 Conclusions

The combination of results from this and the preceding experiment demonstrated that enrichment was greatest in the 'per protocol' methods (figure 59).The experiments have shown that the 'per protocol' methods are the most reliable for obtaining consistently enriched RNA which is in proportion to total RNA. However it was not possible to consistently demonstrate species specificity in binding. However all binding appeared to be affected by the presence of all components of the 'double probe' technique. It is possible that mRNA may interfere with binding by interference with available poly-T sites (i.e. those not taken up with a CA-Clamp after hybridisation).

6.5 Pure Culture Experiment 4: Poly-A Wash

6.5.1 Aims

To examine the effects of non-specific binding of mRNA, or other material to Poly-T sites on paramagnetic particles, as a potential for hindering probe specificity of SIP protocol.

6.5.2 Materials and Methods

18 aliquots of highly ^{13}C enriched *E.coli* total RNA were prepared in eppendorfs with 50µls (2.5mg) of RNA and 50µl of hybridisation buffer (40% formamide). These were hybridised at 70^0C for 10min and then at 25^0C for 30min. Nine of these eppendorfs were prepared 'per protocol' (each with a parallel eppendorf of 4mg paramagnetic particles washed three times, hybridised with 5µl (1.78nmol) of CA-clamp, washed again three times and then suspended in 800uls of hybridisation buffer) three with 5µl (1.78nmol) of modified *E. coli* probe (CCCCCCTTTGGTCTTGCCCCCCGGGTGGGTGGGTGGGTGGG), three with modified eubacterial probe (Bact-338) and three with modified *Bifidobacterial* probe (Bif-164).

For the Poly wash arm x9 eppendorfs of RNA/buffer were prepared as the per protocol arm (3 modified *E.coli*, 3 Bact 338, 3 Bif 164). After washing, hybridisation with CA-Clamp and re-washing, the paramagnetic particles were then hybridised again to a second oligo-nucleotide probe 5µl (1.78nmol) 'poly-A' (AAAAAAAAAAAAAAAAAAAAAAAA). This was done at 25^0C with gentle mixing overnight. After this second step the particles were then washed three times before being resuspended in 800µls of hybridisation buffer. All 18 RNA mixtures were added to their paired particles and incubated together at 25^0C for 2hr. The particles were then removed, washed three times and then resuspended in 100µls ultrapure water for harvesting in the peltier thermocycler. This was done at 75^0C for 6min (figure 59). Particles were washed once more with 100µls of ultrapure water to give a total of 200µls supernatant. Analysis of samples for relative ^{13}C encorporation was by LC-IRMS.

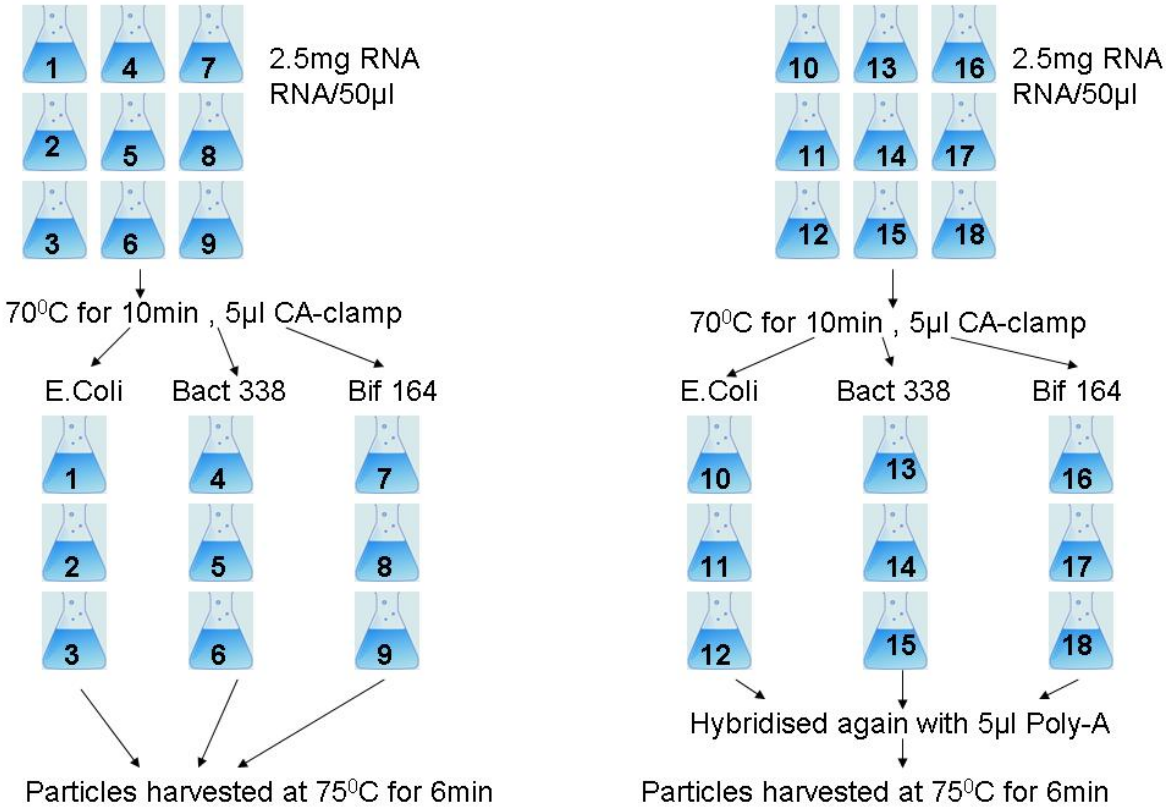

Figure 59: Schematic for 'poly-A' experiment

6.5.3 Results

All samples demonstrated modest ^{13}C enrichments above baseline (figure 60) which was on scale with total RNA (8:1). There was no significant difference between the poly-A experiment and the 'per protocol' arms in terms of ^{13}C incorporation. In the poly-A arm there was, again, the suggestion that enrichments were highest in the Bif-164 samples.

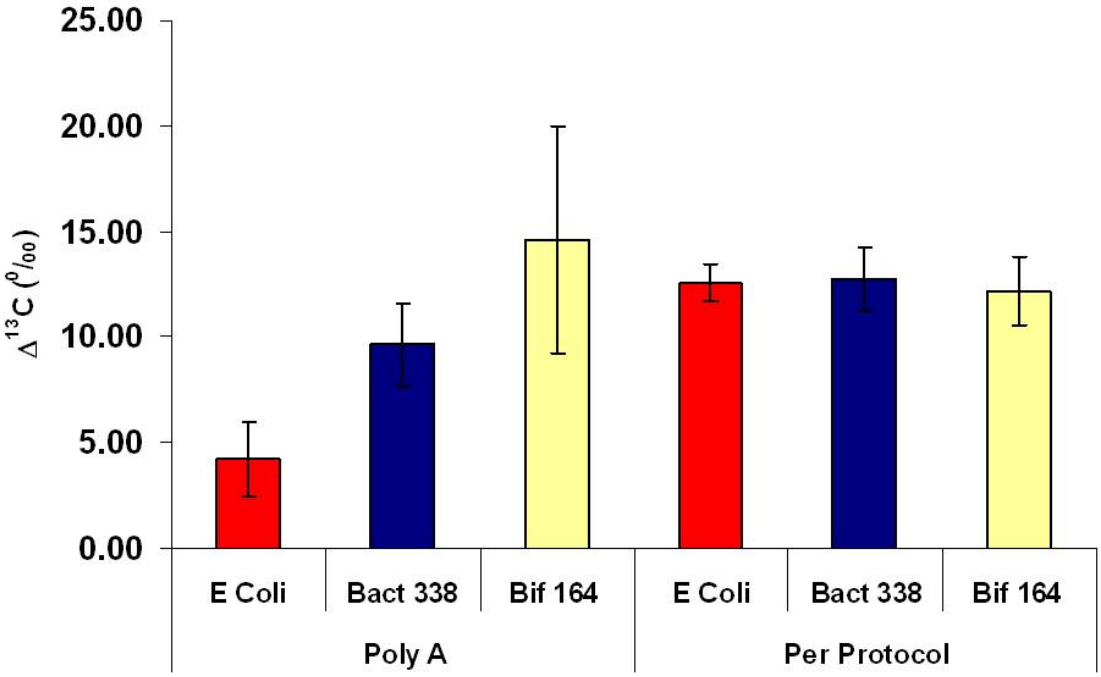

Figure 60: Comparison of $\Delta^{13}C$ ($^{0}/_{00}$) enrichments using the 'poly-A wash' technique versus 'per protocol' (parts/mille) of 16s rRNA using modified *E.coli*, eubacterial and *Bifidobacterial* probes

6.5.4 Conclusion

Species, or probe specific, specificity was not demonstrated. Again there was a suggestion that enrichments were greater in the *Bifidobacteria* probed specimens. This was counter to our understanding of the probe capture technique. The lack of effect of adding the poly-A wash step the protocol demonstrates that these limitations in the protocol are not related to non specific binding of mRNA alone.

6.6 Conclusions of Pure Culture Experiments

The current research on pure cultures demonstrated that ^{13}C isolation and analysis of bacterial RNA was possible, and enriched material could be reliably isolated. Highly enriched supernatants were isolated, only from highly enriched components of bacterial RNA using this *E. coli* model. Although not optimised the 'per protocol' method yielded significantly better results than other variations carried out suggesting a degree of specificity in the binding of material to the double probe. In addition demonstration of a reduction of enrichment of particles after thermocycler harvesting suggested some efficiency of the disassociation methods.

Repeated analysis failed to resolve dilution of signal and specificity of binding, particularly at a group level. As the purity of the startingRNA product was confirmed by gel electrophoresis and UV spectrometry, it was presumed that starting material was relatively pure bacterial RNA; therefore contaminants could have come only from reagents used in the protocol.

Buffer constituents or other components of the RNA preparation are unlikely to bind to poly-T sites significantly and contamination with these is unlikely as particles were triple washed on two occasions prior to analysis. The melting temperature of probes, as opposed to the bacterial RNA, may vary according to base pair length and GC content of 16s rRNA and probes. However, even if harvest temperatures exceed probe melting points resulting in significant dilution with un-enriched probe material, it would be expected that the dilution factor would have been relatively constant when using the same type of probes. This was not the case as marked variations in enrichments between identical experimental conditions occurred. Non-specific binding of material remains an unanswered issue and is a likely source of loss of specificity. However it can be assumed that enrichment of other sources of RNA (mRNA) might achieve relatively uniform enrichment at a consortia level; again this would not fit with variations between identical experimental conditions.

Specificity of binding remained a key issue as, even with 16s rRNA oligonucleotide probes, target RNAs will come from a ‘range’ of sequences rather than one sequence. Recently work combining SIP with FISH analysis has shown that as more about unculturable bacteria subspecies is learnt, what were initially thought of as sub-group specific probes will isolate a consortia of related sequences (434). To learn more about the phylogeny of such bacteria the authors suggested that a panel of related probes be used to characterise relative binding specificity. Miyatake et al (435) have improved SIP methodology for specific functional SIP in marine sediments by applying oligonucleotide probes with additional probes that are complementary to the sequences directly upstream and downstream of target sequences. Such data suggest that designer oligonucleotides will never achieve absolute specificity, but rather an ‘optimal profile’ for considered work. Such relative specificity may hamper experiments where differential incorporation of tracer into related species are being studied.

Clearly this methodology of SIP has generated significant results, but further work to predictably enrich 16s RNA, improve ^{13}C signal above background and between groups and confirm groups/species specificity is required before SIP can be used to perform clinical observations in inflammatory diseases.

7 Conclusions and Future Research

The hypotheses formulated and tested by the research outlined in this thesis (section 1.15) were

1. Probiotics prevent NEC in at risk infants of very low birth weight (VLBW).

2. The human gut microbiota can be labelled by stable isotope probing (SIP) to measure metabolic activity.

3. Quantitative measurement of the metabolic activity of the unculturable gut microbiota is a useful way of studying changes in the microbiota, compared with measures of bacterial diversity, and may enlighten our understanding of bacterially mediated inflammatory stimuli in inflammatory gut diseases of childhood.

7.1 Summary of Principle Findings of the Systematic Review

The research described in chapter 2 has demonstrated that:

- The administration of prophylactic enteral probiotics to VLBW and <33wk infants appear to significantly reduce the risk of proven (Bell's stage ≥ 2) NEC.

- The administration of prophylactic enteral probiotics to VLBW and <33wk infants appears to significantly reduce all cause mortality.

- Insufficient data exist to determine whether probiotics reduce NEC related mortality in VLBW and <33wk infants.

- Greater numbers of subjects are required to determine the safety of enteral probiotics administration to VLBW and <33wk infants.

- Optimisation of specific type, dosage and timing of oral probiotics for the prevention of NEC is required.

The current evidence broadly supports the use of prophylactic enteral probiotics for the prevention of NEC in VLBW and <33wk infants. Yet this clinical application lacks the foundations of adequate knowledge of the principle mechanisms by which orally administered bacteria establish themselves as 'residents' in the gastrointestinal tract, their primary interactions with the host environment or their therapeutic mechanisms of action. Nevertheless, in conjunction with evidence from studies of infectious diarrhoea, atopic dermatitis (section 1.4) and pouchitis in IBD (section 1.8.3), this research supports the clinical applications for nutritional therapy. The gold standard of double blinded placebo trials, in testing food products in humans, are limited (as illustrated in sections 1.4 and 1.8.3), not only because of lack of volition by manufacturers to test them properly, but also because of difficulties in generating appropriate placebos for trials (436), or defining the active ingredient which may involve components or metabolites of bacteria. At present faecal markers, such as biochemical changes in stool parameters (SCFAs or Ig levels) are the only commonly used methods, yet the site of desired action of probiotics may be remote from site of administration within the gastrointestinal tract, or indeed in another organ systems of the body.

Probiotic studies to date lack scientific rigour; there is no standardisation of preparations and probiotics acceptance and scientific use is threatened by pressure from the commercial purveyors of these products as 'health foods'. The marketing of 'functional foods' or 'nutraceuticals' remains a contentious and difficult area to legislate. The 'General Food Law Regulation' of the European Parliament 2002 is complex and is ambiguous in determining whether certain products should be regulated under this act or by medicinal Law (437). In the USA the Food and Drug Administration defines live bacteria as 'live biotherapies' but this definition falls short of the standards required for the scientific definition of probiotic agents (438). Identification and enumeration of live bacteria in a complex matrix is difficult, time consuming and costly (439). The Fermented Milks and Lactic Acid Bacteria Beverages Association of Japan has set a minimum of 10^7 bacteria to be considered a 'dose' of a probiotic (440), although others have suggested dosages as low as 10^5 (441). At present there is little incentive for manufacturers of commercial products to adhere to pharmaceutical standards for commercially available produce (442). Where the possibility of untruthful or misleading marketing of products is possible the integrity of the science of probiotics as a whole suffers.

Man has been consuming bacterially augmented food since at least 3500BC (443), yet it is only now in the 21st century that the science of probiotics may have come of age. The establishment of the routine use of enteral probiotics for the prevention of NEC, as the

leading contender for clinical application, may serve as the crucible for the legitimisation of live microbial therapy within the medical community. However the establishment of probiotic therapy within the armoury of clinical therapies requires recognition of the threats posed by the commercialisation of this science and the adherence to the required principles outlined namely; identification, enumeration; mechanisms of action (both *in vitro* and *in vivo*), efficacy and safety in well designed double blind placebo trials in human subjects.

7.2 Novel Techniques to Sample the Gut Microbiota and SIP

The research described in chapters 3-6 of this thesis represented a logical series of experiments that have demonstrated that:

- Faecal bacterial RNA can be reliably obtained from human faecal samples (section 3.1).

- Faecal bacterial RNA can be enriched with sufficient ^{13}C for detection by IRMS (section 3.2).

- ^{13}C enrichment of bacterial total RNA in human faecal samples can be modified predictably with stimuli for metabolic activity (section 3.2), and that these changes in incorporation have been validated against another marker of bacterial metabolic activity (3.3).

- Capture of bacterial 16s rRNA from human faecal samples is possible using oligo-dT particles and a double probe capture technique (sections 4.5-4.6).

- ^{13}C incorporations can be detected in 16s rRNA and correlated with total RNA incorporation (section 5.1).

- ^{13}C urea was the most reliable candidate tracer for SIP experiments at the RNA and 16s rRNA levels (section 5.1).

- 16s rRNA SIP of human faecal samples currently lacks species specificity to identify group specific changes in ^{13}C incorporation (Section 6.2).

- Optimal ^{13}C incorporation using the techniques described achieved some specificity in overall binding capacity (section 6.4).

Methods to study the gut microbiota have expanded rapidly in the last five years (as detailed in sections 1.9-1.12). Novel culture techniques, such as microbead culture, allow simultaneous single cell culture of multiple individual microbes (444), expanding our knowledge of the contents of this complex ecosystem. Yet the majority of these bacteria remain uncultured.16s rRNA has been key to the characterisation of unculturable bacteria and has enlightened us to the wealth of information that remains undisclosed about the gut microbiota. The development of 'high throughput' methods of community profiling such as FISH-MAR and microarray has demonstrated correlations between host factors and microbial populations (445). However, defining the metabolic consequences of these bacteria remains beyond the scope of such methods. The birth of meta 'omic' sciences has led to insights into the activity of unculturable bacterial communities. Here screening for biomarkers of activity in DNA, mRNA or protein metabolites can define the metabolic 'potential' of a bacterial community (446). However analysis of these markers still requires the analysis of functionality outwith the ecosystem, such as by transference of genes to vectors such as *E. coli* (447). SIP, in concert with other meta 'omic' approaches shows the potential to link the metabolic activity of the gut microbiota to diversity both *in vitro* and *in vivo*.

The hypothesis that changes in bacterial metabolic activity can be used as a disease marker in faecal samples (section 1.15) has been advanced by the research described in this thesis by the application of SIP-IRMS to human faecal microbiota. The principle of achieving ^{13}C incorporation into faecal bacterial RNA by virtue of their metabolic functioning was reliably demonstrated. The use of probe specific hybridisations and LC-IRMS analysis has overcome the requirement for high levels of ^{13}C enrichment of bacterial RNA for separation of isotope labelled RNA or DNA by centrifugal SIP methods (362;367). This order of magnitude of improvement in sensitivity allows the study of changes of bacterial metabolic activity according to stimuli in concert with other markers of bacterial metabolic activity in human faecal samples. The scale of such incorporation is such that experiments could be envisaged in ecosystems where there is a large flux of substrate and subsequent dilution of tracer, such as the human large bowel. SIP is uniquely placed to link function to phylogeny without the confounders of colonocyte metabolism and *in vitro* effects of metabolite analysis. The use of ^{13}C urea as a tracer for delivery of labelled carbon to bacterial RNA shows promise as a reliable marker of activity which is cheap, safe and deliverable to the human gut microbiota *in vivo*. The use of a novel 'double probe'

oligonucleotide probe capture technique for isolation and extraction of 16s rRNA with *a priori* knowledge of bacterial groups opens up the potential study of the changes of metabolic activity of individual species or higher level of taxa within complex consortia. Differences between the whole consortia incorporations and *Bifidobacteria* were demonstrated and appeared to relate to stimuli for metabolic activity. Such individual changes may be important in terms of substrate availability for the whole ecosystem or metabolite effects on the responses of the gut immune system.

The complexity of these interactions will undoubtedly require observations of bacterial numbers and activity to study effects on the host. As new candidate anti-inflammatory bacteria are identified by molecular techniques (448), their phylotype can only be inferred by genetic sequencing. Likewise the metabolic profile that infers such properties will need to be linked to culture independent technology. It is the combination of these techniques that will allow us to determine how bacteria respond to their environment and how these metabolic reponses confer protection or risk in diseases such as IBD.

7.3 Current Limitations in SIP and Immediate Research Agenda

The methods developed in chapters 3-6 have shown that it is possible to reliably obtain ^{13}C enriched bacterial RNA on a scale appropriate for analysis by LC-IRMS. These enrichments have been demonstrated to be related to biochemical stimuli for metabolic activity and have been validated against other markers of metabolism in *in vitro* models. The current methods, as yet, are unable to clearly demonstrate specificity at bacterial group level whilst utilising 16s rRNA probe capture technology. The complexity of the SIP lies in the difficulty in hybridising, isolating and harvesting specific sequences of lengthy RNA (~1500 base pairs). However the varying yields of ^{13}C in 16s rRNA experiments suggests that there are factors other than the limitations of binding capacity in the current SIP methodology. While there are several potential rate-limiting steps which remain unidentified, optimisation of the ^{13}C signal at the16s rRNA level needs to be achieved, as dilution by unenriched carbon to the degree seen these experiments will hinder analysis of genuine changes in metabolic activity. Greater specificity of oligonucleotide binding is also required to enable valid observations at the species specific level.

The immediate future work into the optimisation of SIP should focus on hybridisation of RNA and hybridisation conditions, particularly melting points of probe binding and

capture particles. Probe efficiency may also be improved by establishing methods to reduce non specific binding of non 16s rRNA to oligonucleotide probes. With optimisation of the current SIP protocol from as little as 6g of faecal material would yield adequate RNA to perform multiple probe capture experiments. Using probes for the ten dominant bacterial phylotypes in human faeces, >95% gene coverage of samples enabling analysis of major group changes in activity in comparison to the whole consortia. Such a scale would make the possibility of studying diseases using small stool samples from children. Analyses of both species-specific changes and the consortia in patients with IBD in relapse and remission versus controls would be the immediate goal of a programme of research using methods. Success with such an approach would provide the platform for the study of gastrointestinal diseases where the gut microbiota are important in the aetiology such as NEC and allergic diseases.

7.4 Towards SIP in vivo

The opportunity to apply SIP *in vivo* will emerge as the methodology and applications mature and the obstacles encountered in *in vitro* studies are overcome. The technical challenges involved in enriching and isolating 16s rRNA are all resolvable. Progress will be driven by the recognition that the complex environmental and immunological factors which influence bacterial activity *in vivo* are difficult to replicate *in vitro* or in animal models. The potential to deliver a SIP tracer to an appropriate site within the gut will lead to the opportunity to biopsy or collect luminal and/or faecal contents and link metabolically active phylogenic groups through their degree of nucleic acid enrichment. This key goal requires validation, but in due course may reveal the functional significance of different groups of bacteria in inflammatory and other gastrointestinal diseases.

Fuller understanding of the metabolic activity of the gut microbiota in IBD could ultimately lead to new, targeted therapies. For instance the delivery of ‘designer’ non-digestible carbohydrates that select for and induce the proliferation of particular bacterial species in the colon could modulate the microbiota in favourable ways through the production of anti-inflammatory products with cyto-protective and mucosal-healing properties. The identification of unculturable bacteria as anti-inflammatory or probiotic may in turn be delineated by culture independent assays of function. Such an approach requires integration of our growing understanding of the metabolic activity and diversity of the gut flora within the trinity of aetiological factors that include the host genotype and immune response (see section 1.6). Current research is transforming the simple interrelations of genetics, immunology and gut microbiota into a hierarchical network of

metabolic pathways, immune interactions and genetic regulations (figure 61) which represents a real working hypothesis upon which we can continue to develop focussed studies and targeted therapies exploiting the role of the microbial flora in IBD.

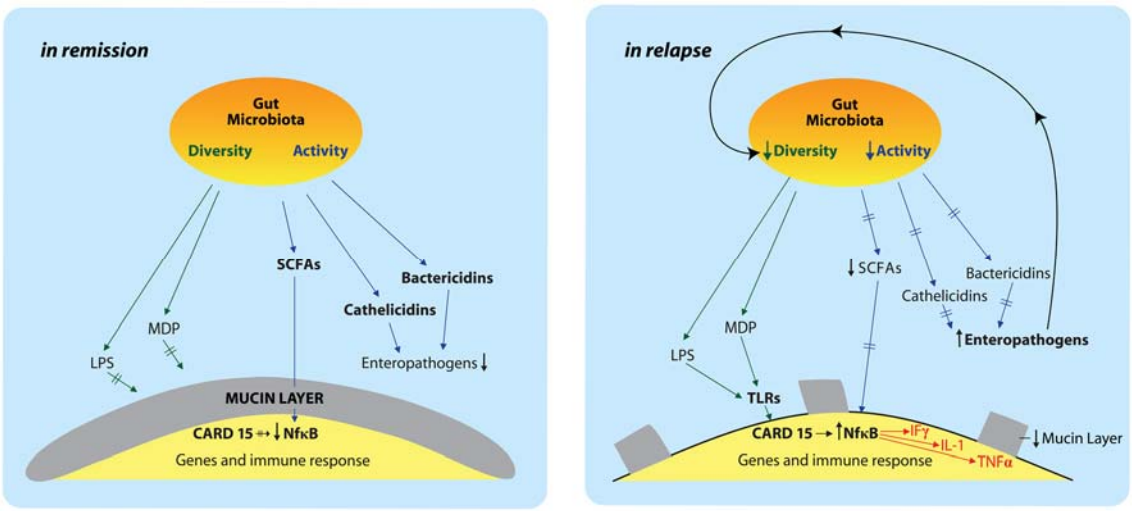

Figure 61: Hypothesised interrelationship between bacterial diversity and activity in relapsing IBD

Appendices 1: Initial 16s rRNA probe capture protocol

Step 1 ***Faecal enrichment***

a. 6g fresh stool + 30mls sterile PBS

b. Homogenise for 2 min (max speed) in stomacher

c. Centrifuge at 20,000g for *30* minutes.

d. Remove as much liquid as possible but do not disturb the creamy layer

e. Vortex the pellet vigorously for at least 1 minute

Step 2 **Total RNA recovery**

a. Remove all remaining liquid from 1e and transfer to a micro-centrifuge tube.

b. Add 1 volume (or as much as possible) of Prep-man ultra reagent.

c. Heat in boiling waterbath for 10 minutes and cool at room temperature for 2 minutes.

d. Centrifuge at 20,000g for 10 minutes.

e. Transfer supernatant to a fresh 2ml micro-centrifuge tube.

f. Add an equal volume of phenol: chloroform: isoamylalcohol (25:24:1)

g. Mix (by inversion) until emulsion forms.

h. Centrifuge at 20,000g for 5 mins until the phases are well separated.

i. With a pipette, remove the aqueous phase (not yellow coloured layer)

j. Repeat stage f-i two times removing as much protein as possible.

k. Add an equal volume of chloroform and repeat steps g-i.

l. To the resultant sample add 0.1 volumes of 3M-sodium acetate and 2 volumes of ethanol.

m. Incubate on ice for 30 minutes.

n. Recover nucleic acid by centrifugation at >10,000g (max speed) for 15 mins at 4°C

o. Remove liquid and dry the pellet by leaving open on petri dish with cover over.

p. Re-suspend the pellet in 266 of TE buffer

Step 3 ***Removal of DNA***

a. Add 30µls of 10x Reaction buffer
b. Add 40 µls of DNAse ONE (1 U/ µl)
c. Mix and incubate for 1 hour at 37°C
d. Remove enzyme by ethanol precipitation. (See step 2L to 20) and resuspend in 300 µls of ultrapure water

SStep 4 ***Hybridisation***

50uls of total RNA solution is mixed with 50uls of 2 times hybridisation buffer. This is made up as follows:

5mls of 20 x SSC buffer	10x
20 µls of N-Lauroyl sarcosine solution	0.2%
20 mgs of NaCl	0.2%
4 mgs of SDS	0.04%
4 mls of formamide	40%

Made up to lOmIs with ultrapure water.
This is hybridised at 70°C for 10 minutes then incubated at room temperature for 30 minutes.

Biotinylated capture probe is added (1.78nmols) 5 µls and the hybridisation mixture is incubated overnight at room temperature under gentle mixing.

Step 5 ***Harvesting of 16S rRNA by Magnetic Separation.***

a. Harvesting is performed using 8.9mg of M-280 paramagnetic streptavidin coated beads (Dynal Biotech, Wirral, UK). Take the amount of beads that you need. I.e. At 10mg beads/ml that is 890uls per reaction and wash them in water using the magnets for separation. **Discard the supernatant each time.** Do this three times

b. Re-suspend after washing in 900 µls of hybridization buffer without formamide., I.e. substituting water for the formamide part of the buffer.

c. Add 900 µls of beads to the 105 uls of RNA solution from Step 4 and incubate on rotation for 2 hours at room temperature.
d. Wash the beads 3 times in equal volumes of ultrapure water, **discarding the supernatant** each time and then add 100 µls of water after the final wash.
e. The 16S rRNA is harvested by incubation at 90°C for three minutes followed by the magnetic removal of the beads. **The supernatant contains the purified RNA.**

f. Add 100 µls of product to a pre-weighed tin capsule and dry at 50°C. Weigh capsule afterwards.

Appendix 2: Final 16s rRNA probe capture protocol

Step 1 ***Faecal enrichment***

f. 6g fresh stool + 30mls sterile PBS

g. Homogenise for 2 min (max speed) in stomacher

h. Centrifuge at 20,000g for *30* minutes.

i. Remove as much liquid as possible but do not disturb the creamy layer

j. Vortex the pellet vigorously for at least 1 minute

S

Step 2 **Total nucleic acid recovery**

q. Remove all remaining liquid from 1e and transfer to a micro-centrifuge tube.

r. Add 1 volume (or as much as possible) of Prep-man ultra reagent.

s. Heat in boiling waterbath for 10 minutes and cool at room temperature for 2 minutes.

t. Centrifuge at 20,000g for 10 minutes.

u. Transfer supernatant to a fresh 2ml micro-centrifuge tube.

v. Add an equal volume of phenol: chloroform: isoamylalcohol (25:24:1)

w. Mix (by inversion) until emulsion forms.

x. Centrifuge at 20,000g for 5 mins until the phases are well separated.

y. With a pipette, remove the aqueous phase (not yellow coloured layer)

z. Repeat stage f-i two times removing as much protein as possible.

aa. Add an equal volume of chloroform and repeat steps g-i.

bb. To the resultant sample add 0.1 volumes of 3M-sodium acetate and 2 volumes of ethanol.

cc. Incubate on ice for 30 minutes.

dd. Recover nucleic acid by centrifugation at >10,000g (max speed) for 15 mins at 4°C

ee. Remove liquid and dry the pellet by leaving open on petri dish with cover over.

ff. Re-suspend the pellet in 266 of TE buffer

Step 3 ***Removal of DNA***

d. Add 30µls of 10x Reaction buffer
e. Add 40 µls of DNAse ONE (1 U/ µl)
f. Mix and incubate for 1 hour at 37°C
d. Remove enzyme by ethanol precipitation. (See step 2L to 20) and resuspend in 300 µls of ultrapure water

Step 4 ***Hybridisation***

50uls (2.5mg) of total RNA solution is mixed with 50uls of 2times hybridisation buffer. This is made up as follows:

5mls of 20 x SSC buffer	10x
20 µls of N-Lauroyl sarcosine solution	0.2%
20 mgs of NaCl	0.2%
4mgs of SDS	0.04%
4 mls of formamide	40%

Made up to lOmIs with ultrapure water.
This is hybridised at 70°C for 10 minutes then incubated at room temperature for 30 minutes.

Biotinylated capture probe is added (1.78nmols) 5 µls and the hybridisation mixture is incubated overnight at room temperature under gentle mixing.

Step 5 ***Harvesting of 16S rRNA by Magnetic Separation.***

a. Harvesting is performed using 4mg of novagen Oligo-dt particles . Take the amount of beads that you need. I.e. At 10mg particles/ml that is 400uls per reaction and wash them in water using the magnets for separation. **Discard the supernatant each time.** Do this three times.

b. Re-suspend after washing in 800µls of hybridization buffer without formamide., I.e. substituting water for the formamide part of the buffer.
c. Particles are hybridised with 1.78nmols 5µls of CA-Clamp modified probe by inucabation overnight at room temperature under gentle mixing.

d. Add 805µls of particles to the 105 uls of RNA solution from Step 4 and incubate on rotation for 2 hours at room temperature.
e. Wash the partilces 3 times in equal volumes of ultrapure water, **discarding the supernatant** each time and then add 100 µls of water after the final wash.
e. The 16S rRNA is harvested by incubation at 75°C for 6 minutes followed by the magnetic removal of the particles. **The supernatant contains the purified RNA.**

f. 100ul supernatant is removed and particles are washed with a further 100uls of water, which is removed to give a final 200ul sample for analysis.

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