A Study of the Intestinal Microbiota in Health and Disease

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ABSTRACT

The intestinal microbiota is a massive and complex community, essential to the human host for good health and well-being. However, this population has been associated with gastrointestinal disease, and remains poorly understood. The aim of this study was to develop and validate DNA-based assays for the intestinal microbiota and to apply these methodologies to faecal samples collected from healthy volunteers and patients with gastrointestinal disease. Over 250 faecal samples were analysed using temporal temperature gradient gel electrophoresis (TTGE) and real time PCR. Validated assays had high sensitivity and reproducibility. Healthy individuals displayed a high level of temporal stability during short term studies (≤ 6 weeks) and long term studies (1-4 years). Analysis of faecal samples provided by identical and fraternal twins demonstrated an influence of host genetics over the composition of the predominant bacteria in children. Two intervention studies, bowel lavage and the Atkins' diet, were carried out to monitor the impact of environmental change on the population's stability in healthy volunteers. Following bowel lavage, microbial populations rapidly recovered to control densities, however the stability of the population was disturbed. Introduction of the Atkins' diet, led to a significant change in the composition of the microbial population. A preliminary study of the intestinal microbiota in disease groups was undertaken. Significant differences were detected between inflammatory bowel disease groups and controls. Cluster analysis in these patients indicated a potential association between the composition of the predominant bacterial population and disease localisation. The studies reported here demonstrate that the faecal microbiota in healthy individuals is a highly stable population under the influence of both host genetics and environmental variables, however the population present in patients with inflammatory bowel disease exhibits differences compared to healthy controls.

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CHAPTER 1: INTRODUCTION

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1.1.1 HISTORICAL BACKGROUND

Van Leeuwenhoek first observed bacteria derived from the gut over 300 years ago in a faecal sample viewed under a microscope (Prescott *et al.*, 1999). Nearly two centuries later Louis Pasteur developed the Germ Theory of disease and also postulated that our health was related to the commensal organisms inhabiting our bodies (Schottelius M, 1902). However, during the 19th century the microbial population of the colon was regarded as having an adverse effect on the well being of the human host. Concepts such as 'autointoxication' suggested that toxins derived from bacterial protein fermentation were detrimental to health, and led to the practice of surgical removal of the colon. The surgeon Arbuthnott Lane even endorsed removal of healthy colons as a preventative measure (Parry, 2004). In 1907 Metchnikoff observed that Bulgarian peasants who consumed large amounts of fermented milk, had an extended life span (Metchnikoff, 1907; Metchnikoff, 1908). These findings prompted Metchnikoff to abandon surgical removal of the colon, and instead advocate the consumption of health promoting live bacteria to improve patient's health and life span (Metchnikoff, 1907; Metchnikoff, 1908). The faecal microbiota received little interest during much of the 20th century, with most microbiological research focused on pathogenic organisms. However in the late 1960s and 1970s interest returned to the indigenous colonic microbiota and their role in human health and disease.

1.1.2 THE RESIDENT MICROBIAL POPULATION

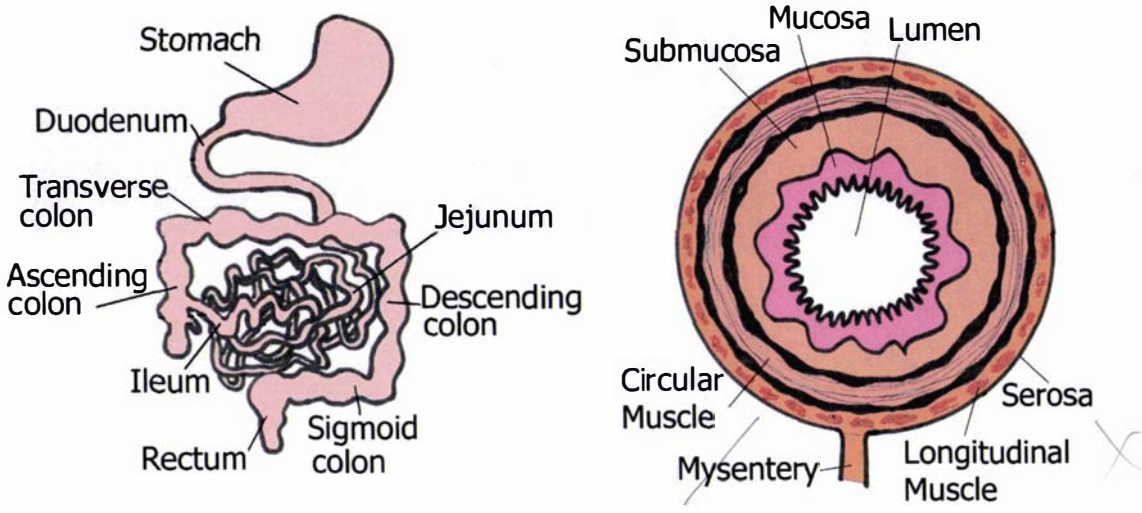
The resident microbial population inhabiting the gut is known as the intestinal microbiota. The intestinal microbiota is composed of autochthonous species that colonise the intestine and allochthonous species that are present only transiently. The

relationship between the host and the microbiota is one of mutualism, either commensalism or symbiosis. The population is complex with an estimated diversity of 400-500 species (Moore and Holdeman, 1974) derived from the kingdoms (domains) of *Bacteria* and *Archaea*. Oganisms derived from these kingdoms are referred to as bacteria and archaeobacteria respectively. Recent diversity estimate models predict that greater than 7000 strains comprise the gut microbiota (Backhed *et al*., 2005). The community is dominated by 30-40 species that account for as much as 99% of the microbial mass (Drasar, 1986). The ten most common species identified in culture-based studies are *Bacteroides vulgatus, Bacteroides thetaiotaomicron, Bacteroides distasonis, Bifidobacterium adolescentis, Coprococcus eutactus, Eubacterium aerofaciens, Eubacterium elignes, Fusobacterium prausnitzii, Peptostreptococcus productus* and *Ruminococcus bromii* (Drasar, 1986).

Surprisingly little is known about the microbiota due to the difficulties associated with studying this population. Ninety nine percent of the bacteria inhabiting the intestine are obligate anaerobes (Hao and Lee, 2004) that require fastidious culturing conditions. From microscopic examinations of faecal samples and subsequent culturing, it has been estimated that the culture conditions are known for only 60% of the intestinal microbiota (Moore and Holdeman, 1974). Other estimates suggest that the majority of the organisms in the ecosystem have not been cultured *ex vivo* (Eckburg *et al*., 2005). The inability to culture such a substantial part of the population adds an inherent bias to culture studies used to describe the intestinal microbiota.

1.1.3 THE INTESTINAL HABITAT

The gastrointestinal tract is an ideal environment for microbial populations. It is comprised of the mouth, oesophagus, stomach, small intestine, colon, and rectum. In general the tract is made up of layers of muscle and is lined with mucosa (Figure 1.1). The combined mucus membranes of the small and large intestine have an enormous surface area of approximately 400 m^2 (Macdonald and Monteleone, 2005). The constant supply of nutrients and removal of waste, the temperature, and redox conditions, and the assortment of habitats provided by the structure of the gut wall and the changing pH conditions along the tract provide diverse habitats for microbial populations.



Anatomy of the Gastrointestinal Tract Intestinal Cross Section

Figure 1.1. Organisms of the microbiota can be found throughout the length of the gastrointestinal tract, with each organ providing different conditions for growth. Significant populations of commensals are found in the lumen and associated with the mucosa of the gut, but do not penetrate beyond these tissues in healthy individuals.

1.1.4 DISTRIBUTION WITHIN THE GASTROINTESTINAL TRACT

The microbiota populates the length of the tract, with unique communities found in different regions. Differences in microbial distribution can be found in each microenvironment of the intestine. For example the lumen, the unstirred mucus that coats the epithelial cells, the crypts of Lieberkuhn, and the surface of the epithelial cells all provide different environments (Berg, 1996). The microbiota also varies from section to section with vast differences seen in the diversity and size of populations from the stomach to the colon.

In the stomach, the low pH conditions generally prevent colonisation by micro-organisms, but some bacteria can be found populating the mucosa. Gastric biopsy samples examined for bacterial DNA were found to contain Helicobacter, Enterococcus, Pseudomonas, Streptococcus, Staphylococcus, Stomatococcus, Acinetobacter,

Brevundimonas, Enterobacter, Propionibacterium, and Rhizobium species (Monstein *et al.*, 2000). The presence of bacterial DNA in the stomach mucosa provides no information about the metabolic activity of these organisms, it is quite possible that they may represent ingested bacteria originating from the oral cavity or respiratory tract, rather than a genuine gastric population (Monstein *et al.*, 2000). Bile reflux can increase the pH in the stomach, and increased bacterial numbers in gastric juice collected from reflux patients have been reported (Zhang *et al.*, 2004a).

The microbial population remains low in the small intestine as a result of the low pH and rapid transit (4-6 hours), and the population varies proximally and distally. The proximal small intestine has an overall population of 10^5 to 10^7 organisms per ml of fluid with acid-tolerant lactobacilli and streptococci predominating (Mackie *et al.*, 1999). A ten-fold increase in microbial numbers can be found in the distal small intestine accompanied by greater diversity. The ileum is considered a transition zone between the colon and small intestine (Mackie *et al.*, 1999).

The largest microbial population in the body is found in the colon with 10^{11} bacteria per ml of faeces, and bacteria comprising an estimated 55% of faecal solids (Stephen and Cummings, 1980), although dead bacteria may account for as much as 1/3 of the total bacterial DNA in faeces (Apajalahti *et al.*, 2003). Such a large population exists due to the favourable growth conditions found in the colon; slow transit time (60-70 hours), more favourable pH conditions, and an abundance of undigested fibre. In addition to faeces, bacteria can also be found in close association with the mucosa of the gastrointestinal tract. It has been shown these populations differ in their composition in terms of anaerobes to aerobes and also at the species level (Zoetendal *et al.*, 2002).

Some examples of bacteria commonly isolated from the large intestine using culture techniques are given in Table 1.1. Examples of uncultivated or novel organisms isolated using molecular methodologies are given in Table 1.2. This data highlights the broader picture provided by the use of molecular methodologies to study the intestinal ecosystem.

Table 1.1 A selection of characterised bacterial species commonly isolated from the intestine in culture studies. Table adapted from Drasar, 1986.

Bifidobacterium	**Streptococcus**	**Other**
Bifidobacterium adolescentis	*Streptococcus agalactide*	*Escherichia coli*
Bifidobacterium angulatum	*Streptococcus anginosus*	*Citrobactor freundii*
Bifidobacterium bifidum	*Streptococcus avium*	*Klebsiella pnuemoniae*
Bifidobacterium breve	*Streptococcus bovis*	*Enterobacter cloacae*
Bifidobacterium catenulatum	*Streptococcus cemoris*	*Enterobacter aerogenes*
Bifidobacterium cornutum	*Streptococcus equisimilius*	*Proteus mirabilis*
Bifidobacterium dentium	*Streptococcus faecalis*	*Ptoteus morganii*
Bifidobacterium infantis	*Streptococcus faecium*	*Acidaminococcus fermantans*
Bifidobacterium longum	*Streptococcus lactis*	*Megasphera elsdenii*
Bifidobacterium pseudolongum	*Streptococcus mitior*	*Peptococcus asaccharolyticus*
Clostridium	*Streptococcus mutans*	*Peptococcus magnus*
Clostridium bejerinkii	*Streptococcus salivarius*	*Peptococcus prevotti*
Clostridium butyricum	*Streptococcus sangius*	*Peptostreptococcus productus*
Clostridium cadaveris	*Streptococcus constellatus*	*Ruminococcus albus*
Clostridium celatum	*Streptococcus intermedius*	*Ruminococcus bromii*
Clostridium clostidiiforme	*Streptococcus morbillorium*	*Ruminococcus flavefaciens*
Clostridium difficile	*Staphylococcus epidermidis*	*Sarcina ventriculi*
Clostridium innocum	**Bacteroides**	*Veillonella parvula*
Clostridium leptum	*Bacteroides assacharolyticus*	*Coprococcus cutactus*
Clostridium malenominatum	*Bacteroides capillosus*	*Coprococcus catus*
Clostridium nexile	*Bacteroides coagulans*	*Coprococcus comes*
Clostridium paraputrificum	*Bacteroides distasonis*	*Gemiger formicilis*
Clostridium perfringens	*Bacteroides eggerthii*	*Lachnospira multiparus*
Clostridium ramosum	*Bacteroides fragilis*	*Propionobacterium acnes*
Clostridium tertium	*Bacteroides furcosus*	*Propionobacterium granulosum*
Eubacterium	*Bacteroides hypermegas*	*Propionobacterium jensenii*
Eubacterium aerofaciens	*Bacteroides melaninogenicus ss melaninogenicus*	*Desulfomonas pigra*
Eubacterium contortum		*Leptotrichia buccalis*
Eubacterium cylindroides	*Bacteroides multiacidus*	*Butyrivibrio fibriosolven*
Eubacterium lentum	*Bacteroides ovalis*	*Sucinimonas amylolytia*
Eubacterium limosum	*Bacteroides ovatus*	*Vibrio succinogenes*
Eubacterium rectale	*Bacteroides praeacutus*	
Eubacterium ruminantium	*Bacteroides putredinis*	
Eubacterium tenue	*Bacteroides ruminicola ss brevis*	
Eubacterium tortuosum	*Bacteroides ruminicola ss ruminicola*	
Eubacterium ventriosum	*Bacteroides splanchinicus*	
Lactobacillus	*Bacteroides thetaiotaomicron*	
Lactobacillus acidophilus	*Bacteroides uniformis*	
Lactobacillus brevis	*Bacteroides vulgatus*	
Lactobacillus casei	**Fusobacterium**	
Lactobacillus fermentum	*Fusobacterium mortiferum*	
Lactobacillus leichmannii	*Fusobacterium naviforme*	
Lactobacillus minutus	*Fusobacterium necrogenes*	
Lactobacillus plantareum	*Fusobacterium nucleatum*	
Lactobacillus rogosae	*Fusobacterium plauti*	
Lactobacillus ruminis	*Fusobacterium prausnitzii*	
Lactobacillus salivarius	*Fusobacterium russi*	
	Fusobacterium symbiosum	
	Fusobacterium varium	

Table 1.2. Examples of uncultivated or novel organisms isolated from the human gastrointestinal tract using molecular methodologies. (Adapted from Bonnet *et al*, 2002, and Eckburg *et al*, 2005, supplementary material)

Uncultivated or Novel Organism's Accession Number	Reference	Closest Full Length Neighbour			
		Similarity (%)	Accession Number	Description	Source
AY916298	Eckburg	87.5	AF371945	Uncultured bacterium clone p-5324-2Wa3	swine intestine
AY916386	*et al*, 2005	88.1	AY239411	Uncultured bacterium clone rc4-2(2)	rat faeces
AY916186		89.3	AF371525	Uncultured bacterium clone p-2601-9F5	swine intestine
AY916333		89.5	U81762	Unidentified eubacterium clone vadinHA42	anaerobic digestor
AY916249		90	AY239409	Uncultured bacterium clone rc2-30(4)	rat faeces
AY916146		90.4	AY239409	Uncultured bacterium clone rc2-30(4)	rat faeces
AY916367		90.6	AF481224	Peptococcus sp. oral clone MCE10_265	oral clone
AY916248		90.7	L16496	*Bacteroides splanchnicus* NCTC10825	type strain
AY916331		90.7	X71852	*Clostridium papyrosolvens*	type strain
AY916206		91.1	U42221	*Desulfovibrio fairfieldensis*	liver abscess
AY916159		91.4	AF030452	*Ruminococcus albus* strain OR108	type strain
AY916302		91.5	AF371866	Uncultured bacterium clone p-1030-a5	swine intestine
AY916351		91.6	AF332709	Uncultured bacterium clone UASB_brew_B8	brewery wastewater
AY916330		91.6	AY538684	Bacteroidetes bacterium Smarlab 3301186	unclear
AY916310		91.6	L16496	*Bacteroides splanchnicus* NCTC 10825	type strain
AY916339		91.7	AF371527	Uncultured bacterium clone p-3870-23G5	swine intestine
AY916231		91.7	AF371905	Uncultured Bacteroides sp.	human faeces
AY916358		91.8	AF371829	Uncultured bacterium clone p-837-a5	swine intestine
AY916316		91.9	AY244942	Uncultured rumen bacterium clone BE28	cow rumen
AY916388		92	AF371866	Uncultured bacterium clone p-1030-a5	swine intestine
AY916359		92.1	AF192154	*Desulfovibrio desulfuricans*	type strain
AY916172		92.1	AF153865	Uncultured bacterium adhufec77.25	human faeces
AY916148		92.2	AY323524	*Prevotella denticola*	type strain
AY916179		92.4	AJ295664	Bacterium Irt-JG1-53	uranium mining waste
AY916328		92.6	AF371530	Uncultured bacterium clone p-1735-b3	swine intestine
AY916205		92.6	AF371829	Uncultured bacterium clone p-837-a5	swine intestine
AY916362		92.7	M23730	*Streptococcus pleomorphus*	type strain
AY916176		92.8	AF153865	Uncultured bacterium adhufec77.25	human faeces
AY916202		93	AB009219	Unidentified rumen bacterium RFN71	cow rumen
AY916278		93	AF371866	Uncultured bacterium clone p-1030-a5	swine intestine
AY916234		93.1	AF101240	*Slackia exigua*	type strain
AY916369		93.1	AY212765	Uncultured bacterium clone wet62	equine manure
AY916269		93.1	AY862393	Uncultured bacterium clone HuDI53	human intestinal tract
AY916149		93.1	AF137279	Uncultured bacterium adhufec73	human faeces
AY916272		93.2	AF371800	Uncultured bacterium clone p- 2053-s959-5	swine intestine
AY916377		93.2	AF371866	Uncultured bacterium clone p- 1030-a5	swine intestine
AY916381		93.4	AF371822	Uncultured bacterium clone p-1014-a5	swine intestine
AY916304		93.5	AB185758	Uncultured rumen bacterium clone U29-B09	cow rumen
AY916142		93.6	AF371684	Uncultured bacterium clone p- 2722-65A5	swine intestine
AY916294		93.6	AY858462	Uncultured bacterium clone ZEBRA_23	zebra intestine
AY916260		93.6	AF371759	Uncultured bacterium clone p- 2506-18B5	swine intestine
AY916256		93.7	AF371800	Uncultured bacterium clone p- 2053-s959-5	swine intestine
AY916363		93.7	AF371822	Uncultured bacterium clone p- 1014-a5	swine intestine
AY916140		93.7	AF028350	*Clostridium cocleatum*	type strain
AY916343		93.8	AJ408963	Uncultured bacterium clone HuCA7	human colon
AY916347		93.8	AB009219	Unidentified rumen bacterium RFN71	cow rumen
AY916354		93.9	AF132279	Uncultured bacterium adhufec73	human faeces
AY916379		93.9	Btv crosst	*Butyrivibrio crossotus*	type strain
AY916266		93.9	AF371572	Uncultured bacterium clone p- 1028-a5	swine intestine
AY916240		93.9	AJ576348	uncultured bacterium clone PeH31	hindgut of larva
AY916175		94	AF028350	*Clostridium cocleatum*	type strain
AF153873	Bonnet	99	AY985031	Uncultured bacterium clone BA35	human intestinal tract
AF153872	*et al*, 2002	99	AY169429	*Faecalibacterium prausnitzii* clone 1-84	Greenland glacier ice
AF153871		96	AY916167	Uncultured bacterium clone M484	human intestinal tract
AF153870		99	AY980892	Uncultured bacterium clone LQ42	human intestinal tract
AF153869		98	AB200218	*Bacteroides plebeius*	human faeces
AF153868		98	AY979765	Uncultured bacterium clone M526	human intestinal tract
AF153867		99	AF132272	Uncultured bacterium adhufec43	human intestinal tract
AF153866		99	AF132282	Uncultured bacterium adhufec94	human intestinal tract
AF153865		91	AY985661	Uncultured bacterium clone C667	human intestinal tract
AF153864		99	AY684418	Uncultured bacterium clone HuDI01	human intestinal tract

1.1.5 COLONISATION OF THE LARGE INTESTINE

Prior to birth the gastrointestinal tract is free of micro-organisms and sterile. The colonisation of the newborn's intestine begins immediately after a vaginal delivery with enterobacteria and streptococci species (Favier *et al.*, 2002). Caesarean deliveries permit less contact between the maternal microbiota populations (vaginal and faecal) and the infant, increasing the likelihood that the initial contact with bacteria will be from environmental sources. It has been suggested that the important role of the initial colonising aerobic species is to alter the redox conditions of the gut allowing future colonisation by anaerobic bacteria (Stark and Lee, 1982).

Following the initial exposure to micro-organisms new bacterial species enter the infant's gastrointestinal tract through feeding, and hence the development of the microbiota is strongly influenced by diet (Mackie *et al.*, 1999). Formula-fed babies develop a diverse population including bifidobacteria, streptococci, bacteroides, and clostridia species (Favier *et al.*, 2002). Breast-fed babies are most frequently exposed to staphylococci, streptococci, corynebacteria, lactobacilli, micrococci, propionibacteria, and bifidobacteria from the milk duct, nipple, and surrounding skin (Mackie *et al.*, 1999). Bifidobacteria dominates the flora of breast-fed infants, and a more diverse population, resembling that found in formula fed-babies, does not develop until dietary supplementation begins (Mackie *et al.*, 1999). A succession of bacterial species then continues to populate the gut until a population resembling that found in adults is established (Stark and Lee, 1982).

A molecular study on the colonisation patterns in two infants found the same general order of succession identified in culture studies (Favier *et al.*, 2002). The study also identified clostridia (an anaerobic bacterium) as the first dominant coloniser in one infant rather than an aerobic organism. Bacteroides failed to be detected in both infants, and ruminococcal species were present in both infants' faeces (Favier *et al.*, 2002).

Little is known of the bacteria-host and bacteria-bacteria interactions that are required to allow colonisation and succession in the community, and the subsequent stability of the climax community. The intestinal tract is lined with host-derived carbohydrates that can provide attachment sites for bacteria expressing adhesins (Bourlioux *et al.*, 2003).

Bacteria can also ferment these carbohydrate structures, and it has been hypothesised that an initial nutrient foundation provided by the host may determine the pattern of colonisation at weaning (Hooper *et al.*, 1999). *Bacteroides thetaiotaomicron*, a member of the mouse and human microbiota, produces a signal in response to low levels of environmental fucose (Hooper *et al.*, 1999). This signal has been shown to induce mouse epithelial cells to produce fucosylated glycans, a nutritional source for the bacterium (Hooper *et al.*, 1999). This interaction is likely to be a key factor in the organism's ability to become a stable member of the ecosystem. A further study has shown *B. thetaiotaomicron* mutants that cannot express fucose on the bacterial surface are unable to colonise the host, suggesting simultaneous expression of this sugar by both host and the bacterium is required for colonisation (Coyne *et al.*, 2005). It is probable that an intricate system operates to establish the climax community, where the metabolic activities, and the bacteria-bacteria and bacteria-host communication of founding members creates an environment and nutrient foundation suitable for new species. These species then further modify the environment through their own activities and interactions with other bacteria, permitting more colonisation and complexity in the population (Hooper and Gordon, 2001). Ultimately the ability of a species to compete for resources will determine what organisms form the population, with poor competitors quickly excluded from the community.

1.1.6 CHARACTERISTICS OF THE FAECAL MICROBIOTA

The microbiota is considered to be adult-like by the time a child is 2 years old (Stark and Lee, 1982), however structural changes to the population occur during ageing (Hopkins *et al.*, 2001). Children harbour higher numbers of enterobacteria, bifidobacteria, clostridia, and bacteroides-porphyromonas-prevotella species than adults, indicating a less complex microbiota (Hopkins *et al.*, 2001). The elderly also possess a slightly different flora to younger adults with increased enterobacteria numbers, accompanied by declining anaerobe populations (Hopkins *et al.*, 2001). Reductions in bifidobacteria and bacteroides, accompanied by reduced species diversity have also been described in the elderly (Woodmansey *et al.*, 2004).

In 1976 Holdeman, Good, and Moore carried out culture studies on the faecal microbiota of 23 people to determine the species present and their frequency. They

observed large person-to-person variation in the flora and concluded that none of the 23 specimens were alike. The greatest similarity was observed in samples collected from the same individual. However in contrast to these results, a culture based study on butyrate-producing bacteria found significant differences in this population in three individuals over a period of 1 year (Barcenilla *et al.*, 2000). Temporal studies on Lactobacillus species diversity also found a variable stability in 2 out of the 3 subjects assessed (Heilig *et al.*, 2002). Therefore despite the stability found amongst predominant members of the faecal flora, variation has been reported to occur amongst the minor bacterial groups.

Stress, infection, and medical treatments such as antibiotics, chemotherapy, or radiation can curtail the stability of the faecal flora (Holdeman *et al.*, 1976; Levy, 2000). This may disturb the balance of colonic bacteria by removing protective species such as lactobacilli and bifidobacteria, and allowing unaffected bacteria to overgrow, potentially causing diarrhoea and bacterial translocation across the mucosa (Berg, 1996). Reduced stability has also been described in patients suffering from irritable bowel syndrome (Matto *et al.*, 2005).

1.1.7 THE ROLE OF THE MICROBIOTA IN HOST PHYSIOLOGY

The presence of bacteria in the intestine is crucial to the normal development of several aspects of host physiology. Colonisation of neonates with commensal bacteria is crucial in the development of the systemic immune system, in particular these organisms lead to an expansion of natural and anti-microbial antibodies in the circulatory system (Cebra, 1999).

The presence of commensal bacteria in the gut can also strengthen the mucosal barrier against pathogens. For example, microarray analyses have shown that the colonisation of germfree mice by *Bacteroides thetaiotaomicron* can induce host expression of a variety of genes important in defence (Hooper *et al.*, 2001). The same study also found that *Bacteroides thetaiotaomicron* colonisation leads to the expression of genes involved in postnatal maturation, angiogenesis, nutrient uptake and metabolism, and the processing of xenobiotics. The faecal microbiota can also influence the permeability of the mucosa . *Lactobacillus brevis* has a protective effect on the mucosa by slightly

reducing permeability (Garcia-Lafuente *et al.*, 2001), however other members of the faecal flora can have a detrimental effect on the integrity of the mucosal barrier. *Escherichia coli, Klebsiella pneumoniae,* and *Streptococcus viridans* can significantly increase the permeability of the mucosa to small molecular weight proteins (Garcia-Lafuente *et al.*, 2001).

Members of the microbiota protect the health of the host through their participation in colonisation resistance. They compete with pathogenic bacteria for resources such as attachment sites and nutrition, and some can also secrete bacterocins that are toxic to other species of bacteria (Levy, 2000).

In the colon, bacteria ferment undigested carbohydrate into short chain fatty acids (SCFA), which can be rapidly absorbed by the host and used as energy (Cummings and Macfarlane, 1997). Germ free rodents require a 30% higher caloric intake than normal mice to make up for the lack of energy derived from the microbiota (Wostmann *et al.*, 1983).

Bacteria that colonise the intestinal mucosa are also involved in shaping the underlying microvasculature of the host's intestinal tissues. This process is regulated by the paneth cell, and involves signalling through this bacteria sensing epithelial cell (Stappenbeck *et al.*, 2002). In this way, the bacterial population of the gut increases the absorption capacity of the intestine by increasing the microvasculature (Stappenbeck *et al.*, 2002).

1.1.8 FERMENTATION BY THE INTESTINAL MICROBIOTA

The colonic microbiota is a substantial microbial population with massive metabolic potential, and is an important 'organ' of the body in its own right (Hooper *et al.*, 2002). Nutrients that have failed to be absorbed by the small intestine, pass into the colon and provide a source of carbon for microbial fermentation. The majority of simple carbohydrates and sugars are absorbed by the small intestine. Some sugars such as raffinose, stachyose, lactulose, sorbitol and xylitol pass undigested into the colon with resistant starches, plant cell wall polysaccharides, oligosaccharides, and proteins and peptides. Host mucopolysaccharides present on the mucosa also provide an energy source for the microbiota. Bacterial enzymes degrade the complex macromolecules into

simple sugars, and amino acids that can then be fermented to produce SCFA, and H_2 and CO_2. The nutrient environment in the colon is in constant flux; daily changes in food consumption produce a variable nutrient supply, and as new nutrients enter the colon, others are broken down, and others are utilised by the microbiota. This produces a dynamic environment for micro-organisms to live in, and promotes the survival of species that can utilise variable carbon sources.

The major SCFAs produced from bacterial fermentation are acetate, propionate, and butyrate. The host absorbs as much as 99% of the SCFAs, which can be used as an energy source for enterocytes (Scheppach, 1994). Amino acid fermentation is the primary source of energy for some species of the intestinal microbiota such as *Acidaminococcus* (Rogosa, 1969). The major products of amino acid fermentation are also SCFAs, however ammonia, amines, phenols, indoles, organic acids, alcohols and H_2 and CO_2 gases are also produced.

Hydrogen is important in the fermentation reactions of anaerobic bacteria, where it is used as an electron sink for catabolism of sugars and amino acids (Levitt, 1995). Hydrogen gas accumulates in the intestine, and is removed by the host on the breath or through flatus. Several microbial groups carry out H_2 disposal including dissimilatory nitrate reducing bacteria, dissimilatory sulfate reducing bacteria, methanogens, acetogens, and amino acid fermenting bacteria.

Hydrogen gas disposal by methanogens and sulfate reducing bacteria is of particular interest as these non-pathogenic organisms have been linked to gastrointestinal disorders, including inflammatory bowel disease (Roediger *et al.*, 1997) and cancer (Pique *et al.*, 1984).

Two methanogenic archaeobacterial species have been identified from human faeces, the predominant *Methanobrevibacter smithii* (Miller *et al.*, 1982), and the less common *Methanosphaera stadtmaniae* (Miller and Wolin, 1985). These two methanogenic organisms are strict anaerobes and produce methane from H_2 and CO_2, and H_2 and methanol respectively. The conversion of 4 moles of H_2 to 1 mole of CH_4 and 2 moles of H_2O effectively reduces gas volume. The reaction is a safe method of disposing of

hydrogen gas from within the gut, as methane is not toxic and can be readily excreted on the breath and in flatus.

Sulfate reducing bacteria are phylogenetically diverse anaerobic organisms that belong to both the *Archaea* and the *Bacteria* kingdoms. Five main genera of sulfate reducing bacteria are found in the human intestinal microbiota, the predominant genus is Desulfovibrio, with Desulfobacter, Desulfomonas, Desulfobulbus, and Desulfotomaculum present in lower numbers (Gibson *et al.*, 1988). The common characteristic among all sulfate reducing bacteria is the ability to carry out dissimilarity sulfate reduction, whereby external sulfate is used as a terminal electron acceptor and sulfide is generated using the enzyme APS reductase (Peck, 1962; Stille, 1984). Hydrogen gas can then combine with sulfide to generate toxic hydrogen sulfide (H_2S). This compound can impair colonocyte nutrition by preventing the oxidation of n-butyrate (Roediger *et al.*, 1993) and is therefore an undesirable route for hydrogen disposal in the intestine.

A competitive relationship is thought to exist between methanogens and sulfate reducing bacteria. An inverse relationship has been found between large numbers of sulfate reducing bacteria and methanogenesis in both British and African subjects leading the authors to suggest that these reactions cannot occur concurrently in the colon (Gibson *et al.*, 1988).

1.1.9 DYNAMICS OF METHANOGEN AND SULFATE REDUCING BACTERIA POPULATIONS

Methanogen carriage rates vary in different populations and ethnic groups. Studies of North Americans have reported the presence of methane on the breath of 33% of volunteers (Bond *et al.*, 1971), however in two culture studies the production of methane from faecal cultures was found to occur in 72% of the population (Weaver *et al.*, 1986) and 55% of the population (Miller *et al.*, 1982). Rates of 77% have been found in rural Nigerians (Hudson *et al.*, 1993). Amongst the South African population 72-84% of blacks excreted methane on the breath, however amongst Indians and whites only 41% and 52% respectively were methanogenic (Segal *et al.*, 1988). Culture studies examining the presence of methanogens in Italians found 90% of faecal samples

contained these organisms (Rutili *et al.*, 1993). Amongst the Hungarian population, 55% of the individuals studied were found to be methane excreters (Flatz *et al.*, 1985), while only 44% of Scandinavians are methanogenic (Bjorneklett and Jenssen, 1982).

The factors that inhibit the development of methanogen populations in non-methanogenic individuals could be related to bile acid and transit time (Florin and Woods, 1995; El Oufir *et al.*, 1996). Unlike methane producing ruminants, bile acid can pass into the colon from the small intestine in humans. Studies have demonstrated that bile acid can inhibit methanogenesis, and that the concentration of bile acid is inversely proportional to methane production (Florin and Woods, 1995). A fast transit time could reduce the numbers of methanogens present in the colon by diluting the population, which is slow growing. The influence of genetics over the methanogenic status of an individual has been examined. Analysis of both human twin pairs and rats concluded that a shared environment rather than genetics was responsible for the development of methane on the breath in twins (Florin *et al.*, 2000). Another study found equivalent concordance rates for the excretion of methane on the breath in identical and fraternal twins (Flatz *et al.*, 1985). Gender also appears to influence the carriage of methanogens as numerous studies report higher carriage rates in women than men (Flatz *et al.*, 1985; Segal *et al.*, 1988; Hudson *et al.*, 1993; Florin *et al.*, 2000). However, other studies have reported no difference based upon gender (Bond *et al.*, 1971). Florin *et al*, 2000, suggested that gender differences may be accounted for by the faster transit time seen in men, or alternatively by sex differences in the circulation of bile acid. The carriage of methanogens may also be influenced by the presence of sulfate reducing bacteria, which also utilise H_2 gas (Gibson *et al.*, 1988).

Estimates of the prevalence of sulfate reducing bacteria in the population vary amongst different groups. In a British study 70% of faecal samples contained sulfate reducing bacteria, however in rural black South Africans only 15% of the population harboured these organisms (Gibson *et al.*, 1988). Further reports indicate carriage rates of 65% in healthy adults (Gibson *et al.*, 1993a), however rates amongst Americans have been reported to be as low as 30% (Strocchi *et al.*, 1993).

Numerous studies have been conducted to determine if sulfate reducing bacteria or methanogens are the best competitors for hydrogen gas, and what factors may enable

one of these groups to dominate over the other. Studies carried out in methanogenic individuals demonstrated a decline in breath methane and in methanogen densities during dietary supplementation with sulfate. This was accompanied by an increase in sulfate reduction rates (Christl *et al.*, 1992). It has been hypothesised that this may be due to the oxidation of H_2 being more thermodynamically favourable for sulfate reducing bacteria than methanogens (Gibson *et al.*, 1993b). These findings suggested that sulfate reducing bacteria could out-compete methanogens in the presence of abundant sulfate. However, other studies in which faeces containing sulfate reducing bacteria and faeces containing methanogens were mixed found that methanogens dominated, even in the presence of abundant sulfate (Strocchi *et al.*, 1994).

In addition, physiological processes of the host may affect the predominance of one group of hydrogen gas consuming organisms over another. Modification of transit time through cisapride or loperamide treatment in healthy volunteers led to fluctuations in methanogen and sulfate reducing bacteria populations (El Oufir *et al.*, 1996). In this study, carriage of large numbers of methanogens was associated with a slow transit time, and large numbers of sulfate reducing bacteria were associated with a fast transit time (El Oufir *et al.*, 1996). A study of children suffering from chronic constipation also linked the presence of breath methane to a prolonged colonic transit time (Soares *et al.*, 2005).

Methanogens and sulfate reducing bacteria are not the only hydrogen gas-consuming organisms in the microbiota. Acetogens generate acetate from CO_2 and H_2 and given the uncharacterised diversity of the gastrointestinal ecosystem, other groups may also be present. The formation of acetate has been documented in the presence of methanogenesis (Lajoie *et al.*, 1988), however greatest densities of the acetogens have been found when methanogenesis is absent (Dore *et al.*, 1995). However, methane production and sulfate reduction are theoretically more energetically favourable than acetogenesis (Gibson *et al.*, 1993b), making it unlikely that acetogens could out compete these groups for their common substrate.

1.2 THE IMMUNE SYSTEM AND THE INTESTINAL MICROBIOTA

The organisms of the faecal microbiota are commonly referred to as commensals, though the relationship between the host and the microbial population may be better described as mutualism. Micro-organisms benefit from the intestinal habitat provided by the host, and the host derives benefit through the utilisation of fermentation products and the development of a robust mucosal immune system. The immune mechanisms that permit carriage of the microbiota while successfully eradicating pathogens are slowly beginning to be understood.

1.2.1 INNATE IMMUNITY

The epithelial cells lining the lower digestive tract carry out two important functions; they permit the entrance of digestive products and ions into the body, but prevent commensals, pathogens, and macromolecules from gaining access (Macdonald and Monteleone, 2005). A crucial component of the host's innate immunity is the intestinal barrier that separates the epithelial lining of the intestine from the milieu of the lumen. The intestinal barrier is created by simple epithelia. These cells are sealed together at the apical membrane by tight junctions forming an effective diffusion barrier. Several structural components of the cells, including the microvilli and the glycocalyx, and local secretions of mucin prevent commensals, pathogens, and macromolecules from coming in contact and adhering to the plasma membrane (Sansonetti, 2004).

Additional innate defence mechanisms exist to prevent pathogens gaining a foothold in the gut. The peristaltic motion of the gut limits the growth of micro-organisms. Epithelial cells are sloughed off the intestinal wall every 2-5 days along with any adherent organisms. Paneth cells in the small intestine produce a plethora of substances that are toxic to bacteria including lysozyme, defensins, phospholipase A2, and cathelicidins (Ayabe *et al.*, 2004).

1.2.1.1 Toll-Like Receptors

Toll-like receptors (TLRs) act as an essential tool through which the innate immune system can detect and respond to the presence of microbial pathogens. They are expressed on numerous cells in the human intestine including intestinal epithelial cells, macrophages of the lamina propria, and dendritic cells (Abreu *et al.*, 2005). TLRs belong to a group of receptors known as pattern recognition receptors (PRR). The receptors detect pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), flagellin, CpG-rich DNA motifs and muramyl dipeptide. PAMPs act as ideal and efficient markers for sensing pathogens; they are only produced by microbes, are essential for microbial survival, and do not vary within bacterial classes. Therefore all bacteria express PAMPs, including the indigenous, commensal microbiota of the host (Abreu *et al.*, 2005).

TLRs 1-10 have been identified in humans, and recently TLR11 was characterised in mice (Zhang *et al.*, 2004b), although no homologue appears to be present in humans. Colonic epithelial cells have been shown to express TLRs 1-9 (Otte *et al.*, 2004). *In vitro* studies have shown that colonic epithelial cells exposed to prolonged LPS exposure develop tolerance and cross-tolerance to other PAMPs (Otte *et al.*, 2004). This mechanism appears to function through reduced TLR surface expression, decreased IL-1R associated kinase (IRAK) activity (an essential enzyme in TLR signal transduction), and increased expression of the TLR signalling inhibitor, Tollip (Abreu *et al.*, 2005). This may explain the unresponsiveness of the host to the intestinal microbiota. TLR-5 has been reported to be expressed on the basolateral membrane of colonic epithelial cells (Gewirtz *et al.*, 2001), and Nod1 and Nod2 are intracellular PPRs (Viala *et al.*, 2004). The localisation of these receptors suggests that they cannot be activated by commensal organisms present in the lumen, and rather, are situated for the detection of invasive bacteria (Viala *et al.*, 2004; Abreu *et al.*, 2005). The signalling pathways initiated by TLRs regulate the gene expression of factors important in mediating host defence such as cytokines, for example TLR-5 activation stimulates the secretion of IL-8 and MIP3α (Rhee *et al.*, 2004). TLR signalling has also been shown to lead to the secretion of antimicrobial peptides (Thoma-Uszynski *et al.*, 2001), and the induction of DC maturation (Kaisho *et al.*, 2001). In addition to inducing pro-inflammatory responses, TLR activation by commensal bacteria has been shown to

attenuate inflammation (Neish *et al.*, 2000; Kelly *et al.*, 2004), and protect from intestinal injury (Rakoff-Nahoum *et al.*, 2004).

1.2.2 AQUIRED IMMUNITY

1.2.2.1 Gut Associated Lymphoid Tissue

The gut-associated lymphoid tissue (GALT) is a specialised tissue of the immune system that samples antigen from the intestine and produces antigen reactive IgA (Nagler-Anderson, 2001). The GALT is divided into inductive and effector sites. The best-characterised inductive sites of the intestine are Peyer's patches and these are where the immune system first encounters antigen through M cells. Recently, intestinal villous M cells have been characterised and these cells may provide an alternative gateway for the induction of antigen-specific immune responses (Jang *et al.*, 2004). Following the transport of antigen across the intestinal barrier by M cells, APC phagocytose the antigen and present the processed antigen to CD4+ lymphocytes, leading to the secretion of cytokines that promote the production of IgA (Prescott *et al.*, 1999). The IgA+ B cells then travel to the lamina propria of the gut via the lymph and blood circulatory systems where they undergo terminal differentiation to become IgA secreting plasma cells. Antigen reactive IgA is then transported into the lumen via epithelial cells.

Unlike pathogenic bacteria, commensal organisms are not transported into the Peyer's patch, as they generally remain trapped in the mucus layer (Nagler-Anderson, 2001). In addition to intestinal villous M cells, a new mechanism has been described whereby commensal organisms are regularly sampled from the intestinal milieu by dendritic cells (Rescigno *et al.*, 2001). The population of dendritic cells in the lamina propria penetrates the tight junctions of the intestinal barrier and directly accesses antigen in the lumen. In mice, this process appears to depend on the chemokine receptor CX3CR1 (Niess *et al.*, 2005). These DCs can sample both pathogenic and non-pathogenic antigen, however only DCs carrying pathogenic bacteria are found deeper in the lamina propria at the base of the villi (Rescigno *et al.*, 2001). This may indicate that commensal species are unable to stimulate the maturation and migration of DCs, whereas pathogenic bacteria can (Nagler-Anderson, 2001).

An alternative pathway for the development of IgA secreting plasma cells has been proposed. A study carried out using T cell-deficient mice found that the IgA derived from B1 peritoneal cells was reactive to specific antigens of the commensal microbiota and at the same time was produced in a T cell independent manner (Macpherson *et al.*, 2000). This lead the authors to hypothesise that B1 cells may represent a primitive pathway for specific immune defence that evolved prior to the T cell dependent IgA induction pathway.

Stromal cells of the lamina propria can facilitate the switching of IgM+ B cells to IgA+ B cells, and the differentiation of B cells to plasma cells (Fagarasan *et al.*, 2001). In concert with the actions of stromal cells, peritoneal B cells, and dendritic cells may permit T cell independent, antigen driven production of commensal specific IgA, thereby allowing the lamina propria to act as both effector and inductor sites in the GALT (Fagarasan *et al.*, 2001) (Figure 1.2). Thus there may be both T cell dependent and independent antigen driven pathways within the lamina propria and Peyer's patches that can initiate specific IgA secretion against commensal organisms and pathogenic bacteria respectively.

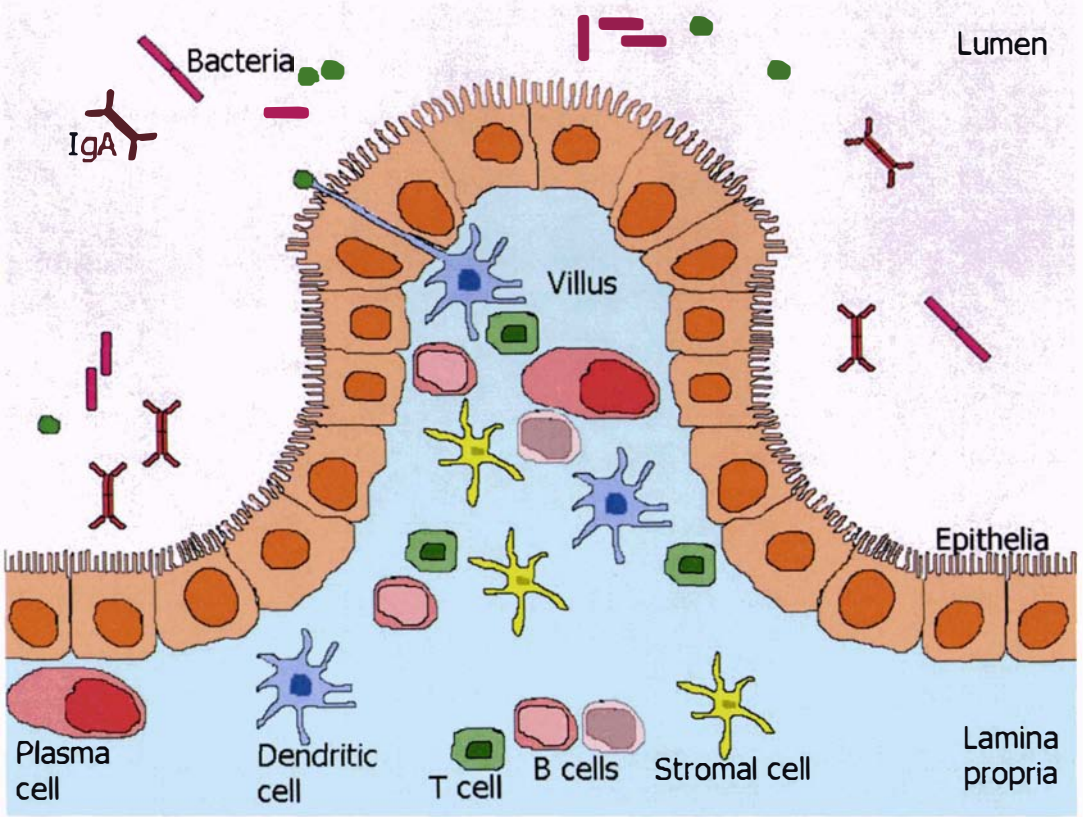


Figure 1.2. Dendritic cells present in the lamina propria can initiate the production of antigen driven IgA independently of Peyer's patches. Dendritic cells penetrate the epithelial layer and sample antigen from the lumen. Antigen can be presented directly to B cells in the lamina propria. Stromal cells can then facilitate B cells class switching and differentiation *in situ* to generate IgA secreting plasma cells. Adapted from Fagarasan and Honjo, 2003.

1.2.2.2 IgA

IgA is a secretory antibody that protects the host from pathogens, but also has a role in regulating the intestinal microbiota. The continuous secretion of IgA onto mucosal surfaces to maintain the immune barrier is not a constitutive function of mammals as both newborn and germ free mammals have few IgA secreting cells in the lamina propria of the gut (Berg, 2001). Significant IgA production, and maturation of the gut's mucosal immune system, appears to develop in response to the colonisation of the gut with members of the normal microbiota (Talham *et al.*, 1999), and hence the IgA that is secreted contains antibodies that are reactive with specific members of the normal gut microbiota.

A considerable portion of the IgA found within the gut is not produced in response to, and does not react with a particular food or pathogen derived antigen. This type of IgA is referred to as natural IgA (Shimoda *et al.*, 1999). It is possible for IgA to inhibit bacteria in a non-specific manner via an interaction with bacterial adhesins. The carbohydrate side chain found on the H-chain constant regions of IgA can bind to lectin-like bacterial adhesins (Wold *et al.*, 1990). This inhibits bacteria from binding to the carbohydrate-containing receptors of intestinal epithelial cells, thereby inhibiting invasion of host tissues.

Evidence of an altered microbiota has been found in activation-induced cytidine deaminase (AID) deficient mice (Fagarasan *et al.*, 2002). AID is important in class switching B cells from IgM production to IgA production, and this finding suggests that IgA production may be important in establishing homeostasis amongst the gut microbiota (Fagarasan and Honjo, 2004).

1.2.3 IMMUNE TOLERANCE TO THE INTESTINAL MICROBIOTA

Tolerance to the commensal microbiota is not well characterised, however there are several features of the gastrointestinal environment that may allow this microbial population to persist in the gut without generating a marked inflammatory response. The environment in the healthy gut is geared towards avoiding unnecessary inflammation. Antigen specific CD4+ cells produce the anti-inflammatory cytokines IL-10 and TGF-β (Groux *et al.*, 1997), and macrophages and stromal cells produce the anti-inflammatory mediator PGE2. Compared to other immunoglobulin classes, IgA is relatively non-inflammatory. It fails to activate complement and is the predominant immunoglobulin class found in the gut. As mentioned earlier, IgA bound to bacteria can inhibit attachment to colonic cells (Wold *et al.*, 1990). The dendritic cells within Peyer's patches also secrete high levels of IL-4 and IL-10 (Iwasaki and Kelsall, 1999; Iwasaki and Kelsall, 2001), which also help establish the anti-inflammatory environment of the gut.

As previously described, the distribution of bacterial sensors on epithelial cells may also contribute to the inability of these organisms to trigger an inflammatory response

(Gewirtz *et al.*, 2001). Recent studies show that TLR-2 and TLR-4 are only expressed by crypt epithelial cells, and TLR-3 is only expressed by mature epithelia (Furrie *et al.*, 2005b). This permits tolerance to the commensal microbiota as these organisms usually do not penetrate into the crypts, and TLR-3 is not activated by bacteria, rather it detects viral pathogens (Furrie *et al.*, 2005b). Nagler-Anderson C, 2001, has hypothesised that commensal bacteria may have a PAMP that stimulates the immune system to produce anti-inflammatory cytokines, thereby counteracting any pro-inflammatory stimulus, or alternatively commensals may lack a particular PAMP that is found only in invasive pathogenic bacteria (Nagler-Anderson, 2001). It has also been suggested that while all bacteria may share the same PAMP, a second danger signal may be required to initiate an immune response against pathogens (Matzinger, 1998; Matzinger, 2002).

Recent work on the organism *Bacteroides fragilis* has offered some explanation as to how organisms can survive in the gut despite the presence of targeted IgA (Krinos *et al.*, 2001). The bacterium has eight distinct capsular polysaccharides and their expression at the cell's surface can be controlled through 13 reversible DNA inversions of their promoter. This feature could lead to as many as 256 distinct polysaccharide combinations. Experiments utilising antibodies against each polysaccharide found only portions of *Bacteroides fragilis* cultures bound antibody (Krinos *et al.*, 2001). It is likely that the organism can persist in the gut due to the variable expression of capsular polysaccharides mediated by DNA inversion factors (Coyne *et al.*, 2003; Weinacht *et al.*, 2004), which creates a 'moving target' for the immune system.

A different approach is employed by 2 non-pathogenic Salmonella strains to avoid eradication by the immune system (Neish *et al.*, 2000). *In vitro* experiments demonstrate that *Salmonella typhimurium* $PhoP^c$ and *Salmonella pullorum* can abolish the epithelial synthesis of inflammatory cytokines by preventing the degradation of IκB, an inhibitor of the major transcription factor involved in cytokine production, NFκB (Neish *et al.*, 2000).

Therefore avoiding unnecessary inflammation in the gut appears to result from the activities of both the host and commensal organisms of the microbiota.

1.3 THE INTESTINAL MICROBIOTA AND DISEASE

Several human diseases of the gastrointestinal tract have been linked to the intestinal microbiota. The failure of the intestinal barrier to prevent bacterial translocation, dysbiosis of the bacterial population, products of microbial fermentation, and the role of the microbiota in stimulating the development of a robust immune system have been linked to both diseases of the gastrointestinal tract and allergic and autoimmune conditions (Guarner and Malagelada, 2003).

Ulcerative colitis and Crohn's disease are chronic gastrointestinal diseases that are collectively called inflammatory bowel disease (IBD). Both diseases are characterised by inflammation of the bowel and have been associated with an immune response to the intestinal microbiota. In ulcerative colitis, inflammation can be found along the complete length of the colon, mainly affecting the mucosa. The commonest symptoms described in ulcerative colitis are the passage of blood and mucus with stools, and lower abdominal pain that increases during the passage of stools. In contrast, Crohn's disease inflammation is found in both the mucosa and submucosa and can involve any part of the gastrointestinal tract. Crohn's disease patients often display normal tissue between sites of inflammation. As the disease can affect the length of the gastrointestinal tract, symptoms vary depending upon the area affected. Gastroduodenal Crohn's disease causes early satiety, nausea, emesis, dysphagial, and postprandial pain. Diseased small bowel leads to symptoms of diffuse abdominal pain, anorexia, and diarrhoea, and colonic Crohn's disease mimics the symptoms of ulcerative colitis. Symptoms are treated with anti-inflammatory drugs and immunosuppressive therapies.

Familial clustering and twin studies suggest that susceptibility to IBD may be conferred by an inherited predisposition (Ahmad *et al.*, 2001). Several susceptibility regions for IBD have been identified through linkage analyses (Schreiber *et al.*, 2004). Polymorphisms in LPS receptor gene CD14 have been associated with ulcerative colitis (Obana *et al.*, 2002). The promoter polymorphism leads to increased expression of CD14 and through this may propagate inflammation (Obana *et al.*, 2002). Increased susceptibility to Crohn's disease has been associated with mutations in the gene

encoding Nod2 (Hampe *et al.*, 2001; Hugot *et al.*, 2001), a cytosolic PRR involved in regulating the inflammatory response to bacterial products. Polymorphisms in TLR-4 have been associated with IBD (Franchimont *et al.*, 2004) and polymorphisms in the TNFα promoter gene have also been associated with Crohn's disease (Levine *et al.*, 2005).

Indirect evidence suggests that the intestinal microbiota act as an antigenic trigger for inflammation in IBD. Experimental colitis in HLA-B27 mice cannot be induced in the germfree state (Rath *et al.*, 2001). Colitis is induced by a subset of the enteric microbiota, and a complex interaction of both anaerobic and aerobic bacteria provide the antigenic drive necessary for chronic inflammation (Rath *et al.*, 2001). Studies have also demonstrated that the faecal stream is responsible for maintaining inflammation in Crohn's disease patients (Harper *et al.*, 1985). Inflammatory bowel disease patients exhibit a loss of tolerance to the microbiota (Marteau *et al.*, 2004), however evidence suggests this is not a global loss of tolerance, rather patients are immunologically reactive to a subset of microbial antigens (Landers *et al.*, 2002). Antibiotic treatment has been shown to enhance ulcerative colitis patient's response to conventional therapy (Turunen *et al.*, 1998). Probiotic therapy has also demonstrated merit in maintaining remission in IBD (Gionchetti *et al.*, 2000), leading to a reduction in mucosal permeability and decreased inflammation in ulcerative colitis patients (Kennedy *et al.*, 2002; Furrie *et al.*, 2005a), and achieving remission in some patients failing to respond to conventional therapy for ulcerative colitis (Bibiloni *et al.*, 2005).

Sulfate reducing bacteria are potentially involved in ulcerative colitis through the generation of toxic H_2S. The carriage rate of sulfate reducing bacteria amongst ulcerative colitis patients has been reported to be as high as 96% (Pitcher MCL, 1994). Ulcerative colitis patients have higher densities of the these organisms than controls (Pitcher and Cummings, 1996) and appear to produce more H_2S than controls (Pitcher *et al.*, 2000), a metabolic product that may damage the integrity of the intestinal barrier by impairing fatty acid oxidation (Roediger *et al.*, 1993). However in contrast to these findings, recent work has found that counts and carriage of sulfate reducing bacteria are not significantly different from controls (Pitcher *et al.*, 2000) and that luminal hydrogen sulfide is not elevated in ulcerative colitis (Moore *et al.*, 1998).

The microbiota have also been associated with other diseases such as irritable bowel syndrome (IBS). IBS is not well understood, and patients may suffer from a range of symptoms including constipation and/or diarrhoea, abdominal distension, bloating, the passage of mucus, urgency, and the sensation of incomplete evacuation (Thompson *et al.*, 1999). These symptoms can be used to categorise patients into three symptom subgroups, diarrhoea predominant IBS, constipation predominant IBS, and IBS with alternating bowel habits (Thompson *et al.*, 1999).

The flora of IBS patients is significantly different to controls with respect to total anaerobes and bacteroides species, and the total bacterial concentrations in mucosal biopsies have been reported to be higher than controls (Swidsinski *et al.*, 2002). Reduced levels of Lactobacillus species and increased levels of Viellonella species have been associated with diarrhoea and constipation predominant IBS, respectively (Malinen *et al.*, 2005). Bacillary dysentery has been identified as a causative factor in post-infectious IBS (Wang *et al.*, 2004), and antibiotic use has been associated with a 3-fold increase in the risk of developing functional bowel complaints (Maxwell *et al.*, 2002). The microbiota of IBS patients also exhibits temporal instability compared to controls (Matto *et al.*, 2005). Evidence for activation of the mucosal immune system has also been reported in IBS patients (Chadwick *et al.*, 2002), which may be triggered by antigens derived from the microbiota. Modification of the microbiota through the introduction of probiotic bacteria has been associated with a reduction in IBS symptoms (Nobaek *et al.*, 2000).

Fermentation products generated by the microbiota may also contribute to IBS symptoms, such as bloating. Studies using abdominal radiographs have found larger quantities of bowel gas in IBS patients than in controls (Koide *et al.*, 2000) and IBS symptoms such as abdominal distension and increased flatulence are often seen in patients with a disturbed intestinal flora (Nobaek *et al.*, 2000). Ineffective gas propulsion in the small bowel (Salvioli *et al.*, 2005) and failure of distension related reflexes (Passos *et al.*, 2005) may impair evacuation of intestinal gas, and have also been associated with bloating and distension.

IBS patients with an alternating bowel habit have been found to have rapid transit through the small bowel, coupled with a failure to rapidly clear the ascending colon

(Hebden *et al.*, 2002). This may lead to large amounts of unabsorbed carbohydrate being available for bacterial fermentation in the colon, and this in concert with poor clearance could feasibly cause symptoms of pain and distension (Hebden *et al.*, 2002). The production of hydrogen gas has been shown to be greater in IBS patients than in controls (King *et al.*, 1998). An exclusion diet resulted in improvement in patient symptoms and also led to a reduction in hydrogen (King *et al.*, 1998). As hydrogen measurements were made following lactulose consumption these changes are likely to reflect changes in the fermentation characteristics by the microbiota rather than a substrate effect (King *et al.*, 1998). Reduction in fermentation through antibiotic use and reduced fibre diet has also been shown to improve abdominal symptoms associated with IBS (Dear *et al.*, 2005). Methanogens can effectively reduce the H_2 gas volume through the production of CH_4 and may be beneficial in reducing bloating. However, methanogens have been associated with constipation predominant IBS (Pimental *et al.*, 2003), in whom a significant symptom is bloating.

1.4 METHODS FOR STUDYING THE MICROBIOTA

Previously the study of diversity and dynamics of natural microbial ecosystems relied on non-molecular techniques such as microscopy and cultivation. These techniques permitted only limited identification and classification of microbial organisms, and the task of analysing a single faecal sample was laborious and time consuming. The fundamental problem with these approaches is that the majority of microbial organisms in environmental samples have not been isolated in pure culture (Amann *et al.*, 1995), presenting two problems. Firstly, these organisms are unavailable for detailed studies as they cannot be cultured. Secondly, culture based studies carried out on populations containing these organisms will fail to detect them, producing biased results about the composition of the ecosystem. These issues can be overcome by the use of culture independent methods for studying microbial populations.

1.4.1 TEMPORAL TEMPERATURE GRADIENT GEL ELECTROPHORESIS

In 1993, Muyzer *et al*, introduced denaturing gradient electrophoresis as a powerful molecular tool to examine the diversity of environmental microbial populations at the genetic level. Temporal temperature gradient gel electrophoresis (TTGE) belongs to the denaturing gel electrophoresis family, which also includes denaturing gradient gel electrophoresis (DGGE). These techniques use denaturants and electrophoresis to physically separate DNA fragments based upon sequence differences to generate genetic fingerprints (Muyzer and Smalla, 1998; Muyzer, 1999). Denaturing gel electrophoresis has been used to analyse microbial communities found in numerous environmental samples including a multipond solar saltern (Casamayor *et al*., 2002), costal sand dunes (Kowalchuk *et al*., 1997b), fish farm sediments (McCaig *et al*., 1999) and activated sludge (Watanabe *et al*., 1998). These methods have also been used to examine bacteria associated with other organisms including coral (Bourne and Munn, 2005), marine diatoms (Grossart *et al*., 2005), marram grass (Kowalchuk *et al*., 1997a) and humans (Satokari *et al*., 2001; Schabereiter-Gurtner *et al*., 2002; Zoetendal *et al*., 2002; Siqueira *et al*., 2004). Denaturing gradient gels have also been utilised in the food industry to monitor bacterial populations important in fermented foods (Lee *et al*., 2005; Fontana *et al*., 2005).

The 16S rRNA gene is a molecular marker routinely used to identify and classify organisms based upon constant and variable regions within the gene (Woese, 1987). This feature can be exploited, such that all microbial 16S rRNA genes present in an environmental DNA sample can be separated based upon their unique DNA sequences. This produces a genetic fingerprint of the numerically predominant members of a microbial population (Muyzer and Smalla, 1998).

The genetic fingerprint can permit immediate side-by-side comparison of populations isolated from different habitats or treated under different experimental conditions. Analysis of banding patterns can vary from simple observations to the generation of data through statistical analyses (Fromin *et al*., 2002). Relationships between band changes, such as the appearance or disappearance of a band, can be associated with environmental variables. Based upon factors such as shared and unique bands or proportional band intensity, whole profile analysis can be carried out to determine

diversity indices and similarity matrices. Generation of a similarity matrix also permits the generation of dendrograms and application of clustering techniques. While these analyses provide information about the microbial population without characterising the species present, it is also possible to identify bands through DNA sequencing. However, environmental populations are generally poorly characterised, therefore a band's DNA sequence may not necessarily be present in genetic databanks for comparison purposes.

TTGE involves the formation of a denaturing polyacrylamide gel and the application of a temperature gradient during electrophoresis. During TTGE double stranded PCR products pass through the gel. Due to the denaturing environment (urea and temperature), DNA melts in discrete segments called melting domains. When the Tm of the lowest melting domain is reached, DNA becomes partially melted and the mobility of the DNA is reduced. The sequence of the DNA determines the melting point, with GC rich regions having a higher Tm than AT rich regions. To ensure that the migration of melted DNA through the gel is halted, a GC-clamp is attached to fragments during PCR amplification. The GC-clamp contains 30-40 bps of guanine and cytosine residues causing it to only melt in extreme denaturing conditions. Therefore, in the denaturing conditions of TTGE, the clamp does not denature while the lowest melting domain of the fragment does. In this partially melted form migration becomes significantly retarded, and optimal resolution of DNA is achieved (Sheffield *et al.*, 1989). The GC-clamp only permits melting of the lowest melting domain before mobility is restricted, allowing separation of DNA fragments that only differ by a single base pair (Sheffield *et al.*, 1989) (Figure 1.3).

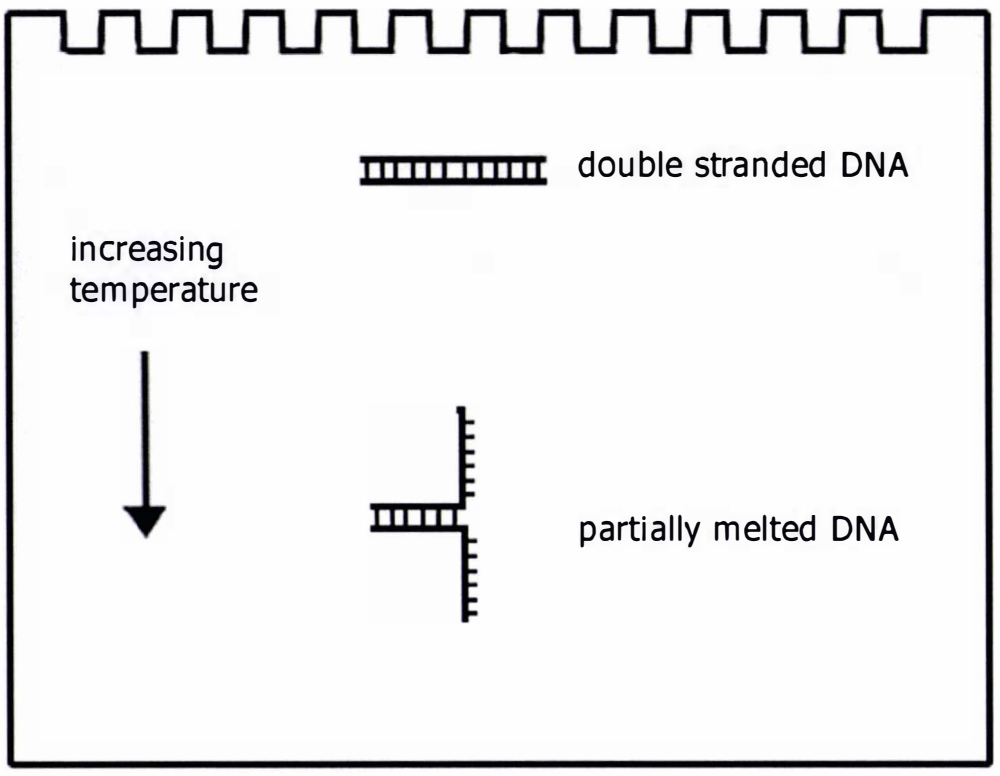


Figure 1.3. Double stranded PCR products containing a GC-clamp are loaded into the TTGE gel. During electrophoresis the temperature increases, establishing a denaturant gradient down the gel. Based upon the DNA sequence, the double stranded structure will begin to dissociate due to the denaturing environment. The GC-clamp remains intact, creating a forked structure. In this partially melted state the DNA has reduced mobility in the gel, and therefore forms a DNA band.

There are some universal limitations common to denaturing gradient gel systems. The use of degenerate primers can lead to the production of multiple bands on gels from a single DNA template, as the system can discriminate between single base changes located in the primer (Kowalchuk *et al.*, 1997b). An identical problem arises if the gene studied has multiple operons that contain small sequence differences. This problem has been encountered in 16S rRNA gene studies in which a pure culture of *Paenibacillus polymyxa* produced ten bands rather than one on a TGGE gel (Nubel *et al.*, 1996). It is also possible that two distinct microbial species with completely different sequences may exhibit an identical migration pattern. Some bacterial DNA sequences may contain multiple melting domains, giving rise to a smeared band in the TTGE gel profile, rather than a discrete band (Kisand *et al.*, 2003). The formation of heteroduplexes or chimeras

during PCR could also lead to the appearance of additional bands on gels that do not represent community species (Qiu *et al.*, 2001). Hairpin loops can also form in the GC-clamp during PCR. This may cause the early termination of elongation, which may give rise to new bands on gels (Nubel *et al.*, 1996). These examples demonstrate that potentially spurious products may generate bands on denaturing gels, or that each band on a gel may not simply represent only one microbial species.

Limitations are also associated with the preliminary stages of the method. Biases can be encountered during DNA extraction, for example bacteria that are lysed readily may be over represented in a population, compared to organisms that resist lysis. Therefore different DNA extraction methods can lead to different band profiles (Maarit Niemi *et al.*, 2001). Further biases may be introduced during PCR through preferential amplification of one template over another, for example GC-rich permutations at priming sites have been found to amplify with higher efficiency than AT-rich permutations (Polz and Cavanaugh, 1998).

1.4.2 REAL TIME POLYMERASE CHAIN REACTION

The quantitative real time polymerase chain reaction permits rapid quantitation of nucleic acids through PCR. Real time PCR has been applied to microbial communities to quantitate the general bacterial load and specific species (Mackay, 2004). The advantages of real time PCR over traditional quantitative methods is that uncultivated or undescribed micro-organisms can be targeted with broad or species specific primers, and the technology is highly sensitive and rapid.

In 2000, Suzuki *et al*, developed probe based real time PCR assays to target ribosomal RNA genes from the domains *Bacteria* and *Archaea* and the genera Synechococcus and Prochlorococcus. The authors demonstrated that the real time PCR assays could accurately estimate the gene abundance in complex nucleic acid mixtures.

Real time PCR has been compared against other methodologies for detecting and quantitating microbial populations. Fang *et al*, 2002, compared probe based real time PCR assays targeting *Mycobacterium avium* subspecies *paratuberculosis* to traditional culture enumeration and nested PCR. They found that real time PCR compared

favourably to these traditional methods. Nadkarni *et al*, 2002, have shown that real time PCR assays achieved 40-fold greater estimation of bacterial numbers in carious dentine than traditional culture methods. This technology has also been applied to the intestinal microbiota of the gut. Huijsdens *et al*, 2002, successfully quantitated *E. coli* and *B. vulgatus* populations in mucosal biopsy samples from the sigmoid colon, and Malinen *et al*, 2003, measured the abundance of 6 species in faecal samples. Real time PCR assays utilising Sybr Green I dye, rather than probes, have also been used to successfully quantitate bacteria in environmental samples (Stubner, 2002; Bach *et al*., 2002), and comparisons have demonstrated that both chemistries have similar sensitivities (Bach *et al*., 2002; Malinen *et al*., 2003).

During PCR amplification the formation of new double stranded DNA molecules follows a sigmoidal curve (Figure 1.4). Initially the reaction proceeds exponentially, with the number of DNA molecules doubling after each cycle. However the reaction eventually becomes exhausted and reaches the plateau phase. In real time PCR the original nucleic acid copy number is deduced from the amount of DNA formed during the exponential phase of the reaction. To achieve this, fluorescent dyes or probes are included in the reactions to bind to the synthesised DNA molecules. The amount of fluorescence produced correlates to the amount of DNA copies present in the sample.

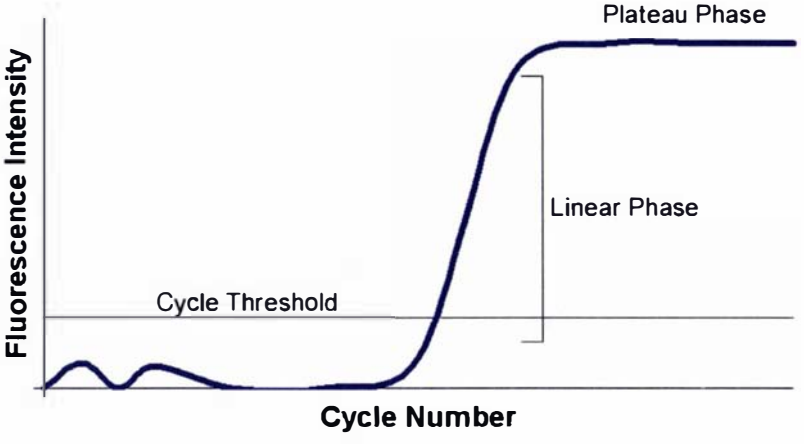


Figure 1.4. The optimal amplification plot during PCR is a sigmoid curve. Initially the accumulation of amplicons is masked by background fluorescence in the reaction. During the linear phase fluorescence derived from the amplicons exceeds the background, this point is known as the cycle threshold, from which the quantity of template is determined. The reaction becomes exhausted in the plateau phase and amplification is inhibited.

Standards of known gene copy number are included in real time PCR experiments. A dilution series of the standards permits the construction of a standard curve, from which unknown samples can be quantitated based upon the measurement of fluorescence. Standards must be amplified under identical reaction conditions and behave similarly to the target gene in order to be successful quantification tools. To achieve this, standards need to contain the same primer sites as the target, and the standard's amplified product should be approximately the same size as the target amplicon.

An inherent problem associated with quantitating organisms based upon gene copies is the variable number of gene copies present in different species. This problem is further complicated in environmental samples, which may contain previously undescribed organisms. The choice of a DNA standard is also affected by this phenomenon. It has been demonstrated that rapidly growing bacteria have approximately twice the number of ribosomal DNA operons than slower growing organisms, potentially leading to over or under estimation of population numbers depending on the standard used (Nadkarni *et al.*, 2002). To overcome these problems Suzuki *et al*, 2000 recommended that bacterial populations should not be expressed as absolute numbers, but rather should be expressed relative to the total ribosomal DNA measured from the domain *Bacteria.* Furthermore the use of plasmid clones containing a single copy of the gene of interest can prevent under or over estimation of total gene copy numbers present in samples (Suzuki *et al.*, 2000).

1.5 AIMS OF THE THESIS

Very little is understood about the intestinal microbiota, and the majority of studies examining this population have been carried out using cultivation techniques. The objective of the present study was to develop and validate molecular methodologies and to apply these techniques to pilot studies examining the faecal microbiota in healthy individuals and patients. TTGE was used to generate a DNA fingerprint of the predominant bacterial organisms in the population, and real time PCR was employed to provide quantitative data on bacteria, methanogen, and sulfate reducing bacteria

populations. These methods were used to characterise the normal temporal stability of the faecal microbiota, and to determine the degree of similarity in the predominant bacterial populations of un/related individuals, and patients with the same disease type. These techniques were also used to monitor the composition of the faecal microbiota following environmental challenges such as a dramatic dietary change, and bowel cleansing.

The specific aims of the thesis were to:

1. Optimise and validate TTGE and real time PCR to provide robust qualitative and quantitative data respectively.
2. Determine population dynamics over the short and long term in healthy individuals.
3. Compare twins and unrelated healthy volunteers to investigate if host genetics influence the composition of the predominant bacteria, and carriage of methanogens and sulfate reducing bacteria.
4. Determine if the population can be altered by an intervention that targets all members of the faecal microbiota; the impact of bowel cleansing and the subsequent reconstitution of the microbiota were monitored.
5. Determine if microbial populations can be altered by an extreme dietary change (the Atkins' diet).
6. Compare faecal microbiota populations amongst controls, and amongst disease groups to determine if a characteristic microbiota is associated with particular disease states.

CHAPTER 2: MATERIALS AND METHODS

The methods described below were developed and optimised in Chapter 3, and were subsequently applied to the studies carried out in Chapters 4 - 8.

2.1. ETHICAL APPROVAL

Ethical approval for this study was granted by the Wellington Ethics Committee, application 99/98: Microbial Factors in Pathogenesis of Irritable Bowel Syndrome and Inflammatory Bowel Disease.

2.2 MICROBIAL DNA FROM FAECAL SAMPLES

2.2.2 COLLECTION AND HOMOGENISATION OF FAECAL SAMPLES

Volunteers collected faecal samples in standard faecal sample tubes, which were stored at -20°C and processed as soon as possible. Samples were homogenised to ensure even distribution of organisms throughout the sample. Potassium phosphate buffer (0.05M, pH 7) was added to the faecal sample in a ratio of 1.3mL to 1g of faeces. Samples were vortexed with 6 glass beads (6mm diameter) for 10 minutes to thoroughly homogenise the sample.

2.2.3 FAECAL DNA EXTRACTIONS

The QIAmp DNA Stool Mini Kit's (Qiagen) "*Protocol for Isolation of DNA from Stool for Pathogen Detection*" was used to extract genomic DNA from homogenised stool samples. The manufacturer's instructions were followed with two minor amendments:

A 500µL aliquot of the homogenate was used as starting material rather than 200µL to account for sample dilution during homogenisation. The lysis temperature (step 3) was increased from 70°C to 95°C to improve lysis of gram-positive bacteria.
DNA concentrations were calculated based upon the sample's absorbance at 260nm, and the genomic DNA was visualised on ethidium bromide stained 1.5% agarose gels using UV light.

2.3 PCR-TTGE

2.3.1 AMPLIFICATION OF BACTERIAL 16S RIBOSOMAL RNA GENES

To selectively amplify the V6-V8 region of 16S rRNA genes from the domain *Bacteria*, the primers U968 and L1401 were used (Nubel *et al.*, 1996) (Table 2.1). Primer U968 has a 40bp GC-clamp attached to the 5' end to produce fragments that do not completely dissociate during TTGE (Sheffield *et al.*, 1989).

Table 2.1. Bacterial V6-V8 Region 16S rRNA Gene Primers. The sequence of the GC clamp attached to primer U968 is shown in italics.

Primers	Primer Sequence (5' to 3')
U968-GC	*CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G*AA CGC GAA GAA CCT TAC
L1401	GCG TGT GTA CAA GAC CC

Faecal DNA was amplified in a 50µL PCR using the Qiagen HotStar*Taq* PCR kit. The reactions were carried out with 2.5mM $MgCl_2$, 200µM dNTPs, 0.2µM of the forward (U968-GC) and reverse (L1401) primers. Cycling was carried out in an MJ Research thermal cycler under the following conditions: Initial denaturation and *Taq* polymerase activation at 95°C for 15 minutes. This was followed by 36 cycles of: denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds and extension at 72°C for 35

seconds. A final extension was carried out at 72°C for 7 minutes. PCR products were visualised on ethidium bromide stained 1.5% agarose gels under UV light.

2.3.2 TTGE CONDITONS

TTGE was carried out using the BioRad DCODE Mutation Detection System. A 6% acrylamide solution was prepared with 37.5:1 acylamide:bis, 8M of urea, 1x TAE, 10% TEMED, and 10% APS and poured between 16cm x 16cm glass plates separated by 1mm spacers. Seven litres of 1x TAE buffer was preheated to 56°C and the temperature controller was allowed at least 15 minutes to stabilise at a ramp rate of 0.7 °C per hour. Bacterial PCR products were mixed with an equal volume of loading dye (70% glycerol, 0.05% bromophenol blue) and then loaded into pre-rinsed gel wells. Gels were run at 80V for 14 hours and 20 minutes. Over this time, the temperature increased by 0.7°C per hour from 56°C to 66°C.

2.3.3 STAINING TTGE GELS

TTGE gels were silver stained. The gel was fixed in 10% ethanol for 5 minutes, then oxidised in 1% nitric acid for 10 minutes. The gel was then rinsed in Milli-Q water for 2 minutes. Staining was carried out by incubating the gel in 0.1% silver nitrate for 15 minutes. The gel was then rinsed in water for 2 minutes and incubated in developing solution (0.019% formalin, 0.28M Na_2CO_3) until bands appeared. The developing solution was changed and replaced with fresh solution as it discoloured. Finally the gel was incubated in fixing solution (10% acetic acid) for 10 minutes.

2.3.4 TTGE QUALITY CONTROL

TTGE profiles were monitored to ensure TTGE bands were optimally resolved. This was achieved by including a bacterial ladder in every run. The bacterial ladder consisted of PCR products of the 16S rRNA gene amplified using the U968-GC and L1401 primer set. The PCR products were derived from five bacterial species; *Clostridium perfringens, Proteus mirabilis, Klebsiella pneumoniae, Listeria*

monocytogenes, and *Escherichia coli.* These PCR products all migrate differently under the denaturing conditions of TTGE, yielding a characteristic TTGE profile. If the characteristic profile was not generated, the TTGE experiment was repeated.

2.3.5 TTGE GEL BAND PURIFICATION

PCR products were run across 5 lanes of the TTGE gel. To avoid fixation of the DNA, the gels were stained in ethidium bromide (10μg/mL) for 15 minutes, followed by 10 minutes to de-stain in dH_2O. The target band was visualised under UV light and excised from each lane using a sterile scalpel blade. Gel fragments were placed together in 1mL of sterile dH_2O. The dH_2O was heated at 95°C for 20 minutes, and left at 40°C for 72 hours to allow DNA to diffuse from the gel fragments into solution. DNA was then purified and concentrated using the QIAquick Gel Extraction Kit (Qiagen).

2.3.6 TTGE PROFILE ANALYSIS

Banding patterns in TTGE gels were scored by two independent investigators and by the 1D Image Analysis Software (Kodak Digital Science). From the scored profiles the software package generated migration and net intensity values for each band.

Sorenson's similarity coefficient (C_s) was calculated to provide a measure of two TTGE profiles' similarity (McCracken *et al.*, 2001). Where 'j' represents the number of shared bands, 'a' represents the total bands in lane 1, and 'b' represents the total bands in lane 2.

$$C_s = \frac{2j}{(a + b)} \times 100$$

Shannon's Index (H') was calculated to measure the proportional abundances of species in the population from the TTGE profile (McCracken *et al.*, 2001), and therefore provided an estimate of community richness. 'P_i' is the proportion of the population

belonging to the *i*th species (ie, the proportion of the total band intensity for the lane, attributable to the *i*th band).

$$H' = - \sum p_i \ln(p_i)$$

C_s and H' values were analysed statistically using T-tests, one-way ANOVA, and Pearson's product moment correlation coefficient. P values were obtained for 2-tailed analyses in all cases. Statistical calculations were carried out using Microsoft Excel 2000 and MINITAB 14.1 (Minitab Inc).

2.4 REAL TIME PCR

Assays were developed to quantitate copy numbers for the bacterial 16S rRNA gene, the archaeobacterial 16S rRNA gene, and the sulfate reducing bacterial APS reductase gene.

2.4.1 REAGENTS AND CONDITIONS

The ABI Prism 7000 Sequence Detection System was used to quantitate target genes present in DNA samples. Reactions were carried out according to the manufacturer's instructions using the Invitrogen Platinum SYBR green qPCR SuperMix UDG kit. ROX reference dye was included in all master mixes to normalise the fluorescent reporter signal. A total volume of 25µL was prepared for each reaction in 96 well plates. The primer set U968-GC and L1401 were used as described in PCR-TTGE assays, however the GC clamp was removed to minimise the likelihood of primer dimer formation and to improve efficiency (Table 2.2). To target methanogens, the archaeobacterial 16S rRNA gene primers Arch 344f (Casamayor *et al.*, 2002) and Arch 806r (Takai *et al.*, 2000) were used, and APSfw and APSrv primers were used to target the APS reductase gene of sulfate reducing bacteria (Deplancke *et al.*, 2000) (Table 2.2). Bacterial and archaeobacterial primer sets were used at a final concentration of

0.1μM, and sulfate reducing bacterial primers were used at 0.5μM for the degenerate forward primer APSfw, and 0.2μM for the reverse primer APSrv.

Table 2.2. Primer sets used in real time PCR assays

Target Gene	Primer Set	Primer Sequence (5' to 3')
Bacterial 16S rRNA gene	U968	AA CGC GAA GAA CCT TAC
	L1401	GCG TGT GTA CAA GAC CC
Archaeobacterial 16S rRNA gene	Arch 344f	AC GGG GY[2]G CAG CAGGCGCGA
	Arch 806r	GGA CTA CCC GGG TAT CTA AT
Sulfate reducing bacteria APS reductase gene	APSfw	TGG CAG ATM[1] ATG ATY[2] M[1]AC GG
	APSrv	GG GCC GTA ACC GTC CTT GAA

M: nucleotide A or C
Y: nucleotide C or T

To ensure all reactions had the same amount of template DNA, samples were quantified using a biophotometer (Eppendorf). All samples were made up to a concentration of 10ng/μL and a 2μL aliquot used in real time PCR assays.

Bacterial and archaeobacterial real time PCR assays was carried out under the following conditions: UDG digestion at 50°C for 2 minutes, followed by inactivation of the enzyme at 95°C for 15 minutes. Cycling was carried out with a denaturation step at 95°C for 15 seconds followed by an annealing step at 63°C for 1 minute.

The sulfate reducing bacteria real time PCR assay was carried out under the same conditions, except that a change was made to the annealing step. Annealing was carried out at 63°C for 30 seconds followed by a further incubation at 85°C for 30 seconds.

2.4.2 QUANTITATION USING EXTERNAL STANDARDS

An absolute quantitation method was used to determine the gene copies present in unknown samples. A seven point, 10 fold dilution series of the external standard was prepared and a standard curve constructed (Ct vs log{copy number}). Based upon the Ct values of unknown samples the gene copies present in these samples were determined from the standard curve.

2.4.3 QUALITY CONTROL

Triplicate reactions were carried out for each unknown sample and each standard sample. The average Ct from these triplicates was used in analysis. A 'no template' control sample was included in each plate to permit detection of any DNA contamination. To ensure the integrity of the standard curve, a threshold of 0.990 was set for the R2 value. Following real time PCR, plates were immediately stored at –20°C to prevent product degradation. To ensure that SYBR green fluorescence was only derived from specific amplification products all samples were assessed on agarose gels stained with ethidium bromide and viewed under UV light. Dissociation curves were analysed for the sulfate reducing bacteria assay to ensure the specific products remained double stranded at 85°C.

2.4.4 STATISTICAL ANLAYSIS

Average values from the triplicates were analysed using t-tests and one-way ANOVA. P values were obtained for 2-tailed analyses in all cases. Statistical calculations were carried out using Microsoft Excel 2000 and MINITAB 14.1 (Minitab Inc).

2.5 SEQUENCING

Prior to sequencing, DNA samples were purified with the QIAquick Gel Extraction Kit (Qiagen). The Alan Wilson Centre Genome Service (Massey University, New Zealand) sequenced purified PCR products and plasmid inserts. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primer set U968 and L1401, APSfw and APSrv, or Arch 344f and Arch 806r. Sequences were compared against sequences in GenBank using BLAST software (National Center for Biotechnology Information, National Library of Medicine, Bldg. 38A, Rm 8N-301-480-9241; email, info@ncbi.nlm.nih.gov; URL, http://www.ncbi.nlm.nih.gov.)

CHAPTER 3: OPTIMISATION AND VALIDATION OF METHODS

3.1 ABSTRACT

PCR-TTGE and real time PCR assays were optimised and validated for the study of bacteria, methanogens and sulfate reducing bacteria in faecal DNA samples. The PCR-TTGE assay successfully targeted the bacterial domain with 16S rRNA gene primers and could detect bacterial DNA comprising as little as 1% of the population. TTGE profiles were analysed to determine the impact of DNA chimeras, *Taq* errors, single stranded DNA and heteroduplexes on the TTGE banding profile. The PCR-TTGE assay was found to generate highly reproducible banding patterns from faecal DNA samples. All real time PCR assays had high reproducibility and precision. External standards were prepared from plasmid DNA to allow quantitation of unknown samples from a standard curve. The use of the plasmid standards was validated by experiments that demonstrated equivalent amplification efficiencies between external standards and samples. The real-time PCR assays were found to specifically amplify target genes in bacteria, methanogens and sulfate reducing bacteria. The assays for methanogens and sulfate reducing bacteria had detection limits of 11 and 4 gene copies respectively.

3.2 INTRODUCTION

PCR-TTGE and real time PCR assays can be used to study uncharacterised microbial populations such as the intestinal microbiota. These methods use nucleic acids to describe populations qualitatively (PCR-TTGE) and quantitatively (real time PCR). This molecular approach permits rapid and sensitive detection of both previously described organisms and uncharacterised species.

PCR-TTGE is a powerful technique that provides a visual snap shot of a population. Samples can be compared to each other to determine the similarity of the species composition and the effect of clinical interventions on the population. While this technique can rapidly provide an enormous amount of information about a population, its power can be reduced by the inherent limitations of multi-template PCR. Heteroduplexes, DNA chimeras, *Taq* mutations, and single stranded DNA can potentially form spurious bands in TTGE profiles (Espejo *et al.*, 1998; Simpson *et al.*, 1999; Qiu *et al.*, 2001; Thompson *et al.*, 2002).

Real time PCR allows the rapid quantitation of microbial populations. By incorporating SYBR green I (a fluorescent dye that binds double stranded DNA) into the reaction the exponential accumulation of DNA can be monitored. From standards of known gene copies the amount of starting template in each sample can be determined.

It is important to optimise storage conditions, DNA extraction and primer specificity to ensure that the protocol is robust and reproducible. To work effectively, the length of PCR products should be minimised with a maximum of 500bp for PCR-TTGE (Myers *et al.*, 1985) or 1000bp for real time PCR (Roche Molecular Biochemicals, 2000). PCR-TTGE primers must also carry a GC-clamp at the 5' end to prevent complete dissociation of double stranded PCR products during TTGE (Sheffield *et al.*, 1989). The presence of non-specific products can distort TTGE profiles and quantitation results leading to over-estimation of diversity.

One PCR-TTGE assay and 3 real time PCR assays were developed and optimised to target the following genes in faecal DNA samples:

1. The 16S rRNA gene of the domain *Bacteria*
2. The 16S rRNA gene of the domain *Archaea*
3. The APS reductase gene of sulfate reducing bacteria

The 16S rRNA gene codes for a component of the 30S subunit of ribosomes. The gene is composed of variable and constant regions, a characteristic that makes it ideal for comparing both distantly related organisms and those that are very closely related. The 16S rRNA gene was used to target the bacterial population in order to analyse species composition in PCR-TTGE and for quantitation of the total bacterial population in real time PCR. In addition, the archaeobacterial 16S rRNA gene was used to detect and quantitate methanogens, the only archaeobacterial organisms known to populate the human gut (Levitt, 1995).

16S rRNA genes could not be used to target the sulfate reducing bacteria as these organisms are a diverse group of both bacterial and archaeobacterial species. They share the metabolic characteristic of being able to dissimilate sulfate into hydrogen sulfide. This evolutionary diversity limits the usefulness of 16S rRNA genes to target all members of this group. A gene ubiquitous to all dissimilatory sulfate reducers encodes the enzyme APS reductase (Peck, 1962; Stille, 1984). This enzyme is responsible for the conversion of adenosine-5'-phosphosulfate (APS) into sulfite and AMP during dissimilatory sulfate reduction (Figure 3.1) and has previously been used to study populations of sulfate reducing bacteria (Deplancke *et al*., 2000). Therefore to quantitate the sulfate reducing bacteria, the APS reductase gene was targeted.

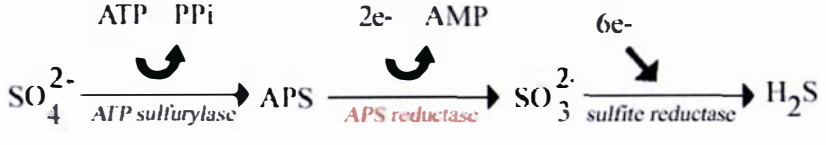


Figure 3.1. The reduction of sulfate to sulfide is dependent upon the enzyme *APS reductase* in sulfate reducing bacteria. Figure adapted from Deplancke *et al*, 2000.

This chapter describes the optimisation and validation of PCR-TTGE and real time PCR assays to generate robust qualitative and quantitative data on faecal populations of bacteria, methanogens and sulfate reducing bacteria.

3.3 MATERIALS AND METHODS

3.3.1 VOLUNTEERS/DNA EXTRACTIONS/PCR-TTGE/REAL TIME PCR/SEQUENCING

Faecal DNA samples provided by 6 healthy volunteers were used in the optimisation and validation of molecular methodologies. DNA extractions, PCR-TTGE, real time PCR, and sequencing methods were carried out as described in Chapter 2: Materials and Methods, unless otherwise indicated.

3.3.2 GEL STAINING METHODS

Four methods were tested to obtain optimal staining of band profiles and minimal background stain (3.3.2.1 – 3.3.2.4)

3.3.2.1 Ethidium Bromide Staining

Gels were incubated in 10μg/mL of ethidium bromide for 15 minutes, followed by 10 minutes to de-stain in dH_2O. Gels were photographed using the 1D Image Analysis Software (Kodak Digital Science).

3.3.2.2 Silver Staining Method 1 (Cairns, 1994)

Gels were incubated in fixing solution (10% ethanol, 0.5% acetic acid) for 3 minutes, followed by incubation in staining solution (10% ethanol, 0.5% acetic acid, 0.2% silver nitrate) for 5 minutes. The gel was then washed once for 20 seconds and once for 2 minutes in Milli-Q water. The gel was incubated in developing solution (3% NaOH, 0.5% formaldehyde) until bands appeared. Finally gels were treated with fixing solution for 5 minutes.

3.3.2.3 Silver Staining Method 2

The gel was fixed in 10% ethanol for 5 minutes, then oxidised in 1% nitric acid for 10 minutes. The gel was then rinsed in Milli-Q water for 2 minutes. Staining was carried

out by incubating the gel in 0.1% silver nitrate for 15 minutes. The gel was then rinsed in water for 2 minutes and incubated in developing solution (0.019% formalin, 0.28M Na_2CO_3) until bands appeared. The developing solution was changed and replaced with fresh solution as it discoloured. Finally the gel was incubated in fixing solution (10% acetic acid) for 10 minutes.

3.3.2.4 Silver Staining Method 3

The gel was fixed in 10% acetic acid for 20 minutes, followed by 3 rinses in Milli-Q water for 2 minutes. The gel was treated with 1% nitric acid for 10 minutes and subsequently rinsed in Milli-Q water for 2 minutes. The gel was stained for 15 minutes in 0.1% silver nitrate, and then rinsed in water for no longer than 10 seconds. The gel was treated with developing solution (0.019% formalin, 0.28M Na_2CO_3) until bands appeared. The developing solution was changed and replaced with fresh solution as it discoloured. Development was stopped by incubating for 10 minutes in fixing solution (10% acetic acid).

3.3.3 T7 ENDONUCLEASE I DIGESTION

Thirty units of T7 Endonuclease I (New England Biolabs Inc) were incubated at 37°C with 25µL PCR products in 1x NEBuffer 4, for 30 minutes. The reaction was stopped using the QIAquick Gel Extraction Kit (Qiagen) protocol for Enzymatic Clean-up.

3.3.4 MUNG BEAN NUCLEASE DIGESTION

One unit of Mung Bean Nuclease (New England Biolabs Inc) was added for every 1µg of PCR product, and incubated in 1x Mung Bean Nuclease buffer at 30°C for 30 minutes. The reaction was stopped using the QIAquick Gel Extraction Kit (Qiagen) protocol for Enzymatic Clean-up.

3.3.5 RE-CONDITIONING PCR

PCR was carried out to amplify bacterial 16S rRNA gene fragments as described in section 2.3.1. Once the cycling was complete, 5µL of the PCR products were removed

and used as template in a new reaction. Cycling conditions for this reaction were altered to 95°C for 15 minutes, followed by 3 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 35 seconds.

3.3.6 ELONGATION PCR

PCR was carried out to amplify bacterial 16S rRNA gene fragments as described previously in section 2.2.1, however the elongation step at 72°C was extended from 35 seconds to 4 minutes for all 36 amplification cycles.

3.3.7 BACTERIAL DNA SAMPLES

Bacterial DNA was used in optimisation and validation of the assays, and construction of a bacterial DNA ladder. Bacterial DNA samples from *Bacteroides vulgatus* (ATCC 29327), *Bacteroides fragilis* (ATCC 25285), *Yersinia enterocolitica* (ATCC 27739), *Clostridium difficile* (ATCC 6989), *Clostridium perfringens* (ATCC 13124), *Escherichia coli* (ATCC 12651), *Proteus mirabilis* (NCTC 5887), *Klebsiella pneumoniae* (ATCC 13883), *Listeria monocytogenes* (ATCC 7302), *Micrococcus lysodeicticus* (ATCC 27523), and *Helicobacter pylori* (ATCC 43526) were used.

3.3.8 PREPARATION OF EXTERNAL STANDARDS FOR REAL TIME PCR

Permission to clone PCR products, derived from human faecal DNA samples, into the plasmid vector pCR4-TOPO was granted by the Genetic Technology Committee (Massey University, Palmerston North, New Zealand), application GMO 03/Mu/19 Molecular Genetic Analysis of Human Intestinal Microbiota.

PCR products amplified from the bacterial 16S rRNA gene, archaeobacterial 16S rRNA gene, and sulfate reducing bacteria's APS reductase gene were prepared from faecal DNA samples as described in section 2.3.1 and 2.4.1. However, for archaeobacteria and sulfate reducing bacteria assays the HotStar*Taq* kit was used rather than the Invitrogen Platinum SYBR green qPCR SuperMix UDG kit. PCR products were cloned in the plasmid vector pCR4-TOPO (Invitrogen) using the TOPO TA Cloning Kit for Sequencing (Invitrogen). The plasmids were then transformed into TOP10 chemically

competent *E. coli* cells (Invitrogen), and plated out onto LB plates containing kanamycin (50μg/mL). Plates were incubated at 37°C overnight. Colonies were used to inoculate LB broth containing kanamycin (50μg/mL) and incubated at 37°C overnight.

Plasmid DNA was extracted from the bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid inserts were sequenced as described in section 2.5.

Plasmid DNA samples were diluted 1:10 and quantified in triplicate using a biophotometer. Gene copy numbers were determined using the average DNA concentration and the total size of the plasmid containing the insert. Aliquots of the 1:10 dilution were prepared and stored at –20°C.

3.4 RESULTS

3.4.1 OPTIMISATION OF DNA EXTRACTIONS FROM FAECAL SAMPLES

The QiaAmp DNA Stool Mini Kit was used for all DNA extractions. To ensure adequate lysis of microbial cells, comparisons were made between aliquots of the same faecal sample, incubated for 2.5, 5, 7.5, 10 and 15 minutes at 95°C in the QiaAmp DNA Stool Mini Kit's ASL buffer (lysis buffer). No additional bands were evident in the TTGE profiles following extended incubation in lysis buffer (Figure 3.2), therefore the 5 minute incubation time was used in all subsequent DNA extractions. In addition, the high level of similarity evident in the TTGE profiles demonstrated the reproducibility of the faecal DNA extraction.

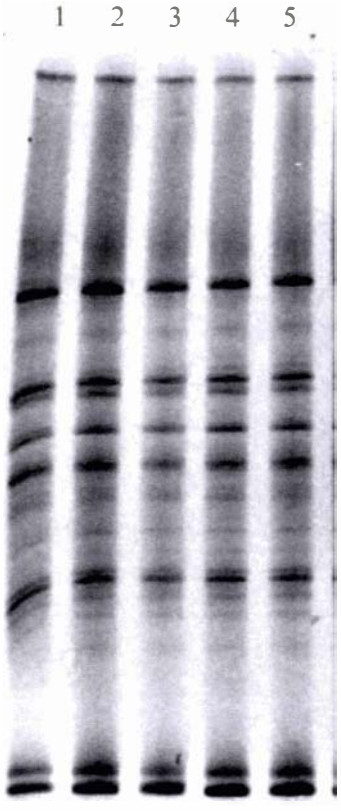


Figure 3.2. Bacterial 16S rRNA genes were amplified and TTGE profiles compared from 5 DNA samples that were prepared using different lysis times at 95°C in ASL buffer (Qiagen). Lane 1, 2.5 minutes; Lane 2, 5 minutes; Lane 3, 7.5 minutes; Lane 4, 10 minutes; Lane 5, 15 minutes.

3.4.2 OPTIMISATION AND APPLICATION OF TTGE

3.4.2.1 Effect of Template Amount on TTGE Profiles

A template gradient was constructed to determine the optimal amount of DNA to be used in PCR-TTGE. Aliquots of 400, 200, 100, 50, 20, and 5ng of template faecal DNA were used in 6 PCRs. The resulting TTGE profiles demonstrated a consistent bacterial banding pattern regardless of the original template amount (Figure 3.3).

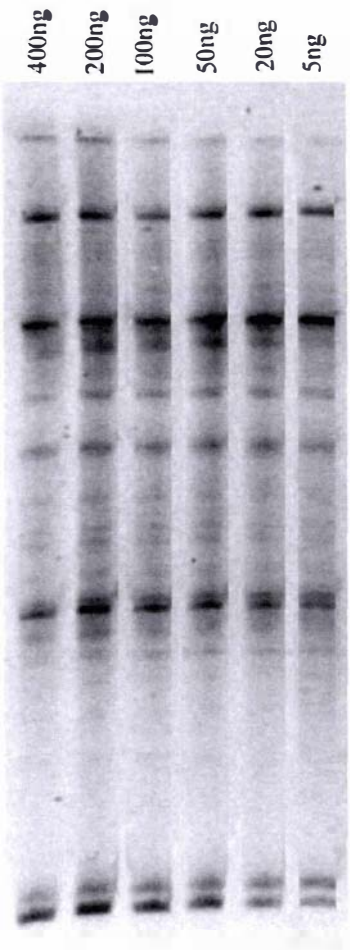


Figure 3.3. Bacterial TTGE profiles prepared with a template gradient of faecal DNA in the PCR.

3.4.2.2 Specificity of PCR-TTGE Assay

The specificity of the bacterial primer set was tested by amplifying template DNA from several bacterial species, and the archaeobacterial species, *Methanococcus voltae*. The specific product (~ 470bp) was formed from all templates except that of *Methanococcus voltae* (Figure 3.4).

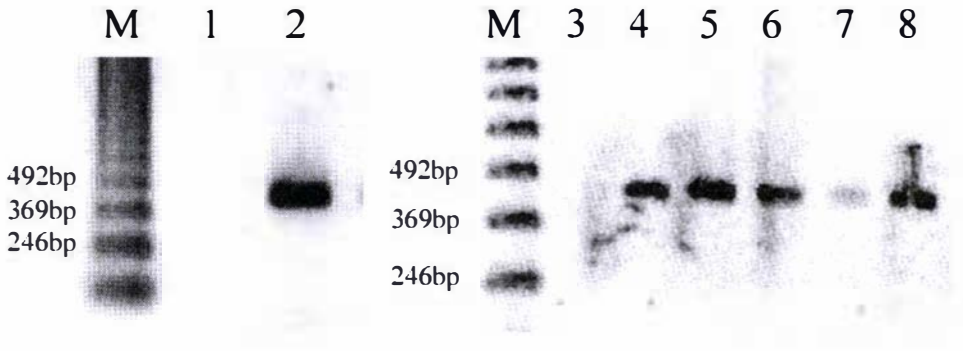


Figure 3.4. The specificity of the bacterial primer set U968-GC and L1401 was tested against target and not target templates. Lane M, 123bp DNA ladder; Lane 1, no template control; Lane 2, faecal DNA sample; Lane 3, *M. voltae* (archaeobacterial species); Lane 4, *C. difficile*; Lane 5, *C. perfringens*; Lane 6, *E. coli*; Lane 7, *Y. enterocolitica*; and Lane 8, *L. monocytogenes*.

To ensure the bands within the TTGE profile represented bacterial organisms, sequencing was carried out. A faecal DNA sample from a healthy volunteer was amplified using the U968-GC and L1401 primers, and the PCR products were run on a TTGE gel. Bands were excised from the gel, and the DNA purified as described in section 2.3.5. Sequence analysis found that the products all had the greatest identity to bacterial clones or bacterial species that had all previously been isolated from the human gastrointestinal tract (Figure 3.5).

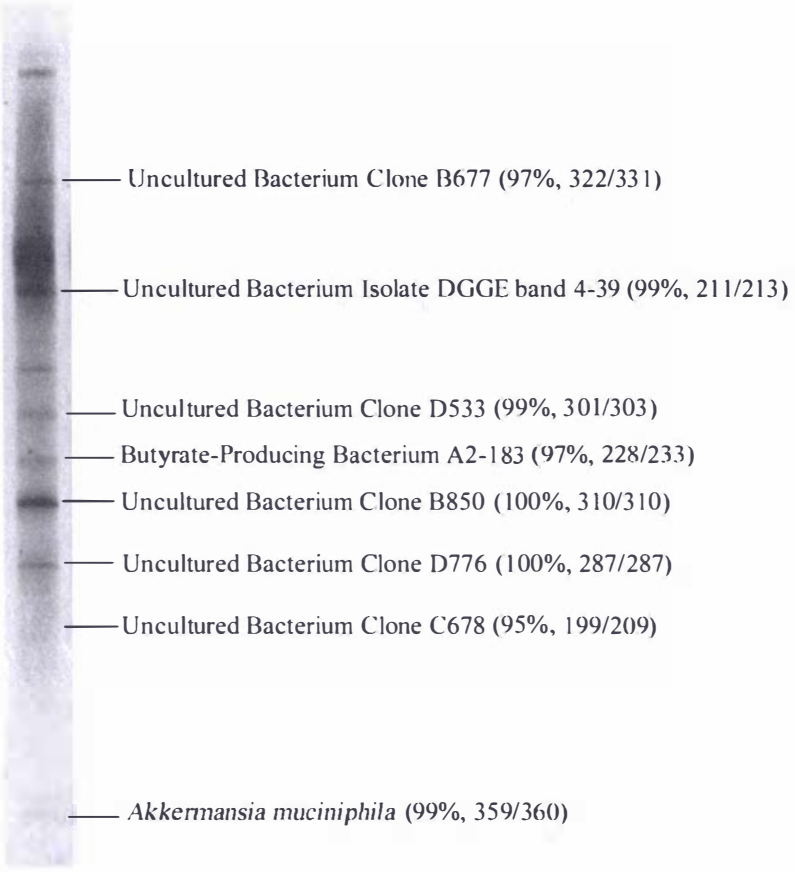


Figure 3.5. Bacterial TTGE bands were excised from the gel and the DNA purified. DNA was sequenced and organisms with greatest identity were obtained through BLAST searches of GenBank. Percentage values refer to the identity between the purified bands' DNA sequence and the sequence present in GenBank. Ratios show the number of shared nucleotides between the two sequences with respect to the total number of nucleotides compared.

3.4.2.3 Reproducibility of the PCR-TTGE Assay

TTGE profiles produced from 8 duplicate PCR reactions were compared to test the reproducibility of the PCR-TTGE assays. All 8 PCR reactions produced highly similar TTGE profiles (Figure 3.6). Determination of Sorenson's similarity co-efficients revealed a range from 94-100%, with a median similarity of 96% in the profiles (data not shown).

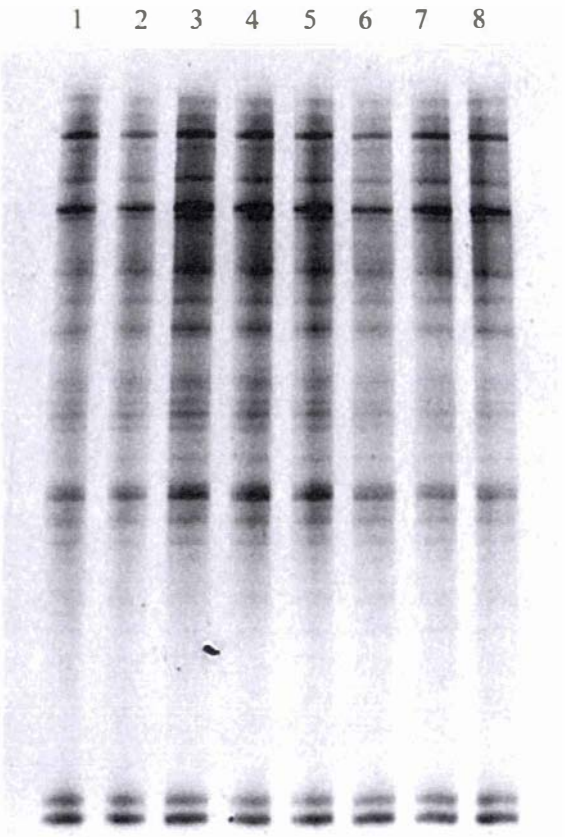


Figure 3.6. Eight separate PCRs were performed (Lanes 1-8) on a single faecal DNA sample to amplify the V6-V8 region of the bacterial 16S rRNA gene. The products were analysed on TTGE to assess the reproducibility of the PCR

3.4.2.4 Sensitivity of the PCR-TTGE Assay

An experiment was carried out to determine if the DNA of an individual species could be detected even if it comprised only 1% of the total DNA template. A mixture of *Clostridium difficile, Proteus mirabilis*, and *Klebsiella pneumoniae* template DNA was made, where the amount of *P. mirabilis* DNA varied from 33%-1% of the total DNA template. *P. mirabilis* band could be detected down to 1% of the population (Figure 3.7).

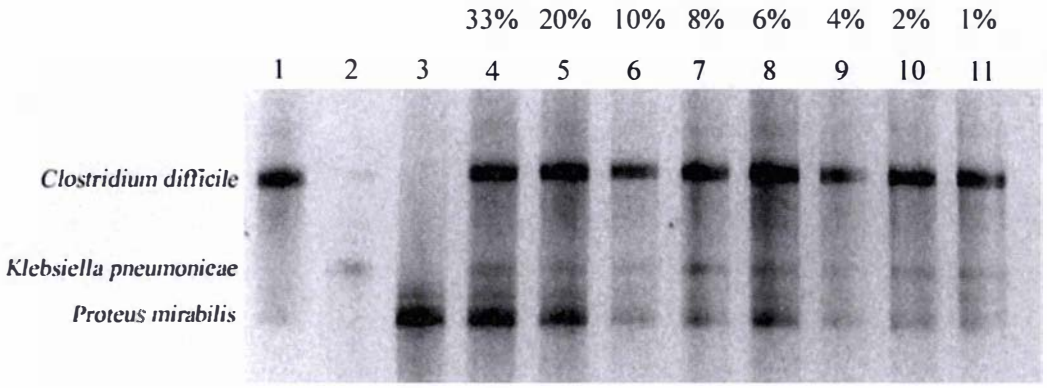


Figure 3.7. PCR-TTGE profiles prepared from a mixture of genomic DNA from 3 bacteria. Each PCR was prepared with different proportions of *Proteus mirabilis* starting template, ranging from 33% to 1%. Lane 1 contains *C. difficile* alone; Lane 2 contains *K. pneumoniae* alone; Lane 3 contains *P. mirabilis* alone. Lanes 4-11 contain DNA from all 3 organisms, and decreasing amounts of *P. mirabilis* (as shown).

3.4.2.5 TTGE Gel Staining

Comparisons of ethidium bromide staining and three silver staining methods (Methods 1, 2 and 3) were carried out to assess suitability for monitoring banding patterns in TTGE gels. The same sample was run in 4 lanes spaced apart on a single TTGE gel. Following electrophoresis the gel was cut into 4 separate pieces and each section was stained using a different method (Figure 3.8). Ethidium bromide staining proved to be less sensitive than all three silver stain methods, with some bands almost undetectable compared to silver staining. The best silver stained gel was prepared using method 2, which had the clearest banding profile. The bands were more readily identifiable in the native gel, than in the printed image.

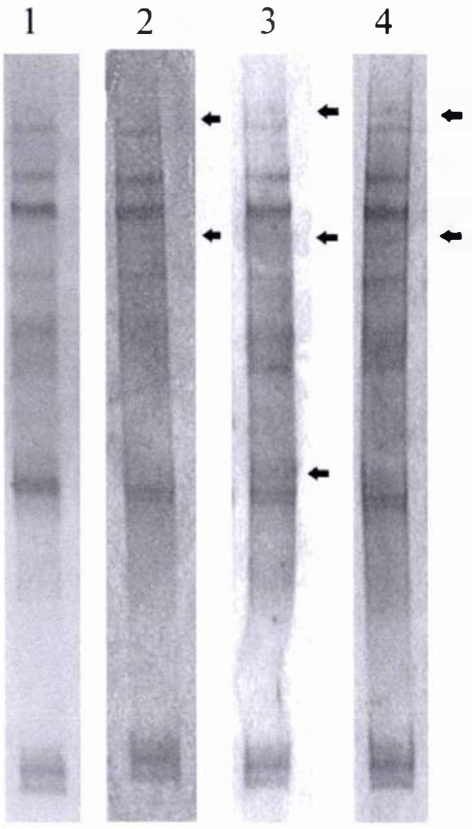


Figure 3.8. The same bacterial PCR products were electrophoresed in 4 lanes on a single gel. The gel was cut into 4 pieces and each lane was stained using a different method. Lane 1, ethidium bromide; Lane 2, silver staining method 1; Lane 3, silver staining method 2; Lane 4, silver staining method 3. Arrows indicate bands that are more readily detectable using silver staining rather than ethidium bromide staining.

3.4.2.6 Identification of TTGE Gel Artefacts

Two methods were used to determine if heteroduplexes contributed significantly to TTGE profiles. The enzyme T7 endonuclease I was used to digest heteroduplexes in PCR products (Qiu *et al.*, 2001) prior to TTGE analysis. Comparisons to an untreated sample showed that the enzyme had selectively digested a smear, and a band located at the very top of the gel (Figure 3.9A). An alternative method called re-conditioning PCR (Thompson *et al.*, 2002) was also employed where a sample of PCR products was diluted 10 fold in a new PCR reaction, and cycling carried out for only 3 cycles. In this new reaction, the primer excess should prevent non-homologous DNA sequences from

annealing to one another. This method also removed the smear at the very top of the TTGE gel, and increased the relative intensity of the upper band (Figure 3.9A).

An attempt to minimise chimera formation was made by extending the elongation cycle during PCR to 4 minutes (Qiu *et al.*, 2001). This did not alter the TTGE profile (Figure 3.9A).

Single stranded DNA has been reported to reduce the visibility of TTGE banding patterns (Simpson *et al.*, 1999). Mung Bean Nuclease was used to selectively digest single stranded DNA in PCR products. This treatment removed the uppermost band present in the TTGE profile, but failed to enhance the visibility of any additional bands (Figure 3.9B).

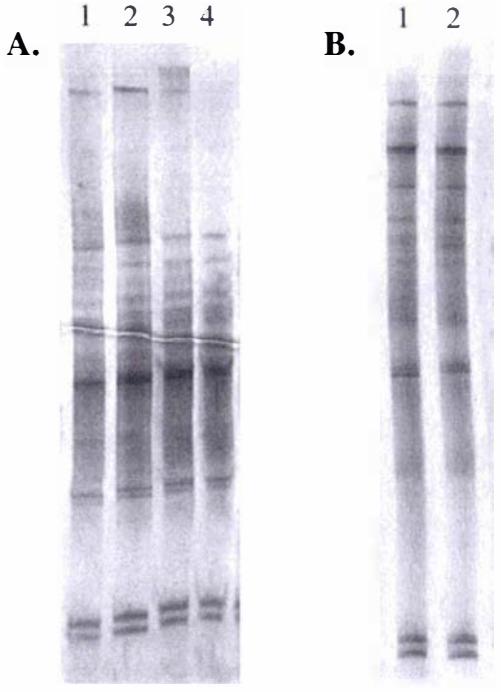


Figure 3.9. A. Bacterial 16S rRNA PCR products were divided into 3 aliquots and treated to reduce heteroduplexes and chimeras. A sample was subjected to reconditioning PCR to remove heteroduplexes through primer excess (Lane 1). A PCR reaction was also carried out with an increased elongation time of 4 minutes to minimise chimera formation (Lane 2). One sample was left untreated (Lane 3). One sample was treated with T7 Endonuclease I to selectively digest heteroduplexes (Lane 4). B. Bacterial 16S rRNA products prepared from a different DNA sample were divided into 2 aliquots, and one was treated with Mung Bean Nuclease to remove single stranded DNA (Lane 1), the other was untreated (Lane 2).

To assess the impact of random *Taq* polymerase errors on TTGE profiles, the reproducibility of the PCR-TTGE assay was reviewed (Figure 3.6). The high degree of reproducibility suggests that random *Taq* mutations have no significant effect on the TTGE profiles generated from this assay.

3.4.2.7 Construction of a Bacterial Ladder

In order to monitor the quality of band separation during TTGE experiments a bacterial DNA ladder was developed. The ladder was constructed from bacterial DNA samples available in the laboratory. Using the bacterial GC-clamped primers, DNA was amplified from *Bacteroides vulgatus, Bacteroides fragilis, Yersinia enterocolitica, Clostridium difficile, Clostridium perfringens, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus lysodeicticus,* and *Helicobacter pylori.* Subsequent analysis of their migration on a TTGE gel lead to the selection of *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Listeria monocytogenes,* and *Clostridium perfringens* species (Figure 3.10).

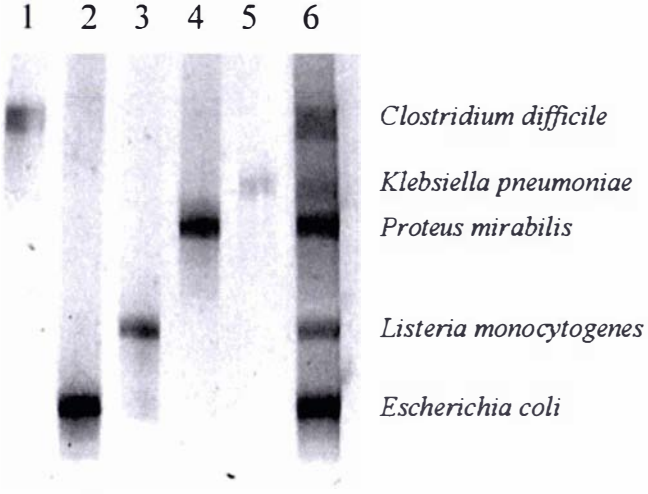


Figure 3.10. Bacterial PCR products from 5 bacteria were used to construct a bacterial ladder for TTGE. Lane 1, *Clostridium perfringens*; Lane 2, *Escherichia coli*; Lane 3, *Listeria monocytogenes*; Lane 4, *Proteus mirabilis*; Lane 5, *Klebsiella pneumoniae*; Lane 6, ladder containing all 5 bacterial species' PCR products.

3.4.3 OPTIMISATION OF REAL TIME PCR ASSAYS

3.4.3.1 Primers

Initial optimisation experiments using the APS reductase gene primers produced non-specific products, despite increased reaction stringency (data not shown). Re-evaluation of the primers using gene alignments to sulfate reducing bacteria revealed continual mismatches at the terminal 3' residue (Table 3.1). Only one species, *Desulfofrigus oceaneus* (gi: 18034222), was found to have the primer's G residue at this position. Given the lack of homology seen at this crucial site it was removed from the primer sequence. Using the altered forward primer for the APS reductase gene the PCR was optimised to produce a single product.

Table 3.1. The APS reductase gene's forward primer (APSfw) sequence was aligned against the DNA sequences from sulfate reducing bacterial species from different genera. Mismatches are highlighted in red.

Organism	Sequence (5' to 3')
Primer APSfw	TGGCAGATMATGATYMACGGG
Desulfobacter postgatei	TGGCAGATCATGATCAACGGT
Desulfococcus biacutus	TGGCAGATCATGATCAACGGC
Desulfofrigus oceaneus	TGGCAGATCATGATCAACGGG
Desulfovibrio vulgaris	TGGCAGATCATGATCAACGGT
Desulfocapsa thiozymogenes	TGGCAGATCATGATYMACGGT
Desulfobulbus elongatus	TGGCAGATCATGATCAACGGC
Desulforhopalus singaporensis	TGGCAGATCATGATCAACGGT
Desulfacinum hydrothermale	TGGCAGATCATGATCAACGGT
Thermodesulforhabdus norvegica	TGGCAGATCATGATCAACGGT
Desulfobacterium anilini	TGGCAGCTCATGATCAACGGT
Desulfovibrio baarsii	TGGCAGATCATGATCAACGGC
Desulfomonile tiedjei	TGGCAGCTCATGATCAACGGC
Desulfotomaculum halophilum	TGGCAGATCATGCTTAACGGT

M: nucleotide A or C
Y: nucleotide C or T

Primers targeting 16S rRNA genes of the domain *Archaea* were selected from published papers (Takai and Horikoshi, 2000; Casamayor *et al.*, 2002). The forward primer Arch344f was developed to target archaeobacteria (Casamayor *et al.*, 2002). The reverse primer Arch 806r also targets archaeobacteia and has been used successfully in real time PCR (Takai and Horikoshi, 2000). This primer, when paired with Arch 344f, yielded a 410bp product, which is a suitable size for real time PCR assays (<1000bp). However, Arch 806r does contain 2 degenerate sites. This primer was specific for both bacteria and archaeobacteria in gene alignments. All of the representative species in the archaeobacterial alignments, including the two species present in the gut - *Methanobrevibacter smithii* and *Methanosphaera stadtmaniae* - contain GG residues at this position (Table 3.2), so the degeneracy was removed from the primer and GG residues selected.

Table 3.2. The archaeobacterial 16S rRNA gene's reverse primer (Arch 806r) sequence was aligned against the DNA sequences of archaeobacterial species. The degenerate nucleotide sites are highlighted in blue. Mismatches are highlighted in red. The two methanogen species isolated from the human gut are in bold type.

Organism	Sequence (5' to 3')
Arch806r (degeneracy present)	ATTAGATACCCVSGTAGTCC
Arch806r (degeneracy removed)	ATTAGATACCCGGGTAGTCC
Thermoplasma volcanium	ATTAGATACCCGGGTAGTCC
Thermoplasma acidophilum	ATTAGATACCCGGGTAGTCC
Thermococcus alcaliphilus	ATTAGATACCCGGGTAGTCC
Thermococcus siculi	ATTAGATACCCGGGTAGTCC
Achaeoglobus fulgidus	ATTAGATACCCGGGTAGTCC
Pyrodicitium abysii	ATTAGATACCCGGGTAGTCC
Desulfurococcus saccharovorans	ATTAGATACCCGGGTAGTCC
Pyrobaculum calidifontis	ATTAGATACCCCTGTAGTCC
Sulfolobus acidocaldarius	ATTAGATACCCCGGTAGTCC
Methanobrevibacter smithii	ATTAGATACCCGGGTAGTCC
Methanosphaera stadtmaniae	ATTAGATACCCGGGTAGTCC
Halobacterium salinarum	ATTAGATACCCGGGTAGTCC
Halococcus qingdaogense	ATTAGATACCCGGGTAGTCC
Methanococcus voltae	ATTAGATACCCGGGTAGTCC

V: nucleotide A or C or G
S: nucleotide C or G

Comparisons were made between the PCR-TTGE bacterial primer set U968 and L1401, with and without the GC-clamp used in PCR-TTGE assays. The GC-clamp increased the Ct from 20.04 to 23.26, therefore unclamped primers were used in future assays to maximise sensitivity and efficiency.

3.4.3.2 Specificity of Primer Sets

Primer sets were tested against target and non-target DNA samples under the optimised real time PCR conditions. The primer sets targeting the sulfate reducing bacteria and the methanogens only amplified target species (Figure 3.11).

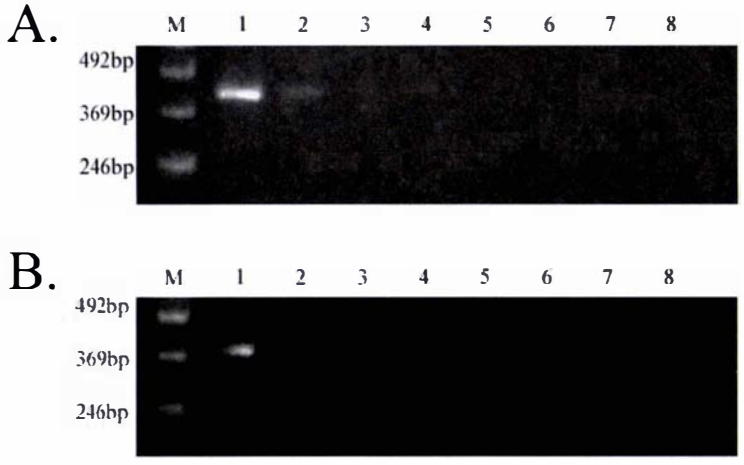


Figure 3.11. Specificity of the primers targeting the archaeobacterial 16S rRNA gene of methanogens (A) and sulfate reducing bacteria's APS reductase gene primers (B). 123bp DNA ladder (lane M), plasmid standard (lane 1), *Methanococcus voltae* (lane 2), *Clostridium difficile* (lane 3), *Proteus mirabilis* (lane 4), *Helicobacter pylori* (lane 5), *Yersinia enterocolitica* (lane 6), *Clostridium perfringens* (lane 7), and no template control (lane 8).

As an alternative to agarose gel electrophoresis, the ABI Prism 7000 can generate a dissociation curve for identifying the presence of specific and non-specific PCR products. Multiple peaks in this curve indicate the presence of multiple products in the reaction. Comparisons in the detection of non-specific products were made between agarose gel electrophoresis and the dissociation curves. Although the dissociation curve

and gel method both identified primer dimers, they differed when larger spurious products were present. The dissociation curve failed to differentiate between a ~ 200bp product and the 410bp specific product of the archaeobacterial 16S rRNA gene, which were readily identifiable on the agarose gel (Figure 3.12). Therefore all real time PCR products were checked on an agarose gel to be certain all the measured fluorescence was derived exclusively from the specific amplification product.

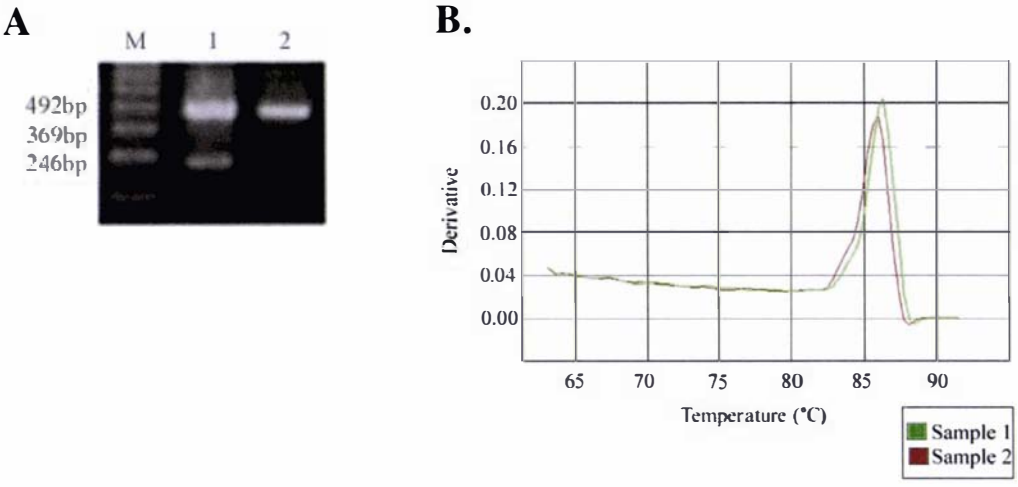


Figure 3.12. Comparisons of non-specific product detection by ABI Prism 7000's dissociation curve and agarose gel electrophoresis. A. Two real time PCR samples run on a 2% agarose gel. 123bp DNA ladder (M), Sample 1 containing a 410bp specific product and a ~ 200bp non-specific product (Lane 1), Sample 2 containing a 410bp specific product only (Lane 2). B. Sample 1 and Sample 2 both produce the same dissociation curve, despite sample 1 containing a non-specific product.

3.4.3.3 Real Time PCR Product Stability

The real time PCR products were found to rapidly degrade if left at room temperature, preventing agarose gel analysis. Real time PCR products have dUTP incorporated during the reaction to allow selective degradation by uracil-DNA glycosylase (UDG) of any carryover DNA in subsequent experiments. To determine if the DNA degradation was due to residual UDG activity, the inactivation step at 95°C was extended from 2

minutes to 15 minutes. The increased incubation prior to amplification was found to markedly improve product stability (data not shown).

3.4.3.4 Preparation of External Standards

PCR products derived from faecal DNA samples were cloned into the plasmid vector pCR4-TOPO to produce external standards for real time PCR assays. The clones were sequenced to identify the organism they were derived from and to accurately measure the length of the amplicon for copy number calculations.

The clone inserts were approximately the same length as the target templates. The sulfate reducing bacterial clone had 98% identity to *Desulfovibrio piger* DSM 749, and the archaeobacterial clone had a sequence identical to *Methanobrevibacter smithii*. The bacterial clone had 99% identity to *Akkermansia muciniphila*.

3.4.3.5 Amplification Efficiencies of External Standards and Faecal Genomic DNA

Standard curves constructed from plasmid DNA dilution series can be used to quantify unknown samples. For this approach to be valid the two sample types, plasmid DNA and genomic DNA, must have equivalent amplification efficiencies during real time PCR.

Assessment of the reaction efficiency for different template types was made using the delta Ct method.* A line graph was prepared from the difference in the template's Ct values in 10-fold dilutions. The efficiencies of the reactions are considered similar enough to permit quantitation if the slope of the trend line is ≤ 0.1. This parameter was met by all three assays (Figure 3.13).

* PRISM, ABI. 7700 Sequence Detection System User Bulletin 2. 1997. pp. 3–10.

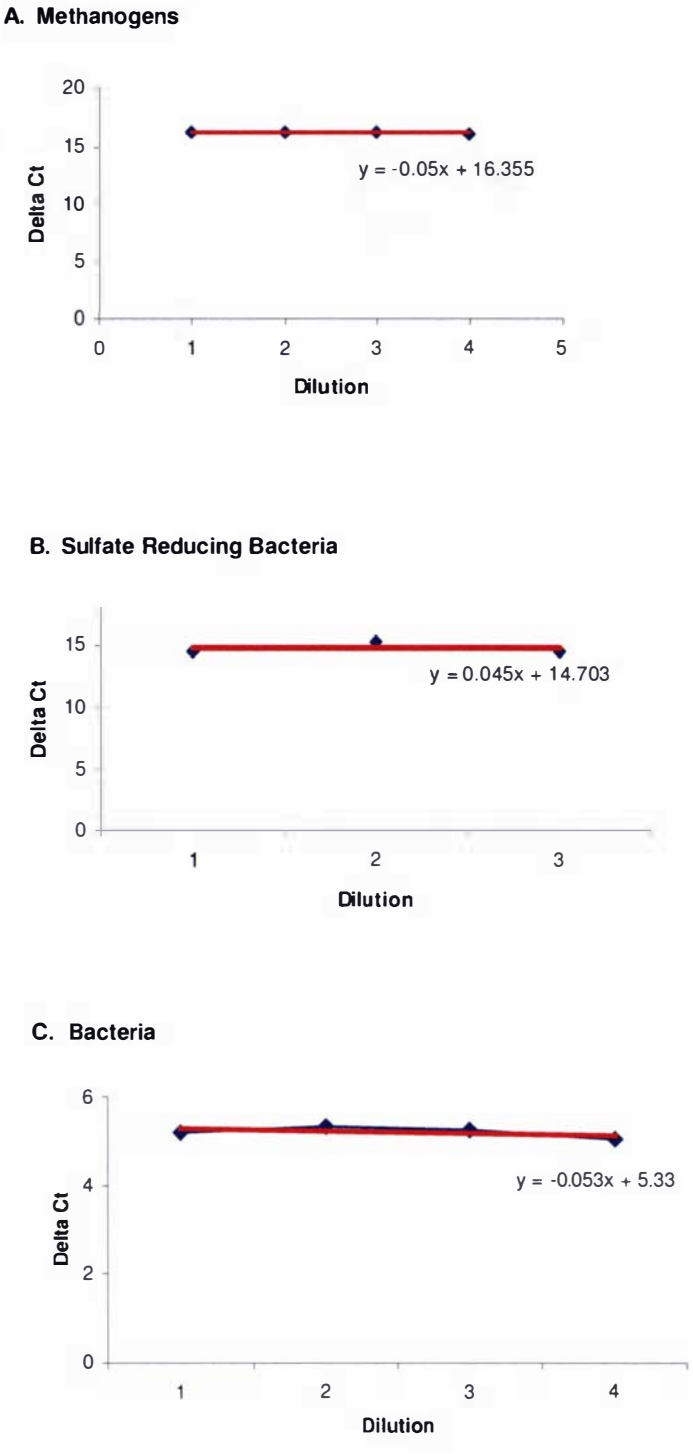


Figure 3.13. The amplification efficiencies of plasmid DNA and faecal genomic DNA were compared for each real time PCR assay using the delta Ct method. For each assay the delta Ct values were plotted against each 10-fold dilution. The formula of the resulting trend lines all had a slope ≤ 0.1.

3.4.3.6 Quantitation of Template DNA

In order to add a constant amount of DNA to each reaction, the total DNA for each sample was quantified using a biophotomer. Five DNA extracts derived from the same faecal sample were used to validate this approach. A total of 10ng of DNA, as measured by the biophotomer, was used in each reaction. When the bacteria in these 5 samples were quantified in triplicate, reproducible results were obtained. The replicates gave very low standard deviations that were ≤ 0.122, and across the five samples the Ct values differed by less than 1 cycle (data not shown).

3.4.3.7 Quantitation Parameters

The precision, reproducibility, and detection limits for each assay were determined by carrying out 10-fold dilution series in triplicate for each assay's external standard.

Detection limits were determined for the methanogen and sulfate reducing bacteria assays. Each clone was serially diluted 10-fold, until extinction of the signal or the reproducibility of the data declined. The amount of plasmid present in the concentrated sample was then determined using a biophotometer, and the gene copy number present in the lowest detectable dilution calculated (Figure 3.14). The detection limit for reliable quantitation in the sulfate reducing bacteria assay was 4 gene copies, and 11 gene copies for the detection of methanogens. The bacterial assay's detection limit was 132 genes.

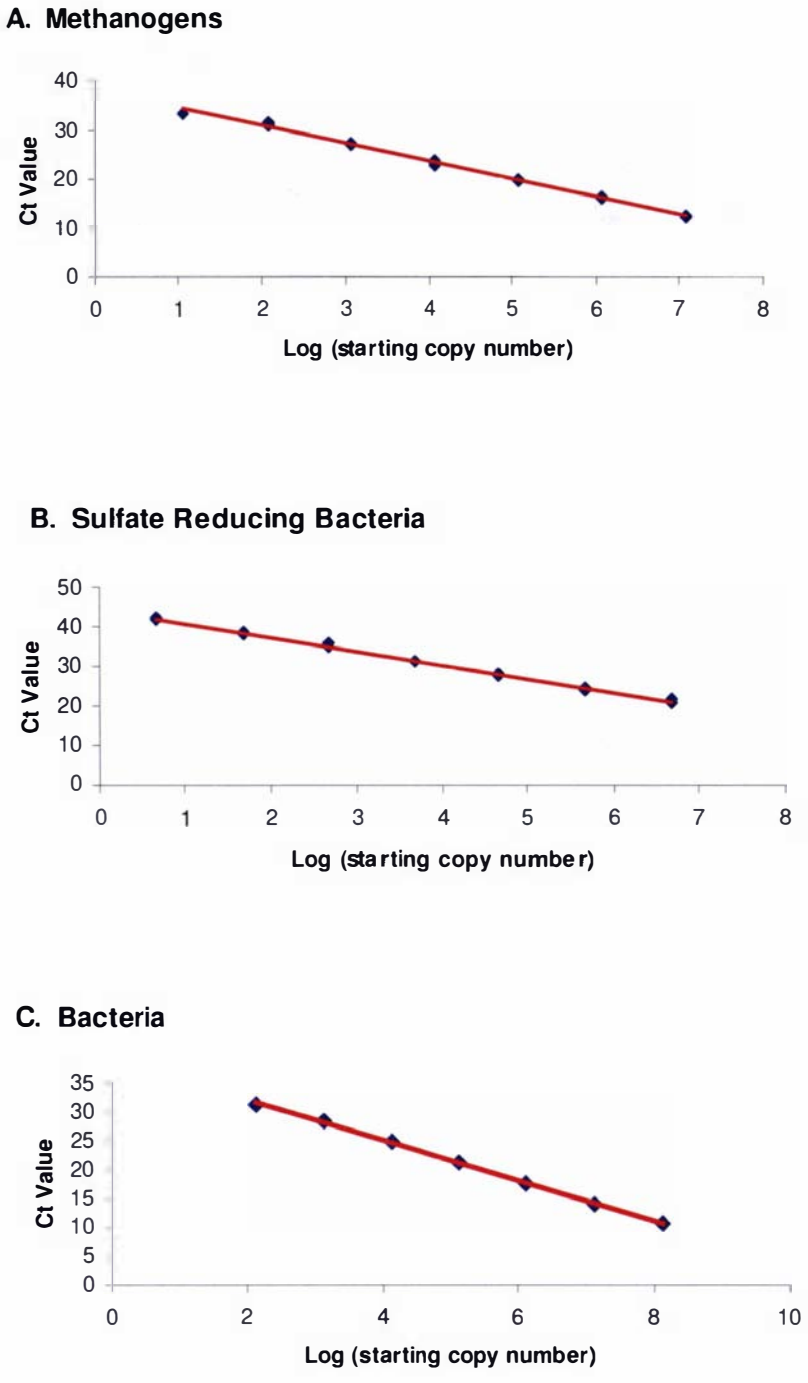


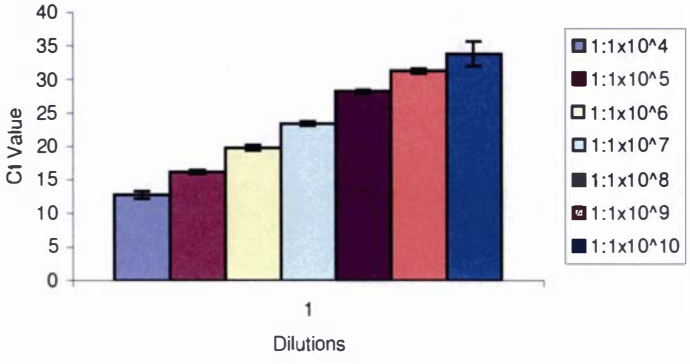
Figure 3.14. Detection limits of the assays were determined by 10 fold serial dilutions of external standards until extinction. Starting copy numbers are plotted against the threshold cycle.

The precision of each assay was measured by calculating the standard error for the 95% confidence interval for the 10-fold dilution series of each external standard (Figure 3.15). The errors for each dilution were low and did not overlap. Co-efficients of variation were ≤ 2.2%. These finding indicate that all three assays had high precision for each dilution studied.

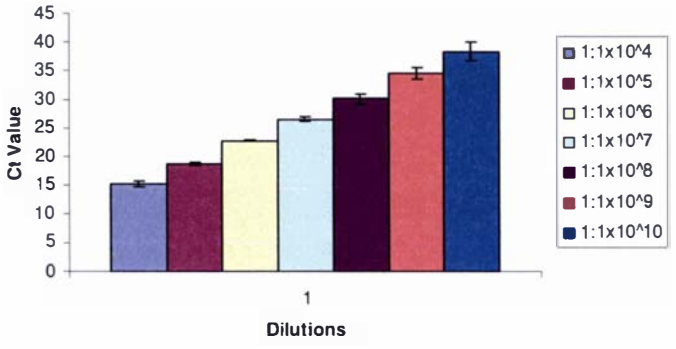
Reproducibility within the assay was monitored through the standard deviations calculated for each triplicate. Generally the standard deviations increased as the samples became more dilute, however sufficient reproducibility was still maintained as demonstrated by the precision calculations.

The variability between different real time PCR experiments was determined for bacteria, methanogen, and sulfate reducing bacteria assays. In each case, the same DNA sample was included in triplicate in 3 different experiments and the Ct value monitored. In the methanogen assay the average Ct value from the 3 experiments was 23.41 ± 0.54 (error values show the 95% confidence interval for the standard error). The average Ct for the sulfate reducing bacteria assay was 24.86 ± 1.09, and multiple experiments in the bacteria assay generated an average Ct of 18.51 ± 0.26. These data show that between different experiments the assays are reproducible, with low variability.

A. Methanogens



B. Sulfate Reducing Bacteria



C. Bacteria

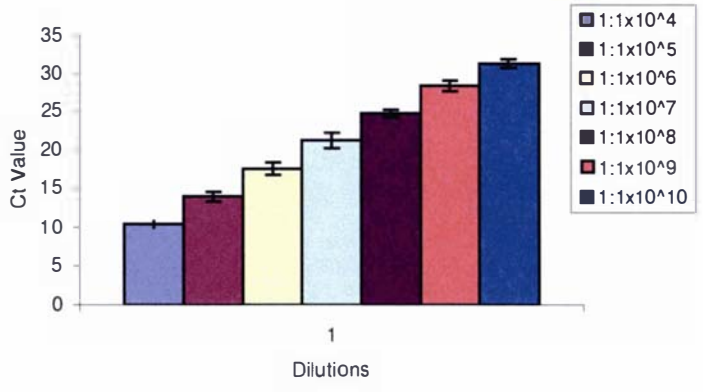


Figure 3.15. The average Ct values and standard errors are shown for each real time PCR assay. External standards were serially diluted and quantitated in triplicate for each assay (raw data Appendix H). A. The real time PCR assay targeting the archaeobacterial 16S rRNA gene of methanogens. B. The real time PCR assay targeting the APS reductase gene of sulfate reducing bacteria. C. The real time PCR assay targeting the 16S rRNA gene of bacteria.

3.5 DISCUSSION

The results reported in this chapter describe the optimisation, validation, and application of PCR-TTGE and real time PCR to monitor the composition of predominant faecal bacterial species, and to quantitate methanogens, sulfate reducing bacteria and bacterial populations.

Faecal DNA extraction using the QIAamp DNA Stool Mini Kit was found to be highly reproducible and increased incubation time at 95°C did not yield any further bands on TTGE gels. Since this study was undertaken the QIAamp DNA Stool Mini Kit has been compared to other commercial kits (McOrist *et al.*, 2002) and bead beating lysis methods (Li *et al.*, 2003) by other research groups. These studies demonstrated that the QIAmp DNA extraction method is the most effective and sensitive commercial kit for faecal samples and it also achieves 95% lysis of bacterial cells, equivalent to the bead beating method.

Analysis of multiple PCRs on a single faecal DNA sample demonstrated that the bacterial PCR-TTGE assay was highly reproducible, and approximately a 100-fold change in template amount did not alter the observed banding pattern. Although there are over 500 different bacterial species present in the microbiota (Moore and Holdeman, 1974), only a fraction of this population is represented on the TTGE gel. A group of 30-40 predominant bacterial species are thought to comprise 99% of the total bacterial population (Drasar, 1986), and therefore these organisms make up a larger proportion of the genomic DNA and come to dominate the TTGE profile. The experiment to determine the sensitivity of the assay demonstrated that DNA from an individual bacterial species must comprise at least 1% of the total template DNA to be detected in TTGE gels.

The sequence analysis of TTGE bands revealed greatest identity to uncharacterised bacterial clones in most cases. This reflects the poor characterisation of the bacterial community present in the gut. Recent work carried out but Eckburg *et al*, 2005, resulted in the production and analysis of 13,355 bacterial clones from the human gastrointestinal tract of multiple volunteers. The majority of clones contained

sequences derived from uncultivated species and novel organisms (Eckburg *et al.*, 2005). Therefore the presence of DNA from uncharacterised organisms in TTGE gel profiles is not surprising.

As the TTGE profile is a representation of the different PCR products formed from a DNA sample, the profile potentially contains spurious products associated with the dynamics of a multi-template PCR. Heteroduplexes commonly form when different templates contain constant and variable regions, thereby permitting the formation of double stranded DNA within the constant regions and nucleotide mismatches in variable regions. This population of double stranded DNA products can lead to overestimation of species diversity in TTGE profiles (Espejo *et al.*, 1998). As the TTGE gel resolves bands based upon the melting characteristics of the double stranded DNA, the mismatches present in heteroduplexes will lead to a lower Tm than genuine products, therefore positioning the heteroduplexes near the top of the TTGE profile. The contribution of heteroduplexes to TTGE profiles was examined using T7 endonuclease I and re-conditioning PCR. Both approaches demonstrated that heteroduplexes contributed very few bands to the TTGE profile, and they were located at the very top of the TTGE pattern as anticipated.

Chimeric DNA can form during PCR when a partially elongated template acts as a primer for a heterologous template sequence. The use of 4 minute elongation steps during PCR cycling has been reported to cause ~ 3 fold reduction in chimera formation (Qiu *et al.*, 2001). Analysis of the faecal DNA samples amplified under normal PCR conditions, and those amplified with extended elongation time demonstrated no change in the resulting TTGE profile.

Random *Taq* mutations are likely to arise in PCR and these sequence changes have the potential to produce additional bands on TTGE profiles (Qiu *et al.*, 2001). However, the TTGE profile was highly reproducible, suggesting that random *Taq* mutations do not significantly affect the TTGE pattern.

Chimeras are likely to form during multi-template PCR and random *Taq* errors are inevitable in PCR. However given that TTGE profiles represent the predominant bacterial templates present in faecal DNA samples, it is reasonable to expect that one-

off random template changes such as chimeras and *Taq* mutations will not be able to amplify to a sufficient level where they will be detectable in TTGE patterns.

Single stranded DNA has been reported to form a smear in TTGE profiles and overshadow regions of the banding pattern (Simpson *et al.*, 1999). The presence of single stranded DNA in TTGE profiles was assessed using Mung Bean Nuclease. This failed to improve the detection of any bands in the profile but did remove a band at the very top of the gel; the position of this band suggests it was likely to be a heteroduplex structure.

Recent research suggests that primer concatamer formation can distort PCR-based community profiles (Osborne *et al.*, 2005). Analysis of the sequence data reported in this thesis revealed no spurious primer sequences.

The concern with artefacts in the TTGE profile is that random changes due to spurious PCR products could lead to the assumption that the population has changed. In addition, artefacts can lead to overestimation of the population diversity. The results demonstrated that using the conditions described here, there are very few artefacts present in the TTGE profiles, and the level of reproducibility generated from one faecal DNA sample is very high. The detection of uncharacterised bacterial species, the rapid assessment of multiple samples and the high reproducibility demonstrate that PCR-TTGE can provide information about bacterial communities that may not be observed through normal culturing techniques.

The real time PCR assays were successfully optimised to allow sensitive quantitation of the sulfate reducing bacteria, methanogens, and bacteria. The primers specifically amplified the target genes, while failing to generate products from non-target templates. The removal of the G residue at the 3' end of the APSfw primer was subsequently shown to produce the correct primer sequence. The G residue was erroneously added to the primer sequence when it was originally published (Friedrich, 2002). All external plasmid DNA standards were found to have equivalent amplification efficiencies to faecal genomic DNA isolates under assay conditions, therefore permitting reliable quantitation of the samples from known concentrations of the external standards. Although the primers that targeted methanogens were shown to be specific in the gut, it should be noted that the primer sequences may amplify archaea or uncharacterised bacteria present in other ecosystems.

Ten fold dilution series of each external standard demonstrated low detection limits, high precision, and high reproducibility in each assay. The bacterial assay's detection limit was set unusually high due to the presence of contaminant bacterial DNA in reagents. The PCR product derived from the contaminant was sequenced and found to have 100% identity to *Pseudomonas* sp. SBW25. Attempts to eliminate the contaminating DNA from reagents failed. However, as the level of Pseudomonas gene copies detected by the system was very low (7 gene copies per reaction), comprising only 0.0000005% of the average bacterial population that this assay measures. There have been several reports of bacterial contamination in *Taq* polymerase preparations (Hughes *et al.*, 1994; Huijsdens *et al.*, 2002; Nadkarni *et al.*, 2002) and Pseudomonas species are common contaminants of water (Martino *et al.*, 1998; Schillinger and Du Vall Knorr, 2004). The presence of the contaminant was not considered a significant problem in this assay due to the high level of bacteria found in faecal DNA samples. The detection limits for the methanogen and sulfate reducing bacteria assays define the limit for reliable quantitation. Below this level it was still possible to detect the presence of target genes through the formation of an amplification plot and the identification of specific products on agarose gels. However, the reproducibility and precision were poor below the assay's detection limits due to the stochastic limitations associated with reliably pipetting, for example, a single gene copy. Therefore not all positive samples can necessarily be quantitated.

The reliability of a SYBR green I based real time PCR assay is dependent upon the detection of any spurious products generated during the reaction. As SYBR green I binds to double stranded DNA, the formation of non-specific products will give rise to additional fluorescence, and therefore lead to an overestimation of the number of target gene copies present. A rapid approach to screening for non-specific products is incorporated into most real time PCR machines, where a dissociation curve is generated based upon the Tm of the double stranded PCR products present in the sample. However, this method is only useful if the specific and non-specific PCR products have a markedly different Tm. The results of this study found that the dissociation curve could not reveal the presence of non-specific products and therefore in order to ensure that false positive results were not generated by the assays, all samples were checked on agarose gels following cycling.

In summary, PCR-TTGE and real time PCR assays have been successfully optimised and validated to target bacteria, methanogens and sulfate reducing bacteria. The assays were all found to be robust, sensitive, and highly reproducible. Chapters 4 - 8 describe the application of these techniques to investigate bacteria, methanogens and sulfate reducing bacteria in human faecal samples.

CHAPTER 4: LONG AND SHORT TERM STABILITY OF THE FAECAL MICROBIOTA

4.1 ABSTRACT

The temporal stability of the predominant bacteria, and populations of methanogens and sulfate reducing bacteria were monitored in healthy volunteers using PCR-TTGE and real time PCR. The composition of the predominant bacteria, and the carriage rates and densities of methanogens and sulfate reducing bacteria were assessed to determine population stability in both the long and short term. The composition of bacterial TTGE profiles was examined in 8 individuals over periods ranging from several weeks to several years. Over a period of 4 to 6 weeks, 6 volunteers provided multiple faecal samples. The bacterial population remained relatively constant over time with a median of 87% similarity. Long term data collected over periods ranging from 1-4 years in 5 different volunteers also found high stability, with a median similarity value of 82%. Methanogen and sulfate reducing bacteria were present in 5/12 and 7/12 volunteers respectively. A study of the short and long term stability of these populations carried out amongst 6 volunteers found that carriage of these organisms was maintained over a period of up to 4 years. However, the densities of methanogens and sulfate reducing bacteria were found to fluctuate by as much as 2-logs during both short term periods (4 weeks) and long term periods (1 - 4 years).

4.2 INTRODUCTION

The faecal microbiota is a large and complex population, with an estimated 500 different bacterial species (Moore and Holdeman, 1974). PCR-TTGE and real time PCR permit rapid analysis of microbial communities and the use of kingdom and group specific primers allow specific populations to be targeted. PCR-TTGE can only identify the predominant bacterial species present in an environmental sample. The remaining population is likely to be comprised of hundreds of bacterial species, including those that are commonly found in all individuals and species that are only harboured in a proportion of individuals.

Studies examining the stability of the predominant bacteria over a period of 6-7 months have shown that this population is relatively stable (Zoetendal *et al.*, 1998), and organisms found in culture based studies have also been found to exhibit high stability (Holdeman *et al.*, 1976). No study has examined the stability of the bacteria over a period of several years. Methanogens and sulfate reducing bacteria have not been studied in the New Zealand population, and the stability of these populations has not been determined in faecal samples over the long term.

It is important to understand the inherent variability of the faecal microbiota in healthy individuals, before investigations on the effect of external factors can be carried out. Therefore, in this study the temporal stability of the predominant bacteria and methanogen and sulfate reducing bacterial populations were investigated using PCR-TTGE and real time PCR assays. Faecal samples collected from volunteers over a period of weeks were used in short term studies, and samples collected over 1-4 years were used in long term studies.

4.3 METHODS

4.3.1 VOLUNTEERS

4.3.1.1 Volunteers for PCR-TTGE Study

Eight healthy volunteers provided faecal samples collected over different time periods. Seven different volunteers provided a single faecal sample each.

4.3.1.2 Volunteers for Real Time PCR Study

Twelve healthy volunteers provided a faecal sample and an alveolar breath sample. Ten volunteers provided faecal samples collected over different time periods to investigate temporal changes.

4.3.2 BREATH METHANE MEASUREMENTS

Volunteers consumed a standard meal of 225g of baked beans and 2 slices of toast. Three hours later they provided an alveolar breath sample, using the AlveoSampler (Quintron Instrument Company). Methane present in this sample was measured using the Quintron Microlyzer SC.

4.3.3 FAECAL DNA EXTRACTION/PCR-TTGE/REAL TIME PCR/SEQUENCING

The storage, homogenisation and subsequent extraction of DNA from faecal samples, application of PCR-TTGE and real time PCR, and sequencing of microbial DNA were carried out as previously described in Chapter 2: Materials and Methods.

4.4 RESULTS

4.4.1 APPLICATION OF PCR-TTGE TO ASSESS VARIABILITY IN THE FAECAL MICROBIOTA BETWEEN DIFFERENT INDIVIDUALS

Seven different volunteers provided a single faecal sample for TTGE analysis (Figure 4.1). The TTGE profile representing the predominant bacteria was different for each volunteer, however there were common bands present in all profiles. The number of bands per sample ranged from 12 - 25 with a median of 21.5. The similarity between these samples ranged from 36% - 78% with a median value of 55% (Appendix A.1).

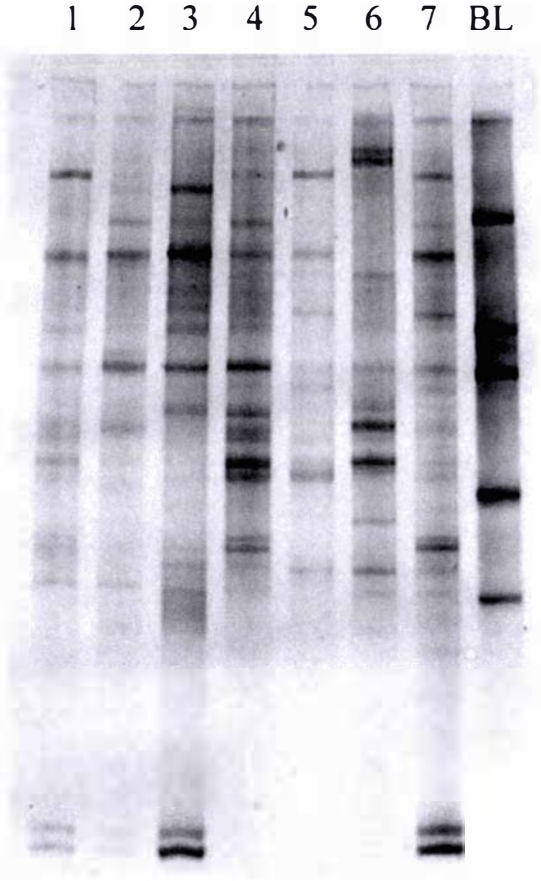


Figure 4.1. Bacterial TTGE profiles were prepared from the faecal samples of seven different volunteers (Lanes 1-7). Lane BL, bacterial ladder.

4.4.2 APPLICATION OF PCR-TTGE TO ASSESS TEMPORAL VARIATION IN THE BACTERIAL POPULATION

The temporal stability of the bacterial population was examined in 8 volunteers. Volunteers either provided samples collected over the short term (several weeks) and/or samples collected over the long term (several years). In all cases TTGE profiles were prepared to allow comparisons between the first sample collected and samples collected in the subsequent weeks or years.

4.4.2.1 Short Term Stability of the Bacterial Population's Composition

Five volunteers provided 4 faecal samples each that were collected over a 4 week period, and an additional volunteer provided 10 faecal samples collected over 6 weeks (Figure 4.2). Samples were analysed to determine the temporal variability of the bacterial population in the short term. The median number of bands per sample was 16, with a range from 12 - 20. Analysis of the TTGE profiles revealed a stable population with Sorenson's similarity co-efficient values ranging from 100% - 75% and a median value of 87% (Appendix A.2).

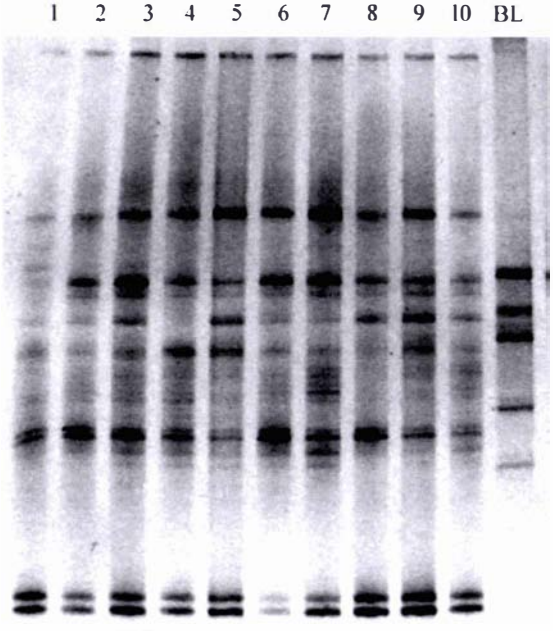


Figure 4.2. Bacterial TTGE profiles of ten faecal samples collected over 6 weeks from one individual (Lanes 1-10). Each sample was collected approximately 4 days apart. Lane BL, bacterial ladder.

4.4.2.2 Long Term Stability of the Bacterial Population's Composition

TTGE profiles of samples collected over a 1 - 4 year period were analysed to determine the temporal variability of the bacterial population in the long term. The median number of bands per sample was 15.5, with a range from 13-20. Amongst all 5 volunteers, Sorenson's similarity co-efficient values ranged from 59% - 89% and a median value of 82% (Appendix A.3) (Figure 4.3).

To determine if increased variability is present in samples collected over the long term, Sorenson's similarity co-efficients from the short and long term studies were compared. No significant difference was observed, indicating that the predominant bacterial population maintains an equivalent degree of stability in both the short and long term.

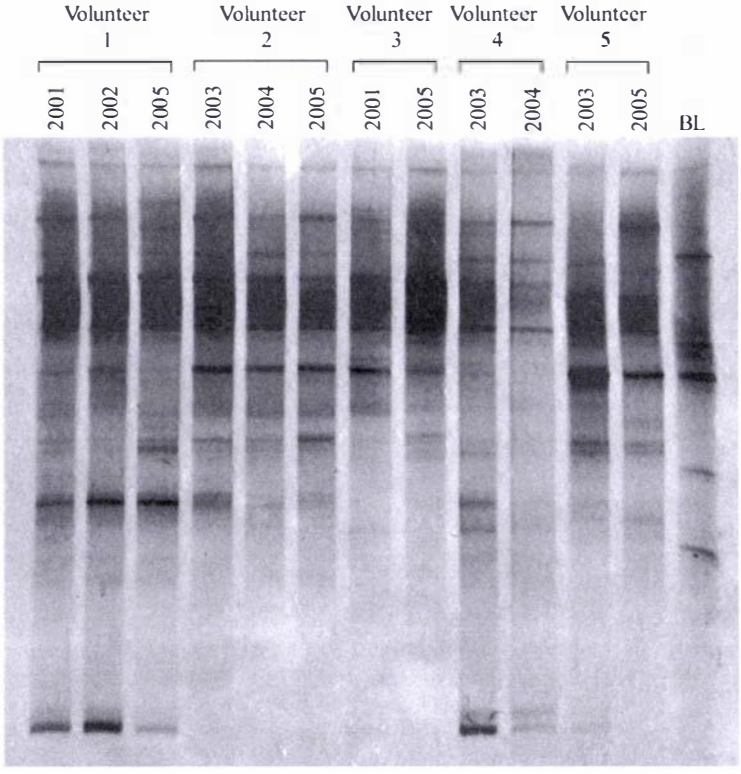


Figure 4.3. Five volunteers provide 2 or 3 faecal samples over a period of 1-4 years. Each TTGE gel lane is dated with the year the sample was collected. BL, bacterial ladder.

4.4.3 APPLICATION OF REAL TIME PCR TO ASSESS CARRIAGE AND TEMPORAL VARIATION OF METHANOGEN AND SULFATE REDUCING BACTERIA POPULATIONS

4.4.3.1 Carriage Rates and Densities of Methanogens and Sulfate Reducing Bacteria in New Zealand Adults

Sulfate reducing bacteria and methanogens are not ubiquitous in adult microbiota and their carriage rates vary amongst different ethnic groups. The sulfate reducing bacteria gene APS reductase was detected in 7/12 New Zealand adults, at concentrations covering a range from 4.42×10^7 to 3.92×10^9 genes/g stool (wet weight), however in one volunteer the amplification plot lay beyond the final dilution of the external standard, therefore the concentration could not be determined (Table 4.1, Volunteer 2). Archaeobacterial 16S rRNA genes were detected in 5/12 New Zealand adults and concentrations varied from $7.45 \times 10^5 - 4.91 \times 10^7$ genes/g stool (wet weight) (Table 4.1).

Numbers of sulfate reducing bacteria and methanogens were also expressed with respect to the total density of all organisms measured in the samples (bacteria + methanogens) (Table 4.1) to normalise for factors such as faecal water content. This generated a value that describes the number of methanogens or sulfate reducing bacteria as a percentage of the total population. These values led to some large changes in inter-person comparisons, for example volunteer 5 and 12 had only a 1.1 fold difference in methanogen numbers, however when expressed as a proportion of the total population this increased to an 11 fold difference.

All positive real time PCR samples were sequenced to validate the assays' specificity. Sequence analysis of positive samples from the 12 adult volunteers found the archaeobacterial 16S rRNA gene products to be derived from the methanogen *Methanobrevibacter smithii* and APS reductase gene products were found to have the greatest identity to *Desulfovibrio piger* or *Desulfovibrio intestinalis* sequences (Table 4.1).

4.4.3.2 Comparison of Real Time PCR and Breath Methane Testing

Methane was detected on the breath of 3/12 volunteers. Real time PCR and sequencing showed that 2 volunteers had *Methanobrevibacter smithii* DNA present in faecal samples but failed to produce sufficient methane on the breath for detection (Table 4.1). These volunteers had the two lowest concentrations of methanogen 16S rRNA genes, only accounting for 0.0003% and 0.0007% of the total population.

Table 4.1. Carriage rates and quantitation of methanogens and sulfate reducing bacteria in faecal samples from New Zealand adults. Samples were quantitated as gene copies/g stool (wet weight) and were also expressed as a percentage of the total microbiota (bacteria + methanogens) to normalise for factors such as faecal water content (raw data Appendix D.1 and E.1)

Volunteer	Breath CH4 (ppm)	Methanogens			Sulfate Reducing Bacteria		
		Genes/g Stool (Wet Weight)	Expressed as a Percentage of Total Microbiota	Sequencing	Genes/g Stool (Wet Weight)	Expressed as a Percentage of Total Microbiota	Sequencing
1	-	1.69E+06	0.0003%	*M. smithii* (100%, 290/290)	4.42E+07	0.0083%	*D. intestinalis* (94%, 299/317)
2	-	7.45E+05	0.0007%	*M. smithii* (100%, 338/338)	not determined	-	*D. intestinalis* (94%, 363/386)
3	-	-	-	-	4.44E+08	1.5837%	*D. intestinalis* (94%, 303/322)
4	24	4.91E+07	0.1685%	*M. smithii* (100%, 362/362)	3.14E+08	1.0782%	*D. intestinalis* (93%, 303/325)
5	4	1.86E+07	0.0189%	*M. smithii* (100%, 269/269)	9.82E+08	0.9979%	*D. piger* (98%, 324/329)
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	4.06E+08	3.2219%	*D. piger* (100%, 329/329)
9	-	-	-	-	3.92E+09	5.5681%	*D. piger* (98%, 321/327)
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	17	2.05E+07	0.2152%	*M. smithii* (100%, 362/362)	-	-	-

'-' indicates not detected

4.4.4 APPLICATION OF REAL TIME PCR TO ASSESS TEMPORAL VARIATION IN BACTERIA POPULATIONS

4.4.4.1 Short Term Variability in the Densities of Bacteria

Short term variability in the bacteria population was assessed in 7 individuals. Six volunteers collected 4 faecal samples, each 1 week apart. The 7th volunteer collected 4 faecal samples over a period of 4 days. The bacterial densities ranged from 7.06 x10^{8} to 4.72 x10^{11} gene copies/g stool (wet weight), with a median of 3.08 x10^{10} gene copies/g stool (wet weight) (raw data Appendix C.2). Within each volunteer the largest difference in bacterial densities between the samples ranged from 1.7-fold to 24-fold (data not shown).

4.4.4.2 Long Term Variability in the Densities of Bacteria

Long term variability in the bacteria population was monitored in 6 individuals that collected faecal samples over a period of 1 to 4 years. The bacterial densities ranged from 7.06 x10^{8} to 4.72 x10^{11} gene copies/g stool (wet weight), with a median of 3.77 x10^{10} gene copies/g stool (wet weight) (raw data Appendix C.3). Within each volunteer the difference between the samples ranged from 1.2 to 300-fold (data not shown).

Comparisons between the bacterial densities measured over the short term and long term revealed no significant difference ($p = 0.40$).

4.4.5 TEMPORAL STABILITY OF METHANOGEN AND SULFATE REDUCING BACTERIA POPULATIONS

Seven volunteers provided faecal samples over short and long term periods to monitor fluctuations in methanogen and sulfate reducing bacteria populations. Four volunteers harboured both methanogens and sulfate reducing bacteria, 2 volunteers were methanogenic only, and 2 volunteers only carried sulfate reducing bacteria.

4.4.5.1 Short Term Stability of Methanogen Populations

Methanogens were monitored over 4 days in 1 volunteer and over 4 weeks in another. During this time period large fluctuations in methanogen densities occurred (Table 4.2). Over the 4 day period with Volunteer 3, densities were initially very similar at approximately 15% of the total population, however on day 3 there was a marked decline to only 0.83%. The population recovered on day 4 to reach 6.25%. With Volunteer 4, a 10-fold fluctuation was observed between weeks 1 and 2, followed by 100-fold difference between weeks 3 and 4.

Table 4.2. The short term stability of methanogen populations was monitored in 2 volunteers. The density of methanogens is expressed as a percentage of the total organisms measured in the faecal sample (bacteria + methanogens) (Raw data Appendix D.2).

Volunteer	Day 1	Day 2	Day 3	Day4
3	15.352%	15.258%	0.834%	6.249%

Volunteer	Week 1	Week 2	Week 3	Week 4
4	0.025%	0.298%	0.005%	3.695%

4.4.5.2 Long Term Stability of Methanogen Populations

Over the long term, all methanogenic individuals maintained carriage of these organisms, however densities were variable (Table 4.3). Over a period of 3 years one volunteer's population decreased 6-fold, while the population of another increased 12-fold over a 1 year period. The individual that provided two faecal samples 4 years apart was found to have a 54-fold increase in methanogens during this time. The methanogen population in Volunteer 7 underwent a 3-fold increase in population density over a 1 year period.

Table 4.3. The long term stability of methanogen populations was monitored in 4 volunteers. The density of methanogens is expressed as a percentage of the total organisms measured in the faecal sample (bacteria + methanogens) (raw data Appendix D.3).

	Year Faecal Sample Collected				
Volunteer	2001	2002	2003	2004	2005
1	0.003%	-	-	0.019%	-
2	-	-	2.047%	0.168%	-
3	0.284%	-	-	-	15.352%
7	-	-	0.041%	0.111%	-

- sample not collected

4.4.5.3 Short Term Stability of Sulfate Reducing Bacteria Populations

The short term stability of sulfate reducing bacteria populations was monitored in 3 individuals who provided 4 faecal samples each, collected 1 week apart (Table 4.4). All volunteers' populations fluctuated during this time period, ranging from a 1.2-fold to 21-fold difference. In one volunteer no sulfate reducing bacteria could not be quantitated in the sample collected in week 3, while in weeks 2 and 4 these organisms accounted for 0.02% and 0.19% of the total population respectively.

Table 4.4. The short term stability of sulfate reducing bacteria populations was monitored in 3 volunteers. The density of sulfate reducing bacteria is expressed as a percentage of the total organisms measured in the faecal sample (bacteria + methanogens) (raw data Appendix E.2).

Volunteer	Week 1	Week 2	Week 3	Week 4
4	0.009%	0.017%	0.000%	0.188%
5	0.024%	0.052%	0.081%	0.537%
6	0.052%	0.276%	0.439%	0.225%

4.4.5.4 Long Term Stability of Sulfate Reducing Bacteria Populations

Sulfate reducing bacteria were monitored in three individuals over a 1 year period (Table 4.5). Each volunteer provided 2 faecal samples and the differences between these ranged from 7-fold to 174-fold. One volunteer provided two faecal samples over

a 3 year period, in this case there was only a 2-fold difference between the sulfate reducing bacterial densities in these samples. The volunteer that collected 3 faecal samples, each 1 year apart had a 119-fold increase between the first and second samples, followed by a 132-fold decrease between the second and third samples.

Table 4.5. The long term stability of sulfate reducing bacteria populations was monitored in 5 volunteers. The density of sulfate reducing bacteria is expressed as a percentage of the total organisms measured in the faecal sample (bacteria + methanogens) (raw data Appendix E.3).

	Year Faecal Sample Collected				
Volunteer	2001	2002	2003	2004	2005
1	0.524%	-	-	0.998%	-
2	-	-	7.820%	1.078%	-
4	-	-	-	1.584%	0.009%
5	-	-	0.027%	3.222%	0.024%
6	-	-	-	5.568%	0.052%

- sample not collected

4.5 DISCUSSION

Optimised PCR-TTGE and real time PCR assays were applied to study the temporal stability of the predominant bacteria, methanogen, and sulfate reducing bacteria populations.

Previous molecular studies have reported that within an individual the predominant bacterial population is very stable, and that different individuals display unique profiles (Zoetendal *et al.*, 1998). These characteristics were observed amongst the New Zealand population. Quantitative data was generated to describe the degree of similarity found over time, and amongst different individuals. The similarity values were generally lower amongst different people. Higher similarity values were obtained when samples collected from the same individual were compared. The range of values obtained from these two groups was not distinct, and an overlap in similarity values was observed. The lowest value obtained for the samples collected from one individual was 59%, while the greatest similarity value obtained between different people was 78%.

The predominant bacterial population was remarkably stable during the long term. Volunteers had not taken antibiotics or had gastrointestinal infections in the 4 weeks prior to sample collection, however in the interim period between sample collections, it is possible that volunteers were exposed to gastrointestinal infections and antibiotics. Despite this, changes in TTGE profiles did not differ any more than those prepared from samples collected several days apart. The data suggest that the small degree of variability present in the microbiota does not give rise to a drift affect, whereby the microbiota gradually changes over a long period of time. The maintenance of such a high degree of stability over the long term is likely to result from numerous interactions between the colonic environment, the host, and other organisms in the population.

The stability of the predominant bacteria within an individual and the divergence seen amongst different people suggests that PCR-TTGE will be ideally suited to longitudinal studies within an individual, and to comparative studies amongst different individuals grouped by a common quality, for example disease.

The density of bacteria in faecal samples collected from the same volunteer varied by as much as 300-fold. Results from real time PCR optimisations (section 3.4.3.6) indicate that little variability is present in the assay itself (less than 1 PCR cycle, or a 2-fold difference in quantified levels). Bacteria data were expressed with respect to the grams of stool used in the DNA extraction. While this does normalise the data to a degree, characteristics of the faecal sample, such as water content, the amount of fibre present, or low-level inhibitors are likely to contribute to the variability present in bacterial densities.

Analysis of the methanogen and sulfate reducing bacterial populations present in 12 adult volunteers was carried out using real time PCR and methane breath testing. This study demonstrated the merit in developing a molecular methodology to assess the methanogen population, as in two instances *Methanobrevibacter smithii* DNA was successfully quantitated in faecal samples, but no methane was detected in breath samples. It has previously been reported that methanogens must reach a density in the gut of 10^8 organisms/g dry weight before methane will be detected on the breath (Weaver *et al.*, 1986). Assuming a total population of 10^{11} organisms/g dry weight, methanogens would need to comprise 0.01% of the population to produce methane on the breath.

Faecal carriage rates for methanogens and sulfate reducing bacteria among the 12 adult volunteers were comparable to those reported in other Western countries (Bond *et al.*, 1971; Flatz *et al.*, 1985; Weaver *et al.*, 1986; Gibson *et al.*, 1988; Levitt, 1995; Fite *et al.*, 2004). By expressing the number of methanogens and sulfate reducing bacterial gene copies with respect to the total measured genes (bacteria + methanogens) the data were effectively normalised to remove variability associated with the faecal sample (Suzuki *et al.*, 2000). This approach caused some dramatic changes in the data when comparing the levels of genes detected in different volunteers, and was therefore included in future studies. Sequencing revealed all real time PCR products were derived from organisms specifically targeted by the primer sets and further validated the specificity of the assays.

The carriage of both methanogens and sulfate reducing bacteria was maintained in all individuals studied over the long term, however there was up to a 2-log difference in

densities with respect to the total microbiota population. Similar degrees of variability were present in samples collected over the short term and those collected over the long term. A study carried out by Miller and Wolin, 1983, found methanogens were consistently detected in faecal samples collected from two volunteers over 10 months and 13 months. During this period they accounted for a relatively constant proportion of the microbiota, however densities did fluctuate by as much as 100-fold (Miller and Wolin, 1983). Similarly, another study found breath methane in 12 volunteers consistently over a 1 year, however fluctuations in breath methane levels were noted (Bond *et al.*, 1971). In a British study, real time PCR was used to monitor populations of mucosal Desulfovibrio in 4 ulcerative colitis patients over a 12 month period (Fite *et al.*, 2004). During this time there was a constant carriage of these organisms in all volunteers, however in two individuals the concentration was found to vary over several magnitudes, but levels remained stable in the remaining volunteers (Fite *et al.*, 2004).

Together the findings reported here, and the work of the cited authors, demonstrate that the carriage of these organisms is constant over time, however within these populations large changes in the number of organisms present can occur. This variability must be taken into consideration when analysing the effect of perturbations on these populations.

It has been suggested that competition between methanogens and sulfate reducing bacteria for H_2 gas causes one of these groups to predominant in the gut. Gibson *et al*, 1988, reported that in British individuals, breath methane was only found on those persons who did not have detectable levels of sulfate reducing bacteria in their faeces. However, African subjects who had methane on the breath were found to have low numbers of sulfate reducing bacteria in some cases. In a later study, 3 groups of individuals were identified; those who are strongly methanogenic with few sulfate reducing bacteria, those who had low levels of methanogenesis and low levels of sulfate reducing bacteria, and those who have no methanogenesis and high levels of sulfate reducing bacteria (Gibson *et al.*, 1993a). Analysis of this phenomenon amongst the 12 New Zealand volunteers found that one group of organisms did predominate in 6 subjects, however 2 volunteers demonstrated breath methane levels in excess of 1ppm, and actually had higher levels of sulfate reducing bacteria than methanogens in faeces. In a further four volunteers neither of these groups of organisms were detected. Other

authors have described the presence of a high degree of sulfate reducing activity, in stools collected from methanogenic individuals (Pitcher *et al.*, 2000).

The differences observed between the study reported here and the work of Gibson *et al*, 1988, might be related to the different experimental approaches utilised. The measurement of gene copies to gauge population density is prone to over-estimation as there are potentially multiple cistrons for the gene in each organism, and bacterial DNA from both live and dead bacteria are measured. However, these considerations would apply equally to both the methanogens and sulfate reducing bacteria quantitated in the New Zealand volunteers. On the other hand the culturing approach used by Gibson *et al*, 1988, is limited to the study of characterised bacteria, for which the culturing conditions are known. In some cases the sulfate reducing bacteria identified in New Zealanders only shared 94% identity to described species, and therefore may represent previously uncharacterised organisms. However, methane was detected on the breath of volunteers that had a substantial population of sulfate reducing bacteria. These data suggest that the relationship between methanogens and sulfate reducing bacteria is likely to be more complex than previously suggested.

In conclusion, the predominant bacteria present in TTGE profiles is a highly stable population, in both the short and long term, and the carriage of methanogens and sulfate reducing bacteria is maintained over time. There were 10 to 100-fold changes in methanogen and sulfate reducing bacteria populations, which must be considered in studies of population dynamics. The influence of host genetics over the composition of the predominant bacteria, and methanogens and sulfate reducing bacteria was examined in Chapter 5.

CHAPTER 5: INFLUENCE OF HOST GENETICS OVER THE COMPOSITION OF THE FAECAL MICROBIOTA

5.1 ABSTRACT

The bacteria, methanogen, and sulfate reducing bacterial populations in the faecal microbiota of related and unrelated children were studied. PCR-TTGE was used to examine the similarity of the predominant bacterial population between identical twins, fraternal twins, and unrelated paired controls. Significant differences were observed between the identical and fraternal twins ($p = 0.037$) and between fraternal twins and unrelated paired controls ($p = 0.001$). This finding suggests that there is genetic influence over the composition of the faecal microbiota. In addition, the degree of similarity in the microbiota of dizygotic twins was negatively correlated with age, however no correlation was evident amongst identical twins. Methanogens were identified in 10/40 samples; the youngest carrier of this organism was 4 months old. Sulfate reducing bacteria were only identified in 6/40 samples. The low carriage rates for these organisms meant the influence of host genetics over these populations could not be examined. The results of this study indicate that the host genotype influences the composition of the predominant bacterial population of children.

5.2 INTRODUCTION

There is large person-to-person variation in the faecal microbiota (Holdeman *et al.*, 1976; Zoetendal *et al.*, 1998). An exception to these findings was made in the early 1980s, when culture based analyses of the faecal microbiota of twins revealed a greater similarity in the bacterial population of monozygotic twins than dizygotic twins (Van de Merwe *et al.*, 1983). These results suggest that the composition of an individual's faecal flora may be influenced by the host's genetic makeup. These findings have been further supported by denaturing gradient gel electrophoresis studies on related and unrelated individuals, where a positive correlation was observed between increasing genetic relationship and the similarity of the faecal microbiota (Zoetendal *et al*, 2001).

The idea that host genetics may influence the composition of the intestinal flora has been buoyed by studies using mice (Toivanen *et al.*, 2001). Bacterial cellular fatty acid profiles were produced using gas-liquid chromatography from stool samples derived from six mouse strains congenic for the major histocompatibility complex (MHC). Analyses revealed that MHC-encoded genes could significantly influence the composition of the faecal flora in mice with the same background genotype. Additional genes also control the population as different background genotypes produced different flora in these mice. A combination of different MHC and background genotypes produced the most significantly different faecal microbiota population. In humans an association between MHC genes and colonisation levels of the oral cavity with caries-inducing bacteria has been found in African-American women (Acton *et al.*, 1999). These studies suggest that the variation in the intestinal microbiota found in different individuals is likely to be influenced by genetics.

Although molecular studies have examined the genetic control on the predominant members of the bacterial population, a significant difference between monozygotic and dizygotic twins has not been shown. The carriage of minor populations has not been analysed in twins and family groups using a molecular approach.

Methanogens and sulfate reducing bacteria are usually minor components of the human microbiota, and their rates of carriage vary among different ethnic groups (Gibson *et al.*, 1988; Segal *et al.*, 1988), and in different disease states (Roediger *et al.*, 1997; Pimentel *et al.*, 2003). To date, no study has examined genetic control over the carriage of sulfate reducing bacteria. An Australian study found that breath methane status was determined by shared and unique environmental factors in humans and rats, rather than a genetic effect (Florin *et al.*, 2000). Similar findings have been made in other studies examining the presence of methane on breath (Bond *et al.*, 1971; Flatz *et al.*, 1985). To date, the majority of studies have utilised breath methane analysis due to the difficulty associated with culturing methanogens, however in these studies methanogenesis itself may be inhibited or below detection in some individuals, though a methanogen population may be present.

PCR-TTGE and real time PCR were employed to examine the influence of the host genotype on the composition of the predominant bacteria in faecal samples from twins and unrelated New Zealand children. This investigation was then extended to determine if there is any evidence of genetic influence over the carriage of methanogens and sulfate reducing bacteria in these individuals.

5.3 MATERIALS AND METHODS

5.3.1 VOLUNTEERS

Thirteen identical twin pairs, 7 fraternal twin pairs, and 24 unrelated individuals that were paired, provided a single faecal sample for analysis. Volunteers' ages ranged from 4 months to 10 years, the median was 23 months. The 24 unrelated controls were divided into groups of breast/formula fed infants and weaned children. Within these groups individuals were approximately age matched to form unrelated control pairs, generating a median age difference of 1.5 months amongst infants, and 1.1 years amongst weaned children. These unrelated infants and children were all living in separate home environments. All the genetically related volunteers were living in the same home environment at the time of sample collection

5.5.2 TWIN ZYGOSITY

Monozygosity of same sex twins was confirmed by genetic analysis of bucchal swabs (DNA Diagnostics, Auckland, New Zealand). Twins were considered monozygous if DNA matches were obtained at all 15 loci examined (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA). Twins that failed to complete zygosity testing were excluded from the study.

5.5.3 FAECAL DNA EXTRACTION/PCR-TTGE/REAL TIME PCR/SEQUENCING

The storage, homogenisation and subsequent extraction of DNA from faecal samples, application of PCR-TTGE and real time PCR, and sequencing of microbial DNA were carried out as previously described in Chapter 2: Materials and Methods.

5.4 RESULTS

5.4.1 COMPARISON OF THE PREDOMINANT BACTERIAL POPULATION IN RELATED AND UNRELATED INDIVIDUALS

TTGE profile comparisons were made between identical twin pairs, fraternal twin pairs, and unrelated individuals. The number of bands per TTGE profile ranged from 8 – 28, with a median of 16 bands. There was a positive correlation between the number of bands and the age of the volunteer (correlation co-efficient = 0.49, $p < 0.001$) (Figure 5.1). Analysis of Shannon's indices also revealed a positive correlation between community richness and age (correlation co-efficient = 0.361, $p = 0.003$) (Figure 5.2). While every individual's profile was unique, increasing levels of similarity were evident in profiles of genetically related individuals (Figure 5.3).

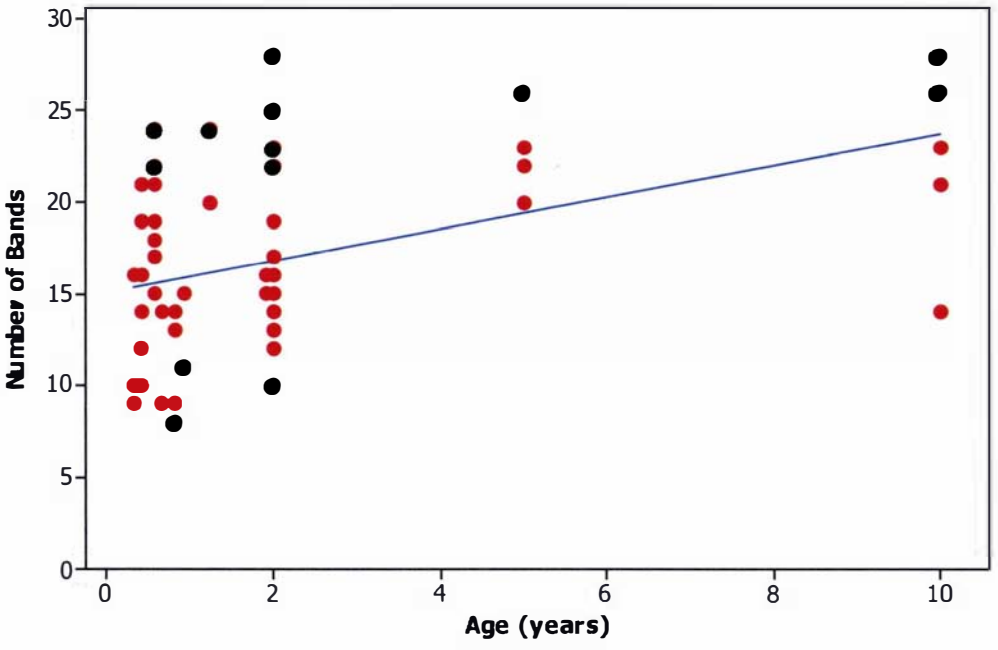


Figure 5.1. Number of bacterial bands detected in children's TTGE profiles with respect to age.

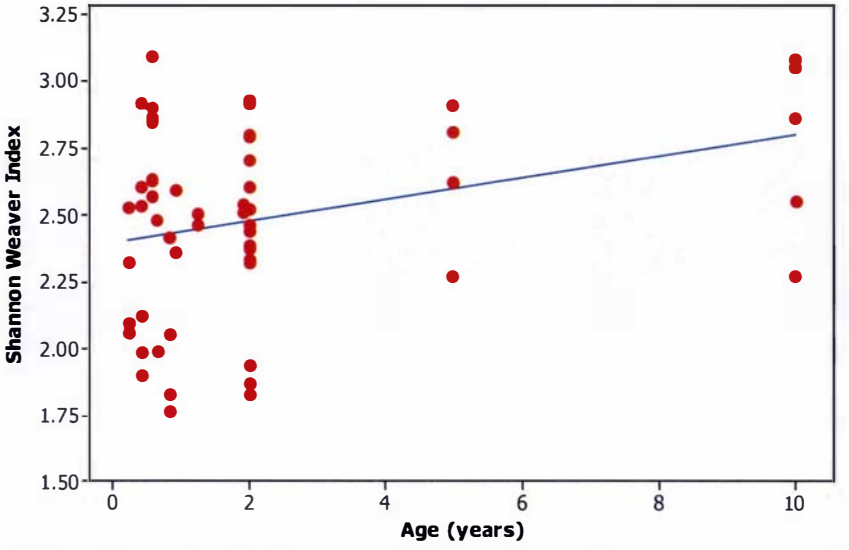


Figure 5.2. Shannon's indices reflecting community richness of children's bacterial TTGE profiles, with respect to age (raw data Appendix B.1).

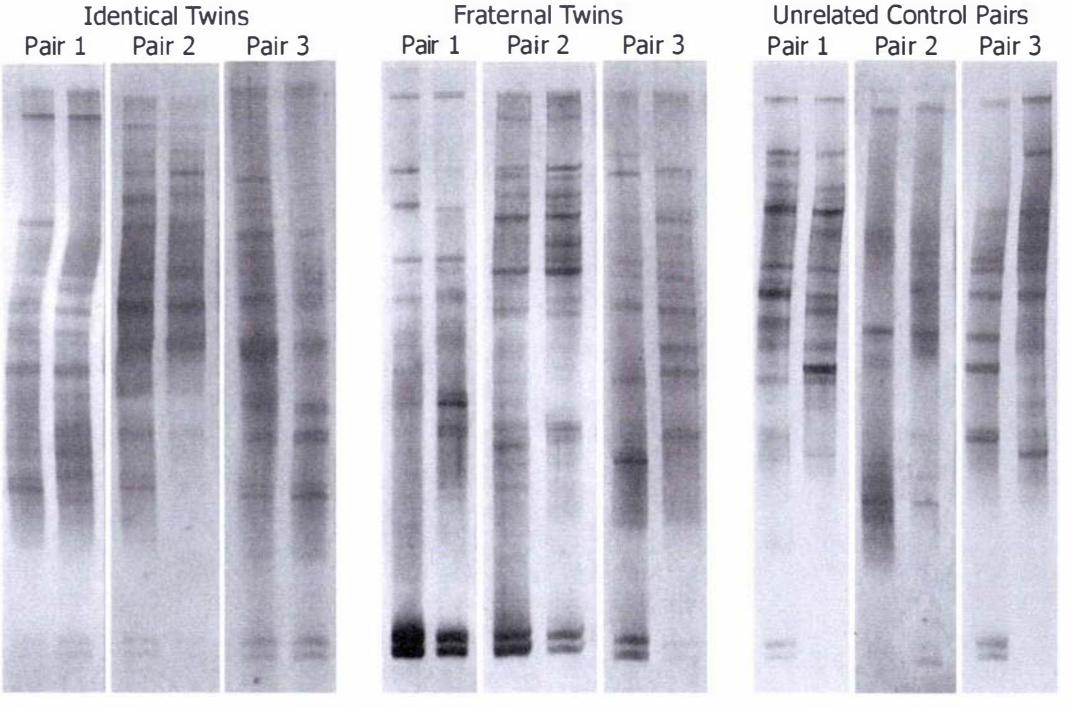


Figure 5.3. Examples of TTGE profiles of the predominant bacterial population present in faecal samples of twin and unrelated control pairs of children. Identical twins: Pair 1, 82.14% similarity, 2 years, males; Pair 2, 75.68% similarity, 0.58 years, females; Pair 3, 91.30% similarity, 0.58 years, females. Fraternal twins: Pair 1, 68.18% similarity, 1.25 years, males; Pair 2, 66.67% similarity, 5 years, mixed genders; Pair 3, 63.64% similarity, 10 years, mixed genders. Unrelated control pairs: Pair 1, 66.67% similarity, 6 years, mixed genders; Pair 2, 34.78% similarity, 1.42 years, males; Pair 3, 46.81% similarity, 2 years, mixed genders.

Sorenson's similarity co-efficient was calculated to assess the similarity of TTGE banding patterns (Figure 5.4). For identical twin pairs the similarity values ranged from 69% - 91%, with a median value of 82%. Fraternal twins ranged from 55-87%, with a median value of 68%. The least similarity was seen amongst unrelated individuals, with a median value of 45%, and a range from 22% - 71%. Analysis using T tests demonstrated a significant difference between identical and fraternal twins ($p = 0.037$) and a significant difference between fraternal twins and unrelated individuals ($p = 0.001$).

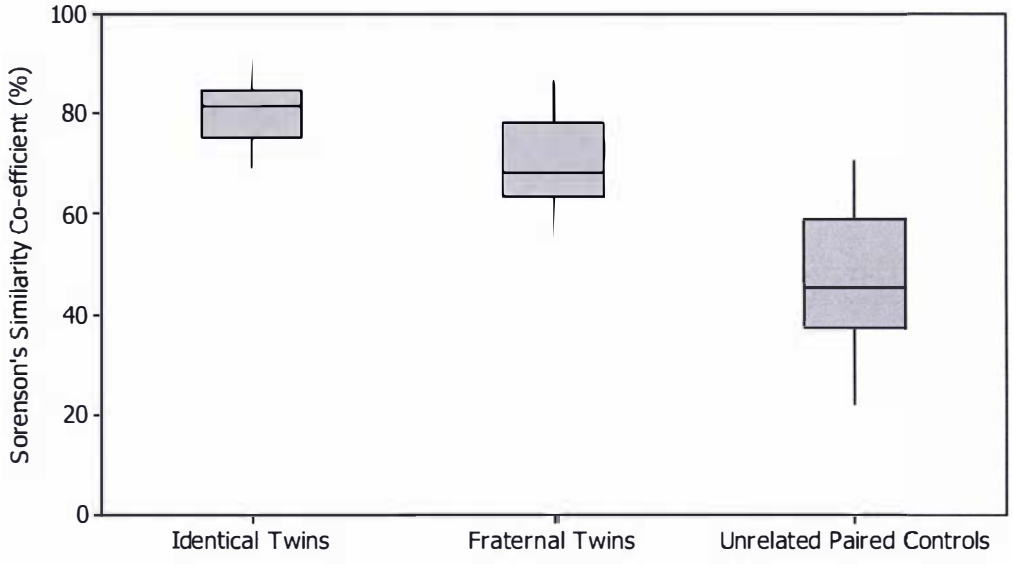


Figure 5.4. Box whisker plot of Sorenson's similarity co-efficients showing the ranges and medians for identical twins, fraternal twins and unrelated paired controls (raw data Appendix A.4).

Similarity co-efficients were examined with respect to the age, gender and diet of the paired individuals (Table 5.1). There was no correlation between the gender of the twin pairs or unrelated controls pairs and their TTGE similarity co-efficients. From the dietary information available, no influence of breast-feeding or formula feeding on TTGE similarity co-efficients was evident. Comparisons between breast/formula fed twins and weaned twins found no significant difference. The diets of weaned twins were classified as identical, similar, or different by their parents. There was no

significant correlation or significant difference in the TTGE similarity values of twins with identical diets and twins with similar diets. Amongst unrelated children, the 2 pairs that were formula fed did not demonstrate increased TTGE profile similarity compared to the pairs consuming different diets.

There was no correlation between TTGE similarity co-efficients and the age of the volunteers amongst identical twin pairs and unrelated control pairs. Analysis of the age difference between unrelated control pairs with respect to TTGE similarity co-efficients revealed no correlation. A large negative correlation between similarity and age was evident in fraternal twins and this was statistically significant (correlation co-efficient = -0.825, $p = 0.022$).

TTGE gel comparisons were also made between 7 sibling pairs and 3 mother-child pairs. Sorenson's similarity values ranged from 38% - 72%, with a median of 58%. Comparisons between these pairs and fraternal twin pairs revealed no significant difference ($p = 0.10$).

Table 5.1. For each group Sorenson's similarity co-efficient, C_s, was analysed with respect to the age and gender of the pair. 100% similarity corresponds to complete identity between the TTGE profiles.

Identical Twins				Fraternal Twins				Unrelated Pairs				
											Breast or Formula Fed	
C(s) (%)	Average Age (years)	Gender	Breast or Formula Fed	C(s) (%)	Average Age (years)	Gender	Breast or Formula Fed	C(s) (%)	Average Age (years)	Gender	Child 1	Child 2
73.68	0.33	females	breast*	86.67	0.42	females	both*	45.45	0.38	mixed	breast†	both†
80.00	0.42	males	both*	78.26	0.66	mixed	formula*	51.43	0.46	females	breast†	both†
75.68	0.58	females	both*	68.18	1.25	males	weaned†	44.44	0.50	mixed	both*	both*
91.30	0.58	females	both*	66.67	5	mixed	weaned†	22.22	0.58	females	both*	both*
82.35	0.83	males	both*	73.47	5	males	weaned†	34.78	1.42	males	weaned‡	weaned‡
84.62	0.92	females	formula*	55.32	10	mixed	weaned†	61.54	1.42	males	weaned‡	weaned‡
90.32	1.92	males	weaned†	63.64	10	mixed	weaned†	45.16	1.42	mixed	weaned‡	weaned‡
69.23	2	females	weaned*					32.00	1.46	mixed	weaned‡	weaned‡
75.00	2	females	weaned†					70.59	1.96	males	weaned‡	weaned‡
81.25	2	males	weaned†					46.81	2	mixed	weaned‡	weaned‡
82.14	2	males	weaned†					44.44	6	females	weaned‡	weaned‡
84.85	2	males	weaned*					66.67	6	mixed	weaned‡	weaned‡
81.63	10	females	weaned*									

* identical diet
† similar diet
‡ different diet

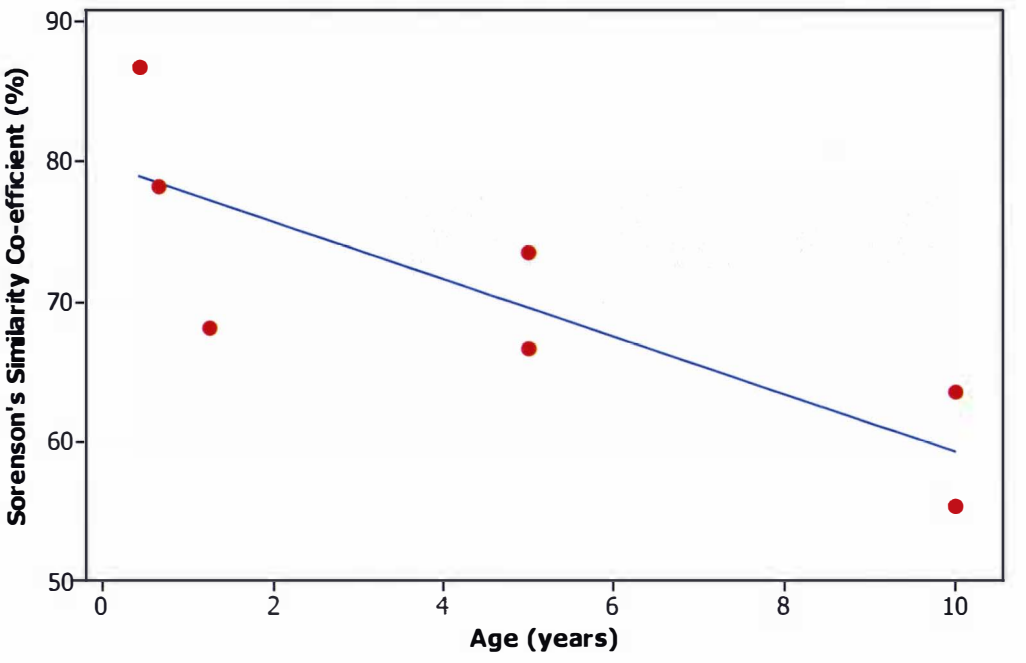


Figure 5.5. Similarity values for the TTGE profiles of fraternal twins, with respect to age.

5.4.2 CONCORDANCE RATES FOR THE CARRIAGE OF METHANOGENS AND SULFATE REDUCING BACTERIA IN IDENTICAL AND FRATERNAL TWINS.

Real time PCR was used to detect methanogens and sulfate reducing bacteria in the faecal DNA samples of identical and fraternal twins. Methanogens were found in 10/40 children, and sulfate reducing bacteria were present in 6/40 children. Bands were sequenced to identify the organisms that amplification products were derived from. All 10 positive methanogen samples had the greatest sequence identity to *Methanobrevibacter* species and sulfate reducing bacteria samples had greatest identity to *Desulfovibrio* species (Table 5.2). Only 3 volunteers were found to be positive for the carriage of both methanogens and sulfate reducing bacteria.

Table 5.2. PCR products that were positive for methanogens or sulfate reducing bacteria were sequenced to identify the organisms that the amplicons were derived from.

Positive Samples	Organism	Identity
Methanogens		
1	*Methanobrevibacter smithii* strain PS (GI:37719327)	367/367 (100%)
2	*Methanobrevibacter smithii* strain PS (GI:37719327)	170/170 (100%)
3	Methanobrevibacter sp. RT (GI:3201652)	270/278 (97%)
4	*Methanobrevibacter smithii* strain PS (GI:37719327)	270/270 (100%)
5	*Methanobrevibacter smithii* strain PS (GI:37719327)	291/291 (100%)
6	*Methanobrevibacter smithii* strain PS (GI:37719327)	345/345 (100%)
7	Methanobrevibacter sp. clone DI_H01 (GI:42602010)	355/355 (100%)
8	*Methanobrevibacter smithii* strain PS (GI:37719327)	147/147 (100%)
9	*Methanobrevibacter smithii* strain PS (GI:37719327)	272/272 (100%)
10	*Methanobrevibacter smithii* strain PS (GI:37719327)	315/315 (100%)
Sulfate Reducing Bacteria		
1	*Desulfovibrio desulfuricans* subsp. *Desulfuricans* (GI:7109233)	378/383 (98%)
2	*Desulfovibrio desulfuricans* subsp. *Desulfuricans* (GI:7109233)	377/383 (98%)
3	*Desulfovibrio desulfuricans* subsp. *Desulfuricans* (GI:7109233)	316/316 (100%)
4	*Desulfovibrio intestinalis* DSM 11275 (GI:18034144)	357/381 (93%)
5	*Desulfovibrio intestinalis* DSM 11275 (GI:18034144)	359/383 (93%)
6	*Desulfovibrio piger* DSM 749 (GI:37719327)	368/375 (98%)

Twin pairs were grouped based upon their methanogen status and sulfate reducing bacteria status (Table 5.3). Twin pairs in which only 1 member carried methanogens/sulfate reducing bacteria were considered to be discordant. Twin pairs in which both members carried methanogens/sulfate reducing bacteria were considered to be concordant. The incidence of concordant twins for the carriage of methanogens or sulfate reducing bacteria was too low to make any determination regarding the host's genetic influence over these groups.

Table 5.3. Concordant and discordant twins for the carriage of methanogens (A.) and sulfate reducing bacteria (B.).

A. Methanogens

Identical Twins	Child 1	Child 2
1	+	+
2	-	-
3	+	-
4	-	-
5	-	-
6	+	-
7	-	+
8	-	-
9	-	-
10	-	-
11	+	-
12	+	-
13	-	-
Fraternal Twins	**Child 1**	**Child 2**
1	-	-
2	-	-
3	+	+
4	-	-
5	-	+
6	-	-
7	-	-

+ indicates organisms present
- indicates organisms not detected

B. Sulfate Reducing Bacteria

Identical Twins	Child 1	Child 2
1	-	-
2	-	+
3	-	-
4	-	-
5	-	-
6	+	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	+
12	-	-
13	-	-
Fraternal Twins	**Child 1**	**Child 2**
1	-	-
2	-	-
3	+	+
4	-	-
5	-	-
6	+	-
7	-	-

+ indicates organisms present
- indicates organisms not detected

5.5 DISCUSSION

In order to determine if host genetics may influence the composition of the predominant bacterial population and the carriage of methanogens or sulfate reducing bacteria, a twin study was undertaken.

It has already been established that there is a positive correlation between the similarity of the predominant bacterial population and the degree of relatedness in adults (Zoetendal *et al*, 2001), and undoubtedly this contributes to the stability of this population. The TTGE results examining the microbiota of children further support this premise, and demonstrate a significant difference between TTGE similarity values in identical twin pairs and fraternal twin pairs, and suggests that genetic influence from the host functions from an early age.

Higher Shannon's indices and greater numbers of bands in TTGE profiles were found in samples from older children. This shows that the predominant bacterial population evident in TTGE banding profiles are increasingly complex, as children grow older. This is likely to reflect the increasing complexity present in the microbiota with age (Hopkins *et al*., 2001).

Amongst fraternal twins, a large negative correlation was observed between the age of the twin pair and the degree of similarity in the TTGE profile. This correlation was statistically significant. Although the numbers of identical and fraternal twins in different age groups is small, one could speculate that identical twin pairs may maintain a constant level of high similarity during development of the microbiota as they are under the same genetic constraints, while a genetic effect in infant fraternal twins may be masked due to low environmental variability. Increased environmental exposure to micro-organisms as fraternal twins grow older, may permit increased divergence due to their different genetic backgrounds. A longitudinal study following the development of the microbiota in infant twins, with adequate controls for factors such as diet and gender, may be warranted to investigate this hypothesis.

Twin pairs undergo simultaneous development of the microbiota in a highly similar environment, which may lead to an over-estimation of the effect of host genetic control. In an attempt to address this issue, comparisons were made between the predominant bacteria amongst 7 sibling pairs (3 adult sibling pairs; living apart, median age 25 years. 4 children sibling pairs; living together, median age 3.5 years) and 3 mother-child pairs (living apart, median age of children 25 years). Like fraternal twins, these pairs have approximately 50% of their genes in common. In contrast to fraternal twins the microbiota of these individuals evolved at different times and therefore with greater environmental variability. Contributory factors to environmental variability include different food types and amounts, infections, and environmental sources of microbes. Comparisons between fraternal twins and the group of sibling pairs and mother-child pairs found no significant difference in TTGE profile similarity values ($p = 0.10$). This suggests that the highly homogeneous environment found amongst twin pairs may not be a major factor determining the increased similarity amongst these groups.

The unrelated paired controls could not be as precisely age matched as twins, and also grew up in different families. These two factors may bias the findings for the unrelated group. Therefore the differences and statistical significance measured between the unrelated pairs and the fraternal twins may have been exaggerated by these factors.

It was hypothesised that the genetic control observed over the predominant bacterial species, may also extend to the minor populations of both methanogens and sulfate reducing bacteria and may be better delineated by molecular methodologies. A genetic effect could contribute to the ability of these organisms to colonise the host and subsequently influence the stability of the emerging population.

The rate of carriage of methanogens amongst the twins was 25%. Reported rates of carriage in children have varied across different studies. Generally breath methane based studies have reported low carriage rates of methanogens; a study in Israel found only 6% of 3 to 4 year olds and 14.3% - 18.2% of 7 to 14 year olds produced methane on the breath (Peled *et al.*, 1985), while studies in Nigeria found rates of 8% in children under 2 years, and 40% in children aged 2 - 6 years (Hudson *et al.*, 1993). However in the Nigerian study the detection of methanogens in under 2 year olds increased to 83% when culturing methods were used. A study in Italy also using culture based methods

identified methanogens in the faeces of 40% of 3 year olds and 60% of 5 years olds, though failed to detect methanogens in children under the age of 27 months (Rutili *et al.*, 1996). To date, only one study has reported the detection of methanogens amongst Western children under 2 years of age; methane was detected in faecal cultures in 15.3% of babies under 3 months of age, and 46.4% in those aged 6 - 10 months in an American study (Belson *et al.*, 2003). In the study reported here, methanogens were identified in faecal DNA samples from 6 children under the age of 1 year (37.5% of this age group), the youngest being only 4 months old. It appears there are divergent findings for the carriage of methanogens in infants and children.

Breath methane measurements have a relatively poor detection limit (Weaver *et al.*, 1986) and this is likely to have contributed to the low carriage rates reported in most breath methane studies. Although real time PCR is very sensitive and can theoretically have a detection limit as down to a single gene copy, this limit applies to the DNA aliquot used in the experiment. The actual detection limit per gram of stool will always be higher than a single gene copy when the level of dilution required for the real time PCR reaction, the volume of the DNA sample, and the inevitable losses during earlier DNA extraction steps are taken into consideration. However, methanogens are also slow growing and difficult to culture, which is likely to compromise the sensitivity of culturing methods. These limitations may explain the divergent carriage rates reported using different methodologies.

Ethnicity may also be a contributing factor. Both African and Italian populations have reported adult carriage rates of 77% (Hudson *et al.*, 1993) and 90% (Brusa T, 1993) respectively, while a study examining both British and African populations using breath methane tests reported rates of 30% and 85% respectively (Gibson *et al.*, 1988).

The median age of New Zealand children in this study was 23 months, an age group that has generally been found to have no methanogens in Western populations. The carriage rate of 25% is lower than rates obtained by culturing for older children in Western populations, but this may be attributable to the lower age of the participants in this group. The demonstration of methanogens in 37.5% of children under the age of 1 year, further supports the findings in American children, showing that these organisms do

colonise the gut in Western children much earlier than the reported 2-3 years of age (Peled *et al.*, 1985; Rutili *et al.*, 1996).

The carriage rate of sulfate reducing bacteria in the faecal DNA samples was only 15%. Very few reports have described the carriage of sulfate reducing bacteria in children. However, in contrast to the findings described in the New Zealand population, it has been reported that species of the *Desulfovibrio* genus are ubiquitous in British infants (Fite *et al.*, 2004). Although a real time PCR approach was used in both studies, different genes were used to target the sulfate reducing bacteria. The APS reductase gene codes for an essential enzyme in sulfate reduction (Peck, 1962; Stille, 1984), and therefore targets the large and diverse family of sulfate reducing bacteria. The British study however, only targeted a single sulfate reducing bacteria genus, *Desulfovibrio*, using primers targeted to the 16S rRNA gene. The complete genome of *Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough* (Gi: 46562128) has five copies of the 16S rRNA gene (Heidelberg *et al.*, 2004). Therefore multiple 16S rRNA genes in the target organisms may have increased the sensitivity of the real time PCR assay. Alternatively the differences in the detection of sulfate reducing bacteria may reflect genuine differences in the carriage of sulfate reducing bacteria in these two populations.

The carriage rates for methanogens and sulfate reducing bacteria in New Zealand children were lower than those obtained for New Zealand adults (section 4.4.3). In both age groups all methanogen PCR products had the greatest identity to Methanobrevibacter species, and sulfate reducing bacteria PCR products had the greatest identity to Desulfovibrio species.

The influence of host genetic control over the carriage of methanogens and sulfate reducing bacteria could not be determined in this study due to the low incidence of methanogens and sulfate reducing bacteria in New Zealand children. In order to determine if host genetics influence the carriage of these organisms a larger study using adults, in which higher carriage rates of these organisms occur, would be required.

Interactions between the human host and the faecal microbiota occur at many levels. Therefore there are likely to be a multitude of genes involved in the host's genetic influence over the composition of the population. The host immune system is in close

contact with the bacterial community, and studies in mice indicate the involvement of MHC genes in shaping the murine faecal microbiota (Toivanen *et al.*, 2001). Less direct aspects of host physiology may also influence the population. There is evidence to suggest that food preference is influenced by human genetics (Falciglia and Norton, 1994; van den Bree *et al.*, 1999), therefore host genes may affect the types of substrates available in the colon for fermentation, which in turn may favour the growth of some bacterial species over others.

The suggestion that host genetics can influence the composition of the predominant bacteria is interesting in the context of inflammatory bowel disease, where patients display a characteristic dysbiotic flora (Van de Merwe *et al.*, 1988). Do patients develop this characteristic flora and thereby over-ride the host's genetic influence over the composition of the faecal flora, or alternatively is the host's genetic influence responsible for the characteristic flora? Studies amongst the children of Crohn's disease patients have described the presence of a "Crohn's-like" flora in 9/26 children who did not display symptoms of Crohn's disease (Van de Merwe *et al.*, 1988). However during a 5 - 7 year follow up period, 3 of these children went on to develop symptoms of gastrointestinal disease, one of which was diagnosed with Crohn's disease. None of the 17 children with "normal" flora developed symptoms that could be attributed to Crohn's disease. Although this study cannot determine whether this was due to the presence or loss of the host's genetic influence, it does suggest that the abnormal flora can precede the onset of inflammatory bowel disease symptoms, and may not simply be a characteristic induced by inflammatory process itself.

The involvement of host genetics in determining the composition of the faecal flora may also be relevant to attempts to therapeutically manipulate the bacterial population of the gut. The consumption of probiotics and prebiotics aims to introduce and promote the growth of favourable organisms. Although the transient presence of these organisms and their by-products may be achieved, the successful introduction of these species into the bacterial population may be inhibited by the influence of host genetics on population composition. The development of "one size fits all" therapeutics may not be appropriate for complex and unique bacterial populations, which are under different constraints by individual host genotypes.

The studies described in Chapter 4 demonstrated that the faecal microbiota is characterised by a high degree of stability over both the short and long term. The composition of the population is also unique in different individuals. The work reported here in Chapter 5 shows a significant difference in the similarity of the microbiota from identical and fraternal twins, arguing that the composition of this population is under genetic control from the host.

Based upon the findings outlined above, investigations were designed to examine how the microbiota responds to environmental challenges. Bowel cleansing is routinely carried out in gastroenterology clinics prior to colonoscopy and may provide a method for modulating the composition of the microbiota. Investigations into the rate of reconstitution and any changes in diversity over time are described in Chapter 6. An extreme dietary change was carried out for 4 weeks in Chapter 7 to determine if alterations in fermentable substrates influenced components of the faecal microbiota.

Gastrointestinal diseases such as inflammatory bowel disease may result from an impaired relationship between the immune system and the microbiota. As the composition of the faecal microbiota is influenced by host genetics, there may be increased similarity in populations from unrelated individuals suffering from the same disease. Therefore the presence or absence of a characteristic microbiota in particular disease states was investigated in Chapter 8.

CHAPTER 6: RECONSTITUTION AND STABILITY OF THE FAECAL MICROBIOTA AFTER INTESTINAL LAVAGE

6.1 ABSTRACT

A study was undertaken in healthy individuals to investigate if bowel cleansing, using Fleet Phospho-Soda, can alter populations in the faecal microbiota. Faecal samples collected before and after lavage were analysed using PCR-TTGE to monitor changes in the predominant bacterial population, and real time PCR was used to measure changes in the densities of methanogens and sulfate reducing bacteria. Faecal samples collected from 10 healthy volunteers and faecal lavage fluid collected from 10 additional volunteers during bowel cleansing were compared to determine the reduction in bacterial numbers. Faecal lavage fluid had on average a 96% reduction in the concentration of bacteria compared to faecal samples. A further ten volunteers provided a control faecal sample prior to the intestinal lavage, and subsequently collected a faecal sample from each bowel movement for the next 7 days. Re-constitution of the bowel microbiota occurred rapidly following lavage. Bacteria, methanogen and sulfate reducing bacteria populations rapidly returned to the densities measured in controls. Intestinal lavage did not appear to affect populations of methanogens or sulfate reducing bacteria. However, calculation of Sorenson's similarity co-efficients demonstrated that the predominant bacterial population was more variable during the post lavage period, compared to the normal level of stability present in this population ($p = 0.0003$). A significant difference was also observed between the volunteers themselves ($p = 0.0004$), with some individuals displaying relatively stable populations in the post lavage period, and others exhibiting more differences. These findings indicate that there is a rapid restoration of bacterial densities following lavage and the normal temporal stability of the faecal microbiota can be disrupted by Fleet intestinal lavage.

6.2 INTRODUCTION

The gastrointestinal system usually exists in a steady state, with an average faecal output of approximately 120g per day (Macfarlane and Macfarlane, 1997) and a stable population of faecal bacteria. The population is composed of indigenous species that occupy habitats within the intestinal ecosystem.

Bowel cleansing is routinely carried out in gastroenterology clinics to prepare the bowel for colonoscopy and effectively clears faecal material from the bowels. Fleet Phospho-Soda is an osmotic laxative, which is taken orally and causes water to be drawn into the lower bowel (Fleet Pharmaceuticals, 2005). This intestinal lavage effectively cleanses the bowel by inducing rapid evacuation through frequent liquid stools. The walls of the bowel are left clear of faecal material for medical examination during colonoscopy.

Bacteria account for a significant proportion of faecal solids, and therefore the substantial reduction in faeces that occurs in the bowel must also lead to a significant drop in the amount of faecal micro-organisms present. Following this procedure, gastrointestinal activity rapidly returns to normal leading to the presence of faeces and accompanying faecal bacterial populations in the large bowel.

To date, no molecular study has examined the effect of intestinal lavage on the faecal microbiota, or investigated how long the population takes to recover to control levels. Based upon different growth rates of bacterial species, reconstitution of the microbiota may lead to the appearance of new organisms in the predominant bacteria, or the loss of slow growing organisms such as methanogens. The aims of this study were to use PCR-TTGE and real time PCR to monitor the impact of bowel cleansing, the rate of reconstitution and the composition of the population following this procedure. If bowel cleansing modulates the composition of the microbiota, it may represent a therapeutic method for treating dysbiotic populations common in gastrointestinal disorders.

6.3 METHODS

6.3.1 VOLUNTEERS

Ten faecal samples and ten faecal lavage fluid samples (unpaired) were collected from healthy volunteers to compare bacterial densities in these sample types.

Ten additional healthy volunteers provided a faecal sample prior to intestinal lavage, and subsequently collected a faecal sample from every bowel movement for the next 7 days.

6.3.2 INTESTINAL LAVAGE

Prior to intestinal lavage, volunteers consumed a liquid diet for one day (for example pureed fruit, jellies, and soup). In the late afternoon volunteers consumed 45mL of Fleet Phospho-soda solution (Fleet Pharmaceuticals) with 2 glasses of fluid and continued to consume only liquid foods. The Fleet Phospho-soda solution induced watery diarrhoea within 2-6 hours. Volunteers then consumed a further 45mL of Fleet Phospho-soda solution in the morning, with 2 glasses of fluid. Once volunteers' stools were clear, the lavage was complete, and normal eating was resumed.

6.3.3 FAECAL DNA EXTRACTION/PCR-TTGE/REAL TIME PCR/SEQUENCING

The storage, homogenisation and subsequent extraction of DNA from faecal samples, application of PCR-TTGE and real time PCR, and sequencing of microbial DNA were carried out as previously described in Chapter 2: Materials and Methods.

6.4 RESULTS

6.4.1 QUANTITATION OF BACTERIAL DENSITIES IN FAECES AND FAECAL LAVAGE FLUID SAMPLES

Differences in bacterial densities between 10 faecal samples and 10 faecal lavage fluid samples (unpaired) were determined to examine if intestinal lavage reduces the bacterial density in the gut lumen. The bacterial population present in these samples was enumerated using real time PCR. The range of densities obtained for both sample sets overlapped (Figure 6.1), and the median density in faecal samples was 5.30×10^{10} bacterial genes/g stool (wet weight), while the median density for lavage samples was 2.56×10^{9} bacterial genes/g stool (wet weight). There was an average 25-fold difference in bacterial densities. At the completion of the lavage, there is no visible faecal material left in the bowel. (Figure 6.2).

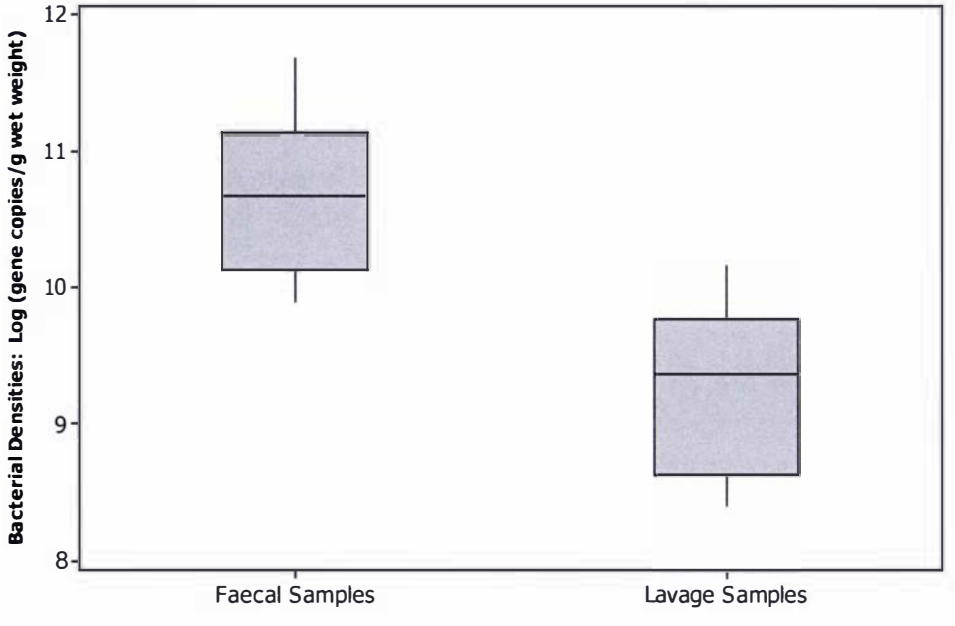


Figure 6.1. The density of bacteria was determined in a set of 10 faecal samples and a set of 10 lavage samples (unpaired) using real time PCR.

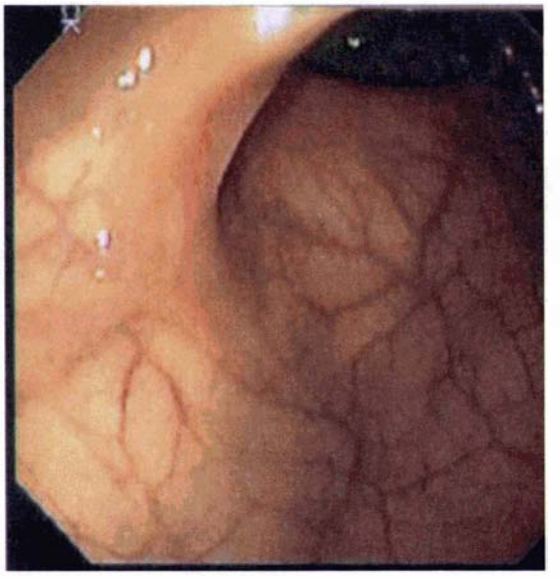


Figure 6.2. Photograph of mucosa lining the wall of the large bowel following intestinal lavage with Fleet Phospho-soda. Photograph provided by Professor Vinton S. Chadwick.

6.4.2 ANALYSIS OF BACTERIAL TTGE PROFILES BEFORE AND AFTER LAVAGE

6.4.2.1 Band Comparisons between Samples Collected from the Same Individual

Post lavage samples from healthy individuals showed some differences in TTGE banding patterns compared to their control samples, however the appearance of new bands, and disappearance of others did not follow an identifiable pattern amongst the volunteers. For example, a characteristic doublet band located at the bottom of the TTGE profile was present in 8/10 individuals. The doublet bands were found to disappear from 1 volunteer's profiles following lavage and to appear in 2 others following lavage. In a further volunteer the doublet appeared for 2 days and then disappeared. However, in 4 individuals the presence of the doublet band was maintained throughout the sampling period. Examples of the effect of the lavage on the doublet band are shown in Figure 6.3. Both bands of the doublet were sequenced in multiple volunteers. Both bands were found to have the greatest identity to *Akkermansia muciniphila* (Appendix F). Therefore this bacterial species appears to be a

predominant member of the faecal microbiota in the gastrointestinal tract in 80% of the volunteers studied, however it was affected differently by the lavage procedure. This example demonstrates that the differences in TTGE profiles did not follow a characteristic pattern amongst the individuals studied.

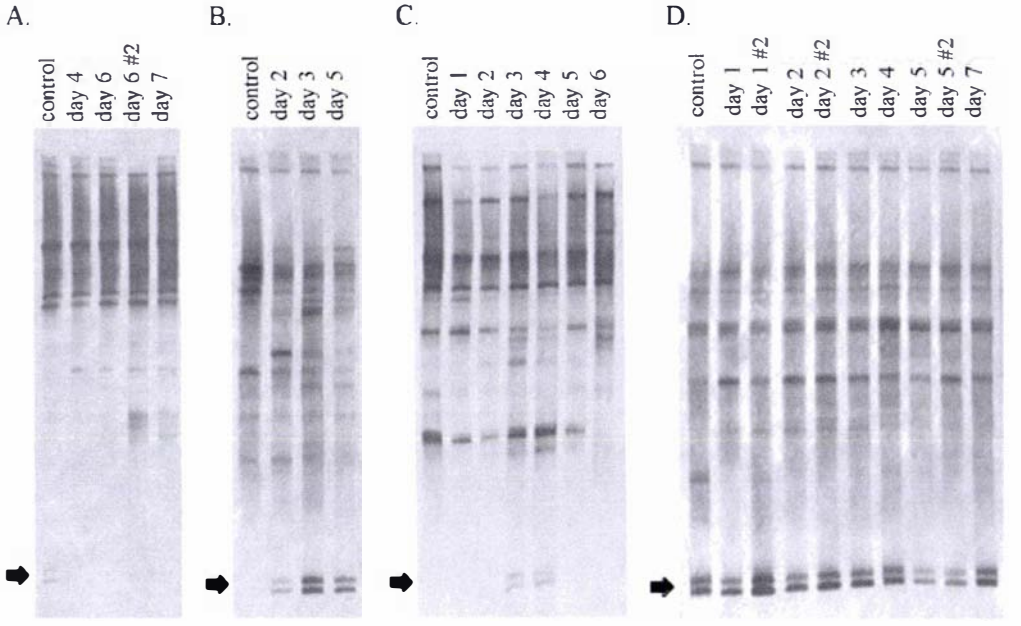


Figure 6.3. Four volunteers' TTGE profiles of faecal samples collected during the lavage study (A-D). Individuals collected varying numbers of samples in the post lavage period based upon the frequency of their bowel movements. The doublet band located at the bottom of the profile (marked with an arrow) disappeared from the TTGE profile in TTGE A. In TTGE B the doublet appeared in the profile following lavage. The doublet both appeared and disappeared following lavage in TTGE C and remained stable throughout the sampling period in TTGE D. Both bands of the doublet have been shown to have greatest identity to the faecal bacterium *Akkermansia muciniphila.*

6.4.2.2 The Effect of the Frequency of Bowel Movements on TTGE Profile Variability

Analysis was carried out to determine if the failure to observe a characteristic change was due to the different number of samples collected. The number of samples provided by healthy volunteers following lavage varied based upon the different frequency of bowel movements. Some individuals collected samples every day, while others

collected multiple samples in one day or no samples for several days (Figure 6.3). One-way ANOVA found no significant difference in the number of band differences observed in faecal samples collected on the same day, collected one day apart, or collected more than one day apart ($p = 0.32$), indicating similar levels of variability between samples, regardless of the time interval between their collection.

6.4.2.3 Sequencing Variable TTGE Bands in One Healthy Volunteer

The TTGE profile of Volunteer 4 was further examined by sequencing (Figure 6.4). The band that migrated the furthest in the TTGE profile on days 3 and 4 was identified as having 100% identity to the recently characterised organism *Akkermansia muciniphila*. Three other bands sequenced from the profile were found to have varying degrees of identity to uncultured bacteria. The band sequenced from the faecal sample collected 1 day after lavage was found to have 94% identity to uncultured bacterium All, which has previously been isolated from human faecal samples. The band analysed from Day 3 post lavage was found to have 99% identity to a butyrate producing bacterium, A2-183, isolated from human faeces. The uncultured bacterial clone Thompsons23, which has previously been isolated from the wild herbivore gut (Nelson *et al.*, 2003), was found to have 97% identity to the band analysed from the Day 4 post lavage TTGE profile.

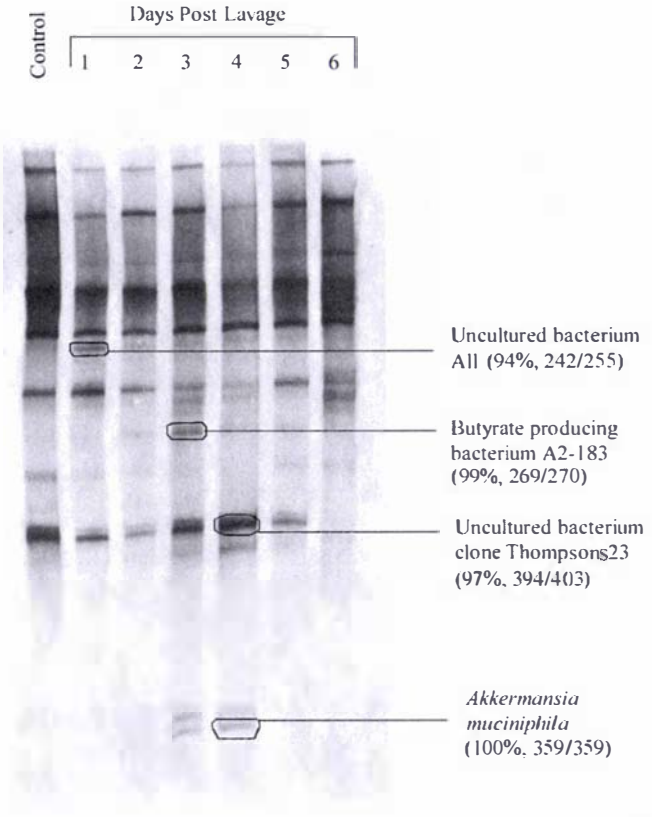


Figure 6.4. Bands that appeared or disappeared in the TTGE profile were excised, the DNA purified and subsequently sequenced to identify the bacterial species the bands represented.

6.4.2.4 Effect of Lavage on Predominant Bacterial Populations in the Seven Days Post Lavage

For the ten volunteers the TTGE profiles generated from the control faecal samples were compared to the post lavage samples using Sorenson's similarity co-efficient. In addition community richness was measured for each sample from the TTGE profile using the Shannon's index.

Amongst the 10 healthy volunteers sampled for 7 days following lavage, the Sorenson's similarity co-efficients ranged from 44% similarity to 97% (Table 6.1), with a median value of 81%.

Table 6.1. Sorenson's similarity co-efficients were calculated by comparing control and post lavage TTGE profiles (raw data Appendix A.5). Some volunteers have two similarity values as two faecal samples were collected in one day.

Healthy Volunteers	Days Post Lavage						
	1	2	3	4	5	6	7
10	-	-	44%	67%	61%	65%	-
6	83%	71%	72%	64%	67%	80%	-
8	-	72%	-	68%	63%	-	-
7	76% 88%	81% 80%	86% -	83% -	76% 84%	- -	79% -
1	- -	- -	- -	76% -	- -	76% 77%	77% -
3	-	90%	85%	88%	83%	85%	81%
9	-	-	83%	-	-	-	71%
2	97%	94%	85%	67%	82%	78%	76%
5	-	78%	82%	86%	85%	-	83%
4	89%	93%	84%	83%	93%	52%	-

- sample not collected

Trend lines of the similarity co-efficients were plotted for each volunteer, however there was no consistent trend over time; some individuals showed declining similarity, others were stable, while others had TTGE profiles become increasingly similar to the control (Figure 6.5). Further analysis using one-way ANOVA demonstrated no significant difference between days 1-7 post lavage ($p = 0.32$). However one-way ANOVA did find a significant difference between the sets of similarity values obtained for each of the 10 volunteers ($p = 0.0004$).

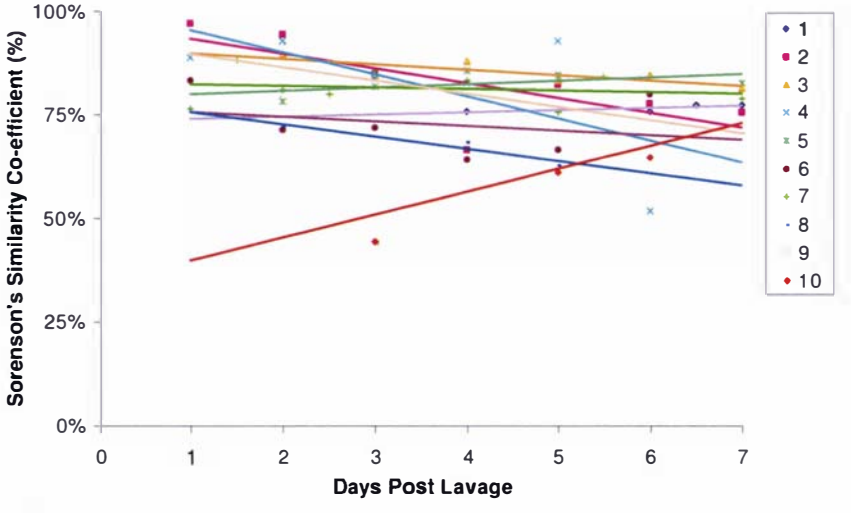


Figure 6.5. A trend line was plotted for each healthy volunteer to determine how the similarity co-efficient changed with respect to time in the post lavage period.

Figure 6.6 shows Sorenson's similarity co-efficients from healthy volunteers compared to the normal temporal variation measured in 5 individuals in the short term, as determined in Chapter 4. The samples collected in the 7 days post lavage from ten healthy volunteers were found to be significantly different to normal temporal variation ($p = 0.0003$).

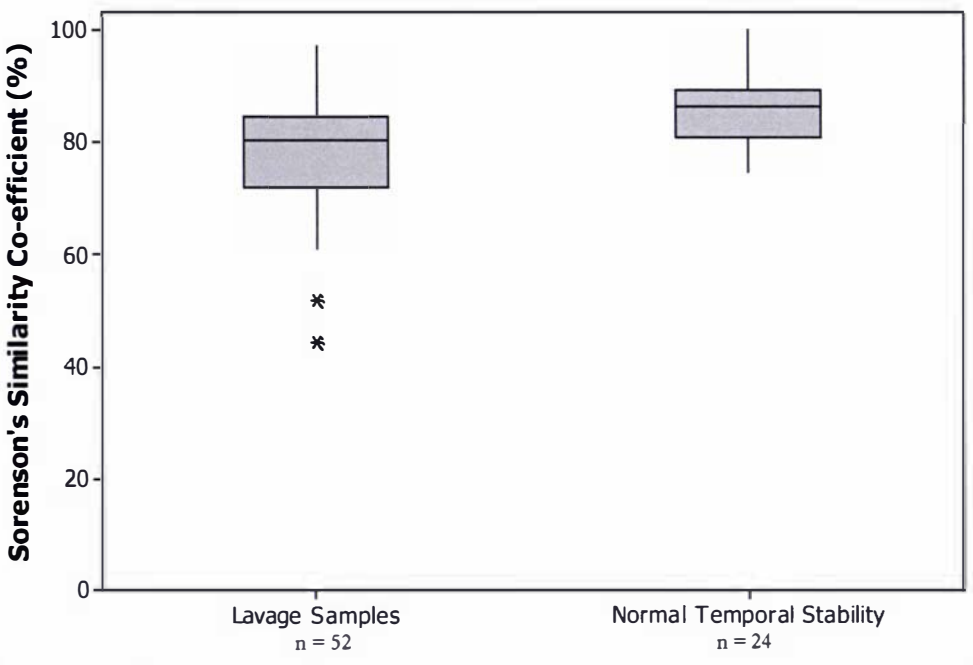


Figure 6.6. Sorenson's similarity co-efficients from TTGE gels showed increased variability in the bacteria population following lavage compared to the normal temporal stability found in the bacteria over the short term. Normal temporal stability values were obtained from the short term stability study in Chapter 4. * indicates the presence of outliers.

6.4.2.5 Effect of Lavage on Community Richness

As a measure of community richness the Shannon's index was calculated from the TTGE profiles of control and post lavage samples collected from ten healthy volunteers (Table 6.2). One-way ANOVA analysis of the Shannon's indices showed that the community richness of the samples before and after lavage was not significantly different ($p = 0.94$)

Table 6.2. Shannon's indices were calculated to establish if the intestinal lavage led to a reduction in community richness (raw data Appendix B.2). No significant difference was detected between the control and post-lavage samples.

		Days Post Lavage						
Volunteer	Control	1	2	3	4	5	6	7
1	2.28				2.18		1.97 2.54	2.45
2	2.61	2.64	2.52	2.56	1.93	2.62	2.53	2.75
3	2.42		2.57	2.50	2.39	2.26	2.33	2.26
4	2.45	2.24	2.10	2.51	2.36	2.38	2.31	
5	1.52		1.74	2.06	2.41	2.13		2.51
6	2.32	2.29	2.60	2.23	2.56	2.07	2.51	
7	2.48	1.98 2.51	2.78 2.50	2.63	2.52	2.80 2.84		2.65
8	2.37		2.95		2.88	2.59		
9	2.70			2.34				2.57
10	2.64			2.57	2.54	2.65	2.61	

- sample not collected

6.4.3 IMPACT OF LAVAGE ON POPULATION DENSITIES

6.4.3.1 Bacterial Densities in Control and Post Lavage Samples

The density of bacteria in the 10 healthy volunteer's control and post lavage samples was measured using real time PCR. The densities were found to vary both between different individuals and within the same individual during the sampling period (Figure 6.7). Analysis of the data using one-way ANOVA found no significant difference between the volunteers, and no significant difference between the samples with respect to time.

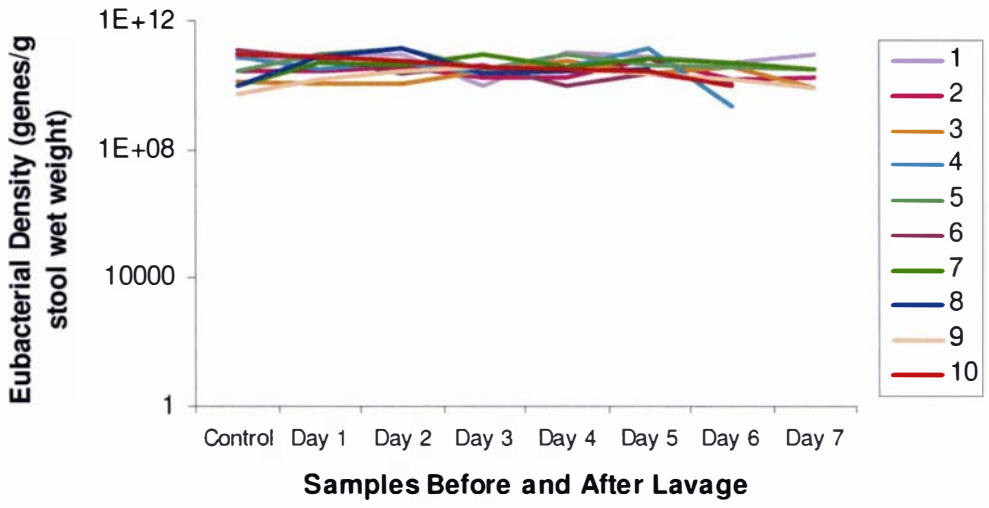


Figure 6.7. The density of bacteria was determined using real time PCR, for the control samples collected before intestinal lavage, and in the samples collected in the post lavage period, from Volunteers 1-10 (raw data Appendix C.4).

6.4.3.2 Methanogen Densities in Control and Post Lavage Samples

Amongst the 10 volunteers, methanogens were present in control samples of Volunteer 5, 8, and 10. The density of methanogens was monitored in the post lavage period within these individuals, and values were expressed as a proportion of the total population density as described in chapter 3 (3.4.3.7) (Figure 6.8). Volunteer 8's methanogen population dropped below the detection limit of the real time PCR assay following the lavage, and were not detected again during the sampling period. Methanogen densities in Volunteer 5 and 10 fluctuated throughout the sampling period. Both volunteers had a higher density in their last sample compared to the control; Volunteer 5 increased from 0.168% of the total population to 0.376%, and Volunteer 10 increased from 0.0189% to 0.439%. One-way ANOVA found no significant difference between the population changes seen in the 3 volunteers ($p = 0.56$).

Analysis of methanogen density changes with respect to time could not be performed across all time points, as these volunteers did not collect faecal samples on the same days. Therefore one-way ANOVA was carried out to compare the densities measured

in the control samples and post lavage samples from day 2, 3, 4, and 5. No significant difference was found between these time points ($p = 0.37$).

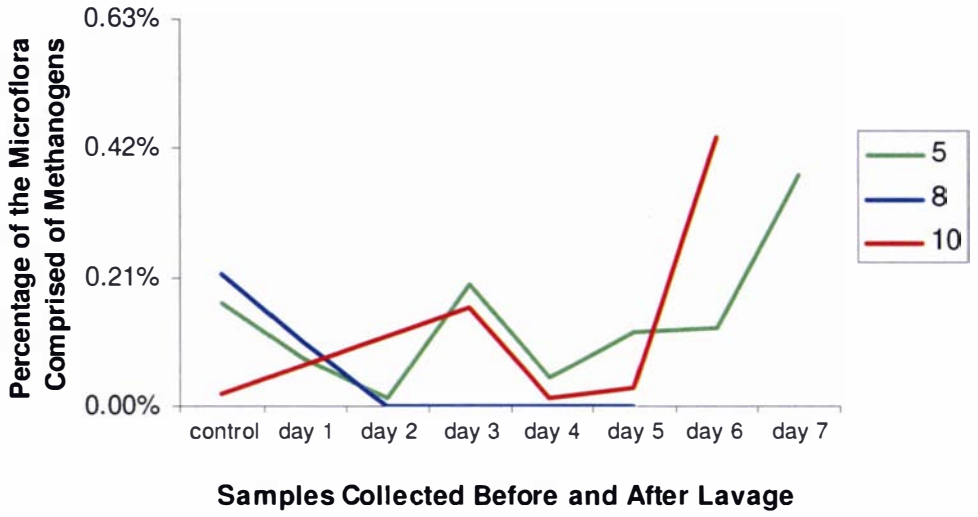


Figure 6.8. Volunteer 5, Volunteer 8 and Volunteer 10 all harboured methanogens. The density of methanogens was monitored before and after lavage in these individuals (raw data Appendix D.4).

6.4.3.3 Sulfate Reducing Bacteria Densities in Control and Post Lavage Samples

Sulfate reducing bacteria were present in the control samples of Volunteer 1, 2, 3, 5, and 10. The density of sulfate reducing bacteria was monitored in the post lavage period within these individuals, and values were expressed as a proportion of the total population density as described in chapter 3 (3.4.3.7) (Figure 6.9). The lavage did not uniformly affect the volunteer's sulfate reducing bacteria populations; 4 of the volunteers maintained a relatively stable population before and after the lavage. In contrast, Volunteer 10 showed large changes in population numbers on a day-by-day basis. One-way ANOVA analysis revealed a significant difference between the 5 volunteers ($p = 0.007$), however no significant difference was observed with respect to the sulfate reducing bacteria proportions measured for each time point ($p = 0.26$).

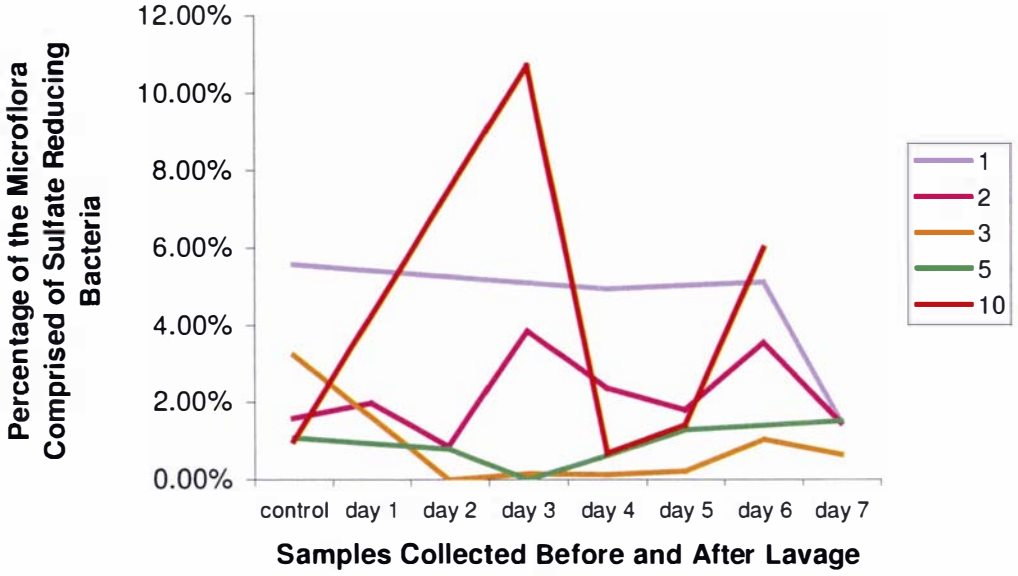


Figure 6.9. Volunteer 1, Volunteer 2, Volunteer 3, Volunteer 5, and Volunteer 10 all harboured sulfate reducing bacteria. The density of sulfate reducing bacteria was measured before and after intestinal lavage in these individuals (raw data Appendix E.4).

6.5 DISCUSSION

Faecal samples collected from healthy volunteers before and after intestinal lavage were examined to determine the impact of Fleet Phospho-Soda induced lavage on the faecal microbiota, and to monitor how the population recovers.

To date, no molecular study has been undertaken to investigate the effect of orally administered Fleet Phospho-Soda osmotic laxative on the faecal microbiota. This study demonstrated a reduction in the density of bacteria present in faecal lavage fluid samples compared to faecal samples. Similar findings were made in a study examining preoperative preparation of the bowel using whole gut irrigation (van den Bogaard *et al.*, 1986). The authors found a reduction of 2-3 logs in faecal aerobes, and a 4-5 log reduction in anaerobes. Bacterial densities were expressed as the number of colony forming units per gram or per mL of faeces. The authors also reported that the anaerobic flora recovered within 24 hours, however aerobes underwent a transient overgrowth for 2 days. A study examining the effect of polyethylene glycol-electrolyte lavage solution (Golytely) on the faecal and colonic microbiota, found no significant difference in the number of bacteria present in faecal and lavage fluid samples (Morotomi *et al.*, 1989). While this result is in contrast to the significant difference detected in the study described here, the findings from the study by Morotomi *et al*, are likely to reflect the fact that bacterial numbers were expressed with respect to dry faecal weights thereby eliminating the dilution effect of lavage. These findings indicate that transient changes in the colonic microbiota do occur in response to intestinal lavage.

Measurements of bacterial densities in faecal samples demonstrated that the microbiota recovered very rapidly following intestinal lavage. In 40% of volunteers a bowel motion was passed in the day following Fleet Phospho-soda administration, and the density of bacteria was equivalent to control levels. This suggests significant bacterial growth over a 24-hour period. Comparison of faecal samples and faecal lavage fluid demonstrated a 25-fold reduction in bacterial densities, therefore densities could be restored to control levels after 5 doublings of the population. However assuming normal faecal output of approximately 120g (Macfarlane and Macfarlane, 1997), further

growth would be required to achieve this mass. During the 24 hours between bowel cleansing and the first bowel movement sufficient time should have been available for the recovery of the population to control densities.

TTGE profiles were generated from faecal samples collected from healthy volunteers before and after lavage to monitor the impact of this procedure on the composition of the faecal microbiota. The time-course study did not show a gradual restoration of the TTGE profile following disruption by the lavage. Rather the microbiota appeared to be highly similar to the control sample at the first time point (in some cases within 24 hours). There were random fluctuations in TTGE profiles over the 7-day monitoring period, which led to no overall trend with time.

Two bands that were present as a doublet at the bottom of the TTGE profile in 80% of the volunteers were sequenced. The upper band from 3 gels was sequenced and the lower band from 5 gels was sequenced. In all cases the bands were found to have the greatest identity to *Akkermansia muciniphila.* The two bands may be derived from different 16S rRNA gene cistrons in this organism's genomic DNA (Nubel *et al.*, 1996). Alternatively one of the bands may represent a truncated PCR product, which has been shown to arise through the formation of hairpin loop structure in the GC-clamp during PCR (Nubel *et al.*, 1996).

TTGE similarity values measured in individuals following lavage were compared to the normal temporal stability observed in healthy individuals that have not undergone this procedure. A significant difference between these groups was detected. This suggests that Fleet intestinal lavage can perturb the bacterial population and reduce the normal level of stability present in the population. Comparisons between the sample sets collected by each volunteer revealed statistical differences. This suggests that Fleet lavage may have affected volunteers differently, with some individuals demonstrating very little change in the faecal microbiota, while others had more marked alterations in the post-lavage TTGE profiles. There was no evidence to suggest that volunteers failed to comply with the Fleet Phospho-Soda protocol, therefore its unlikely that these differences can be explained by poor compliance. The predominant bacterial population in some individuals may be more resilient to perturbation than others.

The lavage procedure did not appear to have a uniform effect on methanogen or sulfate reducing bacterial populations. Despite being slow growing organisms, methanogens were detected in the first faecal samples collected by 2/3 volunteers following the lavage. However, in Volunteer 8, methanogens were not detected in any post lavage samples, despite having the highest density amongst the 3 volunteers in control samples. The drop in methanogens in Volunteer 8 was also accompanied by reduced stability in the TTGE profile, however reduced stability was also evident in one of the volunteers in whom methanogen populations were maintained.

The fact that TTGE bacterial populations following the lavage were so similar to control samples was not completely unexpected. While lavage removes a significant proportion of faecal material and faecal bacteria from the bowel, it does not completely eradicate bacteria from the bowel. Therefore rather than re-colonisation of the bowel by organisms ingested from the environment, residual members of the microbiota are likely to remain both in the lumen and within the mucosal tissue of the gut wall. The bacterial populations present in mucosa and within the faecal lavage fluid have been shown to differ from the faecal microbiota (Zoetendal *et al.*, 2002) (personal communication, Professor Vinton S Chadwick, Wakefield Gastroenterology Research Institute, Wellington, New Zealand), however it is likely that organisms from both these populations can act as a reservoir or founding population from which the unique, host-specific faecal microbiota re-emerges.

Faecal microbial densities recovered rapidly, within 24 hours, following Fleet intestinal lavage, and the composition of the predominant bacterial population was found to exhibit more variability than what is usually present over time. These findings indicate that the normal temporal stability of the faecal microbiota can be disrupted by Fleet intestinal lavage. To determine if an environmental change carried out for a longer period has a more significant effect on the faecal microbiota, the impact of a major dietary change was investigated in Chapter 7.

CHAPTER 7: COMPOSITION AND STABILITY OF THE FAECAL MICROBIOTA AFTER DIETARY CHANGE

7.1 ABSTRACT

A study was undertaken to determine if the composition of the faecal microbiota can be altered through a dramatic dietary change (the Atkins' diet). Faecal samples collected before and after the introduction of the Atkins' diet were analysed. PCR-TTGE was used to monitor changes in the predominant bacterial population, and real time PCR was used to measure changes in the densities of methanogens and sulfate reducing bacteria. Six healthy volunteers collected 4 faecal samples while consuming their normal Western diet for 4 weeks, and a further 4 faecal samples were collected during consumption of the Atkins' diet for 4 weeks.

Increased dietary protein and constipation have both been shown to influence methanogen and sulfate reducing bacteria populations. High protein consumption occurs while following the Atkins' diet and 5/6 volunteers reported constipation during the diet. However methanogen and sulfate reducing bacteria populations were comparable to those seen during consumption of the volunteers' normal Western diets, suggesting that the Atkins' diet does not influence these populations. The Atkins' diet was found to cause a significant change in the faecal microbiota present in TTGE gel profiles ($p = 0.0001$). These findings indicate that the predominant bacterial population can be altered by the introduction of the Atkins' diet.

7.2 INTRODUCTION

Undigested components of the diet pass from the small intestine into the colon and can be readily metabolised by the intestinal microbiota. Individuals consuming a normal Western diet are thought to ingest approximately 20g of plant structural material (Tannock, 1999), which cannot be digested by the human gut. In addition an estimated 10% - 15% of undigested starch passes into the colon (Tannock, 1999). These substrates are fermented by the intestinal microbiota leading to the production of short chain fatty acids (SCFA), H_2, and CO_2. Protein also passes into the large bowel and generates SCFA, however, protein can also be metabolised by the microbiota into toxic by-products such as ammonia, phenols, indoles and H_2S by the microbiota.

Changes in the sorts of dietary substrates reaching the colon are likely to influence the bacterial populations present in the gut, and therefore could potentially be utilised to alter the composition of the microbiota for therapeutic benefit. Major alterations to diet, such as moving from a Western diet to a vegan diet have been shown to cause a significant change in the faecal microbiota (Peltonen *et al.*, 1997). Studies of individuals consuming Western diets, rural Japanese diets, and rural South African diets have also shown variation in the faecal microbiota between these groups (Moore and Moore, 1995). However, minor changes in food consumption do not necessarily affect the microbiota. The introduction of oligosaccharide-containing biscuits (Tannock *et al.*, 2004), eating a controlled Western diet rather than a "free" Western diet, or the introduction of black tea drinking did not significantly alter the faecal microbiota (Mai, 2004). A study introducing oligofructose and inulin to the diet did detect increases in eubacteria, lactobacilli, and bifidobacteria (Langlands *et al.*, 2004).

The Atkins' diet book (Atkins, 2002) has sold more than 45 million copies, and is becoming increasingly popular as Western societies face an obesity epidemic (Astrup *et al.*, 2004). The Atkins' diet prescribes a radical change in macronutrient consumption to reduce weight. The diet is based around restricted carbohydrate consumption; no more than 20g a day during the induction phase (equivalent to 1 potato), while protein and fats can be consumed liberally (Atkins, 2002). This dietary regime leads to the

emptying of glycogen stores and the body subsequently enters ketosis, using ketone bodies as the primary fuel. Ketosis can suppress appetite and high protein intake leads to satiety (Porrini *et al.*, 1997).

While the diet is based around the consumption of net carbohydrates (dietary fibre is not included in the 20g limit) the marked reduction in the consumption of fruit and grains, and the consumption of sugar alcohols in many commercial low-carb foods (artificial sweeteners that are undigested by the human gut) is likely to significantly alter the types of fibre present in the diet. Increased protein consumption is also likely to increase the amount of dietary sulfate delivered to the colon. The extreme dietary change is likely to alter the predominant bacterial population (Moore and Moore, 1995; Peltonen *et al.*, 1997), and changes in dietary sulfate may lead to shifts in methanogen and sulfate reducing bacteria populations.

To examine if the introduction of the Atkins' diet can alter the composition of the faecal microbiota, samples were collected from volunteers before and after this dietary change and compared.

7.3 METHODS

7.3.1. VOLUNTEERS AND SAMPLE COLLECTION

Eight healthy volunteers originally agreed to participate in this study. Two volunteers withdrew at the start of the Atkins' Diet as the diet was too restrictive.

Volunteers provided one faecal sample per week for a period of 4 weeks while consuming their normal Western diet. Directly prior to the commencement of the Atkins' diet volunteers collected a faecal sample (Day 0). Additional samples were collected after 2 weeks, 3 weeks and 4 weeks of eating according to the Atkins' diet.

Blood samples were collected from volunteers in the week prior to initiation of the Atkins' diet, and on the last day of the Atkins' diet. Weight measurements were also made at these times.

7.3.2 THE ATKINS' DIET

Volunteers followed the Atkins' diet eating plan, by initially consuming no more than 20g of carbohydrate per day for 2 weeks, known as the Initiation Phase of the diet. In accordance with the Atkins' diet guidelines volunteers increased their carbohydrate allowance to 25g per day during the 3rd week. In the 4th week the carbohydrate intake was raised to 30g per day. Examples of low carbohydrate meals suitable for the Atkins' diet are given in Appendix G. At the conclusion of week 4 the study was complete and volunteers were free to return to their normal diet or continue with the Atkins' diet.

7.3.3 FAECAL DNA EXTRACTION/PCR-TTGE/REAL TIME PCR/SEQUENCING

The storage, homogenisation and subsequent extraction of DNA from faecal samples, application of PCR-TTGE and real time PCR, and sequencing of microbial DNA were carried out as previously described in Chapter 2: Materials and Methods.

7.4 RESULTS

7.4.1 VOLUNTEER DROP-OUT AND COMPLIANCE

Initially 8 healthy individuals volunteered to participate. Following distribution of Volunteer Packs one volunteer withdrew from the study on the grounds that the food choices were too limited for vegetarians. After one week on the Atkins' diet a further volunteer withdrew after failing to comply with the diet's requirements. The remaining 6 volunteers completed the Atkins' diet as detailed in the methods section.

Compliance amongst volunteers was monitored through ketone sticks (Keto-Diastix, Bayer Diagnostics). Every morning volunteers passed a ketone stick through their urine stream and recorded the stick's colour change. Ketosis was indicated by the development of a pink-purple shade. All 6 volunteers were in ketosis following 2 days of eating according to the induction phase of the Atkins' diet ($\leq$ 20g carbohydrate per day) and in all volunteers ketones were detected in urine throughout the 4 weeks of the study.

7.4.2 THE EFFECT OF THE ATKINS' DIET ON BLOOD AND WEIGHT MEASUREMENTS

Prior to the commencement of the Atkins' diet volunteers underwent blood tests and weight measurements. Levels of liver enzymes, blood lipids, fasting glucose and body weight were determined to monitor the impact of the low carbohydrate eating plan on these variables (Table 7.1). Prior to the resumption of the volunteers' normal eating regime, a further set of blood samples was collected and weight measurements made.

At the conclusion of the study all of the volunteers had lost weight. As a proportion of their original weight, volunteers lost from 4.6% - 8.1% of their total mass, with a median of 5.9%.

The effect of the diet on blood lipids, liver enzymes and fasting glucose varied amongst the different volunteers. Total cholesterol increased in 3 volunteers, and fell in 3 volunteers, which was mirrored in the cholesterol/HDL ratios. Fasting glucose was reduced in 5/6 volunteers. GGTP levels dropped in all volunteers and total protein, globulins, alkaline phosphatase and AST dropped in 5/6 volunteers, however none of these changes were statistically significant.

Table 7.1. Blood samples and weight measurements were obtained from the 6 volunteers before and after the Atkins' diet to monitor the impact of the low carbohydrate eating plan on fasting glucose, liver function, blood lipids, and weight loss.

	Normal	Volunteer 1		Volunteer 2		Volunteer 3		Volunteer 4		Volunteer 5		Volunteer 6	
	Range	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Glucose Fasting (mmol/L)	3-6	5.5	4.9	4.8	4.4	5.8	5.3	5.2	4.7	5.3	4.7	4.6	4.8
Total Protein (g/L)	60-83	80	78	75	68	72	70	77	79	77	71	72	70
Albumin (g/L)	34-50	47	47	51	46	44	45	44	43	48	45	43	43
Globulins (g/L)	20-35	34	31	24	21	27	25	32	35	29	25	29	28
Bilirubin (umol/L)	0-20	6	11	13	7	13	15	7	8	13	13	24	12
GGTP (iu/L)	0-36	36	16	16	13	27	20	9	8	102	15	19	15
Alkaline Phosphatase (iu/L)	20-110	65	51	53	41	61	61	51	43	29	26	32	30
ALT (iu/L)	0-30	27	29	19	26	42	54	10	17	31	18	17	26
AST (iu/L)	0-30	22	25	24	23	29	41	17	20	21	20	24	38
Cholesterol (mmol/L)	3-5	5.6	4.4	5.1	6	3.4	3.2	5.6	6	6.1	5.3	5.4	6
Triglyerides (mmol/L)	0.2-2	1.3	0.8	0.4	0.5	0.5	0.7	1.2	1.3	4.8	1.4	1.1	0.8
HDL Cholesterol (mmol/L)	1-3	1.89	1.72	2.76	2.94	1.78	1.75	1.65	1.72	1.09	1.23	1.88	1.88
LDL cholesterol (mmol/L)	2-3.5	3.1	2.3	2.2	2.8	1.4	1.1	3.4	3.7	2.8	3.4	3	3.8
Cholesterol/HDL (ratio)	2.5-4.5	3	2.6	1.8	2	1.9	1.8	3.4	3.5	5.6	4.3	2.9	3.2
Weight (Kg)		80	75	54	49.9	86.5	82.1	69.4	65.5	99	91	66	63

7.4.3 BACTERIAL TTGE PROFILES DURING CONSUMPTION OF A WESTERN DIET AND CONSUMPTION OF THE ATKINS' DIET

From each volunteer, 1 faecal sample was collected per week over a 4 week period while consuming a normal Western diet. The first sample collected was compared to the subsequent 3 samples to determine the normal variation in the faecal microbiota of the volunteers. The first Atkins' diet sample collected on Day 0 was compared to the 3 samples collected during the Atkins' diet. TTGE profiles were generated to determine the effect of dietary change on the predominant bacterial population. The samples collected while on a normal Western diet appeared relatively stable, with only small changes in the TTGE profiles over the 4 week collection period. During the Atkins' diet the TTGE profiles showed some increased variability in some volunteers, while others appeared to have a stable population throughout this period (Figure 7.1). There did not appear to be a characteristic pattern to the changes in TTGE profiles across all volunteers. Volunteer 6 showed the most changes during the Atkins' diet, therefore bands were sequenced from Volunteer 6 to identify the bacterial species that fluctuated during consumption of the Atkins' diet (Figure 7.2).

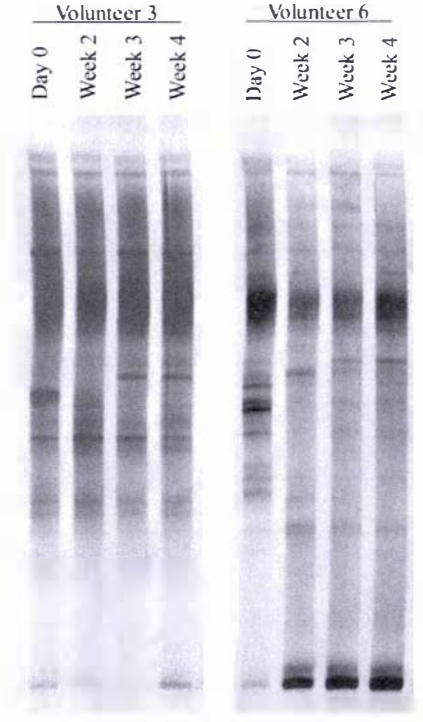


Figure 7.1. TTGE profiles of faecal samples collected from Volunteer 3 and Volunteer 6 before (Day 0) and during consumption of the Atkins' diet (Weeks 2-4). Few changes were evident in Volunteer 3, however more differences were present in Volunteer 6.

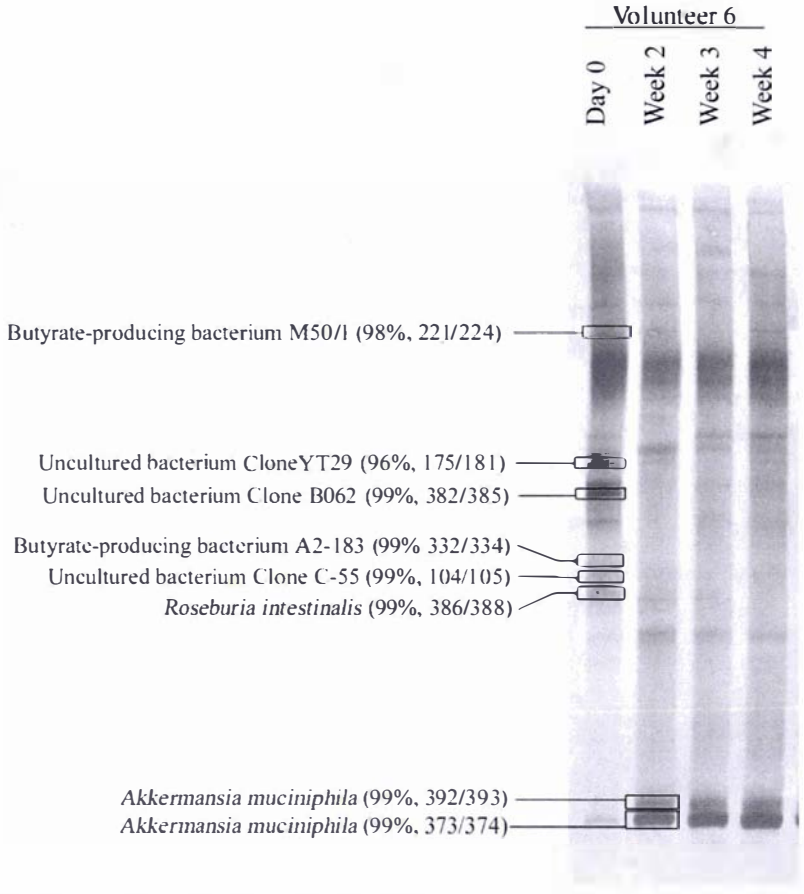


Figure 7.2. Selected bands from the TTGE profile of Volunteer 6 were sequenced. Organisms with the greatest sequence identity to the bands are indicated.

7.4.4 ANALYSIS OF TTGE PROFILES WITH SORENSON'S SIMILARITY CO-EFFICIENT

For each volunteer, Sorenson's similarity co-efficient was calculated to determine the degree of similarity present in samples collected during the Western diet and the degree of similarity present in the samples collected during the Atkins' diet. In each case the first sample collected was compared to the subsequent 3 samples collected during that dietary regime. The data from each diet period were pooled (Figure 7.2). The similarity values obtained from Western diet samples were high, ranging from 72% - 100% with a median of 83%. Amongst the Atkins' diet samples the similarity values were found to cover a larger range from 53% to 91% with a median of 68%.

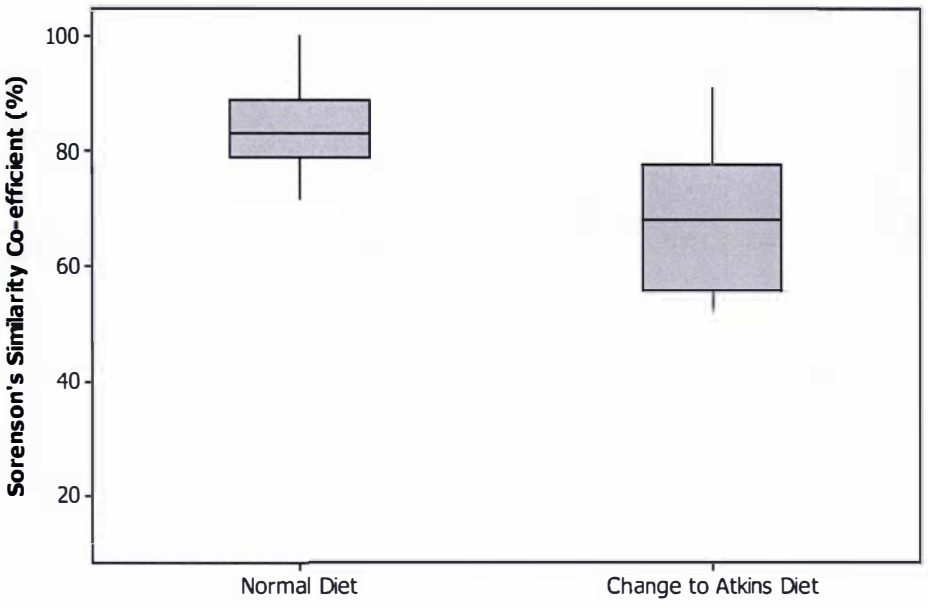


Figure 7.3. Box whisker plot of the Sorenson's Similarity Co-efficient Values for TTGE profiles of the faecal microbiota, obtained from six volunteers consuming a normal Western diet and the Atkins' diet (Raw data Appendix A.6 and A.7).

Comparisons between the pooled control data and the pooled Atkins' diet data revealed a significant difference ($p = 0.0001$), indicating that the initiation of the Atkins' diet causes a significant change in the composition of the predominant bacterial population.

The trend in similarity values over time and amongst different volunteers were analysed during both dietary regimes (Figure 7.4). One-way ANOVA revealed no significant difference in TTGE profile similarity with respect to time for either the Western diet or the Atkins' diet. Pearson's product moment correlation did detect a negative correlation between similarity and time for both the Western diet and the Atkins' diet, however this was not statistically significant.

Data sets were also analysed to determine if the bacterial populations in each volunteer behaved similarly. During the normal Western diet the volunteers' similarity values were all comparable and no significant difference was found. However the data obtained during the Atkins' diet demonstrated a significant difference between the similarity values obtained from different volunteers ($p = 0.006$). This suggests that the

dietary change did not uniformly affect the faecal microbiota of the different individuals.

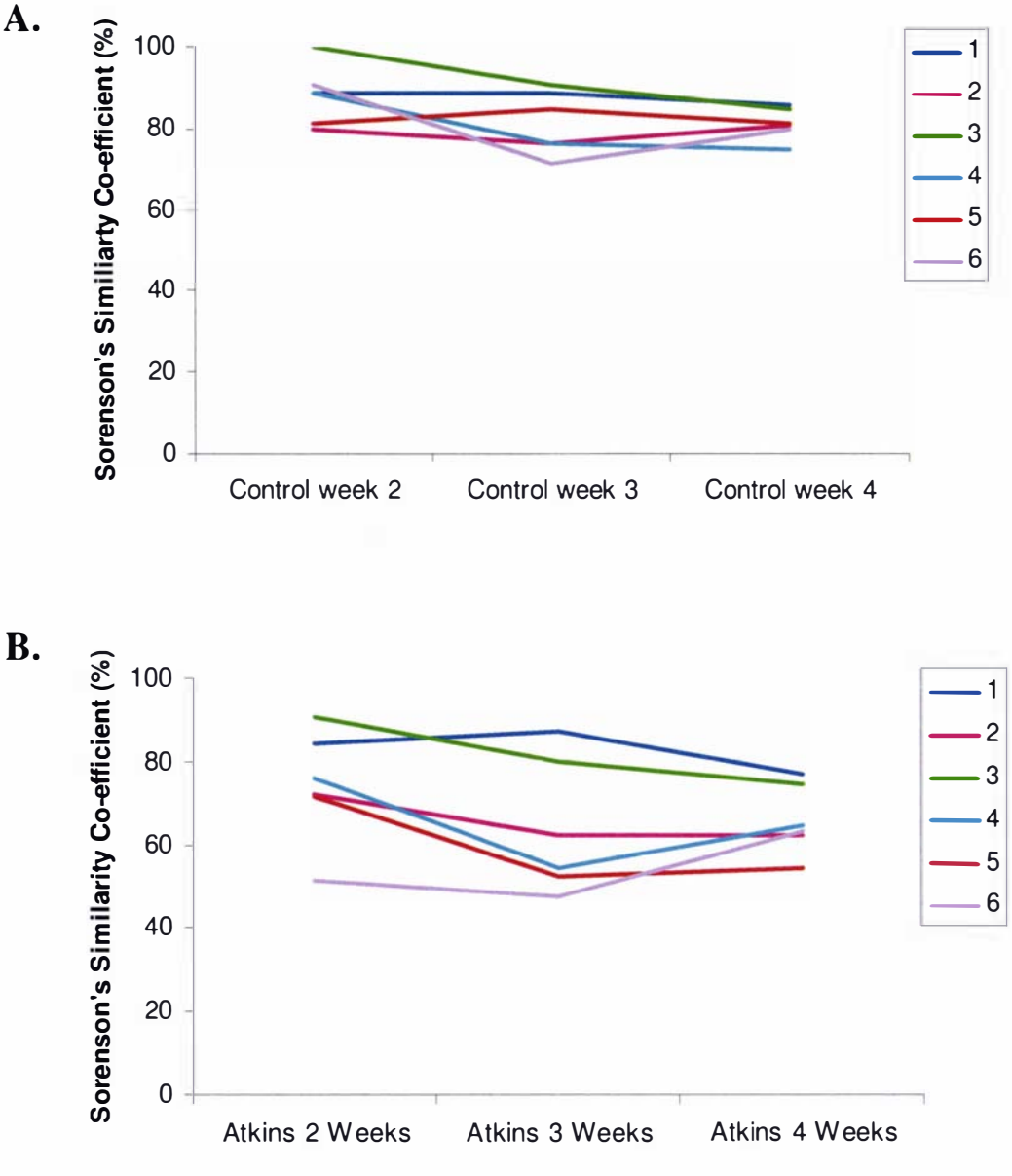


Figure 7.4. The similarity co-efficients obtained from bacterial TTGE profiles were analysed over time and between the volunteers (Volunteers 1-6) during both their normal Western diet (A) and the Atkins' diet (B).

7.4.5 ANALYSIS OF TTGE PROFILES WITH THE SHANNON'S INDICES

The Shannon's index was calculated for each TTGE profile to provide a measure of community richness. Values for volunteers consuming their normal diet ranged from 1.90 – 2.96, with a median of 2.47 (raw data Appendix B.3). Amongst the Atkins' diet samples, values ranged from 2.15 – 2.97, with a median of 2.58 (raw data Appendix B.4). There was no significant difference in community richness between the two diet regimes.

7.4.6 BACTERIAL DENSITIES IN FAECAL SAMPLES COLLECTED WHILE CONSUMING A WESTERN DIET AND WHILE CONSUMING THE ATKINS DIET

Bacterial densities were measured in samples collected from volunteers on their normal Western diet and on the Atkins' diet (raw data Appendix C.4 and C.5). On the Western diet, densities ranged from 1.09×10^{9} – 4.72×10^{11} genes/g stool (wet weight) with a median of 7.49×10^{10} genes/g stool (wet weight). On the Atkins' diet bacterial densities ranged from 2.26×10^{9} – 2.75×10^{11} genes/g stool (wet weight), with a median of 5.94×10^{10} genes/g stool (wet weight). No significant difference was detected between these groups.

7.4.7 METHANOGEN DENSITIES IN FAECAL SAMPLES COLLECTED WHILE CONSUMING A WESTERN DIET AND WHILE CONSUMING THE ATKINS DIET

Amongst the 6 volunteer's Western diet samples, methanogens were only detected in Volunteer 2. Following consumption of the Atkins' diet for 2 weeks methanogen levels dropped markedly in Volunteer 2 (Figure 7.5), however this population shift is similar to those observed during consumption of Volunteer 2's normal diet. This suggests that this change in methanogens could not be directly attributable to the Atkins' diet.

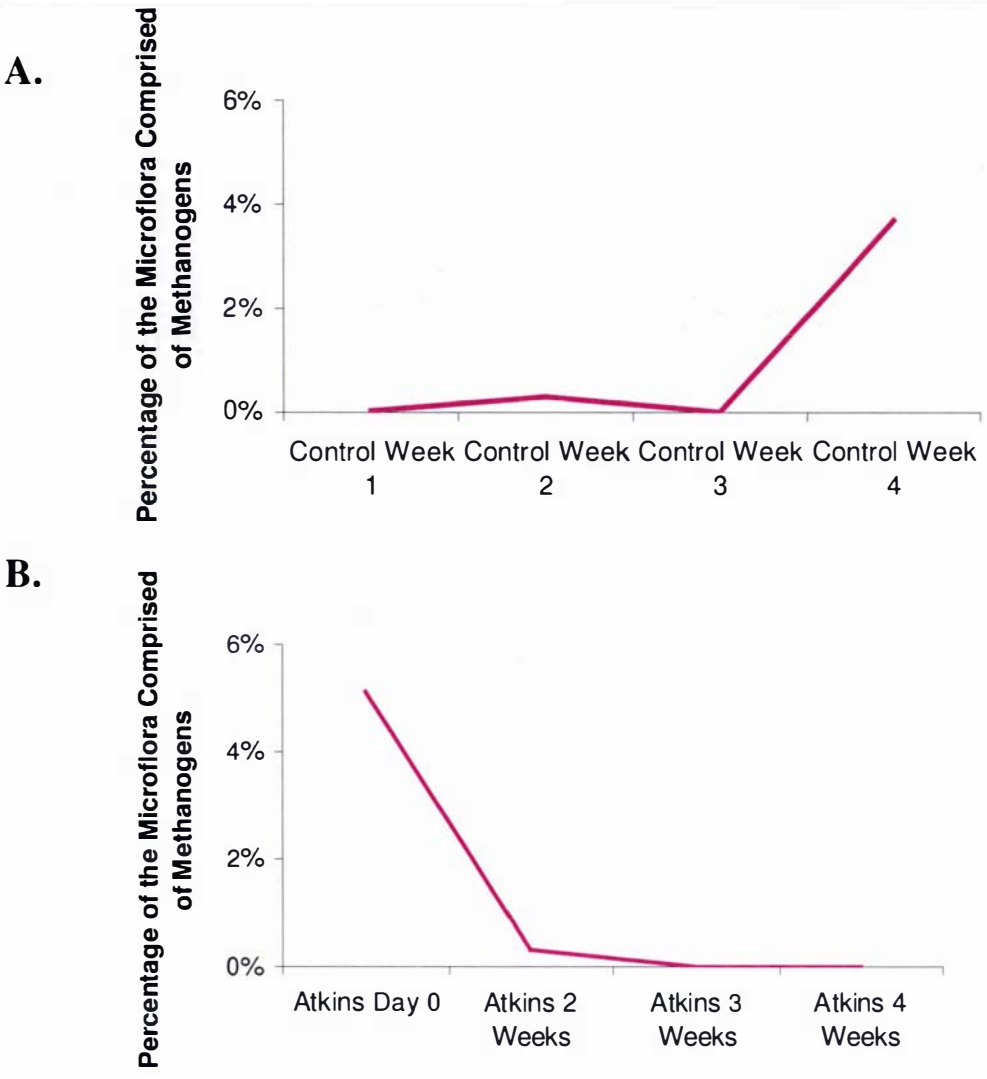


Figure 7.5. Methanogen levels were monitored in Volunteer 2 while consuming a normal Western diet (A) and during a subsequent change to the Atkins' diet (B) (raw data Appendix D.5 and D.6).

7.4.8 SULFATE REDUCING BACTERIA DENSITIES IN FAECAL SAMPLES COLLECTED WHILE CONSUMING A WESTERN DIET AND WHILE CONSUMING THE ATKINS DIET

Sulfate reducing bacteria were detected in Volunteers 1, 2, and 4 during consumption of the Western diet. Sulfate reducing bacteria remained stable throughout the sampling period in Volunteers 2 and 4, however after two weeks of the Atkins' diet there was a

sharp rise in these organisms in Volunteer 1, followed by a subsequent drop back to control levels (Figure 7.6). Analysis of the data collected from Volunteer 1 did not find a significant difference between the densities of sulfate reducing bacteria during the Western diet and during the Atkins' diet.

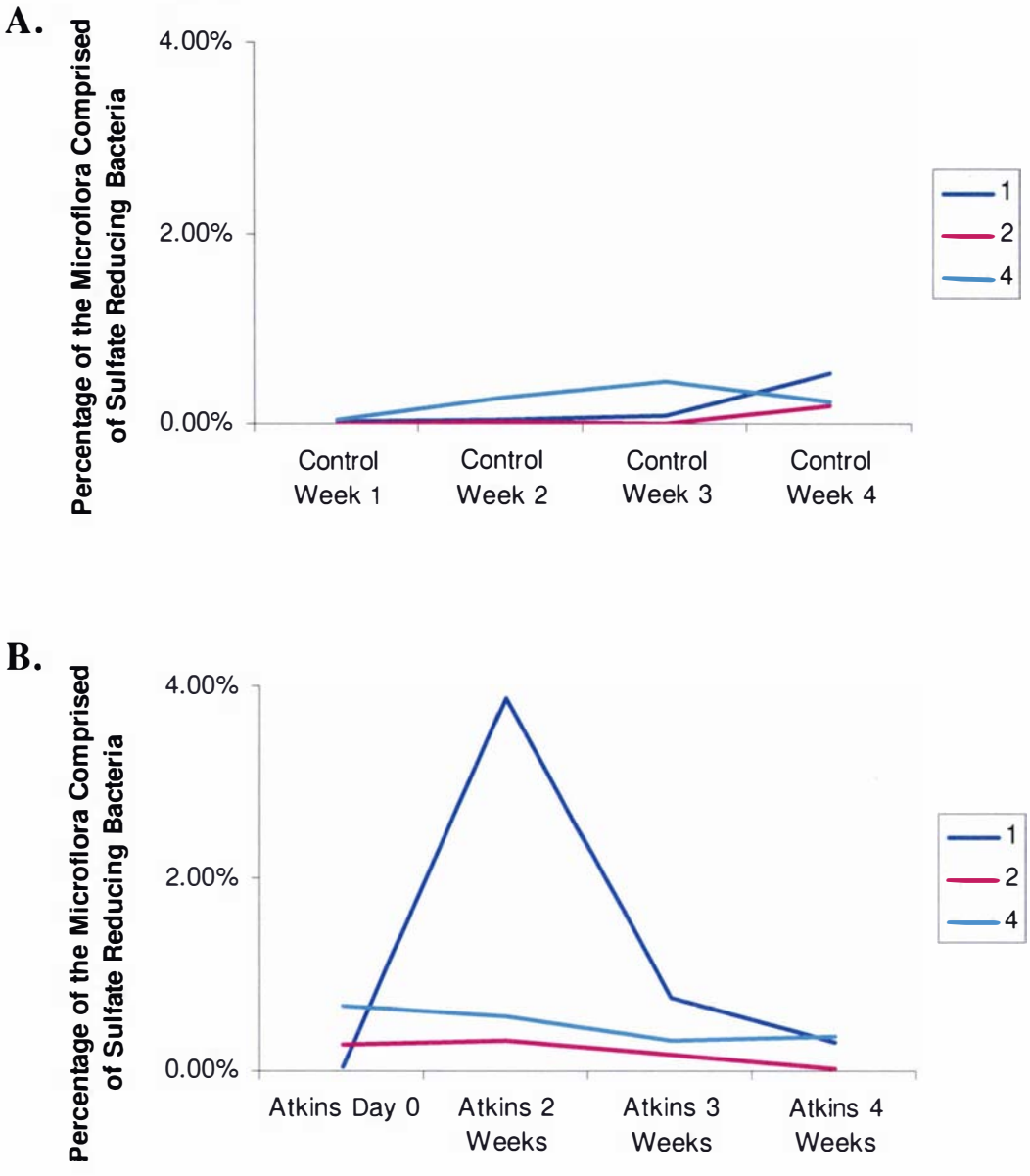


Figure 7.6. Sulfate reducing bacteria levels were monitored in Volunteer 1, 2, and 4 during the consumption of a normal Western diet (A) and during the subsequent change to the Atkins' diet (B) (raw data Appendix E.5 and E.6).

7.5 DISCUSSION

The faecal microbiota was examined in 6 healthy volunteers during the consumption of a normal Western diet, and during the introduction of a low carbohydrate eating plan, the Atkins' diet. The bacterial population was assessed using TTGE to determine if these dietary changes would lead to an altered composition of the predominant bacteria. The effect on methanogens and sulfate reducing bacteria populations were monitored using real time PCR.

Examination of methanogen and sulfate reducing bacteria densities found that these populations were unaffected by the dietary change to the Atkins' diet. A significant difference was observed between the TTGE profile similarity co-efficients from Western diet samples and from Atkins' diet samples. This suggests that the introduction of the Atkins' low carbohydrate eating plan leads to changes in the composition of the faecal microbiota. Sequence analysis of TTGE bands from Volunteer 6 revealed changes in uncharacterised bacteria, and the loss of *Roseburia intestinalis* from the TTGE profile during consumption of the Atkins' diet. *Roseburia intestinalis* has been shown to ferment arabinose, cellobiose, maltose, fructose, raffinose, sucrose, xylose, and starch, but fails to ferment rhamnose, melezitose, mannitol, ribose, inulin, and trehalose (Duncan *et al.*, 2002). Changes in the availability of these substrates on the Atkins' diet may have contributed to the decline of this organism in the faecal microbiota of Volunteer 6.

There were different responses to the Atkins' diet amongst the 6 volunteers. The TTGE profiles of Volunteer 1 and Volunteer 3 remained relatively unaffected by the dietary change, however the remaining volunteers had more variable profiles during this period. Stability in the microbiota despite the dietary perturbation may indicate that the population present in these individuals was more adaptable to changes in available fermentation substrates.

Secondary factors resulting from the dietary change may have also influenced the faecal microbiota. A common complaint associated with the Atkins' diet is constipation,

especially during the restrictive Induction Phase. The colonic environment is likely to be altered by constipation; slow transit time has been associated with decreased faecal weights, SCFA, and breath H_2 and increased faecal pH (El Oufir *et al.*, 1996). During the Atkins' diet, 5/6 volunteers reported constipation during the induction phase, which may have contributed to TTGE profile variability. However, despite constipation favouring the growth of methanogens, and inhibiting the growth of sulfate reducing bacteria populations (El Oufir *et al.*, 1996), no significant changes in these groups were observed.

It has been hypothesised that dietary sulfate can permit the growth of sulfate reducing bacteria populations, while inhibiting the growth of methanogens (Christl *et al.*, 1992). Evidence has also been published which shows increased H_2S and volatile sulfur substances in the faeces of individuals consuming a high protein diet (Geypens, 1997; Magee *et al.*, 2000). In the study reported here, volunteers that did not harbour either methanogens or sulfate reducing bacteria did not establish populations after following the Atkins' diet. Also, amongst volunteers that did harbour these organisms there were no significant changes in the methanogen and sulfate reducing bacterial populations during the Atkins' diet, compared to Western diet samples. It is possible that metabolites of sulfate reducing bacteria could have increased, while population densities remained the same. Also, the study describing the effect of dietary sulfate on the growth of these populations examined methanogenic individuals, in whom the levels of sulfate reducing bacteria were very low. Amongst the volunteers that participated in the Atkins' diet, only 1 volunteer was methanogenic, and they also had a relatively significant population of sulfate reducing bacteria. During the Atkins' diet the methanogen population did decline over time, however with only 1 volunteer harbouring these organisms, conclusions regarding the effect of the Atkins' diet on methanogen populations cannot be made.

In summary, introduction of the Atkins' diet in healthy volunteers led to a significant change in the composition of the predominant bacterial population on TTGE gels, demonstrating that the faecal microbiota is altered by this extreme dietary change. The faecal microbiota is under a degree of genetic control from the human host, however in some individuals it appears possible to modulate the composition of the faecal microbiota through external factors such as bowel cleansing, and dietary changes.

Gastrointestinal diseases such as IBD have been associated with genetic polymorphisms, and the disease process itself may lead to characteristic environmental conditions in gastrointestinal tract of IBD patients. Therefore the faecal flora from individuals with the same disease type were examined in Chapter 8, to determine if a characteristic flora is associated with different gastrointestinal diseases.

CHAPTER 8: THE FAECAL MICROBIOTA IN INFLAMMATORY BOWEL DISEASE

8.1 ABSTRACT

There is increasing evidence to associate inflammatory bowel disease (IBD) to polymorphisms in genes of the immune system. Further evidence suggests that the composition of the microbiota is under a degree of control by the host, and amongst IBD patients the microbiota has been found to be significantly different to controls. To determine if the colonic microbiota from inflammatory bowel disease patients exhibits more similarity than control disease groups and healthy controls, the predominant bacterial TTGE population was examined. Methanogen and sulfate reducing bacteria populations were also monitored.

Similarity values for TTGE gel profiles of ulcerative colitis patients and Crohn's disease patients were found to be significantly different to each other and to healthy controls ($p < 0.03$). Ulcerative colitis patients were also significantly different to all control disease groups ($p < 0.005$), and Crohn's disease patients were significantly different to diverticular disease patients ($p < 0.04$). Differences were characterised by reduced levels of similarity in the IBD groups. In some instances TTGE profiles of IBD patients were found to cluster with respect to disease location. No sulfate reducing bacteria were detected in ulcerative colitis patients.

These findings suggest that the genetic determinants associated with IBD do not appear to influence the composition of the microbiota, or alternatively any influence is overwhelmed by disease related factors such as the inflammatory milieu within the gut. In addition, sulfate reducing bacteria do not appear to be significantly involved in the disease pathogenesis of this group of ulcerative colitis patients.

8.2 INTRODUCTION

The exact mechanisms underlying the pathogenesis of inflammatory bowel disease are unknown, however there is evidence to support a role for the faecal microbiota as environmental triggers for disease onset (Marteau *et al.*, 2004). In addition polymorphisms in genes that regulate the immune system's response to bacteria have been associated with IBD and are likely to predispose individuals to the development of disease (Schreiber *et al.*, 2004).

Transgenic mice predisposed to develop IBD-like colitis have been shown to be free of inflammatory disease when reared in germfree conditions, however reconstitution of the microbiota leads to the development of inflammation and colitis in the intestinal tract (Taurog *et al.*, 1994). In addition, lesions in inflammatory bowel disease generally appear in regions of the gut that have the largest bacterial load (Marteau *et al.*, 2004). Treatments for IBD, such as antibiotic and probiotic therapies (Sartor, 2004), function by modulating the composition of the intestinal microbiota, and therefore implicate this population in disease.

In Crohn's disease, lactobacillus, bifidobacteria, bacteroides, and eubacterium species are significantly reduced compared to control groups (Giaffer, 1991) leading to reduced diversity (Ott *et al.*, 2004). In addition more anaerobic gram-positive coccoid rods and gram negative rods have been found in Crohn's disease patients (Van de Merwe *et al.*, 1988). Breath methane measurements have demonstrated that fewer Crohn's disease patients (6.1%) than healthy individuals (50%) are methanogenic (Peled, 1987).

Some studies investigating the microbiota of ulcerative colitis patients have found that the population resembles that of healthy individuals (Giaffer, 1991). Other studies have found increased numbers of D group streptococci and coliforms, and the presence of invasive *E. coli* (Dickinson, 1980), and reduced counts of bifidobacteria, eubacteria, clostridia, enterobacteria and lactobacilli (Hartley *et al.*, 1992). Sulfate reducing bacteria have also been reported to be at high levels in ulcerative colitis patients (Pitcher

and Cummings, 1996), and the generation of H_2S by these organisms may contribute to impairment of the intestinal barrier in this disease (Roediger *et al.*, 1993).

As described in Chapter 5, the host genotype exerts a degree of control over the composition of the faecal microbiota. These findings are interesting in the context of inflammatory bowel disease, where polymorphisms in bacteria-sensing immune system genes have been associated with the development of disease (Hugot *et al.*, 2001; Obana *et al.*, 2002; Franchimont *et al.*, 2004). The characteristic features of the intestinal microbiota and genetic background associated with IBD, may lead to increased levels of similarity in bacterial TTGE profiles of IBD patients compared to unrelated controls.

The Wakefield Gastroenterology Research Institute (Wellington, New Zealand) has a large sample bank of faecal lavage fluid, collected from gastroenterology patients prior to colonoscopy. Denaturing gradient gel electrophoresis has shown that faecal lavage fluid resembles the bacterial populations of both the colonic mucosa and the faeces (Personal communication, Professor Vinton S Chadwick, Wakefield Gastroenterology Research Institute, Wellington, New Zealand). The similarity present in the predominant bacteria of patients with inflammatory bowel disease, healthy controls, and control disease groups were compared using PCR-TTGE. Real time PCR was also used to establish if significant differences exist between the carriage rates and densities of methanogens and sulfate reducing bacteria in these groups as reported in culture based studies.

8.3 MATERIALS AND METHODS

8.3.1 VOLUNTEERS

Patients routinely undergo colonoscopy to monitor and diagnose conditions including IBD, irritable bowel syndrome, diverticular disease, and polyposis. At the time of colonoscopy, a sample of faecal lavage fluid was collected from 19 IBD patients (9 Crohn's disease patients, 10 ulcerative colitis patients), 10 healthy controls (patients with healthy bowels undergoing routine surveillance for polyps) and 40 individuals with other gastrointestinal disease to act as a control disease group (10 IBS constipation predominant patients, 10 IBS diarrhoea predominant patients, 10 IBS mixed patients, and 10 diverticular disease patients). Samples were immediately stored at –80°C.

8.3.2 FAECAL DNA EXTRACTION/PCR-TTGE/REAL TIME PCR/SEQUENCING

The storage, homogenisation and subsequent extraction of DNA from faecal samples, application of PCR-TTGE and real time PCR, and sequencing of microbial DNA were carried out as previously described in Chapter 2: Materials and Methods.

8.3.3 CLUSTER ANALYSIS AND STATISTICS

Cluster analysis was performed by creating a distance matrix from Sorenson's similarity values. A dendrogram was constructed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA) using Phylodraw version 0.8 (Graphics Application Lab, Pusan National University).

Carriage rates for methanogens and sulfate reducing bacteria were compared using the Fisher Exact Test.

8.4 RESULTS

8.4.1 COMPARISONS OF PREDOMINANT BACTERIAL POPULATION SIMILARITIES AMONGST DIFFERENT PATIENT GROUPS

The number of bands present in the TTGE profiles ranged from 9 - 23, with a median of 17 bands. For both ulcerative colitis and Crohn's disease the median number of bands present in the TTGE profiles of patients was 16. Healthy controls were found to have a median of 17 bands. The control disease group patients with diverticular disease were found to have a median of 18 bands per profile. Amongst the IBS subtypes the median number of bands was 14, 18, and 18 for mixed, constipation predominant, and diarrhoea predominant respectively. One-way ANOVA found no significant difference between the patient groups with respect to the number of bands present in the profiles.

Sorenson's similarity co-efficients were calculated between patients with the same disease type. The medians for each group ranged from 41% - 53% (Figure 8.1). One-way ANOVA was carried out to compare the data obtained for each patient group. This analysis found a significant difference between the groups ($p = 0.0000004$).

Comparisons were made between the healthy controls and the IBD subtypes, IBS subtypes, and diverticular disease. Both the ulcerative colitis and Crohn's disease groups were significantly different to healthy controls, $p = 0.000001$ and $p = 0.03$ respectively. No significant difference was found between the healthy controls and any of the control disease groups.

The similarity amongst TTGE profiles was found to be significantly different between Crohn's disease patients and ulcerative colitis patients ($p = 0.00009$). Crohn's disease patients were also significantly different to the diverticular disease group ($p = 0.04$). Ulcerative colitis patients were significantly different to all IBS subtypes and the diverticular disease group ($p \leq 0.005$).

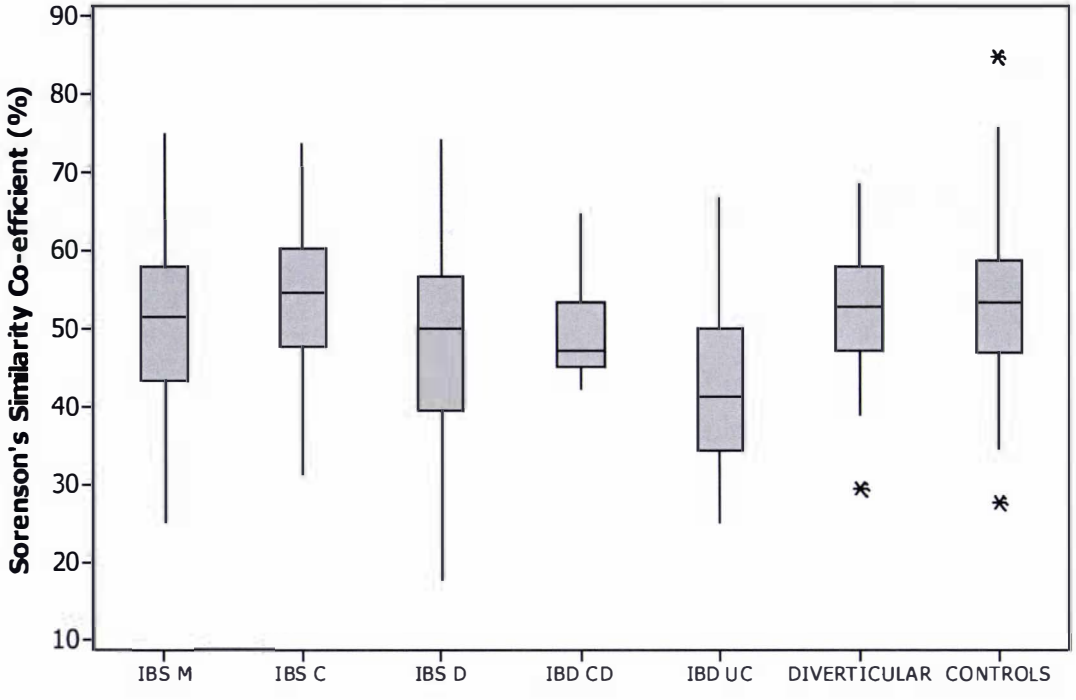


Figure 8.1. TTGE profiles were compared within each patient group to generate Sorenson's similarity co-efficients. The data for each patient group is shown in the box whisker plot (Raw data Appendix A.8 – A.14). IBS M, irritable bowel syndrome mixed subtype; IBS C, irritable bowel syndrome constipation predominant; IBS D, irritable bowel syndrome diarrhoea predominant; IBD CD, inflammatory bowel disease Crohn's disease; IBD UC, inflammatory bowel disease ulcerative colitis; Diverticular, diverticular disease; Controls, polyp patients with healthy bowels. * indicates the presence of an outlier.

8.4.2 CLUSTERING ANALYSIS OF SIMILARITY DATA FROM INFLAMMATORY BOWEL DISEASE PATIENTS

Clustering analysis was performed on IBD patient groups to determine if there was increased similarity between individuals undergoing the same treatment therapy, with the same disease state (active or in remission), or with the same intestinal localisation of disease.

Analysis of the dendrogram for Crohn's disease patients (Figure 8.2) found that patients' bacterial TTGE profiles clustered if they shared the same location of disease in

the intestinal tract. Patients 1, 2, 3, 7, and 9 all showed active disease throughout the colon and these patients were all located in clusters at the top of the dendrogram. Samples lying outside of these clusters were from patients with disease in a single location (patient 6, 8, and 5) or who were in remission (patient 4). Patient 5 had no sign of disease in the colon, but did have active Crohn's disease in the duodenum, this individual was the most different from the patients that had complete colonic involvement. No clustering was evident amongst patients prescribed immunosuppressant drugs and/or anti-inflammatory drugs at the time of sample collection.

The dendrogram generated for the ulcerative colitis patient group demonstrated some similarity in the faecal flora of patients with respect to disease location (Figure 8.3). Patients 1, 7, and 4 were the only patients in the group with proctitis and they were clustered together in the dendrogram. No other associations between the clustering and characteristics of the patient group were evident.

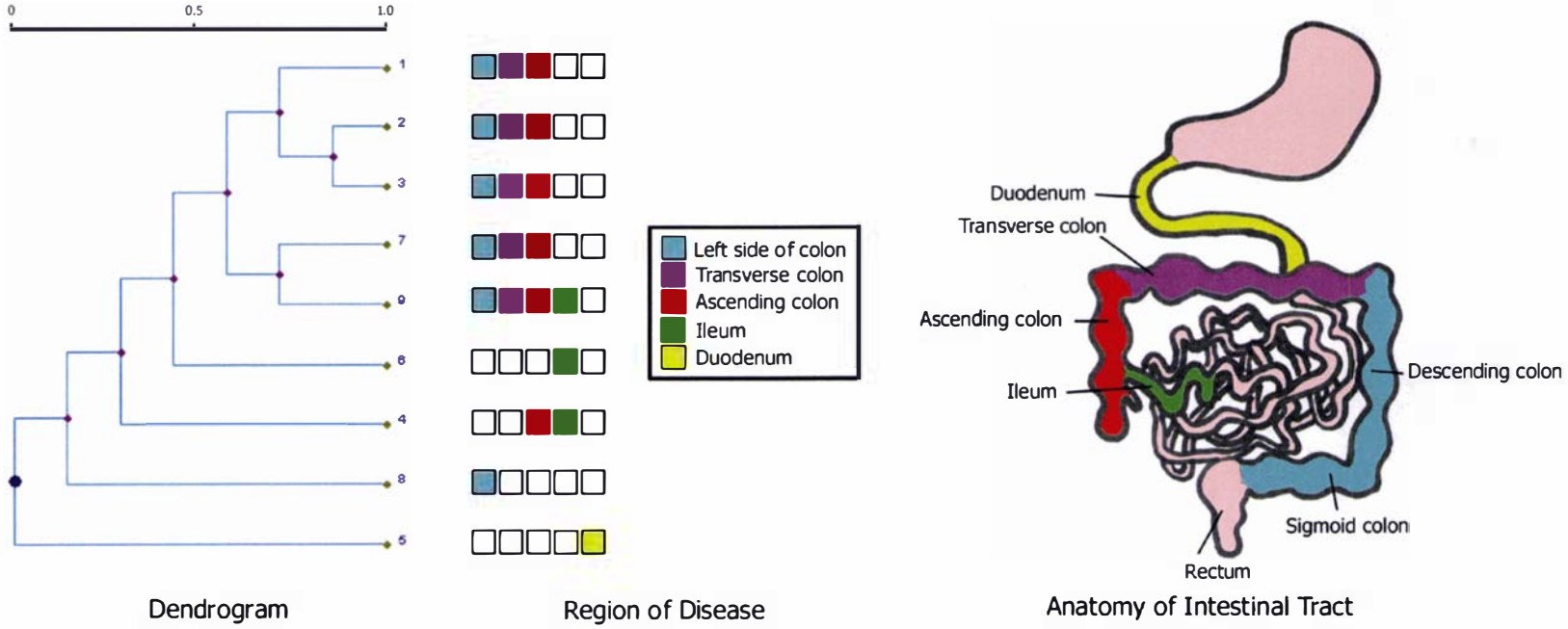


Figure 8.2. Based upon the Sorenson's similarity co-efficients of Crohn's disease patients a dendrogram was constructed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). The resulting clusters were examined with respect to the localisation of disease within the intestinal tract. Patients with disease present throughout the colon were found to be in clusters.

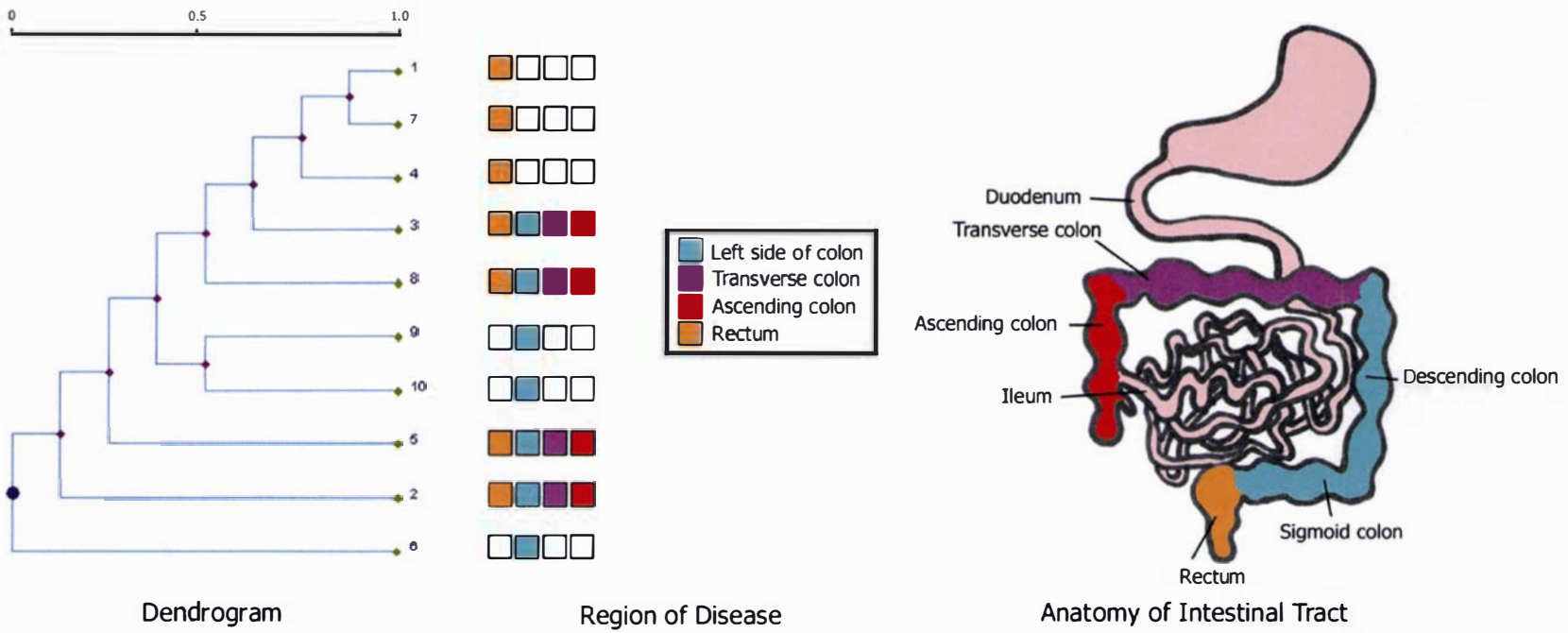


Figure 8.3. Based upon the Sorenson's similarity co-efficients of ulcerative colitis patients a dendrogram was constructed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). The resulting clusters were examined with respect to the localisation of disease within the intestinal tract. Patients with disease affecting the rectum only were found to be in a cluster, however disease affecting other regions of the gut did not cluster together.

8.4.3 COMMUNITY RICHNESS AMONGST INFLAMMATORY BOWEL DISEASE PATIENTS AND CONTROLS

Shannon's indices were calculated for each profile to determine if there were differences in community richness amongst the control and IBD groups. For all the patients, Shannon's values ranged from 1.76 – 2.89, with a median value of 2.53 (Table 8.1). Analysis of the data was carried out with one-way ANOVA. This test found no significant difference in community richness between any of the groups.

Table 8.1. From each TTGE profile the Shannon's index was calculated to provide a measure of community richness for the patient's microbiota (raw data Appendix B.5). IBS M, irritable bowel syndrome mixed subtype; IBS C, irritable bowel syndrome constipation predominant; IBS D, irritable bowel syndrome diarrhoea predominant; IBD CD, inflammatory bowel disease Crohn's disease; IBD UC, inflammatory bowel disease ulcerative colitis; Divert, diverticular disease; Healthy Controls, polyp patients with healthy bowels.

Shannon's Indices						
IBS M	IBS C	IBS D	IBD CD	IBD UC	Divert	Healthy Controls
2.49	2.69	2.41	2.36	2.48	2.08	2.60
2.38	2.58	2.43	2.49	1.87	2.46	2.67
2.41	2.74	2.63	2.78	2.23	2.65	2.43
2.18	2.34	2.37	2.51	2.14	2.52	1.76
2.33	2.21	2.75	2.60	2.78	2.72	2.38
2.49	2.66	2.72	2.73	2.10	2.57	2.30
2.73	2.68	2.73	2.45	2.49	2.61	2.41
2.38	2.85	2.75	2.55	2.86	2.31	2.34
2.65	2.56	2.83	2.41	2.76	2.68	2.56
2.55	2.53	2.77		2.89	2.77	2.27

8.4.4 CARRIAGE RATES OF METHANOGENS AND SULFATE REDUCING BACTERIA

Rates of carriage for methanogens and sulfate reducing bacteria were determined for each patient group (Figure 8.4). Individuals were considered positive for the organisms if the correct sized PCR fragment was generated during real time PCR.

Sulfate reducing bacteria were detected in faecal lavage from 50% of the healthy controls. These organisms were not detected in the samples obtained from ulcerative colitis patients, but 33% of Crohn's disease patients did harbour these organisms. Amongst IBS patients, sulfate reducing bacteria were present in 70% of those patients with diarrhoea predominant and mixed subtypes. Only 40% of constipation predominant patients had these organisms present in faecal lavage fluid. Diverticular disease patient group was found to have carriage rates of 40%. Fishers exact test was used to compare the carriage rates in each group. The ulcerative colitis group was found to be significantly different to healthy controls ($p = 0.03$) and to the diarrhoea predominant and mixed subtypes of IBS ($p = 0.003$).

The carriage of methanogens amongst IBD patients was similar with 33% of Crohn's patients and 40% of ulcerative colitis patients harbouring these organisms. A very high rate of carriage was found in the healthy controls (polyposis patients) where 90% of the individuals were found to have methanogens present in the faecal lavage fluid. Methanogens were found in the samples of 70% of constipation predominant IBS patients, 60% of diarrhoea predominant patients, 50% of IBS patients with mixed symptoms, and 60% of diverticular disease patients. A significant difference between the carriage rates of methanogens in Crohn's disease patients and the healthy controls was found using Fisher's exact test ($p = 0.02$).

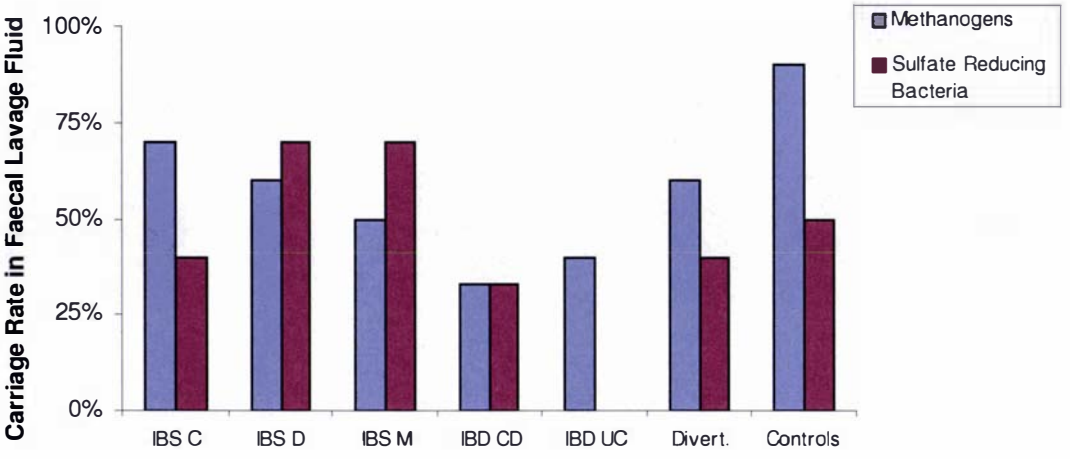


Figure 8.4. The carriage rates of methanogens and sulfate reducing bacteria in the faecal lavage fluid for each patient group were determined using real time PCR (raw data Appendix D.7 and E.7). IBS C, constipation predominant irritable bowel syndrome; IBS D, diarrhoea predominant irritable bowel syndrome; IBS M, mixed type irritable bowel syndrome; IBD CD, inflammatory bowel disease Crohn's disease; IBD UC inflammatory bowel disease ulcerative colitis; Divert, diverticular disease; Controls, polyp patients with healthy bowels.

8.4.5 POPULATION DENSITIES OF BACTERIA, METHANOGENS AND SULFATE REDUCING BACTERIA

Bacterial densities were measured in all faecal lavage fluid samples using real time PCR. The values obtained covered a large range from 2.74 $x10^7$ to 4.53 $x10^{10}$ bacterial genes/g stool (wet weight). Comparisons between each patient group using one way ANOVA found no significant difference between the bacterial densities with respect to disease type.

Using only the samples that had quantitative levels of methanogens and sulfate reducing bacteria, the densities of these organisms in each disease group were compared (Table 8.2). There was a very broad range with methanogens comprising 0.0001% to 9% of the total population, and 0.007% to 42% for sulfate reducing bacteria. One-way ANOVA found a significant difference in the density of methanogens amongst the groups studied ($p = 0.005$) (analysis excluded patients that quantitative data could not be obtained for). Sulfate reducing bacteria densities did not differ significantly amongst the patient groups studied.

Table 8.2. The percentage of the total microflora comprised of methanogens and sulfate reducing bacteria was determined for each disease group using real time PCR (raw data Appendix D.7 and E.7). IBS C, constipation predominant irritable bowel syndrome; IBS D, diarrhoea predominant irritable bowel syndrome; IBS M, mixed type irritable bowel syndrome; Controls, polyp patients with healthy bowels

Percentage of the Total Microflora Comprised of Methanogens			Percentage of the Total Microflora Comprised of Sulfate Reducing Bacteria		
Disease Group	**Range**	**Average**	**Disease Group**	**Range**	**Average**
IBS-C	0.003% - 0.2%	0.06%	IBS-C	0.04% - 16%	5%
IBS-D	0.002% - 2%	0.5%	IBS-D	0.07% - 4%	1%
IBS-M	0.0009% - 0.1%	0.06%	IBS-M	0.007% - 0.2%	0.1%
Crohn's Disease	0.02% - 0.03%	0.02%	Crohn's Disease	0.1% - 0.4%	0.2%
Ulcerative Colitis	-	-	Ulcerative Colitis	-	-
Diverticular Disease	1% - 9%	5%	Diverticular Disease	0.8% - 8%	4%
Controls	0.03% - 1%	0.07%	Controls	0.02% - 42%	11%

- indicates that ≤ 1 sample gave quantitative data, therefore range and average values are not given.

8.5 DISCUSSION

PCR-TTGE and real time PCR were employed to study the microbiota of different patient groups. Comparisons were made between the predominant bacterial TTGE profiles within each group to determine if there is an association between gastrointestinal disease type and the composition of the predominant bacterial microbiota. Carriage rates and densities of methanogens and sulfate reducing bacteria were also determined.

In Chapter 3 it was shown that the degree of similarity between the predominant bacteria of unrelated adults is on average 55%, with a range from 38 - 78%. Other investigators have made similar findings. Zoetendal *et al*, 2001 found an average of 46% similarity between unrelated individuals using PCR-DGGE. To date no study has investigated if unrelated individuals with the same disease exhibit increased levels of similarity compared to unrelated controls. The abnormal microbiota and genetic polymorphisms described in IBD patients may lead to an increased level of similarity in the microbiota of these individuals. The study reported here identified a significant difference between the level of similarity present in the predominant bacterial microbiota of ulcerative colitis patients and Crohn's disease patients. Further it was shown that both these disease groups also differ significantly from healthy controls.

Rather than having more predominant bacterial species in common in TTGE profiles, the ulcerative colitis and Crohn's disease samples displayed more dissimilarity than was observed in control groups. The ulcerative colitis patient group had on average, more dissimilarity than the Crohn's disease patient group, and also a larger range of similarity values.

These results infer that either genetic determinants, which may predispose individuals to Crohn's disease or ulcerative colitis, do not appear to increase similarity in the composition of the intestinal microbiota or alternatively the genetic determinants may influence the bacterial composition, but this effect is overwhelmed by additional factors such as the inflammatory milieu within the diseased gut.

This study has shown that IBD patients have higher levels of heterogeneity in predominant bacterial populations than healthy individuals. A recent study examining the mucosal microbiota found reduced diversity in IBD patients compared to controls (Ott *et al.*, 2004). In the study reported here, the numbers of bands present in TTGE profiles and the values for the Shannon's index did not significantly differ between any of the groups. The different findings may be related to the type of sample examined. The colonic microbiota differs from the faecal microbiota, and the faecal lavage fluid samples utilised in this study have been shown to contain elements of both these populations (personal communication, Professor Vinton S Chadwick, Wakefield Gastroenterology Research Institute, Wellington, New Zealand). The failure to detect a significant difference between the groups and band number or Shannon's index values suggests that community richness and diversity in TTGE profiles of IBD patients were equivalent to those seen in controls. Therefore, the reduced levels of similarity present in the TTGE profiles of these groups are likely to reflect the presence of a larger pool of different bacterial species than what is found amongst healthy individuals. Sequence analysis of bacterial clone libraries would be required to investigate this hypothesis.

Several factors may account for the increased heterogeneity found in the IBD patients compared to controls. The work described here demonstrated clustering of TTGE profile similarities with respect to the location of disease in the intestinal tract. The dendrogram constructed for Crohn's disease patients placed all pan colitis patients together, and amongst ulcerative colitis patients all proctitis cases were also clustered. This suggests that the site of inflammation may influence the composition of the bacterial population. Therefore, overall reduced similarity in the IBD group may reflect the different disease locations in patients. Interestingly, twin studies have shown high concordance for disease location (Halfvarson *et al.*, 2003), and a recent study has shown an association between a TLR4 polymorphism and colonic localisation of disease in Crohn's disease patients (Levine *et al.*, 2005). These studies suggest that the location of disease may be influenced by the host genotype, and the clustering analysis reported here implies that disease location may influence the microbiota composition.

The treatment of IBD involves both anti-bacterial and immunosuppressant drugs. Antibiotics selectively remove large groups of organisms thereby allowing the emergence of other bacterial species in the population. Sulfasalazine and 5-ASA have

been shown to inhibit the activity of sulfate reducing bacteria (Pitcher MCL, 1995; Pitcher *et al.*, 2000) and could also potentially influence other bacterial groups. 5-ASA drugs and antibiotics have both been shown to suppress but not eliminate adherent biofilms in IBD patients, suggesting a general anti-bacterial activity for 5-ASA (Swidsinski *et al.*, 2005). The immune system is in close contact with the bacterial population of the gut and interactions between these components are important in establishing homeostasis. Although the effect of immunosuppressant drugs on the faecal microbiota has not been studied in detail, it is feasible that these drugs may modify the interaction between the host and this population, potentially leading to changes in the bacterial community. The patients examined in this study were not included or excluded based upon their current treatment regime, and therefore their bacterial populations may have been influenced by different drug therapies leading to increased levels of dissimilarity.

Comparisons between Crohn's disease patients with active and inactive disease have shown marked differences in TTGE banding profiles between these two disease states (Seksik *et al.*, 2003) and ulcerative colitis patients have been shown to have a significant reduction in anaerobic bacteria, anaerobic gram-negatives and Lactobacillus species during active disease (Fabia, 1993; Bullock, 2004). All ulcerative colitis patients examined here had active disease, and only 1 Crohn's disease patient was in remission at the time of sample collection.

The surgical history of the patients may also influence the bacterial community of the gut. Ileocolonic resection in Crohn's disease patients has been reported to increase colonisation of the neoterminal ileum (Neut *et al.*, 2002). Only one patient, Volunteer 9 in the ulcerative colitis patient group, had undergone bowel surgery.

Finally, there is evidence for an association between mutations and polymorphisms in immune system genes and the development of IBD (Schreiber *et al.*, 2004). The genetic basis for these conditions is complex and appears to be polygenic with several susceptibility genes described including NOD2 (Hugot *et al.*, 2001), lipopolysaccharide receptor gene CD14 (Obana *et al.*, 2002), and TLR-4 (Franchimont *et al.*, 2004). However only a fraction of the IBD patients examined in these studies were found to be carriers of these polymorphisms. The twin and relative study described in chapter 5

shows that the host genotype is involved in shaping the predominant bacterial population and evidence from mouse studies suggest that genes of the immune system maybe important in this process (Toivanen *et al.*, 2001). Therefore, although the patients involved in this study have all been diagnosed with the same disease they each are likely to have their own unique genetic background of IBD susceptibility. Rather than influencing the bacterial population in the same way, each genetic determinant may influence the intestinal microbiota differently.

Therefore the IBD patients differ with respect to drug therapy, disease state, surgical history, disease location in the intestinal tract, and genetic susceptibility to IBD and these factors may account for the increased heterogeneity present amongst the predominant bacterial population in the IBD patient groups.

Generally the carriage and densities of methanogens and sulfate reducing bacteria in these patient groups were found to support findings made in culture studies, although some differences were present.

Surprisingly, none of the faecal lavage fluid samples from ulcerative colitis patients contained sulfate reducing bacteria, a group of organisms that has been associated with this disease (Pitcher and Cummings, 1996). The real time PCR assay did successfully quantify populations of these organisms in the other disease groups, suggesting that if sulfate reducing bacteria were present in the ulcerative colitis samples they would have been readily detectable by the assay. Sulfasalazine and 5-ASA have been shown to inhibit the production of H_2S by fast growing sulfate reducing bacteria (Pitcher MCL, 1995; Pitcher *et al.*, 2000) and inhibit the growth of some *Desulfovibrio desulfuricans* strains (Dzierzewicz *et al.*, 2004a). Of the ulcerative colitis patients, 60% were prescribed these drugs. A recent report has shown that treatment with metronidazole or ciprofloxacin can cause a decrease in both sulfate reducing bacterial numbers and H_2S (Ohge *et al.*, 2005), however none of the patients in this group were prescribed these medications at the time of faecal lavage fluid collection.

In addition to an absence of sulfate reducing bacteria, the average density of methanogens was very low amongst those ulcerative colitis patients. These findings may suggest that amongst the patients studied here the major route of hydrogen disposal

is not carried out by sulfate reducing bacteria or methanogens. An alternative pathway for hydrogen utilisation is the conversion to acetate by acetogenic bacteria. Acetate, along with butyrate and propionate are the major SCFA found in the gut, and they play an important role in maintaining gut homeostasis (Galvez *et al.*, 2005). Studies have demonstrated a decrease in SCFA concentrations in the lumen of ulcerative colitis patients when compared to controls (Vernia, 1988). If acetogens are present in these individuals the production of acetate could benefit these patients by increasing SCFA concentrations within the lumen. Further studies examining acetogenesis within treated and non-treated patients would be required to investigate this hypothesis.

The carriage rate of methanogens was significantly different between the healthy controls (patients with healthy bowels undergoing surveillance for polyposis) and the Crohn's disease group. A significantly higher proportion of polyposis patients have been found to be methanogenic compared to healthy individuals in breath methane studies (Pique *et al.*, 1984), and the results reported here further support this contention. Individuals with polyps are at a higher risk for the development of colorectal cancer, a disease which has been found to be associated with significantly higher rates of methanogenic individuals than control groups (Haines *et al.*, 1977; Pique *et al.*, 1984). Resection of these tumours leads to a reduction in methanogenic individuals to control group rates (Pique *et al.*, 1984). Other studies have found no association between the proportion of the population that is methanogenic and the risk of developing colorectal cancer (Segal *et al.*, 1988). The reason for higher rates of methanogenic individuals amongst polyposis and colorectal cancer groups remains unknown.

In the constipation predominant IBS group, 70% of the faecal lavage fluid samples were found to contain methanogens. The association of this disease group with the carriage of methanogens is in agreement with findings reported elsewhere (Pimentel *et al.*, 2003). There is evidence in the literature that carriage of methanogens is associated with a long transit time and constipation (El Oufir *et al.*, 1996; Soares *et al.*, 2005), whereas carriage of sulfate reducing bacteria is associated with a shorter transit time and diarrhoeal conditions (El Oufir *et al.*, 1996; Pimentel *et al.*, 2003). The data obtained in this study show that the predominantly diarrhoeal IBD group harboured methanogens at levels that were not significantly different from the IBS constipation group. Furthermore, the carriage rates of sulfate reducing bacteria between the diarrhoea

predominant IBS group and constipation predominant IBS group were not significantly different. Similar findings were made in Chapter 7, where constipation failed to increase methanogen populations. This argues against the premise that the carriage of these organisms is influenced by transit time. This finding is not without precedent as African populations which have been shown to have fast transit times also possess very high rates of methanogenic individuals (Segal *et al.*, 1988).

The measurement of methanogen and sulfate reducing bacterial densities in the faecal lavage fluid may be prone to inaccuracies due to the potential differences in sample dilution. For example, depending upon the compliance of patients the lavage process may cause varying degrees of dilution of the faecal microbiota. Also, samples collected near the end of the bowel cleansing process may be more dilute than those samples collected from the first few bowel movements. This may account for the large variation in bacterial densities measured across the samples. However, by expressing bacterial densities with respect to the total measured microbiota population (bacteria + methanogens) the samples are effectively normalised.

A significant difference in methanogen densities was found between the patient groups. Significance was lost when the diverticular disease group was excluded from the analysis, indicating that the levels of methanogens present within this patient group were significantly higher than other groups. Previous studies have described higher concentrations of methanogens in diverticular disease patients (Weaver *et al.*, 1986). The authors hypothesised that the localisation of both diverticula and methanogens in the left colon may work to provide a sheltered niche for the methanogen population, providing ideal conditions for these slow growing organisms. Interestingly the diverticular disease group also had, on average, the greatest density of sulfate reducing bacteria, although levels of sulfate reducing bacteria in diverticular disease groups were not significantly different to other patient groups.

In conclusion bacterial TTGE profiles of ulcerative colitis patients demonstrated significant differences compared to all other groups studied. Crohn's disease patients were significantly different to all groups except the IBS patient groups. There was no significant difference between the control group and the control disease group. These differences resulted from less similarity amongst the IBD patient groups than in the

control groups, and suggest that the genetic determinants of IBD do not influence the microbiota, or alternatively these factors are overwhelmed by disease-related influences such as the inflammatory milieu. Disease location has been linked to genetic polymorphisms in IBD patients (Halfvarson *et al.*, 2003; Franchimont *et al.*, 2004), and TTGE profiles were clustered with respect to disease location in some instances. The high carriage rates or densities of methanogens measured in polyposis, constipation predominant IBS, and diverticular disease groups mirrored the data obtained from culture based studies. The failure to detect any sulfate reducing bacteria in the ulcerative colitis patients suggests that, amongst the patients studied here, these organisms do not appear to be influencing disease pathogenesis.

CHAPTER 9: CONCLUDING COMMENTS

The intestinal microbiota is a massive and complex bacterial population, comprised of ten times more cells than the human body itself. The aims of the work presented in this thesis were to develop and validate DNA-based assays for colonic bacteria and to assess the potential of these techniques for study of this significant population by performing pilot studies in healthy individuals and patients with gastrointestinal diseases.

The main focus of the research was primarily the predominant intestinal bacteria as this group of ~ 30 species account for as much as 99% of the total microbiota (Drasar, 1986), and undoubtedly these organisms have a profound affect on the ecosystem. A secondary focus was specifically directed at methanogens and sulfate reducing bacteria. These organisms and their metabolic products have been associated with gastrointestinal diseases such as ulcerative colitis (Roediger *et al.*, 1997), irritable bowel syndrome (Pimentel *et al.*, 2003) and colorectal cancer (Pique *et al.*, 1984). There is a need to increase our understanding of these populations and investigate potential methods to modulate their presence in the faecal microbiota.

The principle analytical techniques were temporal temperature gradient gel electrophoresis (TTGE) of PCR products, which allowed qualitative analysis of the predominant bacterial population, and real time PCR, which provided quantitative data on the bacteria, methanogens, and sulfate reducing bacteria. Over 250 faecal samples were analysed over a 4-year period. It would have been difficult to examine such a large number of samples using more labour intensive methods such as culturing techniques.

A major component of the work described in this thesis involved optimisation and validation of TTGE and real time PCR. TTGE was demonstrated to have sufficient sensitivity to detect an organism comprising only 1% of the total template DNA and a high degree of reproducibility was found in the TTGE profiles of replicate PCRs. Each real time PCR assay had high sensitivity. For example, methanogen and sulfate reducing bacteria assays could reliably detect 11 gene copies and 4 gene copies respectively, and the bacterial assay detected 132 gene copies. Determination of each

assay's precision revealed small co-efficients of variation for replicate samples and demonstrated reliable quantitation of gene copies.

Real time PCR was used to quantitate bacterial populations in faecal samples. The sensitivity of the real time PCR assays far exceeded the temporal variation present in the populations studied. Although, the quantitative assays for methanogens and sulfate reducing bacteria had high sensitivity, false negative samples cannot be completely ruled out. Reductions and increases in population densities were readily detected indicating that real time PCR was an appropriate method for monitoring changes in populations of micro-organisms.

TTGE was applied to faecal samples collected from healthy individuals and patients. To obtain data for statistical analysis, banding patterns in TTGE gel profiles were examined and compared. Shannon's indices provided a measure of community richness for each profile, and Sorenson's similarity co-efficient provided information on how similar two banding patterns were. A statistically significant negative correlation was found between Shannon's indices and the age of children in Chapter 5. A high degree of intra-individual stability was observed in TTGE profiles in Chapter 4. Sorenson's similarity co-efficients demonstrated significant differences in composition of the microbiota between identical and fraternal twins (Chapter 5); following bowel lavage (Chapter 6); following introduction of the Atkins' diet (Chapter 7); and between different disease groups (Chapter 8). All these findings are novel, and provide an insight into the dynamics of the intestinal microbiota in health and disease states.

Similarity analysis of TTGE gel banding patterns has its limitations. Band changes observed in TTGE gels reflect alterations in bacterial densities, but these changes merely demonstrate that densities fluctuate above and below the detection limit, rather than a complete appearance or disappearance of the organism from the population. Similarly, only organisms that constitute a substantial proportion of the population can be detected on the TTGE gels, leaving the majority of species present in the population unstudied. In addition, there is the potential that bands with identical migration patterns could represent different bacterial species. Despite these factors, TTGE provides a useful tool to rapidly identify global changes in the predominant members of the population. To obtain additional information this technique needs to be used in concert

with other methodologies. For example, a band change in one sample could be further explored by sequencing the DNA.

TTGE bands that were sequenced predominantly returned sequence information that had greatest identity to uncultivated or novel micro-organisms. In all cases, the Genbank sequences had been isolated from gastrointestinal ecosystems. The sequencing data demonstrated that the majority of organisms identified in TTGE profiles, and therefore the predominant organisms of this ecosystem, are uncharacterised. Such a high level of uncharacterised organisms is not surprising. Recent analysis of 13, 355 ribosomal RNA gene sequences from the gastrointestinal tract revealed that the majority of bacterial sequences were derived from uncultivated or novel organisms (Eckburg *et al.*, 2005). Clinical data collected using culture-based studies over the last 30 years could be complimented by re-examining the predominant microbiota using molecular methods. This is likely to provide additional information on the ecosystem in both health and disease states. When a high proportion of uncultivated and novel organisms in the microbiota become characterised, sequence information will become increasingly informative.

Sorenson's similarity values demonstrated a remarkably high level of temporal stability. A stable community structure may be inevitable in such a complex microbial ecosystem. Relatively constant environmental conditions and the extensive interactions and cross talk that occur between members of the same species, between different bacterial species, and between the host and the bacterial communities are likely to lead to a highly regulated system, which may be difficult to disrupt. However, interactions and inter-dependence may also be a disadvantage. Environmental perturbation may primarily affect a limited number of species, but the loss of fermentation products, metabolic functions, or interactions with these organisms could potentially lead to a secondary effect on dependent species.

Altered TTGE banding patterns were observed following bowel lavage (a procedure that clears the contents of the bowel prior to colonoscopy) and introduction of the Atkins' diet, but no apparent effect on methanogens and sulfate reducing bacteria was observed. TTGE band changes may reflect large fluctuations in species densities as competition in the ecosystem occurs in response to the environmental change.

Some organisms in the microbiota are likely to be more susceptible to bowel cleansing than others. Species that occupy niches that are created by other organisms, or have slow growth rates may struggle to repopulate the bowel following lavage. Fast growing species that do not depend on interactions with other organisms are likely to be more resilient. The location of organisms within the bowel may also minimise the impact of environmental change. The mucus layer may shelter organisms from interventions such as bowel cleansing, thereby reducing their rate of clearance from the bowel during lavage. Organisms in this habitat could then provide a large source of bacteria to inoculate faecal material.

The ability to alter the population through bowel cleansing suggests that this procedure could have potential as a therapeutic strategy in patients with a dysbiotic microbiota, such as IBS patients. A proportion of IBS patients do report symptom resolution following bowel lavage (personal communication, Professor Vinton S Chadwick, Wakefield Gastroenterology Research Institute, Wellington, New Zealand). By removing a substantial fraction of the bacterial load in the intestine through bowel cleansing, the system may be given an opportunity to 'reset' within the confines of host genetics, and subsequently rebuild the faecal microbiota with species that give rise to a stable population. This hypothesis could be explored by carrying out longitudinal studies in IBS patients. Comparisons between the faecal microbiota during asymptomatic phases, during times of bowel symptoms, and subsequently following lavage may highlight population changes associated with bowel complaints, and whether these changes are reversed following bowel cleansing.

Changes in the availability of fermentable substrates are likely to occur following dietary change to the Akins' diet, and this may affect bacterial species differently. For example, organisms that can only grow on a limited number of substrates may struggle to adapt to changes in nutrient availability, while species that can grow optimally on a broad range of substrates are likely to be relatively unaffected by dietary changes. A decline in some species and subsequent loss of their metabolic products from the environment may also affect dependent organisms. Despite changes in substrate and predominant bacterial species, the overall availability of H_2 and CO_2 may remain unaffected, thereby not affecting methanogen and sulfate reducing bacteria.

A high consumption of animal products, and low fibre consumption have been associated with colorectal cancer (O'Keefe *et al.*, 1999). These parameters are likely to be met by the Atkins' diet. Fifteen faecal bacterial species have been associated with a high risk of colorectal cancer (Moore and Moore, 1995), and it would be interesting to determine if long term consumption of the Atkins' diet led to an increase in these populations.

The bowel lavage and the Atkins' diets both succeeded in disrupting the stability of the intestinal microbiota. If resources had permitted, it would have been interesting to monitor volunteers in these studies over a longer time period, to determine how long it took the microbiota to stabilise, and whether the population exhibited resilience stability, by returning to its original composition.

Comparisons of similarity values amongst different groups of individuals found increased similarity with respect to relatedness, but decreased similarity amongst IBD patients. Expanding these studies with larger sample sizes would enable these observations to be investigated further. The study of relatedness was carried out in infants and children. Potentially a similar TTGE based study amongst a group of older twins may detect more marked differences between identical and fraternal twins, as environmental variability is likely to be greater. Amongst IBD patient groups, the profiles that were most similar to each other were obtained from individuals exhibiting the same localisation pattern of disease in the gut. It would be interesting to investigate this effect in large patient groups with different disease localisation patterns, and also to determine if described genetic mutations or polymorphisms are associated with these groups. Although an association may exist between the composition of the faecal microbiota and the location of disease, further studies would be required to determine if the characteristic population results from underlying susceptibility to the disease, or alternatively if the population arises due to active inflammation and disease.

Comparison of methanogen and sulfate reducing bacteria carriage rates between identical and fraternal twins, and between different disease groups were hindered due to the low incidence of these organisms in some groups. The carriage rates for both methanogens and sulfate reducing bacteria amongst the twins were too low to assess the

influence of host genetics over these populations. Recruiting additional twin volunteers to this study was investigated using sample size calculations. Even assuming a distinct difference exists between concordance rates in fraternal and identical twins (50% and 90% respectively), to obtain a 95% confidence interval and 80% power, at least 24 twin pairs would be required in each group. When taking into account the carriage rates of methanogens and sulfate reducing bacteria in children, as many as 160 fraternal twin pairs and 160 identical twin pairs would be required. Resources were not available to undertake a study of this scale. Future studies carried out to investigate genetic control over methanogen or sulfate reducing bacteria populations are more likely to be successful using adult twins because carriage rates are higher than in children.

Sulfate reducing bacteria were not found in faecal lavage fluid collected from ulcerative colitis patients, despite these organisms being readily detected in other disease groups. While some studies report increased carriage rates and counts of sulfate reducing bacteria (Pitcher and Cummings, 1996), and increased stool sulfide in ulcerative colitis patients (Levine *et al.*, 1998), additional studies have described no significant difference to controls (Moore *et al.*, 1998; Pitcher *et al.*, 2000). All the evidence concerning the role of sulfate reducing bacteria in ulcerative colitis is circumstantial. The carriage of sulfate reducing bacteria in healthy individuals suggests that any involvement of H_2S in ulcerative colitis is likely to be associated with the host's failure to detoxify this metabolite. In addition, there are other sources of H_2S in the gut such as amino acid fermentation. Rhodanese is the principal enzyme involved with detoxification of H_2S in the mucosa (Picton *et al.*, 2002), and it would be interesting to investigate the activity of this enzyme in ulcerative colitis patients. If reduced activity were present, subsequent studies could be carried out to determine if IBD associated polymorphisms existed in the rhodanese gene.

The studies presented in this thesis have contributed to the general body of information concerning the faecal microbiota. However, even after more than 50 years of study using cultivation techniques, the work described here shows that the predominant bacterial population of the gut remains largely uncharacterised and poorly understood. Future work, utilising the molecular methodologies applied to the intestinal microbiota in this study, will make significant contributions to our understanding of the colonic microbiota in human health and disease.

APPENDIX A: SORENSON'S SIMILARITY CO-EFFICIENTS

A.1 Unrelated Volunteers from Chapter 4

Volunteers Under Comparison	Bands			Sorenson's Similarity Co-efficient
	Sample 1	Sample 2	Shared	
Volunteer 1 & Volunteer 3	19	24	11	60%
Volunteer 1 & Volunteer 4	19	22	6	78%
Volunteer 1 & Volunteer 6	19	12	2	65%
Volunteer 1 & Volunteer 5	19	21	13	40%
Volunteer 2 & Volunteer 3	21	24	9	67%
Volunteer 2 & Volunteer 4	21	22	11	51%
Volunteer 2 & Volunteer 5	21	12	6	36%
Volunteer 2 & Volunteer 6	21	21	11	48%
Volunteer 3 & Volunteer 4	24	22	7	65%
Volunteer 3 & Volunteer 5	24	12	4	44%
Volunteer 3 & Volunteer 6	24	21	11	44%
Volunteer 3 & Volunteer 7	24	25	10	61%
Volunteer 4 & Volunteer 5	22	12	2	59%
Volunteer 4 & Volunteer 6	22	21	9	56%
Volunteer 4 & Volunteer 7	22	25	8	72%
Volunteer 5 & Volunteer 6	12	21	12	55%
Volunteer 5 & Volunteer 7	12	25	15	54%
Volunteer 6 & Volunteer 7	21	25	15	43%

A.2 Short Term Stability Data from Chapter 4

Volunteer	Samples Under Comparison	Bands			Sorenson's Similarity Co-efficient
		Sample 1	Sample 2	Shared	
1	sample 1 & sample 2	16	16	15	94%
1	sample 1 & sample 3	16	13	13	90%
1	sample 1 & sample 4	16	18	15	88%
1	sample 1 & sample 5	16	17	15	91%
1	sample 1 & sample 6	16	16	15	94%
1	sample 1 & sample 7	16	18	15	88%
1	sample 1 & sample 8	16	17	14	85%
1	sample 1 & sample 9	16	16	14	88%
1	sample 1 & sample 10	16	16	12	75%
2	week 1 & week 2	12	15	12	89%
2	week 1 & week 3	12	15	12	89%
2	week 1 & week 4	12	16	12	86%
5	week 1 & week 2	16	16	16	100%
5	week 1 & week 3	16	17	15	91%
5	week 1 & week 4	16	17	14	85%
6	week 1 & week 2	19	17	16	89%
6	week 1 & week 3	19	15	13	76%
6	week 1 & week 4	19	13	12	75%
7	week 1 & week 2	20	15	14	80%
7	week 1 & week 3	20	14	13	76%
7	week 1 & week 4	20	17	15	81%
8	week 1 & week 2	17	15	13	81%
8	week 1 & week 3	17	16	14	85%
8	week 1 & week 4	17	15	13	81%

A.3 Long Term Stability Data from Chapter 4

Volunteer	Samples Under Comparison	Bands			Sorenson's Similarity Co-efficient
		Sample 1	Sample 2	Shared	
1	2001 & 2002	19	19	17	89%
1	2001 & 2005	19	20	16	82%
2	2003 & 2004	13	16	12	83%
2	2003 & 2005	13	16	12	83%
3	2001 & 2005	15	14	11	76%
4	2003 & 2004	19	15	13	76%
5	2003 & 2004	13	14	8	59%

A.4 Twins and Unrelated Controls from Chapter 5

Relationship	Pair	Bands			Sorenson's Similarity Co-efficient
		Twin 1	Twin 2	Shared	
Identical Twins	1	13	13	10	69%
	2	10	9	7	74%
	3	15	17	12	75%
	4	19	18	14	76%
	5	21	19	16	80%
	6	16	16	13	81%
	7	23	26	20	82%
	8	28	28	23	82%
	9	8	9	7	82%
	10	11	15	11	85%
	11	19	14	14	85%
	12	15	16	13	90%
	13	24	22	21	91%
Fraternal Twins	1	21	26	13	55%
	2	21	23	14	64%
	3	20	22	14	67%
	4	23	26	18	73%
	5	24	20	15	68%
	6	9	14	9	78%
	7	16	14	13	87%
Unrelated Controls	1	21	15	4	22%
	2	11	14	4	32%
	3	13	10	4	35%
	4	14	13	6	44%
	5	17	10	6	44%
	6	16	15	7	45%
	7	10	12	5	45%
	8	25	22	11	47%
	9	16	19	9	51%
	10	14	12	8	62%
	11	28	23	17	67%
	12	15	19	12	71%

A.5 Intestinal Lavage Data from Chapter 6

Volunteer	Samples Under Comparison	Bands			Sorenson's Similarity Co-efficient
		Sample 1	Sample 2	Shared	
1	Control & Day 4	14	15	11	76%
1	Control & Day 6	14	15	11	76%
1	Control & Day 6 #2	14	17	12	77%
1	Control & Day 7	14	17	12	77%
2	Control & Day 1	18	17	17	97%
2	Control & Day 2	18	18	17	94%
2	Control & Day 3	18	15	14	85%
2	Control & Day 4	18	12	10	67%
2	Control & Day 5	18	16	14	82%
2	Control & Day 6	18	18	14	78%
2	Control & Day 7	18	19	14	76%
3	Control & Day 2	13	16	13	90%
3	Control & Day 3	13	13	11	85%
3	Control & Day 4	13	12	11	88%
3	Control & Day 5	13	11	10	83%
3	Control & Day 6	13	13	11	85%
3	Control & Day 7	13	14	11	81%
4	Control & Day 1	14	13	12	89%
4	Control & Day 2	14	14	13	93%
4	Control & Day 3	14	17	13	84%
4	Control & Day 4	14	15	12	83%
4	Control & Day 5	14	14	13	93%
4	Control & Day 6	14	13	7	52%
5	Control & Day 2	12	11	9	78%
5	Control & Day 3	12	10	9	82%
5	Control & Day 4	12	16	12	86%
5	Control & Day 5	12	14	11	85%
5	Control & Day 7	12	17	12	83%
6	Control & Day 1	12	12	10	83%
6	Control & Day 2	12	16	10	71%
6	Control & Day 3	12	13	9	72%
6	Control & Day 4	12	16	9	64%
6	Control & Day 5	12	12	8	67%
6	Control & Day 6	12	13	10	80%
7	Control & Day 1	17	17	13	76%
7	Control & Day 1 #2	17	17	15	88%
7	Control & Day 2	17	20	15	81%
7	Control & Day 2 #2	17	18	14	80%
7	Control & Day 3	17	18	15	86%
7	Control & Day 4	17	19	15	83%
7	Control & Day 5	17	21	15	79%
7	Control & Day 5 #2	17	20	14	76%
7	Control & Day 7	17	21	16	84%
8	Control & Day 2	15	21	13	72%
8	Control & Day 4	15	23	13	68%
8	Control & Day 5	15	20	11	63%
9	Control & Day 3	19	17	15	83%
9	Control & Day 7	19	15	12	71%
10	Control & Day 3	19	17	8	44%
10	Control & Day 4	19	17	12	67%
10	Control & Day 5	19	17	11	61%
10	Control & Day 6	19	15	11	65%

A.6 Western Diet Data from Chapter 6

Volunteer	Samples Under Comparison	Bands			Sorenson's Similarity Co-efficient
		Sample 1	Sample 2	Shared	
1	week 1 & week 2	12	15	12	89%
1	week 1 & week 3	12	15	12	89%
1	week 1 & week 4	12	16	12	86%
2	week 1 & week 2	20	15	14	80%
2	week 1 & week 3	20	14	13	76%
2	week 1 & week 4	20	17	15	81%
3	week 1 & week 2	16	16	16	100%
3	week 1 & week 3	16	17	15	91%
3	week 1 & week 4	16	17	14	85%
4	week 1 & week 2	19	17	16	89%
4	week 1 & week 3	19	15	13	76%
4	week 1 & week 4	19	13	12	75%
5	week 1 & week 2	17	15	13	81%
5	week 1 & week 3	17	16	14	85%
5	week 1 & week 4	17	15	13	81%
6	week 1 & week 2	23	21	20	91%
6	week 1 & week 3	23	16	14	72%
6	week 1 & week 4	23	17	16	80%

A.7 Atkins' Diet Data from Chapter 6

Volunteer	Samples Under Comparison	Bands			Sorenson's Similarity Co-efficient
		Sample 1	Sample 2	Shared	
1	Day 0 & week 2	22	23	19	84%
1	Day 0 & week 3	22	17	17	87%
1	Day 0 & week 4	22	17	15	77%
2	Day 0 & week 2	16	20	13	72%
2	Day 0 & week 3	16	16	10	63%
2	Day 0 & week 4	16	16	10	63%
3	Day 0 & week 2	17	16	15	91%
3	Day 0 & week 3	17	18	14	80%
3	Day 0 & week 4	17	18	13	74%
4	Day 0 & week 2	13	16	11	76%
4	Day 0 & week 3	13	20	9	55%
4	Day 0 & week 4	13	21	11	65%
5	Day 0 & week 2	18	21	14	72%
5	Day 0 & week 3	18	20	10	53%
5	Day 0 & week 4	18	15	9	55%
6	Day 0 & week 2	19	20	10	51%
6	Day 0 & week 3	19	19	9	47%
6	Day 0 & week 4	19	19	12	63%

A.8 Diarrhoea Predominant IBS Data from Chapter 8

Volunteers Under Comparison	Bands Sample 1	Bands Sample 2	Bands Shared	Sorenson's Similarity Co-efficient
1 & 2	14	13	10	74%
1 & 3	14	17	10	65%
1 & 4	14	13	5	37%
1 & 5	14	18	8	50%
1 & 6	14	19	11	67%
1 & 7	14	19	8	48%
1 & 8	14	21	11	63%
1 & 9	14	19	8	48%
1 & 10	14	18	8	50%
2 & 3	13	17	10	67%
2 & 4	13	13	5	38%
2 & 5	13	18	7	45%
2 & 6	13	19	11	69%
2 & 7	13	19	9	56%
2 & 8	13	21	9	53%
2 & 9	13	19	9	56%
2 & 10	13	18	7	45%
3 & 4	17	13	5	33%
3 & 5	17	18	8	46%
3 & 6	17	19	11	61%
3 & 7	17	19	10	56%
3 & 8	17	21	10	53%
3 & 9	17	19	10	56%
3 & 10	17	18	10	57%
4 & 5	13	18	4	26%
4 & 6	13	19	3	19%
4 & 7	13	19	6	38%
4 & 8	13	21	3	18%
4 & 9	13	19	6	38%
4 & 10	13	18	5	32%
5 & 6	18	19	10	54%
5 & 7	18	19	10	54%
5 & 8	18	21	10	51%
5 & 9	18	19	8	43%
5 & 10	18	18	7	39%
6 & 7	19	19	9	47%
6 & 8	19	21	13	65%
6 & 9	19	19	13	68%
6 & 10	19	18	11	59%
7 & 8	19	21	8	40%
7 & 9	19	19	7	37%
7 & 10	19	18	8	43%
8 & 9	21	19	9	45%
8 & 10	21	18	10	51%
9 & 10	19	18	9	49%

A.9 Constipation Predominant IBS Data from Chapter 8

Volunteers Under Comparison	Bands Sample 1	Bands Sample 2	Bands Shared	Sorenson's Similarity Co-efficient
1 & 2	17	14	8	52%
1 & 3	17	19	8	44%
1 & 4	17	14	7	45%
1 & 5	17	15	8	50%
1 & 6	17	18	10	57%
1 & 7	17	18	7	40%
1 & 8	17	20	11	59%
1 & 9	17	19	7	39%
1 & 10	17	21	10	53%
2 & 3	14	19	7	42%
2 & 4	14	14	9	64%
2 & 5	14	15	8	55%
2 & 6	14	18	5	31%
2 & 7	14	18	8	50%
2 & 8	14	20	7	41%
2 & 9	14	19	8	48%
2 & 10	14	21	7	40%
3 & 4	19	14	9	55%
3 & 5	19	15	6	35%
3 & 6	19	18	10	54%
3 & 7	19	18	10	54%
3 & 8	19	20	10	51%
3 & 9	19	19	11	58%
3 & 10	19	21	11	55%
4 & 5	14	15	9	62%
4 & 6	14	18	8	50%
4 & 7	14	18	11	69%
4 & 8	14	20	10	59%
4 & 9	14	19	9	55%
4 & 10	14	21	10	57%
5 & 6	15	18	8	48%
5 & 7	15	18	10	61%
5 & 8	15	20	9	51%
5 & 9	15	19	8	47%
5 & 10	15	21	8	44%
6 & 7	18	18	11	61%
6 & 8	18	20	13	68%
6 & 9	18	19	11	59%
6 & 10	18	21	11	56%
7 & 8	18	20	14	74%
7 & 9	18	19	13	70%
7 & 10	18	21	12	62%
8 & 9	20	19	12	62%
8 & 10	20	21	15	73%
9 & 10	19	21	12	60%

A.10 Mixed IBS Data from Chapter 9

Volunteers Under Comparison	Bands			Sorenson's Similarity Co-efficient
	Sample 1	Sample 2	Shared	
1 & 2	14	14	9	64%
1 & 3	14	14	8	57%
1 & 4	14	10	6	50%
1 & 5	14	14	6	43%
1 & 6	14	14	4	29%
1 & 7	14	18	10	63%
1 & 8	14	21	9	51%
1 & 9	14	18	4	25%
1 & 10	14	16	7	47%
2 & 3	14	14	10	71%
2 & 4	14	10	8	67%
2 & 5	14	14	6	43%
2 & 6	14	14	5	36%
2 & 7	14	18	10	63%
2 & 8	14	21	9	51%
2 & 9	14	18	5	31%
2 & 10	14	16	8	53%
3 & 4	14	10	9	75%
3 & 5	14	14	6	43%
3 & 6	14	14	6	43%
3 & 7	14	18	9	56%
3 & 8	14	21	9	51%
3 & 9	14	18	6	38%
3 & 10	14	16	8	53%
4 & 5	10	14	6	50%
4 & 6	10	14	5	42%
4 & 7	10	18	8	57%
4 & 8	10	21	8	52%
4 & 9	10	18	4	29%
4 & 10	10	16	6	46%
5 & 6	14	14	8	57%
5 & 7	14	18	9	56%
5 & 8	14	21	11	63%
5 & 9	14	18	7	44%
5 & 10	14	16	7	47%
6 & 7	14	18	10	63%
6 & 8	14	21	10	57%
6 & 9	14	18	9	56%
6 & 10	14	16	7	47%
7 & 8	18	21	14	72%
7 & 9	18	18	11	61%
7 & 10	18	16	10	59%
8 & 9	21	18	11	56%
8 & 10	21	16	10	54%
9 & 10	18	16	8	47%

A.11 Diverticular Disease Data from Chapter 9

Volunteers Under Comparison	Bands			Sorenson's Similarity Co-efficient
	Sample 1	Sample 2	Shared	
1 & 2	15	15	9	60%
1 & 3	15	20	11	63%
1 & 4	15	18	7	42%
1 & 5	15	19	10	59%
1 & 6	15	18	11	67%
1 & 7	15	18	9	55%
1 & 8	15	16	9	58%
1 & 9	15	19	9	53%
1 & 10	15	17	9	56%
2 & 3	15	20	12	69%
2 & 4	15	18	8	48%
2 & 5	15	19	8	47%
2 & 6	15	18	10	61%
2 & 7	15	18	8	48%
2 & 8	15	16	7	45%
2 & 9	15	19	10	59%
2 & 10	15	17	8	50%
3 & 4	20	18	8	42%
3 & 5	20	19	10	51%
3 & 6	20	18	11	58%
3 & 7	20	18	10	53%
3 & 8	20	16	10	56%
3 & 9	20	19	12	62%
3 & 10	20	17	10	54%
4 & 5	18	19	8	43%
4 & 6	18	18	10	56%
4 & 7	18	18	7	39%
4 & 8	18	16	5	29%
4 & 9	18	19	11	59%
4 & 10	18	17	8	46%
5 & 6	19	18	10	54%
5 & 7	19	18	10	54%
5 & 8	19	16	8	46%
5 & 9	19	19	11	58%
5 & 10	19	17	10	56%
6 & 7	18	18	9	50%
6 & 8	18	16	8	47%
6 & 9	18	19	9	49%
6 & 10	18	17	9	51%
7 & 8	18	16	9	53%
7 & 9	18	19	8	43%
7 & 10	18	17	10	57%
8 & 9	16	19	8	46%
8 & 10	16	17	8	48%
9 & 10	19	17	12	67%

A.12 Ulcerative Colitis Data from Chapter 9

Volunteers Under Comparison	Bands			Sorenson's Similarity Co-efficient
	Sample 1	Sample 2	Shared	
1 & 2	14	12	6	46%
1 & 3	14	13	8	59%
1 & 4	14	9	7	61%
1 & 5	14	20	7	41%
1 & 6	14	9	4	35%
1 & 7	14	19	11	67%
1 & 8	14	20	9	53%
1 & 9	14	18	4	25%
1 & 10	14	23	8	43%
2 & 3	12	13	4	32%
2 & 4	12	9	5	48%
2 & 5	12	20	8	50%
2 & 6	12	9	4	38%
2 & 7	12	19	5	32%
2 & 8	12	20	8	50%
2 & 9	12	18	5	33%
2 & 10	12	23	6	34%
3 & 4	13	9	6	55%
3 & 5	13	20	6	36%
3 & 6	13	9	4	36%
3 & 7	13	19	8	50%
3 & 8	13	20	9	55%
3 & 9	13	18	5	32%
3 & 10	13	23	7	39%
4 & 5	9	20	4	28%
4 & 6	9	9	3	33%
4 & 7	9	19	7	50%
4 & 8	9	20	5	34%
4 & 9	9	18	6	44%
4 & 10	9	23	6	38%
5 & 6	20	9	5	34%
5 & 7	20	19	8	41%
5 & 8	20	20	8	40%
5 & 9	20	18	8	42%
5 & 10	20	23	9	42%
6 & 7	9	19	6	43%
6 & 8	9	20	5	34%
6 & 9	9	18	4	30%
6 & 10	9	23	4	25%
7 & 8	19	20	10	51%
7 & 9	19	18	6	32%
7 & 10	19	23	12	57%
8 & 9	20	18	9	47%
8 & 10	20	23	11	51%
9 & 10	18	23	10	49%

A.13 Crohn's Disease Data from Chapter 9

Volunteers Under Comparison	Bands			Sorenson's Similarity Co-efficient
	Sample 1	Sample 2	Shared	
1 & 2	15	16	7	45%
1 & 3	15	18	7	42%
1 & 4	15	14	8	55%
1 & 5	15	16	7	45%
1 & 6	15	16	9	58%
1 & 7	15	16	7	45%
1 & 8	15	14	8	55%
1 & 9	15	15	7	47%
1 & 10	16	18	11	65%
2 & 3	16	14	7	47%
2 & 4	16	16	7	44%
2 & 5	16	16	7	44%
2 & 6	16	16	7	44%
2 & 7	16	14	7	47%
2 & 8	16	15	7	45%
2 & 9	18	14	7	44%
2 & 10	18	16	8	47%
3 & 4	18	16	9	53%
3 & 5	18	16	8	47%
3 & 6	18	14	7	44%
3 & 7	18	15	8	48%
3 & 8	14	16	8	53%
3 & 9	14	16	7	47%
3 & 10	14	16	8	53%
4 & 5	14	14	7	50%
4 & 6	14	15	7	48%
4 & 7	16	16	8	50%
4 & 8	16	16	9	56%
4 & 9	16	14	7	47%
4 & 10	16	15	7	45%
5 & 6	16	16	8	50%
5 & 7	16	14	9	60%
5 & 8	16	15	8	52%
5 & 9	16	14	7	47%
5 & 10	16	15	10	65%
6 & 7	14	15	7	48%

A.14 Controls

Volunteers Under Comparison	Bands Sample 1	Bands Sample 2	Bands Shared	Sorenson's Similarity Co-efficient
1 & 2	23	20	13	60%
1 & 3	23	13	7	39%
1 & 4	23	12	8	46%
1 & 5	23	17	9	45%
1 & 6	23	21	12	55%
1 & 7	23	16	7	36%
1 & 8	23	17	10	50%
1 & 9	23	17	9	45%
1 & 10	23	17	10	50%
2 & 3	20	13	9	55%
2 & 4	20	12	8	50%
2 & 5	20	17	10	54%
2 & 6	20	21	13	63%
2 & 7	20	16	11	61%
2 & 8	20	17	14	76%
2 & 9	20	17	13	70%
2 & 10	20	17	9	49%
3 & 4	13	12	7	56%
3 & 5	13	17	8	53%
3 & 6	13	21	8	47%
3 & 7	13	16	7	48%
3 & 8	13	17	6	40%
3 & 9	13	17	8	53%
3 & 10	13	17	7	47%
4 & 5	12	17	8	55%
4 & 6	12	21	9	55%
4 & 7	12	16	6	43%
4 & 8	12	17	6	41%
4 & 9	12	17	5	34%
4 & 10	12	17	4	28%
5 & 6	17	21	10	53%
5 & 7	17	16	9	55%
5 & 8	17	17	9	53%
5 & 9	17	17	9	53%
5 & 10	17	17	10	59%
6 & 7	21	16	12	65%
6 & 8	21	17	13	68%
6 & 9	21	17	11	58%
6 & 10	21	17	10	53%
7 & 8	16	17	14	85%
7 & 9	16	17	11	67%
7 & 10	16	17	9	55%
8 & 9	17	17	12	71%
8 & 10	17	17	10	59%
9 & 10	17	17	10	59%

APPENDIX B: SHANNON'S INDICES

B.1 Twins and Unrelated Controls Data from Chapter 5

IDENTICAL TWINS

Band	Pair 1	Twin 1	Pair 1	Twin 2	Pair 2	Twin 1	Pair 2	Twin 2	Pair 3	Twin 1	Pair 3	Twin 2	Pair 4	Twin 1	Pair 4	Twin 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.030	-0.104	0.072	-0.189	0.053	-0.155	0.160	-0.293	0.026	-0.094	0.018	-0.073	0.018	-0.072	0.047	-0.143
2	0.087	-0.213	0.038	-0.124	0.152	-0.286	0.283	-0.357	0.023	-0.086	0.080	-0.202	0.029	-0.104	0.036	-0.119
3	0.072	-0.189	0.076	-0.196	0.202	-0.323	0.045	-0.140	0.079	-0.200	0.097	-0.226	0.057	-0.164	0.047	-0.144
4	0.055	-0.160	0.072	-0.190	0.128	-0.263	0.077	-0.198	0.113	-0.247	0.096	-0.225	0.067	-0.182	0.103	-0.235
5	0.078	-0.200	0.067	-0.182	0.049	-0.148	0.025	-0.091	0.043	-0.136	0.143	-0.278	0.034	-0.115	0.096	-0.225
6	0.065	-0.177	0.083	-0.207	0.070	-0.185	0.077	-0.198	0.072	-0.189	0.023	-0.085	0.060	-0.169	0.018	-0.074
7	0.036	-0.119	0.048	-0.145	0.124	-0.259	0.101	-0.232	0.085	-0.209	0.058	-0.165	0.084	-0.209	0.057	-0.164
8	0.057	-0.164	0.052	-0.154	0.182	-0.310	0.079	-0.201	0.023	-0.087	0.024	-0.089	0.058	-0.166	0.103	-0.234
9	0.039	-0.126	0.073	-0.191	0.041	-0.131	0.053	-0.156	0.021	-0.080	0.127	-0.262	0.055	-0.160	0.039	-0.126
10	0.057	-0.164	0.057	-0.163			0.099	-0.229	0.134	-0.270	0.144	-0.279	0.066	-0.180	0.160	-0.293
11	0.050	-0.149	0.047	-0.143					0.038	-0.125	0.100	-0.231	0.069	-0.185	0.130	-0.265
12	0.162	-0.295	0.147	-0.282					0.086	-0.211	0.039	-0.127	0.175	-0.305	0.014	-0.059
13	0.212	-0.329	0.168	-0.300					0.133	-0.269	0.006	-0.030	0.153	-0.287	0.028	-0.100
14									0.011	-0.050	0.026	-0.096	0.040	-0.129	0.005	-0.025
15									0.067	-0.180	0.018	-0.073	0.016	-0.066	0.046	-0.141
16									0.030	-0.106			0.009	-0.041	0.023	-0.087
17									0.016	-0.066			0.004	-0.021	0.023	-0.086
18													0.004	-0.020	0.013	-0.056
19															0.013	-0.057
	H'	2.39	H'	2.46	H'	2.06	H'	2.09	H'	2.61	H'	2.44	H'	2.57	H'	2.63

Band	Pair 5	Twin 1	Pair 5	Twin 2	Pair 6	Twin 1	Pair 6	Twin 2	Pair 7	Twin 1	Pair 7	Twin 2	Pair 8	Twin 1	Pair 8	Twin 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.003	-0.018	0.005	-0.025	0.007	-0.033	0.011	-0.051	0.054	-0.157	0.034	-0.116	0.200	-0.322	0.199	-0.321
2	0.003	-0.020	0.006	-0.030	0.005	-0.026	0.000	-0.001	0.020	-0.079	0.019	-0.074	0.012	-0.054	0.071	-0.187
3	0.026	-0.096	0.072	-0.189	0.014	-0.060	0.035	-0.118	0.010	-0.045	0.032	-0.111	0.040	-0.129	0.002	-0.013
4	0.125	-0.260	0.080	-0.201	0.014	-0.059	0.023	-0.086	0.006	-0.030	0.011	-0.049	0.073	-0.191	0.005	-0.026
5	0.128	-0.263	0.083	-0.207	0.015	-0.062	0.030	-0.104	0.011	-0.051	0.020	-0.080	0.041	-0.131	0.012	-0.052
6	0.071	-0.188	0.053	-0.155	0.046	-0.141	0.158	-0.292	0.067	-0.181	0.042	-0.133	0.006	-0.032	0.002	-0.012
7	0.076	-0.195	0.070	-0.186	0.052	-0.153	0.120	-0.255	0.045	-0.140	0.025	-0.092	0.009	-0.042	0.003	-0.018
8	0.079	-0.200	0.062	-0.172	0.082	-0.205	0.263	-0.351	0.043	-0.134	0.026	-0.095	0.005	-0.026	0.028	-0.101
9	0.093	-0.221	0.070	-0.186	0.073	-0.191	0.050	-0.150	0.027	-0.098	0.033	-0.113	0.007	-0.036	0.003	-0.018
10	0.138	-0.273	0.036	-0.121	0.277	-0.355	0.038	-0.124	0.081	-0.204	0.030	-0.106	0.004	-0.024	0.003	-0.015
11	0.082	-0.204	0.079	-0.200	0.068	-0.183	0.038	-0.125	0.072	-0.189	0.032	-0.111	0.007	-0.035	0.005	-0.027
12	0.063	-0.175	0.045	-0.140	0.085	-0.210	0.098	-0.228	0.138	-0.273	0.049	-0.148	0.014	-0.061	0.006	-0.030
13	0.026	-0.094	0.025	-0.093	0.030	-0.105	0.029	-0.103	0.029	-0.104	0.150	-0.285	0.063	-0.173	0.005	-0.025
14	0.020	-0.079	0.050	-0.150	0.083	-0.207	0.055	-0.159	0.085	-0.210	0.110	-0.242	0.013	-0.057	0.005	-0.027
15	0.045	-0.139	0.041	-0.130	0.086	-0.211	0.026	-0.094	0.018	-0.074	0.021	-0.080	0.010	-0.048	0.011	-0.048
16	0.000	-0.002	0.033	-0.112	0.066	-0.179	0.025	-0.093	0.106	-0.238	0.057	-0.164	0.014	-0.060	0.012	-0.054
17	0.008	-0.040	0.035	-0.118					0.022	-0.083	0.038	-0.124	0.015	-0.063	0.007	-0.036
18	0.002	-0.011	0.042	-0.133					0.004	-0.024	0.055	-0.160	0.025	-0.091	0.006	-0.029
19	0.012	-0.054	0.048	-0.147					0.025	-0.094	0.020	-0.079	0.026	-0.095	0.007	-0.033
20			0.043	-0.135					0.034	-0.116	0.022	-0.084	0.027	-0.098	0.009	-0.041
21			0.022	-0.084					0.026	-0.095	0.026	-0.094	0.028	-0.101	0.008	-0.040
22									0.037	-0.122	0.022	-0.084	0.024	-0.089	0.064	-0.176
23									0.039	-0.126	0.028	-0.099	0.164	-0.297	0.078	-0.199
24											0.047	-0.144	0.018	-0.073	0.073	-0.191
25											0.024	-0.090	0.018	-0.073	0.053	-0.156
26											0.026	-0.094	0.018	-0.072	0.040	-0.130
27													0.041	-0.131	0.072	-0.189
28													0.076	-0.195	0.212	-0.329
	H'	2.92	H'	2.53	H'	2.38	H'	2.34	H'	2.87	H'	3.05	H'	2.80	H'	2.52

Band	Pair 9	Twin 1	Pair 9	Twin 2	Pair 10	Twin 1	Pair 10	Twin 2	Pair 11	Twin 1	Pair 11	Twin 2	Pair 12	Twin 1	Pair 12	Twin 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.018	-0.073	0.019	-0.075	0.049	-0.149	0.012	-0.052	0.003	-0.019	0.014	-0.059	0.010	-0.046	0.040	-0.129
2	0.127	-0.262	0.019	-0.076	0.091	-0.218	0.025	-0.091	0.058	-0.164	0.099	-0.228	0.100	-0.230	0.125	-0.260
3	0.192	-0.317	0.211	-0.328	0.117	-0.251	0.061	-0.171	0.120	-0.254	0.180	-0.309	0.081	-0.203	0.080	-0.202
4	0.097	-0.226	0.184	-0.312	0.142	-0.277	0.057	-0.163	0.069	-0.185	0.110	-0.242	0.099	-0.229	0.085	-0.210
5	0.113	-0.246	0.247	-0.345	0.065	-0.177	0.078	-0.199	0.041	-0.130	0.038	-0.124	0.041	-0.131	0.051	-0.151
6	0.190	-0.315	0.210	-0.328	0.102	-0.233	0.095	-0.223	0.037	-0.123	0.000	-0.002	0.070	-0.186	0.090	-0.217
7	0.035	-0.118	0.023	-0.088	0.098	-0.228	0.099	-0.229	0.046	-0.141	0.013	-0.057	0.043	-0.136	0.036	-0.120
8	0.124	-0.259	0.087	-0.212	0.088	-0.213	0.101	-0.232	0.030	-0.106	0.027	-0.098	0.028	-0.100	0.044	-0.138
9	0.105	-0.236			0.107	-0.239	0.081	-0.204	0.076	-0.195	0.102	-0.232	0.050	-0.151	0.040	-0.128
10					0.078	-0.200	0.068	-0.182	0.056	-0.161	0.164	-0.297	0.216	-0.331	0.048	-0.146
11					0.063	-0.174	0.113	-0.246	0.092	-0.220	0.093	-0.221	0.054	-0.157	0.190	-0.316
12							0.083	-0.207	0.075	-0.193	0.043	-0.136	0.042	-0.134	0.044	-0.138
13							0.059	-0.166	0.066	-0.179	0.028	-0.100	0.052	-0.153	0.079	-0.201
14							0.045	-0.140	0.068	-0.183	0.090	-0.216	0.042	-0.134	0.030	-0.105
15							0.024	-0.089	0.049	-0.148			0.072	-0.190	0.006	-0.029
16									0.048	-0.145					0.012	-0.053
17									0.039	-0.126						
18									0.011	-0.050						
19									0.017	-0.069						
	H'	2.05	H'	1.76	H'	2.36	H'	2.59	H'	2.79	H'	2.32	H'	2.51	H'	2.54

FRATERNAL TWINS

Band	Pair 13	Twin 1	Pair 13	Twin 2	Pair 1	Twin 1	Pair 1	Twin 2	Pair 2	Twin 1	Pair 2	Twin 2	Pair 3	Twin 1	Pair 3	Twin 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.027	-0.097	0.039	-0.126	0.009	-0.043	0.034	-0.116	0.009	-0.043	0.054	-0.157	0.030	-0.105	0.013	-0.056
2	0.013	-0.057	0.006	-0.029	0.028	-0.101	0.019	-0.074	0.028	-0.101	0.020	-0.079	0.065	-0.178	0.022	-0.083
3	0.042	-0.132	0.007	-0.034	0.075	-0.194	0.032	-0.111	0.075	-0.194	0.010	-0.045	0.040	-0.128	0.035	-0.118
4	0.019	-0.074	0.003	-0.017	0.001	-0.009	0.011	-0.049	0.001	-0.009	0.006	-0.030	0.041	-0.131	0.036	-0.120
5	0.024	-0.088	0.003	-0.018	0.007	-0.034	0.020	-0.080	0.007	-0.034	0.011	-0.051	0.048	-0.145	0.019	-0.075
6	0.037	-0.122	0.030	-0.105	0.020	-0.077	0.042	-0.133	0.020	-0.077	0.067	-0.181	0.070	-0.186	0.076	-0.196
7	0.075	-0.194	0.016	-0.068	0.050	-0.150	0.025	-0.092	0.050	-0.150	0.045	-0.140	0.014	-0.059	0.054	-0.158
8	0.049	-0.147	0.030	-0.105	0.033	-0.112	0.026	-0.095	0.033	-0.112	0.043	-0.134	0.068	-0.183	0.029	-0.102
9	0.034	-0.115	0.037	-0.122	0.005	-0.026	0.033	-0.113	0.005	-0.026	0.027	-0.098	0.061	-0.170	0.025	-0.092
10	0.032	-0.111	0.054	-0.157	0.081	-0.204	0.030	-0.106	0.081	-0.204	0.081	-0.204	0.148	-0.283	0.059	-0.166
11	0.040	-0.128	0.070	-0.187	0.028	-0.099	0.032	-0.111	0.028	-0.099	0.072	-0.189	0.040	-0.130	0.015	-0.064
12	0.038	-0.124	0.022	-0.085	0.065	-0.177	0.049	-0.148	0.065	-0.177	0.138	-0.273	0.045	-0.139	0.015	-0.063
13	0.046	-0.141	0.076	-0.196	0.010	-0.048	0.150	-0.285	0.010	-0.048	0.029	-0.104	0.014	-0.061	0.027	-0.097
14	0.078	-0.199	0.077	-0.197	0.016	-0.067	0.110	-0.242	0.016	-0.067	0.085	-0.210	0.007	-0.033	0.019	-0.077
15	0.053	-0.156	0.081	-0.204	0.017	-0.070	0.021	-0.080	0.017	-0.070	0.018	-0.074	0.027	-0.097	0.015	-0.063
16	0.059	-0.166	0.033	-0.113	0.021	-0.082	0.057	-0.164	0.021	-0.082	0.106	-0.238	0.031	-0.107	0.059	-0.167
17	0.054	-0.158	0.071	-0.187	0.110	-0.242	0.038	-0.124	0.110	-0.242	0.022	-0.083	0.008	-0.038	0.058	-0.165
18	0.065	-0.178	0.057	-0.163	0.033	-0.112	0.055	-0.160	0.033	-0.112	0.004	-0.024	0.015	-0.061	0.042	-0.132
19	0.036	-0.120	0.118	-0.252	0.022	-0.083	0.020	-0.079	0.022	-0.083	0.025	-0.094	0.011	-0.049	0.167	-0.299
20	0.028	-0.100	0.032	-0.111	0.185	-0.312	0.022	-0.084	0.185	-0.312	0.034	-0.116	0.014	-0.061	0.215	-0.331
21	0.061	-0.171	0.077	-0.198	0.184	-0.312	0.026	-0.094	0.184	-0.312	0.026	-0.095	0.114	-0.248		
22	0.015	-0.064	0.061	-0.170			0.022	-0.084			0.037	-0.122	0.091	-0.218		
23	0.047	-0.144					0.028	-0.099			0.039	-0.126				
24	0.030	-0.105					0.047	-0.144								
25							0.024	-0.090								
26							0.026	-0.094								
	H'	3.09	H'	2.84	H'	2.56	H'	3.05	H'	2.56	H'	2.87	H'	2.81	H'	2.62

Band	Pair 4	Twin 1	Pair 4	Twin 2	Pair 5	Twin 1	Pair 5	Twin 2	Pair 6	Twin 1	Pair 6	Twin 2	Pair 7	Twin 1	Pair 7	Twin 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.067	-0.181	0.162	-0.295	0.010	-0.045	0.007	-0.036	0.018	-0.073	0.026	-0.096	0.058	-0.165	0.092	-0.220
2	0.043	-0.134	0.013	-0.056	0.019	-0.075	0.006	-0.029	0.019	-0.076	0.088	-0.214	0.045	-0.140	0.003	-0.020
3	0.031	-0.108	0.013	-0.057	0.002	-0.011	0.017	-0.070	0.051	-0.151	0.263	-0.351	0.038	-0.124	0.011	-0.050
4	0.060	-0.169	0.016	-0.067	0.022	-0.084	0.037	-0.122	0.088	-0.214	0.199	-0.321	0.053	-0.155	0.001	-0.005
5	0.076	-0.196	0.006	-0.031	0.041	-0.132	0.036	-0.120	0.075	-0.195	0.067	-0.181	0.047	-0.144	0.014	-0.060
6	0.005	-0.028	0.030	-0.105	0.027	-0.099	0.023	-0.087	0.052	-0.154	0.063	-0.173	0.018	-0.074	0.143	-0.278
7	0.003	-0.016	0.001	-0.004	0.039	-0.126	0.004	-0.022	0.044	-0.138	0.083	-0.206	0.068	-0.183	0.125	-0.260
8	0.010	-0.046	0.009	-0.042	0.045	-0.139	0.006	-0.031	0.111	-0.244	0.159	-0.292	0.114	-0.247	0.214	-0.330
9	0.030	-0.104	0.010	-0.045	0.015	-0.063	0.027	-0.097	0.168	-0.299	0.052	-0.155	0.165	-0.297	0.165	-0.298
10	0.013	-0.058	0.003	-0.016	0.019	-0.074	0.027	-0.097	0.113	-0.247			0.128	-0.263	0.102	-0.233
11	0.001	-0.004	0.005	-0.026	0.041	-0.130	0.038	-0.124	0.042	-0.134			0.031	-0.107	0.085	-0.210
12	0.001	-0.005	0.001	-0.004	0.050	-0.150	0.004	-0.020	0.090	-0.217			0.056	-0.162	0.035	-0.117
13	0.021	-0.082	0.002	-0.012	0.058	-0.165	0.035	-0.117	0.091	-0.219			0.062	-0.172	0.008	-0.040
14	0.055	-0.159	0.001	-0.010	0.044	-0.137	0.015	-0.061	0.037	-0.121			0.058	-0.166	0.000	-0.003
15	0.042	-0.133	0.002	-0.011	0.018	-0.073	0.014	-0.060					0.033	-0.112		
16	0.040	-0.130	0.066	-0.179	0.050	-0.151	0.037	-0.122					0.027	-0.098		
17	0.005	-0.025	0.064	-0.175	0.032	-0.109	0.031	-0.107								
18	0.032	-0.111	0.042	-0.133	0.017	-0.069	0.034	-0.114								
19	0.027	-0.097	0.008	-0.039	0.235	-0.340	0.028	-0.100								
20	0.029	-0.103	0.060	-0.169	0.216	-0.331	0.018	-0.073								
21	0.062	-0.172	0.041	-0.130			0.023	-0.086								
22	0.042	-0.133	0.246	-0.345			0.015	-0.064								
23	0.030	-0.106	0.201	-0.322			0.258	-0.350								
24	0.024	-0.091					0.261	-0.351								
25	0.133	-0.268														
26	0.118	-0.252														
	H'	2.91	H'	2.27	H'	2.51	H'	2.46	H'	2.48	H'	1.99	H'	2.12	H'	2.61

UNRELATED CONTROL PAIRS

Band	Pair 1	Control 1	Pair 1	Control 2	Pair 2	Control 1	Pair 2	Control 2	Pair 3	Control 1	Pair 3	Control 2	Pair 4	Control 1	Pair 4	Control 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.035	-0.117	0.056	-0.161	0.049	-0.149	0.014	-0.059	0.008	-0.037	0.008	-0.037	0.013	-0.056	0.021	-0.080
2	0.041	-0.130	0.084	-0.208	0.091	-0.218	0.099	-0.228	0.089	-0.216	0.036	-0.120	0.037	-0.123	0.007	-0.035
3	0.047	-0.143	0.034	-0.114	0.117	-0.251	0.180	-0.309	0.158	-0.292	0.191	-0.316	0.064	-0.176	0.064	-0.177
4	0.022	-0.084	0.114	-0.248	0.142	-0.277	0.110	-0.242	0.143	-0.278	0.086	-0.211	0.065	-0.177	0.035	-0.117
5	0.038	-0.125	0.041	-0.132	0.065	-0.177	0.038	-0.124	0.040	-0.129	0.193	-0.318	0.046	-0.142	0.047	-0.144
6	0.138	-0.273	0.057	-0.164	0.102	-0.233	0.000	-0.002	0.063	-0.174	0.303	-0.362	0.063	-0.173	0.058	-0.166
7	0.060	-0.168	0.068	-0.182	0.098	-0.228	0.013	-0.057	0.059	-0.167	0.122	-0.257	0.038	-0.124	0.389	-0.367
8	0.064	-0.176	0.048	-0.145	0.088	-0.213	0.027	-0.098	0.053	-0.156	0.011	-0.051	0.048	-0.147	0.042	-0.134
9	0.085	-0.209	0.053	-0.155	0.107	-0.239	0.102	-0.232	0.085	-0.209	0.048	-0.146	0.063	-0.174	0.217	-0.332
10	0.061	-0.170	0.052	-0.154	0.078	-0.200	0.164	-0.297	0.108	-0.241	0.001	-0.009	0.341	-0.367	0.067	-0.181
11	0.031	-0.107	0.197	-0.320	0.063	-0.174	0.093	-0.221	0.065	-0.177			0.096	-0.225	0.029	-0.104
12	0.047	-0.144	0.040	-0.128			0.043	-0.136	0.092	-0.220			0.070	-0.186	0.017	-0.069
13	0.056	-0.162	0.047	-0.144			0.028	-0.100	0.037	-0.123			0.023	-0.086	0.006	-0.029
14	0.072	-0.190	0.056	-0.160			0.090	-0.216					0.033	-0.113		
15	0.018	-0.073	0.054	-0.157												
16	0.023	-0.087														
17	0.018	-0.073														
18	0.054	-0.158														
19	0.046	-0.141														
20	0.029	-0.102														
21	0.016	-0.066														
	H'	2.90	H'	2.57	H'	2.36	H'	2.32	H'	2.42	H'	1.83	H'	2.27	H'	1.93

Band	Pair 5	Control 1	Pair 5	Control 2	Pair 6	Control 1	Pair 6	Control 2	Pair 7	Control 1	Pair 7	Control 2	Pair 8	Control 1	Pair 8	Control 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.020	-0.080	0.010	-0.045	0.040	-0.129	0.012	-0.052	0.006	-0.029	0.003	-0.015	0.001	-0.006	0.002	-0.014
2	0.007	-0.034	0.038	-0.124	0.125	-0.260	0.025	-0.091	0.017	-0.070	0.006	-0.030	0.006	-0.029	0.015	-0.063
3	0.067	-0.181	0.114	-0.248	0.080	-0.202	0.061	-0.171	0.009	-0.043	0.007	-0.034	0.054	-0.158	0.014	-0.059
4	0.061	-0.170	0.047	-0.143	0.085	-0.210	0.057	-0.163	0.075	-0.195	0.039	-0.127	0.057	-0.164	0.003	-0.019
5	0.066	-0.179	0.041	-0.130	0.051	-0.151	0.078	-0.199	0.088	-0.214	0.102	-0.232	0.036	-0.120	0.001	-0.007
6	0.089	-0.216	0.300	-0.361	0.090	-0.217	0.095	-0.223	0.246	-0.345	0.356	-0.368	0.078	-0.200	0.021	-0.081
7	0.057	-0.164	0.182	-0.310	0.036	-0.120	0.099	-0.229	0.076	-0.197	0.071	-0.187	0.100	-0.230	0.021	-0.081
8	0.037	-0.121	0.038	-0.125	0.044	-0.138	0.101	-0.232	0.066	-0.179	0.062	-0.173	0.083	-0.206	0.041	-0.131
9	0.064	-0.176	0.128	-0.263	0.040	-0.128	0.081	-0.204	0.029	-0.102	0.050	-0.150	0.060	-0.169	0.048	-0.145
10	0.022	-0.084	0.103	-0.235	0.048	-0.146	0.068	-0.182	0.064	-0.175	0.230	-0.338	0.049	-0.148	0.081	-0.204
11	0.016	-0.065			0.190	-0.316	0.113	-0.246	0.144	-0.279	0.045	-0.139	0.127	-0.262	0.073	-0.192
12	0.097	-0.226			0.044	-0.138	0.083	-0.207	0.088	-0.213	0.030	-0.105	0.015	-0.063	0.026	-0.095
13	0.117	-0.251			0.079	-0.201	0.059	-0.166	0.052	-0.153			0.023	-0.087	0.029	-0.102
14	0.142	-0.277			0.030	-0.105	0.045	-0.140	0.041	-0.131			0.024	-0.089	0.037	-0.122
15	0.079	-0.201			0.006	-0.029	0.024	-0.089					0.020	-0.078	0.030	-0.105
16	0.042	-0.133			0.012	-0.053							0.038	-0.123	0.059	-0.166
17	0.018	-0.071											0.038	-0.124	0.060	-0.169
18													0.009	-0.043	0.106	-0.238
19													0.009	-0.042	0.164	-0.297
20													0.030	-0.104	0.000	0.000
21													0.014	-0.059	0.090	-0.217
22													0.014	-0.059	0.078	-0.199
23													0.021	-0.081		
24													0.019	-0.076		
25													0.075	-0.194		
	H'	2.63	H'	1.98	H'	2.54	H'	2.59	H'	2.33	H'	1.90	H'	2.92	H'	2.71

Band	Pair 9	Control 1	Pair 9	Control 2	Pair 10	Control 1	Pair 10	Control 2	Pair 11	Control 1	Pair 11	Control 2	Pair 12	Control 1	Pair 12	Control 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.008	-0.039	0.085	-0.210	0.035	-0.117	0.015	-0.061	0.004	-0.020	0.039	-0.127	0.010	-0.046	0.003	-0.019
2	0.023	-0.086	0.066	-0.180	0.076	-0.197	0.009	-0.041	0.003	-0.016	0.032	-0.109	0.100	-0.230	0.058	-0.164
3	0.075	-0.195	0.052	-0.154	0.131	-0.266	0.055	-0.159	0.048	-0.145	0.059	-0.167	0.081	-0.203	0.120	-0.254
4	0.050	-0.150	0.055	-0.160	0.021	-0.081	0.044	-0.137	0.055	-0.159	0.045	-0.139	0.099	-0.229	0.069	-0.185
5	0.031	-0.108	0.038	-0.124	0.066	-0.179	0.042	-0.132	0.035	-0.117	0.124	-0.259	0.041	-0.131	0.041	-0.130
6	0.055	-0.159	0.038	-0.125	0.492	-0.349	0.458	-0.358	0.049	-0.149	0.081	-0.203	0.070	-0.186	0.037	-0.123
7	0.057	-0.163	0.053	-0.156	0.066	-0.179	0.053	-0.155	0.041	-0.132	0.046	-0.142	0.043	-0.136	0.046	-0.141
8	0.049	-0.147	0.011	-0.051	0.029	-0.103	0.030	-0.106	0.038	-0.125	0.067	-0.180	0.028	-0.100	0.030	-0.106
9	0.084	-0.208	0.018	-0.072	0.015	-0.064	0.152	-0.286	0.037	-0.121	0.064	-0.176	0.050	-0.151	0.076	-0.195
10	0.200	-0.322	0.078	-0.198	0.010	-0.047	0.060	-0.168	0.050	-0.150	0.021	-0.080	0.216	-0.331	0.056	-0.161
11	0.144	-0.279	0.053	-0.156	0.018	-0.072	0.049	-0.148	0.092	-0.220	0.028	-0.100	0.054	-0.157	0.092	-0.220
12	0.090	-0.217	0.074	-0.192	0.014	-0.061	0.034	-0.116	0.051	-0.152	0.038	-0.125	0.042	-0.134	0.075	-0.193
13	0.035	-0.117	0.081	-0.204	0.016	-0.065			0.064	-0.176	0.039	-0.126	0.052	-0.153	0.066	-0.179
14	0.035	-0.117	0.052	-0.154	0.012	-0.052			0.091	-0.218	0.044	-0.138	0.042	-0.134	0.068	-0.183
15	0.039	-0.127	0.075	-0.195					0.077	-0.197	0.016	-0.068	0.072	-0.190	0.049	-0.148
16	0.025	-0.092	0.055	-0.160					0.061	-0.170	0.042	-0.133			0.048	-0.145
17			0.049	-0.148					0.033	-0.113	0.038	-0.125			0.039	-0.126
18			0.035	-0.116					0.023	-0.087	0.070	-0.186			0.011	-0.050
19			0.031	-0.108					0.019	-0.074	0.076	-0.195			0.017	-0.069
20									0.016	-0.065	0.010	-0.045				
21									0.018	-0.071	0.008	-0.038				
22									0.010	-0.047	0.001	-0.008				
23									0.025	-0.093	0.014	-0.058				
24									0.011	-0.048						
25									0.009	-0.041						
26									0.013	-0.056						
27									0.020	-0.080						
28									0.009	-0.044						
	H'	2.53	H'	2.86	H'	1.83	H'	1.87	H'	3.08	H'	2.93	H'	2.51	H'	2.79

B.2 Intestinal Lavage Volunteers Data from Chapter 6

Band	Volunteer 1	Control	Volunteer 1	Day 4	Volunteer 1	Day 6	Volunteer 1	Day 6 #2	Volunteer 1	Day 7	Volunteer 2	Control	Volunteer 2	Day 1
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.037	-0.121	0.020	-0.077	0.005	-0.027	0.009	-0.041	0.044	-0.137	0.087	-0.212	0.099	-0.229
2	0.057	-0.164	0.032	-0.109	0.006	-0.031	0.014	-0.059	0.020	-0.078	0.080	-0.203	0.073	-0.190
3	0.074	-0.192	0.085	-0.210	0.046	-0.143	0.064	-0.175	0.058	-0.164	0.095	-0.224	0.065	-0.178
4	0.077	-0.197	0.092	-0.219	0.078	-0.200	0.084	-0.208	0.090	-0.216	0.045	-0.139	0.073	-0.191
5	0.237	-0.341	0.307	-0.363	0.314	-0.364	0.135	-0.270	0.180	-0.309	0.159	-0.292	0.139	-0.274
6	0.105	-0.236	0.107	-0.240	0.111	-0.244	0.114	-0.248	0.091	-0.218	0.070	-0.187	0.067	-0.181
7	0.162	-0.295	0.137	-0.273	0.118	-0.252	0.123	-0.258	0.144	-0.279	0.091	-0.219	0.092	-0.220
8	0.113	-0.246	0.104	-0.236	0.215	-0.330	0.153	-0.287	0.176	-0.306	0.122	-0.256	0.131	-0.266
9	0.013	-0.057	0.010	-0.046	0.018	-0.072	0.034	-0.116	0.010	-0.047	0.054	-0.158	0.043	-0.135
10	0.009	-0.044	0.023	-0.086	0.007	-0.034	0.021	-0.083	0.021	-0.082	0.027	-0.096	0.026	-0.094
11	0.010	-0.046	0.014	-0.059	0.000	-0.003	0.017	-0.069	0.015	-0.063	0.043	-0.136	0.033	-0.112
12	0.011	-0.051	0.013	-0.055	0.058	-0.165	0.040	-0.128	0.023	-0.088	0.022	-0.085	0.033	-0.113
13	0.038	-0.123	0.043	-0.134	0.007	-0.033	0.022	-0.085	0.016	-0.067	0.025	-0.091	0.021	-0.082
14	0.059	-0.167	0.005	-0.028	0.004	-0.022	0.084	-0.208	0.032	-0.109	0.016	-0.066	0.023	-0.087
15			0.009	-0.042	0.012	-0.054	0.017	-0.068	0.017	-0.069	0.034	-0.115	0.015	-0.063
16							0.027	-0.098	0.021	-0.080	0.022	-0.084	0.026	-0.095
17							0.043	-0.135	0.041	-0.131	0.003	-0.016	0.041	-0.131
18											0.006	-0.029		
	H'	2.28	H'	2.18	H'	1.97	H'	2.54	H'	2.45	H'	2.61	H'	2.64

Band	Volunteer 2	Day 2	Volunteer 2	Day 3	Volunteer 2	Day 4	Volunteer 2	Day 5	Volunteer 2	Day 6	Volunteer 2	Day 7	Volunteer 3	Control
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.101	-0.232	0.100	-0.230	0.076	-0.195	0.082	-0.205	0.056	-0.162	0.071	-0.187	0.034	-0.114
2	0.075	-0.195	0.037	-0.122	0.025	-0.093	0.040	-0.129	0.019	-0.076	0.050	-0.149	0.080	-0.201
3	0.049	-0.147	0.045	-0.140	0.032	-0.109	0.048	-0.145	0.030	-0.105	0.052	-0.153	0.096	-0.225
4	0.089	-0.215	0.173	-0.303	0.353	-0.368	0.068	-0.183	0.048	-0.146	0.039	-0.127	0.080	-0.201
5	0.161	-0.294	0.068	-0.183	0.078	-0.199	0.136	-0.271	0.067	-0.181	0.075	-0.195	0.121	-0.255
6	0.083	-0.207	0.136	-0.271	0.103	-0.235	0.076	-0.196	0.192	-0.317	0.138	-0.273	0.080	-0.202
7	0.187	-0.314	0.064	-0.176	0.196	-0.319	0.079	-0.200	0.059	-0.166	0.047	-0.143	0.080	-0.203
8	0.057	-0.163	0.041	-0.131	0.000	-0.003	0.074	-0.193	0.093	-0.221	0.088	-0.214	0.101	-0.231
9	0.025	-0.093	0.037	-0.121	0.008	-0.037	0.140	-0.276	0.082	-0.206	0.075	-0.194	0.091	-0.218
10	0.022	-0.084	0.049	-0.147	0.008	-0.038	0.073	-0.192	0.182	-0.310	0.128	-0.264	0.156	-0.289
11	0.008	-0.038	0.049	-0.148	0.044	-0.138	0.028	-0.100	0.046	-0.142	0.048	-0.145	0.054	-0.157
12	0.025	-0.092	0.067	-0.181	0.078	-0.198	0.024	-0.088	0.017	-0.070	0.027	-0.099	0.015	-0.064
13	0.041	-0.130	0.029	-0.102			0.017	-0.070	0.011	-0.051	0.020	-0.080	0.014	-0.059
14	0.025	-0.092	0.039	-0.127			0.051	-0.151	0.022	-0.084	0.037	-0.121		
15	0.021	-0.081	0.067	-0.180			0.024	-0.091	0.007	-0.036	0.029	-0.103		
16	0.009	-0.041					0.039	-0.126	0.021	-0.082	0.022	-0.084		
17	0.008	-0.040							0.031	-0.109	0.013	-0.056		
18	0.014	-0.059							0.015	-0.064	0.023	-0.087		
19											0.018	-0.072		
	H'	2.52	H'	2.56	H'	1.93	H'	2.62	H'	2.53	H'	2.75	H'	2.42

Band	Volunteer 3	Day 2	Volunteer 3	Day 3	Volunteer 3	Day 4	Volunteer 3	Day 5	Volunteer 3	Day 6	Volunteer 3	Day 7	Volunteer 4	Control
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.027	-0.098	0.049	-0.148	0.023	-0.087	0.018	-0.072	0.053	-0.157	0.015	-0.062	0.015	-0.062
2	0.127	-0.262	0.075	-0.194	0.078	-0.200	0.109	-0.242	0.099	-0.228	0.164	-0.296	0.074	-0.192
3	0.081	-0.204	0.050	-0.150	0.056	-0.162	0.065	-0.177	0.061	-0.171	0.066	-0.180	0.074	-0.192
4	0.115	-0.249	0.056	-0.162	0.089	-0.215	0.110	-0.242	0.053	-0.157	0.032	-0.110	0.112	-0.245
5	0.133	-0.269	0.063	-0.174	0.047	-0.144	0.186	-0.313	0.138	-0.273	0.097	-0.226	0.130	-0.265
6	0.068	-0.182	0.112	-0.246	0.169	-0.300	0.088	-0.213	0.095	-0.224	0.088	-0.213	0.114	-0.248
7	0.058	-0.165	0.097	-0.227	0.076	-0.195	0.048	-0.146	0.088	-0.214	0.084	-0.208	0.056	-0.162
8	0.072	-0.189	0.085	-0.210	0.096	-0.226	0.087	-0.212	0.092	-0.220	0.267	-0.353	0.113	-0.247
9	0.060	-0.168	0.114	-0.248	0.095	-0.223	0.160	-0.293	0.201	-0.322	0.040	-0.129	0.018	-0.074
10	0.105	-0.237	0.076	-0.196	0.135	-0.271	0.092	-0.219	0.079	-0.201	0.017	-0.070	0.052	-0.154
11	0.046	-0.141	0.028	-0.100	0.074	-0.193	0.038	-0.125	0.024	-0.091	0.075	-0.194	0.030	-0.105
12	0.032	-0.111	0.110	-0.243	0.061	-0.170			0.003	-0.019	0.021	-0.081	0.051	-0.151
13	0.010	-0.047	0.084	-0.208					0.011	-0.050	0.019	-0.076	0.141	-0.277
14	0.028	-0.100									0.015	-0.063	0.020	-0.078
15	0.026	-0.096												
16	0.011	-0.051												
	H'	2.57	H'	2.50	H'	2.39	H'	2.26	H'	2.33	H'	2.26	H'	2.45

Band	Volunteer 4	Day 1	Volunteer 4	Day 2	Volunteer 4	Day 3	Volunteer 4	Day 4	Volunteer 4	Day 5	Volunteer 4	Day 6	Volunteer 5	Control
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.005	-0.025	0.002	-0.010	0.003	-0.020	0.009	-0.044	0.014	-0.061	0.061	-0.170	0.020	-0.078
2	0.029	-0.102	0.049	-0.147	0.025	-0.093	0.033	-0.112	0.078	-0.198	0.167	-0.299	0.061	-0.170
3	0.101	-0.232	0.128	-0.263	0.067	-0.181	0.058	-0.165	0.098	-0.228	0.099	-0.228	0.033	-0.112
4	0.056	-0.161	0.111	-0.243	0.150	-0.285	0.052	-0.153	0.103	-0.234	0.099	-0.229	0.052	-0.155
5	0.206	-0.325	0.260	-0.350	0.158	-0.292	0.083	-0.207	0.151	-0.286	0.029	-0.102	0.073	-0.192
6	0.161	-0.294	0.184	-0.312	0.122	-0.257	0.185	-0.312	0.193	-0.317	0.187	-0.313	0.561	-0.324
7	0.087	-0.212	0.032	-0.111	0.036	-0.120	0.042	-0.132	0.050	-0.149	0.057	-0.163	0.156	-0.290
8	0.130	-0.265	0.093	-0.221	0.053	-0.155	0.049	-0.148	0.106	-0.238	0.030	-0.105	0.003	-0.020
9	0.027	-0.097	0.000	-0.003	0.040	-0.128	0.031	-0.107	0.034	-0.116	0.085	-0.210	0.005	-0.026
10	0.017	-0.068	0.013	-0.056	0.034	-0.115	0.033	-0.114	0.025	-0.093	0.126	-0.261	0.007	-0.035
11	0.024	-0.089	0.006	-0.029	0.064	-0.176	0.023	-0.086	0.032	-0.111	0.033	-0.114	0.006	-0.032
12	0.030	-0.105	0.033	-0.113	0.009	-0.044	0.258	-0.349	0.033	-0.112	0.010	-0.048	0.022	-0.085
13	0.128	-0.263	0.081	-0.204	0.043	-0.136	0.076	-0.196	0.071	-0.188	0.017	-0.069		
14			0.008	-0.040	0.132	-0.268	0.027	-0.098	0.011	-0.049				
15					0.024	-0.089	0.043	-0.134						
16					0.022	-0.084								
17					0.017	-0.070								
	H'	2.24	H'	2.10	H'	2.51	H'	2.36	H'	2.38	H'	2.31	H'	1.52
Band	Volunteer 5	Day 2	Volunteer 5	Day 3	Volunteer 5	Day 4	Volunteer 5	Day 5	Volunteer 5	Day 7	Volunteer 6	Control	Volunteer 6	Day 1
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.009	-0.044	0.034	-0.116	0.029	-0.103	0.014	-0.061	0.036	-0.120	0.039	-0.127	0.058	-0.164
2	0.026	-0.096	0.087	-0.213	0.080	-0.201	0.060	-0.168	0.079	-0.201	0.040	-0.129	0.021	-0.080
3	0.026	-0.095	0.037	-0.122	0.014	-0.061	0.046	-0.141	0.068	-0.183	0.071	-0.187	0.054	-0.158
4	0.032	-0.110	0.096	-0.225	0.046	-0.142	0.036	-0.120	0.079	-0.200	0.139	-0.275	0.147	-0.282
5	0.016	-0.066	0.086	-0.211	0.049	-0.147	0.069	-0.185	0.071	-0.188	0.135	-0.271	0.148	-0.283
6	0.358	-0.368	0.221	-0.334	0.089	-0.215	0.006	-0.029	0.105	-0.237	0.143	-0.278	0.070	-0.186
7	0.142	-0.277	0.223	-0.335	0.026	-0.094	0.166	-0.298	0.027	-0.097	0.183	-0.311	0.243	-0.344
8	0.009	-0.042	0.014	-0.061	0.045	-0.140	0.152	-0.286	0.049	-0.148	0.052	-0.154	0.013	-0.058
9	0.010	-0.046	0.065	-0.178	0.173	-0.304	0.006	-0.032	0.042	-0.133	0.040	-0.129	0.047	-0.144
10	0.116	-0.250	0.136	-0.271	0.134	-0.270	0.015	-0.063	0.156	-0.290	0.065	-0.177	0.045	-0.139
11	0.256	-0.349			0.012	-0.053	0.008	-0.039	0.156	-0.290	0.036	-0.119	0.066	-0.180
12					0.008	-0.037	0.016	-0.067	0.005	-0.029	0.057	-0.164	0.042	-0.133
13					0.012	-0.051	0.174	-0.304	0.006	-0.030			0.046	-0.141
14					0.014	-0.059	0.232	-0.339	0.003	-0.018				
15					0.115	-0.248			0.004	-0.022				
16					0.155	-0.289			0.045	-0.139				
17									0.069	-0.185				
	H'	1.74	H'	2.06	H'	2.41	H'	2.13	H'	2.51	H'	2.32	H'	2.29
Band	Volunteer 6	Day 2	Volunteer 6	Day 3	Volunteer 6	Day 4	Volunteer 6	Day 5	Volunteer 6	Day 6	Volunteer 7	Control	Volunteer 7	Day 1
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.058	-0.164	0.015	-0.064	0.021	-0.080	0.006	-0.030	0.091	-0.218	0.011	-0.050	0.013	-0.055
2	0.056	-0.161	0.051	-0.152	0.069	-0.184	0.051	-0.151	0.068	-0.183	0.008	-0.040	0.010	-0.045
3	0.110	-0.243	0.125	-0.260	0.106	-0.238	0.123	-0.258	0.089	-0.215	0.043	-0.135	0.059	-0.168
4	0.094	-0.223	0.142	-0.277	0.124	-0.259	0.099	-0.229	0.091	-0.217	0.071	-0.188	0.048	-0.145
5	0.063	-0.173	0.093	-0.221	0.048	-0.146	0.085	-0.209	0.042	-0.134	0.067	-0.181	0.084	-0.208
6	0.093	-0.220	0.258	-0.349	0.096	-0.224	0.360	-0.368	0.142	-0.277	0.044	-0.137	0.059	-0.166
7	0.054	-0.158	0.089	-0.215	0.180	-0.309	0.025	-0.091	0.075	-0.194	0.013	-0.057	0.023	-0.088
8	0.007	-0.033	0.068	-0.182	0.045	-0.139	0.034	-0.115	0.066	-0.179	0.047	-0.143	0.019	-0.074
9	0.105	-0.236	0.032	-0.110	0.042	-0.133	0.028	-0.099	0.041	-0.130	0.028	-0.101	0.029	-0.104
10	0.051	-0.151	0.051	-0.152	0.026	-0.094	0.085	-0.210	0.085	-0.209	0.011	-0.050	0.053	-0.156
11	0.028	-0.100	0.035	-0.117	0.076	-0.195	0.067	-0.182	0.040	-0.128	0.038	-0.124	0.051	-0.151
12	0.102	-0.233	0.042	-0.132	0.047	-0.145	0.038	-0.123	0.089	-0.215	0.043	-0.135	0.034	-0.114
13	0.087	-0.212			0.046	-0.142			0.083	-0.207	0.052	-0.153	0.068	-0.183
14	0.019	-0.076			0.040	-0.129					0.111	-0.243	0.040	-0.130
15	0.070	-0.186			0.014	-0.060					0.035	-0.116	0.030	-0.105
16	0.005	-0.026			0.021	-0.083					0.174	-0.304	0.150	-0.284
17											0.205	-0.325	0.231	-0.338
	H'	2.60	H'	2.23	H'	2.56	H'	2.07	H'	2.51	H'	2.48	H'	2.51

Band	Volunteer 7	Day 1 #2	Volunteer 7	Day 2	Volunteer 7	Day 2 #2	Volunteer 7	Day 3	Volunteer 7	Day 4	Volunteer 7	Day 5	Volunteer 7	Day 5 #2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.004	-0.024	0.007	-0.035	0.010	-0.046	0.011	-0.049	0.009	-0.043	0.011	-0.048	0.014	-0.059
2	0.004	-0.020	0.003	-0.019	0.011	-0.051	0.006	-0.030	0.010	-0.047	0.008	-0.040	0.008	-0.038
3	0.019	-0.077	0.038	-0.123	0.062	-0.172	0.049	-0.148	0.035	-0.117	0.059	-0.166	0.052	-0.153
4	0.041	-0.130	0.072	-0.190	0.085	-0.210	0.062	-0.172	0.041	-0.131	0.047	-0.143	0.074	-0.193
5	0.027	-0.097	0.057	-0.162	0.081	-0.204	0.069	-0.185	0.053	-0.156	0.044	-0.137	0.047	-0.144
6	0.015	-0.064	0.058	-0.166	0.056	-0.161	0.036	-0.121	0.064	-0.175	0.073	-0.190	0.055	-0.160
7	0.009	-0.041	0.016	-0.068	0.029	-0.103	0.027	-0.097	0.027	-0.097	0.024	-0.091	0.028	-0.100
8	0.007	-0.034	0.024	-0.090	0.029	-0.103	0.047	-0.143	0.029	-0.102	0.057	-0.163	0.029	-0.103
9	0.021	-0.080	0.045	-0.140	0.040	-0.129	0.046	-0.142	0.074	-0.192	0.087	-0.213	0.078	-0.199
10	0.020	-0.079	0.028	-0.101	0.057	-0.164	0.063	-0.174	0.098	-0.228	0.100	-0.230	0.070	-0.186
11	0.019	-0.076	0.029	-0.104	0.031	-0.109	0.037	-0.122	0.028	-0.100	0.013	-0.058	0.035	-0.118
12	0.025	-0.092	0.041	-0.132	0.051	-0.151	0.066	-0.179	0.041	-0.130	0.015	-0.065	0.016	-0.065
13	0.058	-0.165	0.050	-0.149	0.024	-0.089	0.036	-0.119	0.023	-0.087	0.059	-0.166	0.087	-0.212
14	0.044	-0.138	0.044	-0.138	0.019	-0.075	0.047	-0.143	0.032	-0.111	0.076	-0.196	0.045	-0.140
15	0.045	-0.139	0.054	-0.157	0.024	-0.091	0.027	-0.097	0.020	-0.077	0.040	-0.128	0.050	-0.151
16	0.316	-0.364	0.046	-0.142	0.001	-0.010	0.032	-0.109	0.009	-0.042	0.036	-0.119	0.017	-0.068
17	0.327	-0.365	0.083	-0.206	0.164	-0.296	0.151	-0.286	0.008	-0.038	0.028	-0.100	0.035	-0.117
18			0.039	-0.126	0.225	-0.335	0.189	-0.315	0.199	-0.321	0.029	-0.102	0.015	-0.064
19			0.121	-0.255					0.201	-0.323	0.063	-0.174	0.024	-0.091
20			0.144	-0.279							0.133	-0.268	0.091	-0.218
21													0.130	-0.265
	H'	1.98	H'	2.78	H'	2.50	H'	2.63	H'	2.52	H'	2.80	H'	2.84

Band	Volunteer 7	Day 7	Volunteer 8	Control	Volunteer 8	Day 2	Volunteer 8	Day 4	Volunteer 8	Day 5	Volunteer 9	Control	Volunteer 9	Day 3
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.011	-0.050	0.029	-0.102	0.023	-0.086	0.013	-0.057	0.067	-0.181	0.011	-0.048	0.002	-0.012
2	0.006	-0.032	0.109	-0.242	0.041	-0.132	0.054	-0.157	0.038	-0.123	0.058	-0.165	0.028	-0.101
3	0.058	-0.166	0.019	-0.074	0.047	-0.143	0.017	-0.069	0.062	-0.172	0.052	-0.154	0.049	-0.147
4	0.058	-0.166	0.185	-0.312	0.090	-0.217	0.070	-0.187	0.125	-0.260	0.075	-0.195	0.067	-0.181
5	0.036	-0.121	0.060	-0.169	0.047	-0.143	0.077	-0.197	0.102	-0.233	0.054	-0.157	0.019	-0.074
6	0.034	-0.116	0.219	-0.333	0.072	-0.189	0.087	-0.213	0.054	-0.157	0.055	-0.159	0.038	-0.123
7	0.019	-0.075	0.063	-0.174	0.050	-0.150	0.068	-0.183	0.037	-0.122	0.065	-0.177	0.033	-0.112
8	0.018	-0.073	0.010	-0.047	0.061	-0.170	0.047	-0.144	0.042	-0.133	0.043	-0.135	0.125	-0.260
9	0.039	-0.126	0.035	-0.118	0.050	-0.150	0.034	-0.114	0.026	-0.095	0.063	-0.175	0.040	-0.128
10	0.069	-0.185	0.097	-0.226	0.020	-0.078	0.019	-0.074	0.003	-0.019	0.059	-0.167	0.014	-0.061
11	0.026	-0.095	0.020	-0.078	0.043	-0.136	0.023	-0.086	0.011	-0.048	0.030	-0.106	0.040	-0.129
12	0.030	-0.106	0.028	-0.100	0.084	-0.208	0.023	-0.088	0.022	-0.085	0.041	-0.132	0.040	-0.129
13	0.049	-0.147	0.033	-0.112	0.017	-0.071	0.021	-0.082	0.019	-0.075	0.020	-0.078	0.013	-0.057
14	0.043	-0.136	0.051	-0.152	0.064	-0.175	0.024	-0.091	0.017	-0.071	0.020	-0.080	0.025	-0.092
15	0.041	-0.131	0.042	-0.133	0.042	-0.133	0.033	-0.113	0.008	-0.040	0.012	-0.052	0.014	-0.060
16	0.014	-0.061			0.019	-0.076	0.011	-0.051	0.023	-0.086	0.023	-0.087	0.246	-0.345
17	0.016	-0.066			0.030	-0.105	0.059	-0.167	0.010	-0.044	0.015	-0.063	0.208	-0.327
18	0.023	-0.086			0.078	-0.199	0.017	-0.070	0.013	-0.057	0.162	-0.295		
19	0.019	-0.076			0.028	-0.099	0.037	-0.121	0.144	-0.279	0.142	-0.277		
20	0.179	-0.308			0.042	-0.134	0.013	-0.057	0.177	-0.306				
21	0.209	-0.327			0.052	-0.154	0.012	-0.052						
22							0.126	-0.262						
23							0.114	-0.247						
	H'	2.65	H'	2.37	H'	2.95	H'	2.88	H'	2.59	H'	2.70	H'	2.34

Band	Volunteer 9	Day 7	Volunteer 10	Control	Volunteer 10	Day 3	Volunteer 10	Day 4	Volunteer 10	Day 5	Volunteer 10	Day 6
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.021	-0.082	0.053	-0.155	0.016	-0.065	0.032	-0.110	0.034	-0.115	0.030	-0.105
2	0.052	-0.153	0.043	-0.136	0.026	-0.096	0.076	-0.196	0.068	-0.183	0.098	-0.227
3	0.060	-0.168	0.068	-0.183	0.067	-0.181	0.166	-0.298	0.084	-0.208	0.133	-0.269
4	0.052	-0.153	0.112	-0.245	0.040	-0.130	0.121	-0.256	0.106	-0.238	0.057	-0.164
5	0.085	-0.209	0.074	-0.192	0.062	-0.172	0.083	-0.207	0.106	-0.237	0.079	-0.201
6	0.133	-0.268	0.091	-0.218	0.039	-0.126	0.088	-0.214	0.059	-0.168	0.103	-0.235
7	0.064	-0.177	0.037	-0.123	0.073	-0.191	0.093	-0.221	0.093	-0.221	0.064	-0.177
8	0.052	-0.153	0.073	-0.191	0.042	-0.134	0.068	-0.183	0.071	-0.187	0.059	-0.168
9	0.034	-0.116	0.056	-0.161	0.020	-0.077	0.057	-0.163	0.041	-0.131	0.064	-0.175
10	0.057	-0.163	0.132	-0.267	0.032	-0.110	0.096	-0.225	0.049	-0.147	0.023	-0.086
11	0.047	-0.143	0.114	-0.248	0.050	-0.149	0.030	-0.105	0.050	-0.149	0.036	-0.120
12	0.049	-0.147	0.014	-0.059	0.061	-0.171	0.026	-0.094	0.024	-0.089	0.052	-0.153
13	0.029	-0.102	0.022	-0.085	0.037	-0.123	0.016	-0.067	0.007	-0.036	0.051	-0.151
14	0.137	-0.273	0.017	-0.070	0.056	-0.161	0.004	-0.022	0.014	-0.059	0.054	-0.157
15	0.129	-0.265	0.061	-0.170	0.027	-0.097	0.002	-0.012	0.014	-0.060	0.097	-0.226
16			0.025	-0.092	0.117	-0.251	0.005	-0.025	0.056	-0.162		
17			0.003	-0.019	0.235	-0.340	0.012	-0.052	0.124	-0.259		
18			0.002	-0.013			0.025	-0.093				
19			0.002	-0.012								
	H'	2.57	H'	2.64	H'	2.57	H'	2.54	H'	2.65	H'	2.61

B3 Western Diet Data from Chapter 7

Band	Volunteer 1	Week 1	Volunteer 1	Week 2	Volunteer 1	Week 3	Volunteer 1	Week 4	Volunteer 2	Week 1	Volunteer 2	Week 2	Volunteer 2	Week 3
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.032	-0.110	0.012	-0.054	0.008	-0.038	0.007	-0.035	0.000	-0.003	0.006	-0.029	0.006	-0.03
2	0.075	-0.195	0.059	-0.167	0.041	-0.131	0.002	-0.011	0.004	-0.020	0.028	-0.099	0.022	-0.08
3	0.112	-0.245	0.159	-0.292	0.064	-0.175	0.021	-0.081	0.009	-0.043	0.076	-0.195	0.146	-0.28
4	0.045	-0.140	0.050	-0.149	0.070	-0.186	0.066	-0.180	0.069	-0.184	0.051	-0.151	0.091	-0.22
5	0.074	-0.193	0.066	-0.179	0.064	-0.176	0.033	-0.113	0.024	-0.091	0.109	-0.241	0.070	-0.19
6	0.047	-0.144	0.085	-0.209	0.143	-0.278	0.068	-0.183	0.099	-0.230	0.141	-0.276	0.114	-0.25
7	0.285	-0.358	0.053	-0.155	0.075	-0.194	0.076	-0.196	0.049	-0.147	0.161	-0.294	0.103	-0.23
8	0.089	-0.216	0.038	-0.125	0.052	-0.153	0.067	-0.180	0.113	-0.247	0.114	-0.247	0.095	-0.22
9	0.116	-0.250	0.204	-0.324	0.223	-0.335	0.037	-0.121	0.101	-0.232	0.073	-0.191	0.054	-0.16
10	0.040	-0.128	0.042	-0.132	0.082	-0.206	0.225	-0.336	0.151	-0.285	0.099	-0.229	0.139	-0.27
11	0.038	-0.125	0.043	-0.135	0.092	-0.220	0.043	-0.135	0.035	-0.118	0.025	-0.092	0.055	-0.16
12	0.045	-0.139	0.040	-0.130	0.021	-0.082	0.063	-0.173	0.056	-0.161	0.068	-0.183	0.053	-0.16
13			0.084	-0.209	0.021	-0.081	0.042	-0.134	0.128	-0.264	0.029	-0.101	0.028	-0.1
14			0.031	-0.107	0.014	-0.059	0.015	-0.063	0.038	-0.125	0.004	-0.021	0.022	-0.08
15			0.035	-0.116	0.030	-0.106	0.060	-0.168	0.011	-0.050	0.018	-0.073		
16							0.176	-0.306	0.029	-0.102				
17									0.023	-0.087				
18									0.027	-0.097				
19									0.018	-0.071				
20									0.016	-0.066				
	H'	2.24	H'	2.48	H'	2.42	H'	2.41	H'	2.62	H'	2.42	H'	2.44

Band	Volunteer 2	Week 4	Volunteer 3	Week 1	Volunteer 3	Week 2	Volunteer 3	Week 3	Volunteer 3	Week 4	Volunteer 4	Week 1	Volunteer 4	Week 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.007	-0.035	0.005	-0.028	0.001	-0.006	0.003	-0.017	0.003	-0.016	0.007	-0.034	0.011	-0.05
2	0.027	-0.098	0.003	-0.019	0.002	-0.011	0.004	-0.021	0.004	-0.021	0.010	-0.045	0.010	-0.04
3	0.091	-0.218	0.023	-0.088	0.011	-0.051	0.037	-0.122	0.039	-0.127	0.013	-0.058	0.029	-0.1
4	0.067	-0.182	0.103	-0.234	0.161	-0.294	0.065	-0.178	0.100	-0.230	0.015	-0.064	0.047	-0.14
5	0.071	-0.188	0.016	-0.065	0.047	-0.143	0.035	-0.117	0.075	-0.194	0.087	-0.213	0.096	-0.22
6	0.094	-0.223	0.100	-0.230	0.057	-0.163	0.029	-0.101	0.078	-0.200	0.050	-0.150	0.065	-0.18
7	0.050	-0.150	0.078	-0.199	0.086	-0.210	0.050	-0.150	0.040	-0.128	0.092	-0.220	0.091	-0.22
8	0.046	-0.142	0.099	-0.229	0.119	-0.254	0.081	-0.203	0.076	-0.196	0.075	-0.194	0.109	-0.24
9	0.064	-0.177	0.054	-0.159	0.030	-0.106	0.046	-0.142	0.041	-0.131	0.070	-0.186	0.059	-0.17
10	0.042	-0.134	0.210	-0.328	0.261	-0.351	0.051	-0.152	0.026	-0.095	0.049	-0.147	0.060	-0.17
11	0.026	-0.094	0.056	-0.162	0.032	-0.110	0.220	-0.333	0.194	-0.318	0.056	-0.161	0.034	-0.12
12	0.095	-0.223	0.058	-0.165	0.044	-0.136	0.076	-0.196	0.082	-0.205	0.023	-0.088	0.036	-0.12
13	0.089	-0.215	0.063	-0.175	0.051	-0.151	0.119	-0.253	0.125	-0.260	0.035	-0.118	0.030	-0.11
14	0.112	-0.245	0.047	-0.144	0.056	-0.161	0.083	-0.206	0.071	-0.187	0.030	-0.106	0.052	-0.15
15	0.045	-0.140	0.062	-0.173	0.030	-0.106	0.032	-0.111	0.011	-0.051	0.020	-0.077	0.210	-0.33
16	0.043	-0.136	0.020	-0.078	0.013	-0.057	0.045	-0.140	0.017	-0.071	0.298	-0.361	0.038	-0.12
17	0.030	-0.104					0.024	-0.090	0.017	-0.069	0.044	-0.137	0.024	-0.09
18											0.002	-0.010		
19											0.025	-0.091		
	H'	2.70	H'	2.48	H'	2.31	H'	2.53	H'	2.50	H'	2.46	H'	2.57

Band	Volunteer 4	Week 3	Volunteer 4	Week 4	Volunteer 5	Week 1	Volunteer 5	Week 2	Volunteer 5	Week 3	Volunteer 5	Week 4	Volunteer 6	Week 1
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.004	-0.023	0.013	-0.058	0.010	-0.047	0.025	-0.092	0.020	-0.079	0.004	-0.024	0.021	-0.08
2	0.003	-0.017	0.004	-0.021	0.040	-0.128	0.030	-0.106	0.047	-0.143	0.018	-0.074	0.016	-0.07
3	0.002	-0.013	0.107	-0.239	0.052	-0.154	0.046	-0.142	0.034	-0.116	0.017	-0.068	0.052	-0.15
4	0.105	-0.236	0.039	-0.127	0.111	-0.244	0.054	-0.158	0.024	-0.089	0.019	-0.076	0.046	-0.14
5	0.024	-0.091	0.064	-0.177	0.071	-0.188	0.079	-0.200	0.039	-0.127	0.025	-0.091	0.083	-0.21
6	0.044	-0.138	0.025	-0.091	0.092	-0.219	0.091	-0.218	0.071	-0.188	0.066	-0.179	0.018	-0.07
7	0.157	-0.290	0.017	-0.070	0.042	-0.134	0.039	-0.127	0.051	-0.152	0.017	-0.069	0.028	-0.1
8	0.045	-0.140	0.015	-0.062	0.042	-0.134	0.305	-0.362	0.333	-0.366	0.165	-0.297	0.051	-0.15
9	0.028	-0.101	0.038	-0.125	0.210	-0.328	0.031	-0.107	0.035	-0.118	0.013	-0.058	0.063	-0.17
10	0.030	-0.105	0.272	-0.354	0.054	-0.157	0.055	-0.159	0.030	-0.106	0.014	-0.059	0.121	-0.26
11	0.018	-0.072	0.011	-0.051	0.045	-0.139	0.047	-0.143	0.046	-0.141	0.015	-0.064	0.026	-0.09
12	0.020	-0.079	0.078	-0.198	0.023	-0.086	0.036	-0.120	0.043	-0.136	0.003	-0.017	0.021	-0.08
13	0.149	-0.283	0.317	-0.364	0.090	-0.217	0.101	-0.231	0.012	-0.052	0.063	-0.173	0.038	-0.12
14	0.042	-0.134			0.061	-0.171	0.017	-0.069	0.047	-0.143	0.150	-0.285	0.048	-0.15
15	0.328	-0.366			0.008	-0.039	0.044	-0.137	0.042	-0.134	0.411	-0.365	0.036	-0.12
16					0.024	-0.088			0.125	-0.260			0.028	-0.1
17					0.026	-0.094							0.080	-0.2
18													0.032	-0.11
19													0.082	-0.21
20													0.019	-0.07
21													0.012	-0.05
22													0.010	-0.05
23													0.069	-0.19
	H'	2.09	H'	1.94	H'	2.57	H'	2.37	H'	2.35	H'	1.90	H'	2.95

Band	Volunteer 6	Week 2	Volunteer 6	Week 3	Volunteer 6	Week 4
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.018	-0.073	0.022	-0.083	0.011	-0.051
2	0.051	-0.151	0.068	-0.183	0.072	-0.190
3	0.050	-0.150	0.079	-0.200	0.047	-0.143
4	0.029	-0.102	0.114	-0.248	0.069	-0.184
5	0.044	-0.137	0.115	-0.249	0.111	-0.244
6	0.072	-0.189	0.100	-0.230	0.089	-0.215
7	0.060	-0.170	0.056	-0.162	0.067	-0.181
8	0.028	-0.100	0.053	-0.156	0.038	-0.125
9	0.056	-0.161	0.044	-0.137	0.043	-0.136
10	0.056	-0.162	0.079	-0.200	0.083	-0.207
11	0.042	-0.133	0.151	-0.285	0.054	-0.157
12	0.076	-0.196	0.069	-0.184	0.091	-0.218
13	0.043	-0.136	0.011	-0.051	0.038	-0.125
14	0.050	-0.150	0.016	-0.068	0.045	-0.139
15	0.072	-0.190	0.014	-0.060	0.034	-0.115
16	0.037	-0.122	0.008	-0.039	0.028	-0.101
17	0.071	-0.188			0.079	-0.200
18	0.026	-0.094				
19	0.025	-0.093				
20	0.016	-0.065				
21	0.077	-0.198				
	H'	2.96	H'	2.54	H'	2.73

B.4 Atkins' Diet Data from Chapter 7

Band	Volunteer 1	Day 0	Volunteer 1	Week 2	Volunteer 1	Week 3	Volunteer 1	Week 4	Volunteer 2	Day 0	Volunteer 2	Week 2	Volunteer 2	Week 3
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.00923	-0.04	0.01284	-0.06	0.01164	-0.05	0.01597	-0.07	0.01416	-0.06	0.01291	-0.06	0.01761	-0.07
2	0.01536	-0.06	0.01599	-0.07	0.02875	-0.1	0.02456	-0.09	0.03533	-0.12	0.02664	-0.1	0.02186	-0.08
3	0.01145	-0.05	0.04649	-0.14	0.0775	-0.2	0.08141	-0.2	0.09626	-0.23	0.05501	-0.16	0.05576	-0.16
4	0.07148	-0.19	0.08186	-0.2	0.08841	-0.21	0.12798	-0.26	0.091	-0.22	0.09226	-0.22	0.16272	-0.3
5	0.13867	-0.27	0.05066	-0.15	0.10575	-0.24	0.1381	-0.27	0.12897	-0.26	0.06061	-0.17	0.16382	-0.3
6	0.03644	-0.12	0.03081	-0.11	0.07601	-0.2	0.12752	-0.26	0.04219	-0.13	0.05607	-0.16	0.08217	-0.21
7	0.06475	-0.18	0.08152	-0.2	0.10404	-0.24	0.08429	-0.21	0.05672	-0.16	0.0633	-0.17	0.09464	-0.22
8	0.08881	-0.22	0.08046	-0.2	0.08748	-0.21	0.0573	-0.16	0.06238	-0.17	0.05544	-0.16	0.09431	-0.22
9	0.08945	-0.22	0.07798	-0.2	0.13315	-0.27	0.05197	-0.15	0.03813	-0.12	0.04706	-0.14	0.05101	-0.15
10	0.07584	-0.2	0.06958	-0.19	0.03133	-0.11	0.07815	-0.2	0.05925	-0.17	0.05325	-0.16	0.04424	-0.14
11	0.05421	-0.16	0.0189	-0.08	0.07289	-0.19	0.05188	-0.15	0.0525	-0.15	0.03112	-0.11	0.03819	-0.12
12	0.03399	-0.11	0.03837	-0.13	0.06152	-0.17	0.01653	-0.07	0.06867	-0.18	0.03415	-0.12	0.02953	-0.1
13	0.02863	-0.1	0.0299	-0.1	0.01689	-0.07	0.01636	-0.07	0.03968	-0.13	0.05515	-0.16	0.03843	-0.13
14	0.04659	-0.14	0.02722	-0.1	0.01643	-0.07	0.05219	-0.15	0.01049	-0.05	0.05188	-0.15	0.0251	-0.09
15	0.03668	-0.12	0.03614	-0.12	0.04189	-0.13	0.01795	-0.07	0.05747	-0.16	0.05182	-0.15	0.03697	-0.12
16	0.03487	-0.12	0.03042	-0.11	0.01173	-0.05	0.02767	-0.1	0.14679	-0.28	0.00859	-0.04	0.04364	-0.14
17	0.02254	-0.09	0.03592	-0.12	0.03459	-0.12	0.03019	-0.11			0.07068	-0.19		
18	0.05291	-0.16	0.01636	-0.07							0.02847	-0.1		
19	0.02101	-0.08	0.04726	-0.14							0.01425	-0.06		
20	0.02122	-0.08	0.02873	-0.1							0.13134	-0.27		
21	0.02263	-0.09	0.03901	-0.13										
22	0.02323	-0.09	0.09511	-0.22										
			0.00845	-0.04										
	H'	2.879	H'	2.973	H'	2.626	H'	2.605	H'	2.608	H'	2.845	H'	2.553

Band	Volunteer 2	Week 4	Volunteer 3	Day 0	Volunteer 3	Week 2	Volunteer 3	Week 3	Volunteer 3	Week 4	Volunteer 4	Day 0	Volunteer 4	Week 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.02565	-0.09	0.00302	-0.02	0.01241	-0.05	0.00561	-0.03	0.01222	-0.05	0.01196	-0.05	0.01424	-0.06
2	0.02777	-0.1	0.00459	-0.02	0.01422	-0.06	0.0159	-0.07	0.03958	-0.13	0.00812	-0.04	0.02361	-0.09
3	0.07359	-0.19	0.01411	-0.06	0.05771	-0.16	0.05704	-0.16	0.12232	-0.26	0.03516	-0.12	0.07383	-0.19
4	0.11983	-0.25	0.06106	-0.17	0.13197	-0.27	0.07611	-0.2	0.08745	-0.21	0.09222	-0.22	0.10152	-0.23
5	0.16421	-0.3	0.13708	-0.27	0.05531	-0.16	0.10563	-0.24	0.08402	-0.21	0.13832	-0.27	0.07315	-0.19
6	0.10804	-0.24	0.07842	-0.2	0.07801	-0.2	0.08604	-0.21	0.06929	-0.18	0.08501	-0.21	0.15474	-0.29
7	0.10291	-0.23	0.13773	-0.27	0.05194	-0.15	0.02575	-0.09	0.04474	-0.14	0.05094	-0.15	0.11305	-0.25
8	0.0738	-0.19	0.13729	-0.27	0.06477	-0.18	0.06935	-0.19	0.02026	-0.08	0.02766	-0.1	0.07746	-0.2
9	0.06591	-0.18	0.03562	-0.12	0.07538	-0.19	0.09354	-0.22	0.07928	-0.2	0.0477	-0.15	0.01997	-0.08
10	0.0457	-0.14	0.05457	-0.16	0.0646	-0.18	0.05356	-0.16	0.04028	-0.13	0.16823	-0.3	0.01557	-0.06
11	0.03497	-0.12	0.07613	-0.2	0.15207	-0.29	0.04428	-0.14	0.0369	-0.12	0.02959	-0.1	0.02259	-0.09
12	0.02513	-0.09	0.06637	-0.18	0.05075	-0.15	0.04192	-0.13	0.0377	-0.12	0.06317	-0.17	0.0039	-0.02
13	0.03142	-0.11	0.02894	-0.1	0.09831	-0.23	0.03166	-0.11	0.04508	-0.14	0.24193	-0.34	0.02694	-0.1
14	0.03533	-0.12	0.06259	-0.17	0.052	-0.15	0.11556	-0.25	0.05062	-0.15			0.02192	-0.08
15	0.036	-0.12	0.05627	-0.16	0.0256	-0.09	0.06812	-0.18	0.0904	-0.22			0.07831	-0.2
16	0.02972	-0.1	0.01093	-0.05	0.01496	-0.06	0.06103	-0.17	0.03666	-0.12			0.1792	-0.31
17			0.03527	-0.12			0.03938	-0.13	0.05135	-0.15				
18							0.00952	-0.04	0.05185	-0.15				
	H'	2.584	H'	2.55	H'	2.585	H'	2.716	H'	2.774	H'	2.231	H'	2.437

Band	Volunteer 4	Week 3	Volunteer 4	Week 4	Volunteer 5	Day 0	Volunteer 5	Week 2	Volunteer 5	Week 3	Volunteer 5	Week 4	Volunteer 6	Day 0
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.02946	-0.1	0.02043	-0.08	0.00321	-0.02	0.00151	-0.01	0.00133	-0.01	0.01366	-0.06	0.01894	-0.08
2	0.03634	-0.12	0.04676	-0.14	0.00387	-0.02	0.00038	-0	0.00123	-0.01	0.00863	-0.04	0.01707	-0.07
3	0.01035	-0.05	0.04007	-0.13	0.00389	-0.02	0.00566	-0.03	0.00484	-0.03	0.04722	-0.14	0.09803	-0.23
4	0.13218	-0.27	0.07607	-0.2	0.0814	-0.2	0.0108	-0.05	0.0131	-0.06	0.08481	-0.21	0.04665	-0.14
5	0.05673	-0.16	0.0953	-0.22	0.04645	-0.14	0.01239	-0.05	0.02967	-0.1	0.07249	-0.19	0.05319	-0.16
6	0.09908	-0.23	0.08178	-0.2	0.09986	-0.23	0.04554	-0.14	0.03329	-0.11	0.09455	-0.22	0.15905	-0.29
7	0.11898	-0.25	0.15234	-0.29	0.09533	-0.22	0.09954	-0.23	0.04753	-0.14	0.03913	-0.13	0.11176	-0.24
8	0.10245	-0.23	0.0578	-0.16	0.09646	-0.23	0.06116	-0.17	0.08985	-0.22	0.04144	-0.13	0.02654	-0.1
9	0.01874	-0.07	0.05366	-0.16	0.1211	-0.26	0.03445	-0.12	0.10242	-0.23	0.00975	-0.05	0.04233	-0.13
10	0.032	-0.11	0.00469	-0.03	0.05093	-0.15	0.03245	-0.11	0.03021	-0.11	0.01785	-0.07	0.05746	-0.16
11	0.02409	-0.09	0.02262	-0.09	0.02792	-0.1	0.01685	-0.07	0.03126	-0.11	0.04941	-0.15	0.05842	-0.17
12	0.02067	-0.08	0.03876	-0.13	0.03943	-0.13	0.03593	-0.12	0.05372	-0.16	0.04817	-0.15	0.07924	-0.2
13	0.02308	-0.09	0.01869	-0.07	0.04155	-0.13	0.03544	-0.12	0.06721	-0.18	0.14247	-0.28	0.04353	-0.14
14	0.01889	-0.07	0.0114	-0.05	0.04887	-0.15	0.01906	-0.08	0.0338	-0.11	0.32936	-0.37	0.04988	-0.15
15	0.0176	-0.07	0.02013	-0.08	0.02366	-0.09	0.02553	-0.09	0.0348	-0.12	0.00107	-0.01	0.01906	-0.08
16	0.04441	-0.14	0.01592	-0.07	0.03791	-0.12	0.05305	-0.16	0.0147	-0.06			0.02782	-0.1
17	0.0234	-0.09	0.06526	-0.18	0.02139	-0.08	0.02798	-0.1	0.03332	-0.11			0.05464	-0.16
18	0.06159	-0.17	0.01569	-0.07	0.15676	-0.29	0.02973	-0.1	0.10601	-0.24			0.00934	-0.04
19	0.0175	-0.07	0.01392	-0.06			0.12697	-0.26	0.26466	-0.35			0.02703	-0.1
20	0.11245	-0.25	0.02036	-0.08			0.32335	-0.37	0.00705	-0.03				
21			0.12835	-0.26			0.00223	-0.01						
	H'	2.72	H'	2.737	H'	2.588	H'	2.391	H'	2.496	H'	2.187	H'	2.731

Band	Volunteer 6	Week 2	Volunteer 6	Week 3	Volunteer 6	Week 4
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.00395	-0.02	0.00145	-0.01	0.00122	-0.01
2	0.01898	-0.08	0.00204	-0.01	0.00175	-0.01
3	0.0286	-0.1	0.00928	-0.04	0.00686	-0.03
4	0.03168	-0.11	0.02945	-0.1	0.01713	-0.07
5	0.10163	-0.23	0.04665	-0.14	0.00957	-0.04
6	0.08163	-0.2	0.15522	-0.29	0.03426	-0.12
7	0.04744	-0.14	0.10403	-0.24	0.1225	-0.26
8	0.02101	-0.08	0.03195	-0.11	0.10345	-0.23
9	0.00711	-0.04	0.01158	-0.05	0.08453	-0.21
10	0.02897	-0.1	0.0487	-0.15	0.04075	-0.13
11	0.06115	-0.17	0.03831	-0.12	0.05851	-0.17
12	0.04731	-0.14	0.01408	-0.06	0.03409	-0.12
13	0.02794	-0.1	0.01191	-0.05	0.01803	-0.07
14	0.01295	-0.06	0.00057	-0	0.05482	-0.16
15	0.01015	-0.05	0.01319	-0.06	0.0352	-0.12
16	0.0203	-0.08	0.00914	-0.04	0.02119	-0.08
17	0.01639	-0.07	0.11975	-0.25	0.09293	-0.22
18	0.00826	-0.04	0.34516	-0.37	0.25716	-0.35
19	0.13212	-0.27	0.00754	-0.04	0.00605	-0.03
20	0.29245	-0.36				
	H'	2.44	H'	2.146	H'	2.428

B.5 Disease Group Patients Data from Chapter 8

Band	IBD-CD	Patient 1	IBD-CD	Patient 2	IBD-CD	Patient 3	IBD-CD	Patient 4	IBD-CD	Patient 5	IBD-CD	Patient 6	IBD-CD	Patient 7
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.009	-0.043	0.010	-0.047	0.026	-0.094	0.021	-0.082	0.014	-0.059	0.034	-0.114	0.005	-0.025
2	0.059	-0.167	0.009	-0.041	0.012	-0.053	0.072	-0.190	0.015	-0.064	0.099	-0.229	0.002	-0.012
3	0.102	-0.233	0.066	-0.179	0.049	-0.148	0.133	-0.269	0.034	-0.116	0.068	-0.183	0.041	-0.131
4	0.043	-0.135	0.172	-0.303	0.106	-0.238	0.097	-0.226	0.102	-0.233	0.056	-0.162	0.068	-0.182
5	0.075	-0.194	0.089	-0.215	0.081	-0.203	0.074	-0.192	0.104	-0.236	0.047	-0.143	0.083	-0.207
6	0.126	-0.261	0.080	-0.202	0.097	-0.227	0.046	-0.142	0.151	-0.285	0.034	-0.115	0.085	-0.209
7	0.152	-0.286	0.088	-0.214	0.064	-0.175	0.064	-0.176	0.037	-0.122	0.056	-0.162	0.061	-0.170
8	0.144	-0.279	0.080	-0.202	0.032	-0.110	0.049	-0.147	0.084	-0.208	0.060	-0.169	0.085	-0.210
9	0.169	-0.300	0.110	-0.242	0.087	-0.212	0.099	-0.229	0.037	-0.122	0.099	-0.229	0.055	-0.160
10	0.020	-0.079	0.081	-0.203	0.039	-0.126	0.054	-0.159	0.047	-0.144	0.061	-0.171	0.063	-0.174
11	0.009	-0.042	0.056	-0.162	0.076	-0.196	0.077	-0.198	0.052	-0.153	0.084	-0.209	0.033	-0.112
12	0.010	-0.047	0.085	-0.209	0.061	-0.171	0.149	-0.284	0.086	-0.212	0.064	-0.175	0.029	-0.104
13	0.023	-0.086	0.018	-0.073	0.045	-0.140	0.025	-0.092	0.066	-0.179	0.076	-0.196	0.020	-0.078
14	0.043	-0.135	0.008	-0.038	0.053	-0.157	0.038	-0.125	0.086	-0.210	0.065	-0.178	0.014	-0.060
15	0.017	-0.068	0.043	-0.136	0.051	-0.152			0.057	-0.164	0.042	-0.134	0.175	-0.305
16			0.005	-0.027	0.070	-0.186			0.027	-0.098	0.053	-0.155	0.182	-0.310
17					0.023	-0.086								
18					0.029	-0.103								
	H'	2.36	H'	2.49	H'	2.78	H'	2.51	H'	2.60	H'	2.73	H'	2.45

Band	IBD-CD	Patient 8	IBD-CD	Patient 9	IBD-UC	Patient 1	IBD-UC	Patient 2	IBD-UC	Patient 3	IBD-UC	Patient 4	IBD-UC	Patient 5
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.009	-0.043	0.060	-0.169	0.036	-0.120	0.061	-0.170	0.023	-0.087	0.049	-0.147	0.013	-0.057
2	0.047	-0.143	0.177	-0.307	0.066	-0.180	0.034	-0.115	0.034	-0.115	0.171	-0.302	0.040	-0.128
3	0.093	-0.221	0.166	-0.298	0.238	-0.342	0.383	-0.368	0.042	-0.134	0.115	-0.249	0.028	-0.099
4	0.111	-0.243	0.083	-0.207	0.060	-0.168	0.184	-0.311	0.061	-0.171	0.100	-0.230	0.016	-0.066
5	0.053	-0.156	0.051	-0.152	0.061	-0.170	0.109	-0.242	0.202	-0.323	0.156	-0.290	0.049	-0.147
6	0.113	-0.246	0.076	-0.196	0.065	-0.179	0.109	-0.242	0.072	-0.189	0.080	-0.202	0.075	-0.194
7	0.070	-0.186	0.019	-0.076	0.079	-0.200	0.007	-0.037	0.058	-0.165	0.139	-0.274	0.099	-0.229
8	0.053	-0.155	0.107	-0.239	0.059	-0.167	0.054	-0.157	0.039	-0.125	0.106	-0.237	0.051	-0.152
9	0.093	-0.220	0.062	-0.172	0.049	-0.148	0.034	-0.116	0.057	-0.164	0.085	-0.210	0.028	-0.099
10	0.080	-0.203	0.102	-0.232	0.074	-0.193	0.001	-0.008	0.286	-0.358			0.183	-0.311
11	0.089	-0.215	0.012	-0.053	0.065	-0.177	0.005	-0.027	0.033	-0.112			0.040	-0.128
12	0.087	-0.212	0.017	-0.068	0.065	-0.178	0.019	-0.076	0.054	-0.157			0.081	-0.204
13	0.056	-0.162	0.004	-0.022	0.048	-0.146			0.040	-0.128			0.040	-0.128
14	0.047	-0.144	0.042	-0.133	0.034	-0.114							0.055	-0.160
15			0.021	-0.083									0.020	-0.080
16													0.017	-0.070
17													0.033	-0.111
18													0.036	-0.119
19													0.047	-0.143
20													0.050	-0.151
	H'	2.55	H'	2.41	H'	2.48	H'	1.87	H'	2.23	H'	2.14	H'	2.78

Band	IBD-UC	Patient 6	IBD-UC	Patient 7	IBD-UC	Patient 8	IBD-UC	Patient 9	IBD-UC	Patient 10	IBS-M	Patient 1	IBS-M	Patient 2
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.044	-0.138	0.008	-0.040	0.010	-0.046	0.022	-0.083	0.032	-0.109	0.042	-0.133	0.025	-0.092
2	0.066	-0.180	0.026	-0.094	0.036	-0.120	0.046	-0.141	0.052	-0.154	0.072	-0.189	0.074	-0.193
3	0.045	-0.140	0.029	-0.103	0.087	-0.213	0.060	-0.169	0.035	-0.117	0.096	-0.225	0.030	-0.104
4	0.136	-0.271	0.096	-0.225	0.095	-0.223	0.079	-0.201	0.036	-0.121	0.071	-0.187	0.229	-0.337
5	0.129	-0.264	0.023	-0.087	0.103	-0.234	0.108	-0.240	0.082	-0.206	0.191	-0.316	0.177	-0.307
6	0.167	-0.299	0.052	-0.154	0.059	-0.167	0.046	-0.142	0.061	-0.170	0.104	-0.236	0.070	-0.186
7	0.173	-0.304	0.018	-0.073	0.069	-0.185	0.037	-0.122	0.068	-0.183	0.092	-0.220	0.059	-0.168
8	0.130	-0.265	0.032	-0.111	0.091	-0.217	0.135	-0.270	0.061	-0.170	0.063	-0.173	0.035	-0.117
9	0.110	-0.243	0.016	-0.066	0.042	-0.133	0.069	-0.185	0.020	-0.077	0.079	-0.200	0.045	-0.140
10			0.026	-0.096	0.046	-0.141	0.051	-0.151	0.019	-0.075	0.027	-0.098	0.030	-0.105
11			0.036	-0.119	0.047	-0.144	0.067	-0.181	0.024	-0.090	0.056	-0.162	0.076	-0.196
12			0.030	-0.106	0.017	-0.069	0.045	-0.139	0.014	-0.061	0.044	-0.137	0.085	-0.209
13			0.052	-0.153	0.037	-0.122	0.075	-0.194	0.021	-0.080	0.023	-0.088	0.039	-0.126
14			0.041	-0.131	0.054	-0.157	0.046	-0.141	0.070	-0.186	0.040	-0.128	0.027	-0.098
15			0.023	-0.087	0.047	-0.144	0.049	-0.148	0.022	-0.085				
16			0.026	-0.095	0.027	-0.097	0.024	-0.091	0.026	-0.096				
17			0.027	-0.097	0.054	-0.158	0.022	-0.084	0.021	-0.080				
18			0.171	-0.302	0.039	-0.128	0.020	-0.079	0.026	-0.095				
19			0.267	-0.353	0.021	-0.081			0.030	-0.106				
20					0.020	-0.079			0.013	-0.057				
21									0.014	-0.059				
22									0.090	-0.217				
23									0.163	-0.295				
	H'	2.10	H'	2.49	H'	2.86	H'	2.76	H'	2.89	H'	2.49	H'	2.38

Band	IBS-M	Patient 3	IBS-M	Patient 4	IBS-M	Patient 5	IBS-M	Patient 6	IBS-M	Patient 7	IBS-M	Patient 8	IBS-M	Patient 9
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.085	-0.210	0.053	-0.156	0.036	-0.119	0.056	-0.162	0.046	-0.142	0.017	-0.069	0.031	-0.107
2	0.066	-0.179	0.077	-0.198	0.108	-0.240	0.045	-0.139	0.040	-0.130	0.096	-0.225	0.040	-0.129
3	0.112	-0.245	0.056	-0.161	0.088	-0.215	0.024	-0.091	0.027	-0.098	0.001	-0.008	0.038	-0.125
4	0.046	-0.141	0.116	-0.250	0.257	-0.349	0.094	-0.223	0.068	-0.183	0.038	-0.124	0.054	-0.158
5	0.146	-0.281	0.120	-0.255	0.131	-0.266	0.076	-0.195	0.102	-0.233	0.304	-0.362	0.109	-0.242
6	0.088	-0.213	0.108	-0.240	0.075	-0.194	0.137	-0.273	0.123	-0.258	0.090	-0.216	0.116	-0.250
7	0.202	-0.323	0.150	-0.285	0.085	-0.209	0.125	-0.260	0.052	-0.155	0.093	-0.221	0.088	-0.215
8	0.020	-0.077	0.204	-0.324	0.070	-0.187	0.130	-0.266	0.099	-0.229	0.053	-0.155	0.089	-0.216
9	0.057	-0.163	0.091	-0.219	0.036	-0.119	0.096	-0.225	0.045	-0.140	0.073	-0.191	0.061	-0.170
10	0.044	-0.137	0.025	-0.092	0.021	-0.082	0.054	-0.157	0.043	-0.135	0.004	-0.021	0.132	-0.267
11	0.026	-0.094			0.012	-0.053	0.065	-0.177	0.046	-0.141	0.046	-0.142	0.038	-0.124
12	0.064	-0.176			0.024	-0.090	0.015	-0.063	0.031	-0.107	0.007	-0.033	0.042	-0.132
13	0.020	-0.077			0.029	-0.103	0.038	-0.124	0.110	-0.242	0.003	-0.016	0.065	-0.178
14	0.026	-0.095			0.028	-0.101	0.045	-0.139	0.062	-0.172	0.007	-0.033	0.051	-0.152
15									0.030	-0.104	0.001	-0.008	0.026	-0.094
16									0.016	-0.067	0.012	-0.054	0.008	-0.037
17									0.010	-0.047	0.016	-0.066	0.002	-0.010
18									0.049	-0.148	0.020	-0.078	0.010	-0.048
19											0.011	-0.050		
20											0.033	-0.113		
21											0.076	-0.196		
	H'	2.41	H'	2.18	H'	2.33	H'	2.49	H'	2.73	H'	2.38	H'	2.65

Band	IBS-M	Patient 10	IBS-C	Patient 1	IBS-C	Patient 2	IBS-C	Patient 3	IBS-C	Patient 4	IBS-C	Patient 5	IBS-C	Patient6
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.038	-0.125	0.043	-0.134	0.080	-0.203	0.016	-0.066	0.039	-0.127	0.200	-0.322	0.015	-0.064
2	0.058	-0.165	0.045	-0.139	0.069	-0.184	0.030	-0.106	0.051	-0.153	0.121	-0.256	0.041	-0.132
3	0.090	-0.217	0.072	-0.189	0.119	-0.253	0.043	-0.135	0.040	-0.128	0.242	-0.343	0.055	-0.159
4	0.069	-0.184	0.086	-0.211	0.087	-0.213	0.053	-0.155	0.034	-0.115	0.120	-0.255	0.036	-0.119
5	0.075	-0.194	0.066	-0.179	0.050	-0.149	0.085	-0.210	0.090	-0.216	0.020	-0.079	0.078	-0.199
6	0.140	-0.275	0.170	-0.301	0.036	-0.119	0.028	-0.099	0.046	-0.141	0.012	-0.052	0.126	-0.261
7	0.098	-0.227	0.088	-0.214	0.104	-0.236	0.049	-0.148	0.042	-0.134	0.001	-0.007	0.100	-0.231
8	0.067	-0.181	0.082	-0.206	0.099	-0.229	0.028	-0.101	0.034	-0.116	0.006	-0.032	0.034	-0.115
9	0.132	-0.267	0.026	-0.095	0.040	-0.128	0.059	-0.167	0.027	-0.097	0.020	-0.077	0.018	-0.072
10	0.068	-0.183	0.030	-0.104	0.062	-0.173	0.045	-0.141	0.023	-0.086	0.026	-0.095	0.036	-0.119
11	0.066	-0.180	0.063	-0.174	0.053	-0.156	0.044	-0.137	0.062	-0.173	0.021	-0.081	0.031	-0.107
12	0.056	-0.162	0.043	-0.135	0.068	-0.182	0.028	-0.100	0.070	-0.185	0.052	-0.155	0.045	-0.139
13	0.013	-0.055	0.040	-0.129	0.046	-0.143	0.018	-0.071	0.225	-0.336	0.063	-0.174	0.037	-0.121
14	0.017	-0.070	0.047	-0.143	0.086	-0.212	0.044	-0.137	0.218	-0.332	0.032	-0.110	0.048	-0.145
15	0.009	-0.042	0.040	-0.128			0.072	-0.189			0.063	-0.175	0.019	-0.077
16	0.003	-0.020	0.028	-0.100			0.020	-0.077					0.016	-0.067
17			0.034	-0.115			0.040	-0.130					0.125	-0.260
18							0.138	-0.274					0.140	-0.275
19							0.160	-0.293						
	H'	2.55	H'	2.69	H'	2.58	H'	2.74	H'	2.34	H'	2.21	H'	2.66

Band	IBS-C	Patient 7	IBS-C	Patient 8	IBS-C	Patient 9	IBS-C	Patient 10	IBS-D	Patient 1	IBS-D	Patient 2	IBS-D	Patient 3
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.024	-0.091	0.021	-0.081	0.039	-0.126	0.044	-0.137	0.019	-0.075	0.023	-0.086	0.023	-0.085
2	0.039	-0.127	0.055	-0.159	0.004	-0.021	0.049	-0.148	0.030	-0.105	0.043	-0.135	0.036	-0.121
3	0.036	-0.119	0.048	-0.145	0.012	-0.051	0.020	-0.079	0.129	-0.264	0.082	-0.205	0.057	-0.164
4	0.074	-0.193	0.018	-0.073	0.077	-0.198	0.022	-0.084	0.155	-0.289	0.126	-0.261	0.059	-0.167
5	0.107	-0.239	0.039	-0.126	0.095	-0.223	0.053	-0.156	0.138	-0.274	0.136	-0.271	0.049	-0.147
6	0.109	-0.242	0.060	-0.169	0.065	-0.178	0.029	-0.102	0.057	-0.164	0.120	-0.254	0.050	-0.149
7	0.048	-0.145	0.061	-0.171	0.044	-0.138	0.034	-0.114	0.020	-0.077	0.074	-0.193	0.073	-0.191
8	0.046	-0.142	0.069	-0.185	0.013	-0.056	0.046	-0.141	0.105	-0.236	0.108	-0.240	0.081	-0.203
9	0.035	-0.117	0.094	-0.223	0.013	-0.058	0.065	-0.178	0.047	-0.144	0.043	-0.136	0.045	-0.139
10	0.037	-0.122	0.041	-0.130	0.024	-0.088	0.022	-0.085	0.105	-0.236	0.103	-0.234	0.144	-0.279
11	0.030	-0.106	0.049	-0.147	0.062	-0.172	0.012	-0.054	0.043	-0.136	0.048	-0.145	0.175	-0.305
12	0.075	-0.194	0.024	-0.089	0.051	-0.153	0.037	-0.121	0.007	-0.036	0.076	-0.196	0.037	-0.122
13	0.011	-0.050	0.011	-0.051	0.010	-0.047	0.027	-0.097	0.045	-0.139	0.018	-0.072	0.065	-0.178
14	0.037	-0.122	0.007	-0.037	0.038	-0.124	0.015	-0.062	0.099	-0.229			0.046	-0.141
15	0.028	-0.100	0.027	-0.098	0.027	-0.098	0.014	-0.060					0.022	-0.085
16	0.014	-0.058	0.047	-0.143	0.035	-0.118	0.014	-0.061					0.025	-0.093
17	0.114	-0.247	0.070	-0.187	0.023	-0.088	0.009	-0.042					0.014	-0.058
18	0.137	-0.272	0.077	-0.198	0.164	-0.296	0.012	-0.052						
19			0.081	-0.204	0.203	-0.324	0.024	-0.089						
20			0.100	-0.230			0.217	-0.331						
21							0.236	-0.341						
	H'	2.68	H'	2.85	H'	2.56	H'	2.53	H'	2.41	H'	2.43	H'	2.63

Band	IBS-D	Patient 4	IBS-D	Patient 5	IBS-D	Patient 6	IBS-D	Patient 7	IBS-D	Patient 8	IBS-D	Patient 9	IBS-D	Patient 10
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.021	-0.081	0.011	-0.048	0.007	-0.035	0.011	-0.049	0.009	-0.041	0.033	-0.112	0.020	-0.078
2	0.033	-0.114	0.054	-0.157	0.025	-0.093	0.013	-0.058	0.016	-0.065	0.041	-0.130	0.026	-0.094
3	0.076	-0.195	0.076	-0.196	0.062	-0.172	0.036	-0.121	0.016	-0.067	0.079	-0.200	0.069	-0.184
4	0.199	-0.321	0.047	-0.144	0.073	-0.191	0.054	-0.158	0.046	-0.142	0.111	-0.244	0.066	-0.180
5	0.126	-0.261	0.043	-0.136	0.034	-0.115	0.041	-0.131	0.038	-0.125	0.066	-0.179	0.111	-0.244
6	0.119	-0.253	0.079	-0.201	0.041	-0.132	0.112	-0.245	0.101	-0.231	0.052	-0.154	0.113	-0.246
7	0.133	-0.268	0.116	-0.250	0.028	-0.101	0.117	-0.251	0.058	-0.165	0.098	-0.227	0.042	-0.132
8	0.059	-0.168	0.087	-0.212	0.054	-0.158	0.102	-0.233	0.055	-0.160	0.046	-0.141	0.053	-0.155
9	0.060	-0.169	0.077	-0.198	0.039	-0.126	0.070	-0.186	0.017	-0.069	0.075	-0.195	0.053	-0.155
10	0.063	-0.175	0.046	-0.143	0.077	-0.197	0.036	-0.121	0.016	-0.066	0.086	-0.210	0.045	-0.139
11	0.040	-0.128	0.023	-0.086	0.042	-0.134	0.111	-0.244	0.024	-0.090	0.036	-0.120	0.037	-0.123
12	0.042	-0.133	0.025	-0.091	0.052	-0.155	0.065	-0.178	0.040	-0.130	0.045	-0.139	0.111	-0.243
13	0.028	-0.101	0.110	-0.243	0.037	-0.122	0.051	-0.153	0.036	-0.119	0.065	-0.177	0.040	-0.129
14			0.067	-0.182	0.025	-0.092	0.051	-0.152	0.015	-0.063	0.033	-0.113	0.052	-0.153
15			0.044	-0.138	0.037	-0.122	0.048	-0.145	0.040	-0.129	0.023	-0.087	0.060	-0.168
16			0.047	-0.143	0.021	-0.080	0.035	-0.118	0.028	-0.101	0.028	-0.101	0.036	-0.119
17			0.023	-0.088	0.032	-0.110	0.020	-0.079	0.041	-0.131	0.027	-0.097	0.048	-0.145
18			0.024	-0.090	0.154	-0.288	0.012	-0.054	0.095	-0.224	0.023	-0.086	0.021	-0.081
19					0.159	-0.292	0.011	-0.051	0.020	-0.080	0.035	-0.116		
20									0.120	-0.254				
21									0.168	-0.300				
	H'	2.37	H'	2.75	H'	2.72	H'	2.73	H'	2.75	H'	2.83	H'	2.77

Band	DIVERT	Patient 1	DIVERT	Patient 2	DIVERT	Patient 3	DIVERT	Patient 4	DIVERT	Patient 5	DIVERT	Patient 6	DIVERT	Patient 7
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.028	-0.101	0.038	-0.125	0.006	-0.029	0.033	-0.113	0.005	-0.028	0.013	-0.055	0.019	-0.076
2	0.029	-0.103	0.048	-0.145	0.031	-0.107	0.016	-0.067	0.012	-0.054	0.051	-0.151	0.027	-0.097
3	0.060	-0.169	0.257	-0.349	0.019	-0.075	0.262	-0.351	0.039	-0.127	0.041	-0.132	0.065	-0.178
4	0.154	-0.288	0.075	-0.195	0.184	-0.312	0.052	-0.153	0.052	-0.153	0.153	-0.287	0.054	-0.157
5	0.069	-0.185	0.060	-0.168	0.062	-0.173	0.052	-0.153	0.057	-0.164	0.120	-0.255	0.058	-0.164
6	0.046	-0.141	0.124	-0.259	0.041	-0.130	0.063	-0.175	0.030	-0.105	0.081	-0.204	0.037	-0.121
7	0.012	-0.052	0.062	-0.173	0.051	-0.152	0.115	-0.249	0.039	-0.127	0.058	-0.165	0.045	-0.139
8	0.024	-0.091	0.053	-0.155	0.054	-0.157	0.066	-0.180	0.049	-0.148	0.063	-0.174	0.064	-0.176
9	0.018	-0.073	0.040	-0.128	0.108	-0.241	0.042	-0.134	0.051	-0.151	0.149	-0.284	0.050	-0.149
10	0.019	-0.075	0.038	-0.125	0.046	-0.141	0.045	-0.140	0.048	-0.145	0.083	-0.206	0.034	-0.114
11	0.019	-0.075	0.041	-0.131	0.030	-0.105	0.091	-0.219	0.045	-0.140	0.072	-0.189	0.031	-0.109
12	0.009	-0.041	0.024	-0.089	0.066	-0.179	0.013	-0.055	0.041	-0.132	0.022	-0.084	0.036	-0.120
13	0.014	-0.059	0.030	-0.104	0.028	-0.101	0.018	-0.073	0.044	-0.136	0.016	-0.067	0.024	-0.089
14	0.129	-0.264	0.046	-0.142	0.013	-0.058	0.026	-0.096	0.050	-0.150	0.014	-0.060	0.041	-0.131
15	0.370	-0.368	0.064	-0.175	0.007	-0.036	0.018	-0.073	0.019	-0.076	0.013	-0.057	0.017	-0.069
16					0.033	-0.113	0.011	-0.049	0.040	-0.128	0.023	-0.087	0.031	-0.108
17					0.016	-0.066	0.023	-0.088	0.063	-0.175	0.009	-0.041	0.138	-0.273
18					0.011	-0.049	0.052	-0.154	0.150	-0.284	0.020	-0.077	0.231	-0.339
19					0.049	-0.148			0.165	-0.297				
20					0.146	-0.281								
	H'	2.08	H'	2.46	H'	2.65	H'	2.52	H'	2.72	H'	2.57	H'	2.61

Band	DIVERT	Patient 8	DIVERT	Patient 9	DIVERT	Patient 10	POLYP	Patient 1	POLYP	Patient 2	POLYP	Patient 3	POLYP	Patient 4
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.011	-0.051	0.010	-0.046	0.040	-0.129	0.036	-0.120	0.018	-0.073	0.050	-0.150	0.016	-0.067
2	0.039	-0.127	0.048	-0.145	0.040	-0.129	0.036	-0.119	0.031	-0.109	0.059	-0.168	0.009	-0.041
3	0.149	-0.284	0.070	-0.187	0.032	-0.111	0.038	-0.123	0.021	-0.081	0.068	-0.183	0.102	-0.232
4	0.143	-0.278	0.086	-0.211	0.057	-0.163	0.109	-0.242	0.089	-0.216	0.119	-0.253	0.120	-0.255
5	0.069	-0.185	0.109	-0.242	0.110	-0.242	0.116	-0.250	0.071	-0.188	0.090	-0.217	0.134	-0.270
6	0.268	-0.353	0.108	-0.240	0.076	-0.196	0.156	-0.289	0.121	-0.256	0.200	-0.322	0.062	-0.172
7	0.083	-0.207	0.084	-0.209	0.040	-0.129	0.113	-0.247	0.053	-0.156	0.074	-0.192	0.004	-0.021
8	0.051	-0.151	0.094	-0.222	0.055	-0.160	0.103	-0.234	0.060	-0.168	0.075	-0.195	0.462	-0.357
9	0.056	-0.162	0.041	-0.130	0.059	-0.168	0.046	-0.141	0.046	-0.141	0.068	-0.183	0.022	-0.083
10	0.029	-0.101	0.024	-0.089	0.048	-0.145	0.031	-0.108	0.013	-0.055	0.089	-0.215	0.018	-0.071
11	0.028	-0.100	0.037	-0.121	0.073	-0.191	0.069	-0.184	0.018	-0.071	0.024	-0.090	0.017	-0.069
12	0.018	-0.073	0.120	-0.254	0.058	-0.165	0.003	-0.016	0.023	-0.088	0.039	-0.126	0.036	-0.119
13	0.008	-0.039	0.059	-0.167	0.034	-0.115	0.003	-0.018	0.096	-0.225	0.045	-0.139		
14	0.013	-0.057	0.029	-0.103	0.061	-0.170	0.007	-0.034	0.029	-0.103				
15	0.015	-0.063	0.011	-0.048	0.040	-0.128	0.015	-0.064	0.014	-0.061				
16	0.019	-0.076	0.039	-0.127	0.098	-0.228	0.006	-0.030	0.005	-0.025				
17			0.006	-0.033	0.079	-0.200	0.002	-0.013	0.008	-0.040				
18			0.008	-0.038			0.000	-0.002	0.022	-0.084				
19			0.017	-0.070			0.006	-0.029	0.114	-0.248				
20							0.010	-0.045	0.148	-0.282				
21							0.006	-0.032						
22							0.014	-0.061						
23							0.075	-0.194						
	H'	2.31	H'	2.68	H'	2.77	H'	2.60	H'	2.67	H'	2.43	H'	1.76

Band	POLYP	Patient 5	POLYP	Patient 6	POLYP	Patient 7	POLYP	Patient 8	POLYP	Patient 9	POLYP	Patient 10
	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)	Pi	Pi Ln(Pi)
1	0.020	-0.077	0.009	-0.044	0.008	-0.039	0.016	-0.065	0.032	-0.111	0.063	-0.174
2	0.066	-0.179	0.001	-0.009	0.043	-0.136	0.020	-0.077	0.019	-0.074	0.201	-0.322
3	0.021	-0.081	0.045	-0.140	0.037	-0.122	0.043	-0.136	0.034	-0.115	0.263	-0.351
4	0.157	-0.291	0.011	-0.050	0.049	-0.147	0.024	-0.088	0.002	-0.013	0.024	-0.090
5	0.198	-0.321	0.164	-0.297	0.146	-0.280	0.075	-0.195	0.084	-0.208	0.061	-0.170
6	0.201	-0.322	0.089	-0.215	0.109	-0.242	0.178	-0.307	0.137	-0.273	0.054	-0.158
7	0.037	-0.122	0.243	-0.344	0.048	-0.146	0.061	-0.170	0.081	-0.203	0.120	-0.255
8	0.029	-0.102	0.106	-0.238	0.005	-0.028	0.078	-0.199	0.009	-0.041	0.057	-0.163
9	0.012	-0.054	0.071	-0.188	0.106	-0.238	0.057	-0.163	0.014	-0.061	0.055	-0.159
10	0.039	-0.126	0.008	-0.040	0.018	-0.073	0.023	-0.086	0.025	-0.092	0.034	-0.115
11	0.037	-0.122	0.003	-0.019	0.034	-0.114	0.032	-0.111	0.068	-0.182	0.003	-0.019
12	0.008	-0.040	0.002	-0.011	0.133	-0.268	0.287	-0.358	0.159	-0.293	0.013	-0.055
13	0.010	-0.048	0.003	-0.018	0.019	-0.075	0.047	-0.144	0.060	-0.168	0.005	-0.026
14	0.046	-0.141	0.134	-0.270	0.006	-0.032	0.032	-0.110	0.061	-0.171	0.008	-0.039
15	0.070	-0.186	0.006	-0.030	0.057	-0.163	0.013	-0.057	0.080	-0.202	0.007	-0.035
16	0.044	-0.137	0.002	-0.013	0.182	-0.310	0.003	-0.018	0.037	-0.122	0.012	-0.054
17	0.006	-0.030	0.015	-0.062			0.012	-0.052	0.098	-0.227	0.021	-0.082
18			0.041	-0.131								
19			0.015	-0.062								
20			0.005	-0.025								
21			0.026	-0.095								
	H'	2.38	H'	2.30	H'	2.41	H'	2.34	H'	2.56	H'	2.27

APPENDIX C: BACTERIAL DENSITIES

C.1 Carriage Rate Data for 12 Healthy Volunteers in Chapter 4

Volunteer	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
1	1.77E+07	0.07	14	1.24E+10	5.34E+11
2	2.79E+06	0.09	18	2.51E+09	1.08E+11
3	9.28E+05	0.07	14	6.50E+08	2.80E+10
4	8.43E+05	0.08	16	6.74E+08	2.91E+10
5	7.60E+06	0.03	6	2.28E+09	9.83E+10
6	1.17E+06	0.01	2	1.17E+08	5.05E+09
7	1.71E+06	0.1	20	1.71E+09	7.38E+10
8	3.24E+05	0.09	18	2.92E+08	1.26E+10
9	2.33E+06	0.07	14	1.63E+09	7.04E+10
10	1.84E+05	0.12	24	2.20E+08	9.51E+09
11	1.58E+05	0.15	30	2.37E+08	1.02E+10
12	1.84E+05	0.12	24	2.20E+08	9.51E+09

C.2 Short Term Stability Data from Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
3	Day 1	3.28E+04	0.05	10	1.64E+07	7.06E+08
3	Day 2	2.76E+05	0.05	10	1.38E+08	5.95E+09
3	Day 3	9.85E+05	0.04	8	3.94E+08	1.70E+10
3	Day 4	2.10E+05	0.04	8	8.40E+07	3.62E+09
4	Week 1	8.42E+06	0.13	26	1.09E+10	4.72E+11
4	Week 2	6.54E+06	0.1	20	6.54E+09	2.82E+11
4	Week 3	5.71E+06	0.09	18	5.14E+09	2.22E+11
4	Week 4	3.86E+06	0.09	18	3.47E+09	1.50E+11
5	Week 1	2.14E+06	0.12	24	2.57E+09	1.11E+11
5	Week 2	3.35E+06	0.13	26	4.36E+09	1.88E+11
5	Week 3	4.06E+06	0.1	20	4.06E+09	1.75E+11
5	Week 4	3.14E+06	0.13	26	4.08E+09	1.76E+11
6	Week 1	7.10E+05	0.04	8	2.84E+08	1.22E+10
6	Week 2	2.06E+06	0.03	6	6.18E+08	2.67E+10
6	Week 3	1.07E+06	0.03	6	3.21E+08	1.38E+10
6	Week 4	1.19E+06	0.02	4	2.38E+08	1.03E+10
8	Week 1	1.45E+07	0.03	6	4.35E+09	1.88E+11
8	Week 2	2.75E+06	0.01	2	2.75E+08	1.19E+10
8	Week 3	4.04E+06	0.02	4	8.08E+08	3.49E+10
8	Week 4	2.48E+06	0.01	2	2.48E+08	1.07E+10
9	Week 1	5.94E+06	0.06	12	3.56E+09	1.54E+11
9	Week 2	6.39E+06	0.05	10	3.20E+09	1.38E+11
9	Week 3	1.16E+06	0.11	22	1.28E+09	5.50E+10
9	Week 4	2.44E+06	0.09	18	2.20E+09	9.47E+10
10	Week 1	3.68E+06	0.01	2	3.68E+08	1.59E+10
10	Week 2	5.94E+06	0.01	2	5.94E+08	2.56E+10
10	Week 3	5.58E+06	0.01	2	5.58E+08	2.41E+10
10	Week 4	1.27E+05	0.02	4	2.54E+07	1.09E+09

C.3 Long Term Stability Data from Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
1	2001	1.89E+07	0.01	2	1.89E+09	8.15E+10
1	2004	7.60E+06	0.03	6	2.28E+09	9.83E+10
2	2003	1.24E+06	0.08	16	9.92E+08	4.28E+10
2	2004	8.43E+05	0.08	16	6.74E+08	2.91E+10
3	2001	3.33E+06	0.01	2	3.33E+08	1.44E+10
3	2005	3.28E+04	0.05	10	1.64E+07	7.06E+08
4	2004	9.28E+05	0.07	14	6.50E+08	2.80E+10
4	2005	8.42E+06	0.13	26	1.09E+10	4.72E+11
5	2003	4.61E+07	0.19	38	8.76E+10	3.78E+12
5	2004	3.24E+05	0.09	18	2.92E+08	1.26E+10
5	2005	2.14E+06	0.12	24	2.57E+09	1.11E+11
6	2004	2.33E+06	0.07	14	1.63E+09	7.04E+10
6	2005	7.10E+05	0.04	8	2.84E+08	1.22E+10
7	2003	6.72E+05	0.13	26	8.74E+08	3.77E+10
7	2004	1.20E+06	0.09	18	1.08E+09	4.66E+10
10	2004	1.17E+06	0.01	2	1.17E+08	5.05E+09
10	2005	3.68E+06	0.01	2	3.68E+08	1.59E+10

C.4 Intestinal Lavage Data from Chapter 6

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
1	control	2.33E+06	0.07	14	1.63E+09	7.04E+10
1	day4	3.27E+06	0.08	16	2.62E+09	1.13E+11
1	day 6	2.33E+06	0.07	14	1.63E+09	7.04E+10
1	day 6#2	2.06E+06	0.03	6	6.18E+08	2.67E+10
1	day 7	4.15E+06	0.05	10	2.08E+09	8.95E+10
2	control	9.28E+05	0.07	14	6.50E+08	2.80E+10
2	day1	5.06E+05	0.12	24	6.07E+08	2.62E+10
2	day 2	1.01E+06	0.08	16	8.08E+08	3.49E+10
2	day 3	4.82E+05	0.08	16	3.85E+08	1.66E+10
2	day 4	4.90E+05	0.08	16	3.92E+08	1.69E+10
2	day 5	1.81E+06	0.09	18	1.63E+09	7.03E+10
2	day 6	5.51E+05	0.06	12	3.30E+08	1.42E+10
2	day 7	6.38E+05	0.06	12	3.83E+08	1.65E+10
3	control	3.24E+05	0.09	18	2.92E+08	1.26E+10
3	day2	3.88E+05	0.07	14	2.71E+08	1.17E+10
3	day3	4.60E+05	0.15	30	6.90E+08	2.98E+10
3	day4	9.12E+05	0.16	32	1.46E+09	6.29E+10
3	day5	8.19E+05	0.07	14	5.74E+08	2.47E+10
3	day6	9.27E+05	0.1	20	9.27E+08	4.00E+10
3	day7	1.51E+05	0.13	26	1.97E+08	8.48E+09
4	control	1.71E+06	0.1	20	1.71E+09	7.38E+10
4	day1	6.62E+05	0.12	24	7.94E+08	3.43E+10
4	day2	1.04E+06	0.12	24	1.25E+09	5.38E+10
4	day3	6.55E+05	0.08	16	5.24E+08	2.26E+10
4	day4	1.32E+06	0.07	14	9.24E+08	3.99E+10
4	day5	2.95E+06	0.12	24	3.54E+09	1.53E+11
4	day6	2.47E+05	0.02	4	4.94E+07	2.13E+09

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
5	control	8.43E+05	0.08	16	6.74E+08	2.91E+10
5	day2	2.76E+06	0.12	24	3.31E+09	1.43E+11
5	day3	6.00E+05	0.13	26	7.79E+08	3.36E+10
5	day4	2.98E+06	0.07	14	2.09E+09	9.00E+10
5	day5	1.68E+06	0.06	12	1.01E+09	4.35E+10
5	day7	1.08E+06	0.07	14	7.56E+08	3.26E+10
6	control	4.16E+06	0.07	14	2.91E+09	1.26E+11
6	day1	1.71E+06	0.09	18	1.54E+09	6.64E+10
6	day2	1.03E+06	0.05	10	5.15E+08	2.22E+10
6	day3	2.09E+06	0.05	10	1.05E+09	4.51E+10
6	day4	5.83E+05	0.04	8	2.33E+08	1.01E+10
6	day5	7.37E+05	0.08	16	5.89E+08	2.54E+10
6	day6	5.11E+05	0.05	10	2.56E+08	1.10E+10
7	control	1.58E+05	0.15	30	2.37E+08	1.02E+10
7	day 1	6.26E+05	0.12	24	7.51E+08	3.24E+10
7	day1#2	1.40E+06	0.11	22	1.54E+09	6.64E+10
7	day2	1.24E+06	0.1	20	1.24E+09	5.35E+10
7	day2#2	7.35E+05	0.12	24	8.82E+08	3.81E+10
7	day3	1.97E+06	0.11	22	2.17E+09	9.35E+10
7	day4	8.48E+05	0.1	20	8.48E+08	3.66E+10
7	day5	1.26E+06	0.08	16	1.01E+09	4.35E+10
7	day5#2	2.85E+06	0.07	14	2.00E+09	8.61E+10
7	day7	8.46E+05	0.09	18	7.61E+08	3.28E+10
8	control	1.84E+05	0.12	24	2.20E+08	9.51E+09
8	day2	3.91E+06	0.09	18	3.52E+09	1.52E+11
8	day3	9.94E+05	0.06	12	5.97E+08	2.57E+10
8	day5	1.23E+06	0.06	12	7.38E+08	3.18E+10
9	control	1.17E+06	0.01	2	1.17E+08	5.05E+09
9	day3	4.48E+06	0.02	4	8.96E+08	3.86E+10
9	day7	1.91E+06	0.01	2	1.91E+08	8.24E+09
10	control	7.60E+06	0.03	6	2.28E+09	9.83E+10
10	day3	1.72E+06	0.05	10	8.60E+08	3.71E+10
10	day4	1.07E+06	0.07	14	7.49E+08	3.23E+10
10	day5	2.01E+06	0.03	6	6.03E+08	2.60E+10
10	day6	1.13E+06	0.02	4	2.26E+08	9.75E+09

C.4 Western Diet Data from Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
1	Week 1	2.14E+06	0.12	24	2.57E+09	1.11E+11
1	Week 2	3.35E+06	0.13	26	4.36E+09	1.88E+11
1	Week 3	4.06E+06	0.1	20	4.06E+09	1.75E+11
1	Week 4	3.14E+06	0.13	26	4.08E+09	1.76E+11
2	Week 1	8.42E+06	0.13	26	1.09E+10	4.72E+11
2	Week 2	6.54E+06	0.1	20	6.54E+09	2.82E+11
2	Week 3	5.71E+06	0.09	18	5.14E+09	2.22E+11
2	Week 4	3.86E+06	0.09	18	3.47E+09	1.50E+11
3	Week 1	5.94E+06	0.06	12	3.56E+09	1.54E+11
3	Week 2	6.39E+06	0.05	10	3.20E+09	1.38E+11
3	Week 3	1.16E+06	0.11	22	1.28E+09	5.50E+10
3	Week 4	2.44E+06	0.09	18	2.20E+09	9.47E+10
4	Week 1	7.10E+05	0.04	8	2.84E+08	1.22E+10
4	Week 2	2.06E+06	0.03	6	6.18E+08	2.67E+10
4	Week 3	1.07E+06	0.03	6	3.21E+08	1.38E+10
4	Week 4	1.19E+06	0.02	4	2.38E+08	1.03E+10
5	Week 1	1.45E+07	0.03	6	4.35E+09	1.88E+11
5	Week 2	2.75E+06	0.01	2	2.75E+08	1.19E+10
5	Week 3	4.04E+06	0.02	4	8.08E+08	3.49E+10
5	Week 4	2.48E+06	0.01	2	2.48E+08	1.07E+10
6	Week 1	3.68E+06	0.01	2	3.68E+08	1.59E+10
6	Week 2	5.94E+06	0.01	2	5.94E+08	2.56E+10
6	Week 3	5.58E+06	0.01	2	5.58E+08	2.41E+10
6	Week 4	1.27E+05	0.02	4	2.54E+07	1.09E+09

C.5 Atkins' Diet Data from Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
1	Day 0	1.22E+06	0.11	22	1.34E+09	5.79E+10
1	Week 2	7.75E+05	0.04	8	3.10E+08	1.34E+10
1	Week 3	5.10E+05	0.07	14	3.57E+08	1.54E+10
1	Week 4	1.06E+06	0.02	4	2.12E+09	9.14E+10
2	Day 0	7.26E+06	0.04	8	2.90E+09	1.25E+11
2	Week 2	8.70E+06	0.05	10	4.35E+09	1.88E+11
2	Week 3	5.35E+06	0.08	16	4.28E+09	1.85E+11
2	Week 4	7.09E+06	0.09	18	6.38E+09	2.75E+11
3	Day 0	4.07E+06	0.06	12	2.44E+09	1.05E+11
3	Week 2	7.41E+05	0.08	16	5.93E+08	2.56E+10
3	Week 3	6.13E+06	0.03	6	1.84E+09	7.93E+10
3	Week 4	1.12E+07	0.02	4	2.24E+09	9.66E+10
4	Day 0	6.65E+04	0.02	4	1.33E+08	5.74E+09
4	Week 2	3.53E+05	0.04	8	1.41E+09	6.09E+10
4	Week 3	4.94E+06	0.02	4	9.88E+08	4.26E+10
4	Week 4	2.03E+06	0.02	4	4.06E+08	1.75E+10
5	Day 0	9.17E+05	0.01	2	9.17E+07	3.96E+09
5	Week 2	5.93E+06	0.02	4	1.19E+09	5.12E+10
5	Week 3	1.02E+07	0.02	4	2.04E+09	8.80E+10
5	Week 4	1.56E+06	0.01	2	1.56E+09	6.73E+10
6	Day 0	1.41E+07	0.01	2	1.41E+09	6.08E+10
6	Week 2	7.21E+04	0.01	2	7.21E+07	3.11E+09
6	Week 3	1.76E+05	0.01	2	1.76E+08	7.59E+09
6	Week 4	5.23E+05	0.01	2	5.23E+07	2.26E+09

C.6 Disease Groups Data from Chapter 8

Disease Group	Volunteer	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
IBD-CD	1	6.93E+05	0.04	8	2.77E+08	5.26E+09
	2	2.56E+06	0.01	2	2.56E+08	4.85E+09
	3	3.27E+05	0.01	2	3.27E+07	6.20E+08
	4	1.63E+06	0.01	2	1.63E+08	3.09E+09
	5	6.39E+05	0.01	2	6.39E+07	1.21E+09
	6	1.71E+06	0.01	2	1.71E+08	3.24E+09
	7	2.37E+04	0.01	2	2.37E+06	4.50E+07
	8	1.21E+05	0.01	2	1.21E+07	2.30E+08
	9	9.34E+05	0.09	18	8.41E+08	1.59E+10
IBD-UC	1	1.58E+06	0.03	6	4.74E+08	8.99E+09
	2	1.44E+04	0.01	2	1.44E+06	2.74E+07
	3	6.96E+05	0.02	4	1.39E+08	2.64E+09
	4	7.52E+04	0.01	2	7.52E+06	1.43E+08
	5	4.67E+05	0.02	4	9.35E+07	1.77E+09
	6	2.45E+05	0.01	2	2.45E+07	4.64E+08
	7	1.42E+06	0.01	2	1.42E+08	2.69E+09
	8	2.06E+06	0.01	2	2.06E+08	3.91E+09
	9	8.36E+05	0.01	2	8.36E+07	1.59E+09
	10	3.63E+06	0.04	8	1.45E+09	2.75E+10
IBS M	1	4.69E+04	0.01	2	4.69E+06	8.89E+07
	2	3.53E+04	0.01	2	3.53E+06	6.69E+07
	3	6.59E+06	0.01	2	6.59E+08	1.25E+10
	4	2.55E+06	0.01	2	2.55E+08	4.83E+09
	5	6.19E+06	0.04	8	2.48E+09	4.69E+10
	6	1.20E+06	0.01	2	1.20E+08	2.28E+09
	7	6.21E+06	0.01	2	6.21E+08	1.18E+10
	8	7.22E+05	0.01	2	7.22E+07	1.37E+09
	9	1.05E+07	0.01	2	1.05E+09	1.99E+10
	10	1.96E+05	0.01	2	1.96E+07	3.72E+08
IBS C	1	2.02E+05	0.02	4	4.03E+07	7.65E+08
	2	4.60E+05	0.02	4	9.21E+07	1.75E+09
	3	1.21E+06	0.01	2	1.21E+08	2.29E+09
	4	6.56E+05	0.01	2	6.56E+07	1.24E+09
	5	1.71E+05	0.03	6	5.14E+07	9.74E+08
	6	1.22E+06	0.01	2	1.22E+08	2.31E+09
	7	1.50E+06	0.02	4	3.00E+08	5.69E+09
	8	4.36E+05	0.04	8	1.74E+08	3.31E+09
	9	4.51E+04	0.005	1	2.25E+06	4.27E+07
	10	1.66E+05	0.02	4	3.31E+07	6.28E+08

Disease Group	Volunteer	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight
IBS D	1	3.48E+06	0.01	2	3.48E+08	6.60E+09
	2	3.18E+06	0.03	6	9.54E+08	1.81E+10
	3	1.73E+06	0.02	4	3.46E+08	6.56E+09
	4	1.41E+05	0.01	2	1.41E+07	2.68E+08
	5	1.23E+05	0.01	2	1.23E+07	2.34E+08
	6	4.29E+06	0.02	4	8.58E+08	1.63E+10
	7	2.46E+06	0.05	10	1.23E+09	2.33E+10
	8	9.72E+05	0.01	2	9.72E+07	1.84E+09
	9	3.63E+06	0.01	2	3.63E+08	6.88E+09
	10	8.62E+05	0.04	8	3.45E+08	6.53E+09
Diverticula	1	7.12E+06	0.01	2	7.12E+08	1.35E+10
	2	4.62E+05	0.01	2	4.62E+07	8.76E+08
	3	7.97E+06	0.03	6	2.39E+09	4.53E+10
	4	9.12E+05	0.01	2	9.12E+07	1.73E+09
	5	2.87E+06	0.01	2	2.87E+08	5.44E+09
	6	3.44E+06	0.02	4	6.88E+08	1.30E+10
	7	3.18E+06	0.01	2	3.18E+08	6.03E+09
	8	4.68E+05	0.01	2	4.68E+07	8.87E+08
	9	3.50E+06	0.01	2	3.50E+08	6.64E+09
	10	2.52E+05	0.01	2	2.52E+07	4.78E+08
Polyps	1	2.63E+05	0.01	2	2.63E+07	4.98E+08
	2	1.36E+05	0.01	2	1.36E+07	2.58E+08
	3	3.73E+06	0.02	4	7.46E+08	1.41E+10
	4	4.27E+06	0.01	2	4.27E+08	8.10E+09
	5	5.25E+05	0.01	2	5.25E+07	9.95E+08
	6	1.91E+06	0.01	2	1.91E+08	3.62E+09
	7	2.81E+06	0.01	2	2.81E+08	5.33E+09
	8	1.46E+05	0.01	2	1.46E+07	2.77E+08
	9	2.55E+06	0.01	2	2.55E+08	4.83E+09
	10	7.91E+05	0.01	2	7.91E+07	1.50E+09

APPENDIX D: METHANOGEN DENSITY

D.1 Carriage Rates Data for 12 Volunteers in Chapter 4

Volunteer	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	5.60E+01	0.07	14	3.92E+04	1.69E+06	5.34E+11	0.0003%
2	1.92E+01	0.09	18	1.73E+04	7.45E+05	1.08E+11	0.0007%
4	1.42E+03	0.08	16	1.14E+06	4.91E+07	2.91E+10	0.1685%
5	1.43E+03	0.03	6	4.30E+05	1.86E+07	9.83E+10	0.0189%
12	3.96E+02	0.12	24	4.75E+05	2.05E+07	9.51E+09	0.2152%

D.2 Short Term Stability Data for Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
3	Day 1	5.94E+03	0.05	10	2.97E+06	1.28E+08	7.06E+08	15.352%
	Day 2	4.96E+04	0.05	10	2.48E+07	1.07E+09	5.95E+09	15.258%
	Day 3	8.29E+03	0.04	8	3.32E+06	1.43E+08	1.70E+10	0.834%
	Day 4	1.40E+04	0.04	8	5.60E+06	2.41E+08	3.62E+09	6.249%
4	Week 1	2.12E+03	0.13	26	2.75E+06	1.19E+08	4.72E+11	0.025%
	Week 2	1.96E+04	0.1	20	1.96E+07	8.44E+08	2.82E+11	0.298%
	Week 3	2.80E+02	0.09	18	2.52E+05	1.09E+07	2.22E+11	0.005%
	Week 4	1.48E+05	0.09	18	1.33E+08	5.75E+09	1.50E+11	3.695%

D.3 Long Term Stability Data for Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	2001	5.85E+02	0.01	2	5.85E+04	2.52E+06	8.15E+10	0.003%
1	2004	1.43E+03	0.03	6	4.30E+05	1.86E+07	9.83E+10	0.019%
2	2003	2.59E+04	0.08	16	2.07E+07	8.94E+08	4.28E+10	2.047%
2	2004	1.42E+03	0.08	16	1.14E+06	4.91E+07	2.91E+10	0.168%
3	2001	9.47E+03	0.01	2	9.47E+05	4.08E+07	1.44E+10	0.284%
3	2005	5.94E+03	0.05	10	2.97E+06	1.28E+08	7.06E+08	15.352%
7	2003	2.77E+02	0.13	26	3.59E+05	1.55E+07	3.77E+10	0.041%
7	2004	1.33E+03	0.09	18	1.20E+06	5.18E+07	4.66E+10	0.111%

D.4 Intestinal Lavage Data for Chapter 6

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
5	control	1.42E+03	0.08	16	1.14E+06	4.91E+07	2.91E+10	0.17%
5	day2	3.90E+02	0.12	24	4.68E+05	2.02E+07	1.43E+11	0.01%
5	day3	1.19E+03	0.13	26	1.54E+06	6.66E+07	3.36E+10	0.20%
5	day4	1.41E+03	0.07	14	9.85E+05	4.25E+07	9.00E+10	0.05%
5	day5	2.03E+03	0.06	12	1.22E+06	5.26E+07	4.35E+10	0.12%
5	day7	4.08E+03	0.07	14	2.85E+06	1.23E+08	3.26E+10	0.38%
8	control	3.96E+02	0.12	24	4.75E+05	2.05E+07	9.51E+09	0.22%
8	day2	0.00E+00				0.00E+00	1.52E+11	0.00%
8	day3	0.00E+00				0.00E+00	2.57E+10	0.00%
8	day5	0.00E+00				0.00E+00	3.18E+10	0.00%
10	control	1.43E+03	0.03	6	4.30E+05	1.86E+07	9.83E+10	0.02%
10	day3	2.79E+03	0.05	10	1.39E+06	6.02E+07	3.71E+10	0.16%
10	day4	1.31E+02	0.07	14	9.19E+04	3.96E+06	3.23E+10	0.01%
10	day 5	6.02E+02	0.03	6	1.81E+05	7.79E+06	2.60E+10	0.03%
10	day6	4.98E+03	0.02	4	9.96E+05	4.30E+07	9.75E+09	0.44%

D.5 Western Diet Data for Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
2	Week 1	2.12E+03	0.13	26	2.75E+06	1.19E+08	4.72E+11	0.025%
2	Week 2	1.96E+04	0.1	20	1.96E+07	8.44E+08	2.82E+11	0.298%
2	Week 3	2.80E+02	0.09	18	2.52E+05	1.09E+07	2.22E+11	0.005%
2	Week 4	1.48E+05	0.09	18	1.33E+08	5.75E+09	1.50E+11	3.695%

D.6 Atkins' Diet Data for Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
2	Day 0	3.92E+05	0.04	8	1.57E+08	6.76E+09	1.25E+11	5.10%
2	Week 2	2.66E+04	0.05	10	1.33E+07	5.73E+08	1.88E+11	0.30%
2	Week 3	not determined				0.00E+00	1.85E+11	0
2	Week 4	not determined				0.00E+00	2.75E+11	0

D.7 Disease Groups Data for Chapter 8

Disease Groups	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
IBD-CD	1	119.8	0.04	8	47920	908563.2	5255292529	0.0173%
	4	518.09	0.01	2	51809	982298.64	3090480000	0.0318%
	6	258.9	0.01	2	25890	490874.4	3242160000	0.0151%
IBD-UC	3	not determined						
	6	not determined						
	9	1.2	0.01	2	120	2275.2	1585083606	0.0001%
	10	not determined						
IBS M	3	229.74	0.01	2	22974	435587.04	12494640000	0.0035%
	4	not determined						
	5	10622.01	0.04	8	4248804	80557323.84	46944960000	0.1713%
	8	not determined						
	10	1.81	0.01	2	181	3431.76	371885706	0.0009%
IBS C	1	89.91	0.02	4	17982	340938.72	764804270.9	0.0446%
	3	12.85	0.01	2	1285	24363.6	2294160000	0.0011%
	4	51.11	0.01	2	5111	96904.56	1244291598	0.0078%
	5	203.07	0.03	6	60921	1155062.16	974226306.2	0.1184%
	8	688.5	0.04	8	275400	5221584	3307963562	0.1576%
	9	15.18	0.005	1	759	14390.64	42737565.36	0.0337%
	10	50.42	0.02	4	10084	191192.64	628306377.1	0.0304%
IBS D	1	934.74	0.01	2	93474	1772267.04	6598080000	0.0269%
	3	55.82	0.02	4	11164	211669.44	6560160000	0.0032%
	4	not determined						
	5	2.88	0.01	2	288	5460.48	234066888	0.0023%
	7	50267.74	0.05	10	25133870	476538175.2	23320800000	2.0025%
	8	not determined						
Diverticula	1	372408	0.01	2	37240800	706085568	13499520000	4.9705%
	3	not determined						
	4	91434.63	0.01	2	9143463	173360058.5	1728579237	9.1149%
	7	3606.17	0.01	2	360617	6837298.32	6029280000	0.1133%
	9	162441.68	0.01	2	16244168	307989425.3	6636000000	4.4353%
	10	not determined						
Polyps	1	not determined						
	2	not determined						
	3	1121.3	0.02	4	224260	4251969.6	14144160000	0.0301%
	4	not determined						
	5	7781.84	0.01	2	778184	14754368.64	995399563.9	1.4606%
	7	not determined						
	8	not determined						
	9	not determined						
	10	not determined						

APPENDIX E: SULFATE REDUCING BACTERIA DENSITIES

E.1 Carriage Rates Data for 12 Volunteers in Chapter 4

Volunteer	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	1.47E+03	0.07	14	1E+06	4.42E+07	5.34E+11	0.008%
2	not determined						
3	1.47E+04	0.07	14	1E+07	4.44E+08	2.80E+10	1.584%
4	9.10E+03	0.08	16	7E+06	3.14E+08	2.91E+10	1.078%
5	7.59E+04	0.03	6	2E+07	9.82E+08	9.83E+10	0.998%
8	1.04E+04	0.09	18	9E+06	4.06E+08	1.26E+10	3.222%
9	1.30E+05	0.07	14	9E+07	3.92E+09	7.04E+10	5.568%

E.2 Short Term Stability Data for Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
4	Week 1	7.65E+02	0.13	26	1E+06	4.29E+07	4.72E+11	0.0091%
	Week 2	1.14E+03	0.1	20	1E+06	4.93E+07	2.82E+11	0.0174%
	Week 3	not determined						
	Week 4	7.55E+03	0.09	18	7E+06	2.93E+08	1.50E+11	0.1884%
5	Week 1	5.24E+02	0.12	24	6E+05	2.71E+07	1.11E+11	0.0245%
	Week 2	1.73E+03	0.13	26	2E+06	9.71E+07	1.88E+11	0.0517%
	Week 3	3.28E+03	0.1	20	3E+06	1.41E+08	1.75E+11	0.0808%
	Week 4	1.69E+04	0.13	26	2E+07	9.45E+08	1.76E+11	0.5369%
6	Week 1	3.70E+02	0.04	8	1E+05	6.38E+06	1.22E+10	0.0521%
	Week 2	5.69E+03	0.03	6	2E+06	7.36E+07	2.67E+10	0.2760%
	Week 3	4.70E+03	0.03	6	1E+06	6.08E+07	1.38E+10	0.4388%
	Week 4	2.68E+03	0.02	4	5E+05	2.31E+07	1.03E+10	0.2252%

E.3 Long Term Stability Data for Chapter 4

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	2001	9.91E+04	0.01	2	1E+07	4.27E+08	8.15E+10	0.5241%
	2004	7.59E+04	0.03	6	2E+07	9.82E+08	9.83E+10	0.9979%
2	2003	9.90E+04	0.08	16	8E+07	3.42E+09	4.28E+10	7.8198%
	2004	9.10E+03	0.08	16	7E+06	3.14E+08	2.91E+10	1.0782%
4	2004	1.47E+04	0.07	14	1E+07	4.44E+08	2.80E+10	1.5837%
	2005	7.65E+02	0.13	26	1E+06	4.29E+07	4.72E+11	0.0091%
5	2003	1.23E+04	0.19	38	2.E+07	1.01E+09	3.78E+12	0.0268%
	2004	1.04E+04	0.09	18	9E+06	4.06E+08	1.26E+10	3.2219%
	2005	5.24E+02	0.12	24	6E+05	2.71E+07	1.11E+11	0.0245%
6	2004	1.30E+05	0.07	14	9E+07	3.92E+09	7.04E+10	5.5681%
	2005	3.70E+02	0.04	8	1E+05	6.38E+06	1.22E+10	0.0521%

E.4 Intestinal Lavage Data for Chapter 6

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	control	1.30E+05	0.07	14	9E+07	3.92E+09	7.04E+10	5.5681%
	day4	1.62E+05	0.08	16	1E+08	5.58E+09	1.13E+11	4.9495%
	day6	1.74E+05	0.07	14	1E+08	5.26E+09	7.04E+10	7.4711%
	day6 #2	1.09E+05	0.03	6	3E+07	1.41E+09	2.67E+10	5.2739%
	day7	6.22E+04	0.05	10	3E+07	1.34E+09	8.95E+10	1.4986%
2	control	1.47E+04	0.07	14	1E+07	4.44E+08	2.80E+10	1.5837%
	day1	1.00E+04	0.12	24	1E+07	5.18E+08	2.62E+10	1.9785%
	day2	8.69E+03	0.08	16	7E+06	3.00E+08	3.49E+10	0.8602%
	day3	1.85E+04	0.08	16	1E+07	6.39E+08	1.66E+10	3.8468%
	day4	1.16E+04	0.08	16	9E+06	4.00E+08	1.69E+10	2.3659%
	day5	3.28E+04	0.09	18	3E+07	1.27E+09	7.03E+10	1.8122%
	day6	1.95E+04	0.06	12	1E+07	5.06E+08	1.42E+10	3.5490%
	day7	9.34E+03	0.06	12	6E+06	2.42E+08	1.65E+10	1.4645%
3	control	1.04E+04	0.09	18	9E+06	4.06E+08	1.26E+10	3.2219%
	day2	not determined						
	day3	7.22E+02	0.15	30	1E+06	4.67E+07	2.98E+10	0.1569%
	day4	1.14E+03	0.16	32	2E+06	7.88E+07	6.29E+10	0.1253%
	day5	1.76E+03	0.07	14	1E+06	5.32E+07	2.47E+10	0.2152%
	day6	9.68E+03	0.1	20	1E+07	4.17E+08	4.00E+10	1.0440%
	day7	9.80E+02	0.13	26	1E+06	5.49E+07	8.48E+09	0.6476%
5	L2 control	9.10E+03	0.08	16	7E+06	3.14E+08	2.91E+10	1.0782%
	L2 day2	2.20E+04	0.12	24	3E+07	1.14E+09	1.43E+11	0.7969%
	L2 day3	not determined						
	L2 day4	1.87E+04	0.07	14	1E+07	5.66E+08	9.00E+10	0.6288%
	L2 day5	2.18E+04	0.06	12	1E+07	5.63E+08	4.35E+10	1.2933%
	L2 day7	1.65E+04	0.07	14	1E+07	5.00E+08	3.26E+10	1.5262%
10	L3 control	7.59E+04	0.03	6	2E+07	9.82E+08	9.83E+10	0.9979%
	L3 day3	1.85E+05	0.05	10	9E+07	3.98E+09	3.71E+10	10.7177%
	L3 day4	7.32E+03	0.07	14	5E+06	2.21E+08	3.23E+10	0.6838%
	L3 day5	2.85E+04	0.03	6	9E+06	3.69E+08	2.60E+10	1.4185%
	L3 day6	6.81E+04	0.02	4	1E+07	5.87E+08	9.75E+09	5.9972%

E.5 Western Diet Data for Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	Week 1	5.24E+02	0.12	24	6E+05	2.71E+07	1.11E+11	0.0245%
1	Week 2	1.73E+03	0.13	26	2E+06	9.71E+07	1.88E+11	0.0517%
1	Week 3	3.28E+03	0.1	20	3E+06	1.41E+08	1.75E+11	0.0808%
1	Week 4	1.69E+04	0.13	26	2E+07	9.45E+08	1.76E+11	0.5369%
2	Week 1	7.65E+02	0.13	26	1E+06	4.29E+07	4.72E+11	0.0091%
2	Week 2	1.14E+03	0.1	20	1E+06	4.93E+07	2.82E+11	0.0174%
2	Week 3	not determined						
2	Week 4	7.55E+03	0.09	18	7E+06	2.93E+08	1.50E+11	0.1884%
4	Week 1	3.70E+02	0.04	8	1E+05	6.38E+06	1.22E+10	0.0521%
4	Week 2	5.69E+03	0.03	6	2E+06	7.36E+07	2.67E+10	0.2760%
4	Week 3	4.70E+03	0.03	6	1E+06	6.08E+07	1.38E+10	0.4388%
4	Week 4	2.68E+03	0.02	4	5E+05	2.31E+07	1.03E+10	0.2252%

E.6 Atkins' Diet Data for Chapter 7

Volunteer	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
1	Day 0	4.07E+02	0.11	22	4E+05	1.93E+07	5.79E+10	0.0334%
1	Week 2	3.01E+04	0.04	8	1E+07	5.19E+08	1.34E+10	3.8786%
1	Week 3	3.90E+03	0.07	14	3E+06	1.18E+08	1.54E+10	0.7647%
1	Week 4	3.22E+03	0.02	4	6E+06	2.78E+08	9.14E+10	0.3035%
2	Day 0	2.02E+04	0.04	8	8E+06	3.49E+08	1.25E+11	0.2643%
2	Week 2	2.76E+04	0.05	10	1E+07	5.94E+08	1.88E+11	0.3157%
2	Week 3	8.62E+03	0.08	16	7E+06	2.98E+08	1.85E+11	0.1612%
2	Week 4	1.23E+03	0.09	18	1E+06	4.77E+07	2.75E+11	0.0173%
4	Day 0	4.54E+02	0.02	4	9E+05	3.92E+07	5.74E+09	0.6833%
4	Week 2	1.99E+03	0.04	8	8E+06	3.43E+08	6.09E+10	0.5630%
4	Week 3	1.54E+04	0.02	4	3E+06	1.33E+08	4.26E+10	0.3116%
4	Week 4	7.38E+03	0.02	4	1E+06	6.37E+07	1.75E+10	0.3636%

E.7 Disease Groups Data for Chapter 8

Disease Groups	Sample	Mean Quantity	Conc. ug/uL	Yield ug	Total Genes	Genes/g Stool Wet Weight	Bacteria Genes/g Stool Wet Weight	Normalised to Total Bacteria and Methanogens
IBD-CD	1	8.05E+02	0.04	8	3E+05	6.10E+06	5.26E+09	0.1161%
	4	7.68E+03	0.01	2	8E+05	1.46E+07	3.09E+09	0.4713%
	7	2.27E+01	0.01	2	2269	4.30E+04	4.50E+07	0.0956%
IBS M	1	not determined						
	3	4.32E+02	0.01	2	43161	8.18E+05	1.25E+10	0.0065%
	6	not determined						
	7	6.03E+02	0.01	2	60337	1.14E+06	1.18E+10	0.0097%
	8	8.48E+02	0.01	2	84832	1.61E+06	1.37E+09	0.1174%
	9	2.94E+04	0.01	2	3E+06	5.58E+07	1.99E+10	0.2801%
	10	not determined						
IBS C	1	7.07E+01	0.02	4	14134	2.68E+05	7.65E+08	0.0350%
	4	1.45E+04	0.01	2	1E+06	2.74E+07	1.24E+09	2.2053%
	6	1.04E+03	0.01	2	1E+05	1.97E+06	2.31E+09	0.0851%
	8	7.13E+04	0.04	8	3E+07	5.40E+08	3.31E+09	16.3115%
IBS D	1	1.38E+05	0.01	2	1E+07	2.62E+08	6.60E+09	3.9650%
	3	1.81E+03	0.02	4	4E+05	6.87E+06	6.56E+09	0.1047%
	4	not determined						
	5	8.76E+01	0.01	2	8761	1.66E+05	2.34E+08	0.0710%
	7	2.40E+04	0.05	10	1E+07	2.28E+08	2.33E+10	0.9566%
	8	1.12E+03	0.01	2	1E+05	2.13E+06	1.84E+09	0.1157%
	9	not determined						
Diverticula	1	1.86E+05	0.01	2	2E+07	3.54E+08	1.35E+10	2.4887%
	7	2.55E+04	0.01	2	3E+06	4.83E+07	6.03E+09	0.8001%
	9	2.96E+05	0.01	2	3E+07	5.62E+08	6.64E+09	8.0933%
	10	1.61E+04	0.01	2	2E+06	3.05E+07	4.78E+08	6.3805%
Polyps	1	5.35E+01	0.01	2	5347	1.01E+05	4.98E+08	0.0203%
	2	2.11E+04	0.01	2	2E+06	4.01E+07	2.58E+08	15.5443%
	5	2.23E+05	0.01	2	2E+07	4.22E+08	9.95E+08	41.7923%
	9	1.05E+03	0.01	2	1E+05	1.98E+06	4.83E+09	0.0410%
	10	9.36E+02	0.01	2	93552	1.77E+06	1.50E+09	0.1183%

APPENDIX F: SEQUENCING DATA FOR *AKKERMANSIA MUCINIPHILA*

The characteristic doublet band located at the bottom of many TTGE gel profiles was sequenced to identify the organism(s) these bands were generated from.

F.1 Identity of Bacteria TTGE Bands from Chapter 3

Lower band of the doublet.
99% identity to *Akkermansia muciniphila*, 359/360 residues match

```
QUERY  1     GTAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1268  GTAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTC  1209

QUERY  61    GCGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGCCCTGGGCATAAGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1208  GCGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGCCCTGGGCATAAGGG  1149

QUERY  121   CCATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1148  CCATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGA  1089

QUERY  181   GTCCCCACCTTTACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCGTTGCTGGACTTAAC  240
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1088  GTCCCCACCTTCACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCGTTGCTGGACTTAAC  1029

QUERY  241   CAAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTGTAACGCCTCCGAAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1028  CAAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTGTAACGCCTCCGAAG  969

QUERY  301   AGTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  968   AGTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  909
```

F.2 Bacteria plasmid clone from Chapter 3

Lower band of the doublet.
100% identity to *Akkermansia muciniphila*, 389/389 residues match.

```
QUERY: 2     TGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACTG 61
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1297  TGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACTG 1238

QUERY: 62    GGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATTG 121
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1237  GGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATTG 1178

QUERY: 122   TAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTCC 181
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1177  TAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTCC 1118

QUERY: 182   TCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTGGCAA 241
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1117  TCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTGGCAA 1058
```

```
QUERY: 242  CAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGGC 301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1057 CAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGGC 998

QUERY: 302  CATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTACA 361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 997  CATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTACA 938

QUERY: 362  TGTCAAGCCCAGGTAAGGTTCTTCGCGTT 390
            |||||||||||||||||||||||||||||
SBJCT: 937  TGTCAAGCCCAGGTAAGGTTCTTCGCGTT 909
```

F.3 Intestinal Lavage Chapter 6, Volunteer 4

Lower band of the doublet.
100% identity to *Akkermansia muciniphila*, 359/359 residues match.

```
QUERY  1     TAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1267  TAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTCG  1208

QUERY  61    CGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1207  CGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGC  1148

QUERY  121   CATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1147  CATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAG  1088

QUERY  181   TCCCCACCTTCACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCGTTGCTGGACTTAACC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1087  TCCCCACCTTCACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCGTTGCTGGACTTAACC  1028

QUERY  241   AAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTGTAACGCCTCCGAAGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1027  AAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTGTAACGCCTCCGAAGA  968

QUERY  301   GTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  967   GTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  909
```

F.4 Intestinal Lavage Chapter 6, Volunteer 7

Upper band of the doublet.
99% identity to *Akkermansia muciniphila*, 371/374 residues match

```
QUERY: 2     ATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACT 61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1298  ATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACT 1239

QUERY: 62    GGGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATT 121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1238  GGGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATT 1179

QUERY: 122   GTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTC 181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1178  GTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTC 1119

QUERY: 182   CTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTTACGTGCTGGTAACTGGCA 241
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
SBJCT: 1118  CTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTGGCA 1059

QUERY: 242   ACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACRAGCTGACGACGG 301
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
SBJCT: 1058  ACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGG 999
```

```
QUERY: 302  CCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTTTTCATTAC 361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
SBJCT: 998  CCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTAC 939

QUERY: 362  ATGTCAAGCCCAGG 375
            |||||||||||||
SBJCT: 938  ATGTCAAGCCCAGG 925
```

Lower band of the doublet.
98% identity to *Akkermansia muciniphila*, 254/257 residues match

```
QUERY: 1    TCGGAGGCGTTACACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTCGTGAGATGTTTGGT 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 971  TCGGAGGCGTTACACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTCGTGAGATGTTTGGT 1030

QUERY: 61   TAAGTCCAGCAACGAGCGCAACCCCTGTTGCCAGTTACCAGCACGTAAAGGTGGGGACTC 120
            ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
SBJCT: 1031 TAAGTCCAGCAACGAGCGCAACCCCTGTTGCCAGTTACCAGCACGTGAAGGTGGGGACTC 1090

QUERY: 121  TGGCRAGACTGCCCARATCAACTGGGAGGAAGGTGGGGACGACGTCAGGTCAGTATGGCC 180
            |||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1091 TGGCGAGACTGCCCAGATCAACTGGGAGGAAGGTGGGGACGACGTCAGGTCAGTATGGCC 1150

QUERY: 181  CTTATGCCCAGGGCTGCACACGTACTACAATGCCCAGTACAGAGGGGGCCGAAGCCGCGA 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1151 CTTATGCCCAGGGCTGCACACGTACTACAATGCCCAGTACAGAGGGGGCCGAAGCCGCGA 1210

QUERY: 241  GGCGGAGGAAATCCTAA 257
            |||||||||||||||||
SBJCT: 1211 GGCGGAGGAAATCCTAA 1227
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F.5 Intestinal Lavage Chapter 6, Volunteer 8

Upper band of the doublet.
98% identity to *Akkermansia muciniphila*, 383/390 residues match

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QUERY: 3    ATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACT 62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1299 ATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACT 1240

QUERY: 63   GGGCCCAGTTTTCAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATT 122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1239 GGGCCCAGTTTTCAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATT 1180

QUERY: 123  GTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTC 182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1179 GTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTC 1120

QUERY: 183  CTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCGACATTACTCGATGGTAACTGGCA 242
            |||||||||||||||||||||||||||||||||||  | | ||  | ||||||||||||
SBJCT: 1119 CTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTGGCA 1060

QUERY: 243  ACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGG 302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1059 ACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGG 1000

QUERY: 303  CCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTAC 362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 999  CCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTAC 940

QUERY: 363  ATGTCAAGCCCAGGTAAGGTTCTTCGCGTT 392
            |||||||||||||||||||||||||||||
SBJCT: 939  ATGTCAAGCCCAGGTAAGGTTCTTCGCGTT 910
```

Lower band of the doublet.
98% identity to *Akkermansia muciniphila*, 387/394 residues match

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QUERY: 3    GCTGATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCG 62
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1303 GCTGATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCG 1244

QUERY: 63   AACTGGGCCCAGTTTTCAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGG 122
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1243 AACTGGGCCCAGTTTTCAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGG 1184

QUERY: 123  CATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCAC 182
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1183 CATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCAC 1124

QUERY: 183  CTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCGACATTACTCGATGGTAACT 242
           ||||||||||||||||||||||||||||||||||||  | | ||  | ||||||||
SBJCT: 1123 CTTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACT 1064

QUERY: 243  GGCAACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACG 302
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1063 GGCAACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACG 1004

QUERY: 303  ACGGCCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCA 362
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 1003 ACGGCCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCA 944

QUERY: 363  TTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT 396
           |||||||||||||||||||||||||||||||||
SBJCT: 943  TTACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT 910
```

F.6 Western Diet Chapter 7, Volunteer 6

Upper band of the doublet.
99% identity to *Akkermansia muciniphila*, 392/393 residues match

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QUERY  1     CTGATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1301  CTGATGCGCCATTACTAGCGATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGA  1242

QUERY  61    ACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1241  ACTGGGCCCAGTTTTTAGGATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGC  1182

QUERY  121   ATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1181  ATTGTAGTACGTGTGCAGCCCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACC  1122

QUERY  181   TTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTTACGTGCTGGTAACTG  240
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
SBJCT  1121  TTCCTCCCAGTTGATCTGGGCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTG  1062

QUERY  241   GCAACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1061  GCAACAGGGGTTGCGCTCGTTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGA  1002

QUERY  301   CGGCCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1001  CGGCCATGCAGCACCTGTGTAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCAT  942

QUERY  361   TACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  393
            |||||||||||||||||||||||||||||||||
SBJCT  941   TACATGTCAAGCCCAGGTAAGGTTCTTCGCGTT  909
```

Lower band of the doublet.
99% identity to *Akkermansia muciniphila*, 373/374 residues match

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QUERY  10    GATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGG  69
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1282  GATTCCGGCTTCGTGTAGGCGGGTTGCAGCCTACAGTCCGAACTGGGCCCAGTTTTTAGG  1223

QUERY  70    ATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGC  129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1222  ATTTCCTCCGCCTCGCGGCTTCGGCCCCCTCTGTACTGGGCATTGTAGTACGTGTGCAGC  1163

QUERY  130   CCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGG  189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1162  CCTGGGCATAAGGGCCATACTGACCTGACGTCGTCCCCACCTTCCTCCCAGTTGATCTGG  1103

QUERY  190   GCAGTCTCGCCAGAGTCCCCACCTTTACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCG  249
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
SBJCT  1102  GCAGTCTCGCCAGAGTCCCCACCTTCACGTGCTGGTAACTGGCAACAGGGGTTGCGCTCG  1043

QUERY  250   TTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTG  309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1042  TTGCTGGACTTAACCAAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTG  983

QUERY  310   TAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTA  369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  982   TAACGCCTCCGAAGAGTCGCATGCTTTCACATGTTGTTCATTACATGTCAAGCCCAGGTA  923

QUERY  370   AGGTTCTTCGCGTT  383
             |||||||||||||
SBJCT  922   AGGTTCTTCGCGTT  909
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APPENDIX G: LOW CAROBYHYDRATE MEALS

Examples of low carbohydrate meals suitable for consumption during the induction phase of the Atkins' diet, Chapter 7.

Meal	Food	Components	Grams of Carbohydrate
Breakfast	Omelette	2xEggs	0.6g/egg
		Mushrooms	1g/0.5 cup
		Cheese	0.4g/0.25 cup
		Cream	0.4g/tbsp
		Spring onions	0.7g/stalk
		TOTAL	**3.8g**
Lunch	quiche	eggs x5	3.0g
		Bacon x3 slices	0.1g
		Broccoli	2.4/cup
		Asparagus x6	2.4g
		Cream 0.5Cup	4g
		Cheese 1cup	1.6g
		Tomato small x1	3.2g
		TOTAL	16.7g
		1/6 portion	**2.8g**
Dinner		Chicken breast	0g
		Stuffed with feta 1 tbsp	0.4g
		Wrapped in bacon	0.1g
		Steamed spinach (2 cups raw)	0.6g
		Broccoli (I cup)	3.4g
		TOTAL	**4.5g**

APPENDIX H: PRECISION CALCULATIONS FOR REAL TIME PCR ASSAYS

Raw data for calculation of real time PCR assay precision, Chapter 3.

Assay	10-Fold Dilution Series	Ct	Average Ct	Standard Deviation	Co-efficient of Variation	Standard Error	95% Confidence Limit
Methanogens	Dilution 1	13.09	12.82	0.23	1.80%	0.13	12.82 +/- 0.56
		12.72					
		12.66					
	Dilution 2	16.14	16.21	0.12	0.73%	0.07	16.21 +/- 0.30
		16.35					
		16.15					
	Dilution 3	20.06	19.87	0.17	0.85%	0.10	19.87 +/- 0.43
		19.74					
		19.81					
	Dilution 4	23.55	23.49	0.09	0.39%	0.07	23.49 +/- 0.30
		23.42					
	Dilution 5	27.16	28.27	0.11	0.38%	0.06	28.27 +/- 0.26
		27.27					
		27.05					
	Dilution 6	30.49	31.24	0.15	0.48%	0.09	31.24 +/- 0.39
		30.38					
		30.19					
	Dilution 7	33.14	33.83	0.74	2.20%	0.43	33.86 +/- 1.85
		33.74					
		34.62					
Sulfate Reducing Bacteria	Dilution 1	15.46	15.22	0.21	1.37%	0.12	15.22 +/- 0.52
		15.07					
		15.14					
	Dilution 2	18.76	18.80	0.13	0.69%	0.07	18.80 +/- 0.32
		18.94					
		18.69					
	Dilution 3	22.82	22.81	0.06	0.24%	0.03	22.81 +/- 0.14
		22.86					
		22.75					
	Dilution 4	26.43	26.61	0.16	0.58%	0.09	26.61 +/- 0.39
		26.72					
		26.67					
	Dilution 5	29.79	30.17	0.33	1.10%	0.19	30.17 +/- 0.83
		30.41					
		30.31					
	Dilution 6	34	34.44	0.39	1.12%	0.22	34.44 +/- 0.96
		34.59					
		34.73					
	Dilution 7	37.57	38.33	0.68	1.77%	0.39	38.33 +/- 1.69
		38.53					
		38.88					

Assay	10-Fold Dilution Series	Ct	Average Ct	Standard Deviation	Co-efficient of Variation	Standard Error	95% Confidence Limit
Bacteria	Dilution 1	10.44 10.37	10.41	0.05	0.48%	0.04	10.41 +/- 0.51
	Dilution 2	14.25 13.95 13.75	13.98	0.25	1.80%	0.15	13.98 +/- 0.63
	Dilution 3	17.92 17.66 17.28	17.62	0.32	1.83%	0.19	17.62 +/-_0.80
	Dilution 4	21.71 20.92 21.23	21.29	0.40	1.87%	0.23	21.29 +/- 0.99
	Dilution 5	24.95 24.87 24.59	24.80	0.19	0.76%	0.11	24.80 +/- 0.47
	Dilution 6	28.67 28.19 28.21	28.36	0.27	0.96%	0.16	28.36 +/- 0.67
	Dilution 7	31.03 31.2 31.48	31.24	0.23	0.73%	0.13	31.24 +/- 0.56

APPENDIX I: PUBLICATIONS ARISING FROM THIS THESIS

Stewart, J., Chadwick, V.S., and Murray, A. (2005). The Human Faecal Microflora: Investigations into Host Genetic Control and Re-constitution. NZ Bio Science, *14*.

Stewart, J.A., Chadwick, V.S., and Murray, A. (2005). Investigations into the influence of host genetics on the predominant eubacteria in the faecal microflora of children. J Med Microbiol (In Press).

Stewart, J.A., Chadwick, V.S., and Murray, A. (2005). Investigations into the genetic control of the colonic microflora in healthy individuals and patients with inflammatory bowel disease. Abstract. In, Inflam Res *54*, suppl 2, 5094.

REFERENCES

Abreu, M.T., Fukata, M., and Arditi, M. (2005). TLR signaling in the gut in health and disease. J Immunol *174*, 4453-60.

Acton, R.T., Dasanayake, A.P., Harrison, R.A., Li, Y., Roseman, J.M., Go, R.C., Wiener, H., and Caufield, P.W. (1999). Associations of MHC genes with levels of caries-inducing organisms and caries severity in African-American women. Hum Immunol *60*, 984-9.

Ahmad, T., Satsangi, J., McGovern, D., Bunce, M., and Jewell, D.P. (2001). Review article: the genetics of inflammatory bowel disease. Aliment Pharmacol Ther *15*, 731-48.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev *59*, 143-69.

Apajalahti, J.H., Kettunen, A., Nurminen, P.H., Jatila, H., and Holben, W.E. (2003). Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl Environ Microbiol *69*, 5731-5.

Astrup, A., Meinert Larsen, T., and Harper, A. (2004). Atkins and other low-carbohydrate diets: hoax or an effective tool for weight loss? Lancet *364*, 897-9.

Atkins, R.C. (2002). Dr Atkins' New Diet Revoluiton (Avon Books, Harper Collins Publishers, New York).

Ayabe, T., Ashida, T., Kohgo, Y., and Kono, T. (2004). The role of Paneth cells and their antimicrobial peptides in innate host defense. Trends Microbiol *12*, 394-8.

Bach, H.J., Tomanova, J., Schloter, M., and Munch, J.C. (2002). Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. J Microbiol Methods *49*, 235-45.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005). Host-bacterial mutualism in the human intestine. Science *307*, 1915-20.

Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., and Flint, H.J. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl Environ Microbiol *66*, 1654-61.

Belson, A., Shetty, A.K., Yorgin, P.D., Bujanover, Y., Peled, Y., Dar, M.H., and Reif, S. (2003). Colonic hydrogen elimination and methane production in infants with and without infantile colic syndrome. Dig Dis Sci *48*, 1762-6.

Berg, R.D. (1996). The indigenous gastrointestinal microflora. Trends Microbiol *4*, 430-5.

Berg, RD. Immunity: Stimulation by Indigenous Gastrointestinal Microflora. Encyclopedia of Life Sciences . Nature Publishing Group. 2001.

Bibiloni, R., Fedorak, R.N., Tannock, G.W., Madsen, K.L., Gionchetti, P., Campieri, M., De Simone, C., and Sartor, B.B. (2005). VSL#3 probiotic mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol *100*, 1539-46.

Bjorneklett, A. and Jenssen, E. (1982). Relationships between hydrogen (H2) and methane (CH4) production in man. Scand J Gastroenterol *17*, 985-92.

Bond, J.H. Jr, Engel, R.R., and Levitt, M.D. (1971). Factors influencing pulmonary methane excretion in man. An indirect method of studying the *in situ* metabolism of the methane-producing colonic bacteria. J Exp Med *133*, 572-88.

Bourlioux, P., Koletzko, B., Guarner, F., and Braesco, V. (2003). The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. Am J Clin Nutr *78*, 675-83.

Bourne, D.G. and Munn, C.B. (2005). Diversity of bacteria associated with the coral Pocillopora damicornis from the Great Barrier Reef. Environ Microbiol *7*, 1162-74.

Brusa, T., Canzi, E., Allievi, L., Del Puppo, E., and Ferrari, A. (1993). Methanogens in the human intestinal tract and oral cavity. Curr Microbiol *27*, 261-265.

Bullock, N.R., Booth, J.C., and Gibson, G.R. (2004). Comparative composition of bacteria in the human intestinal microflora during remission and active ulcerative colitis. Curr Issues Intest Microbiol *5*, 59-64.

Cairns, M.J. and Murray, V. (1994). Rapid Silver Staining and Recovery of PCR Products Separated on Polyacrylamide Gels. BioTechniques *17*, 915-19

Casamayor, E.O., Massana, R., Benlloch, S., Ovreas, L., Diez, B., Goddard, V.J., Gasol, J.M., Joint, I., Rodriguez-Valera, F., and Pedros-Alio, C. (2002). Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. Environ Microbiol *4*, 338-48.

Cebra, J.J. (1999). Influences of microbiota on intestinal immune system development. Am J Clin Nutr *69*, 1046S-1051S.

Chadwick, V.S., Chen, W., Shu, D., Paulus, B., Bethwaite, P., Tie, A., and Wilson, I. (2002). Activation of the mucosal immune system in irritable bowel syndrome. Gastroenterology *122*, 1778-83.

Christl, S.U., Gibson, G.R., and Cummings, J.H. (1992). Role of dietary sulphate in the regulation of methanogenesis in the human large intestine. Gut *33*, 1234-8.

Coyne, M.J., Reinap, B., Lee, M.M., and Comstock, L.E. (2005). Human symbionts use a host-like pathway for surface fucosylation. Science *307*, 1778-81.

Coyne, M.J., Weinacht, K.G., Krinos, C.M., and Comstock, L.E. (2003). Mpi recombinase globally modulates the surface architecture of a human commensal bacterium. Proc Natl Acad Sci U S A *100*, 10446-51.

Cummings, J.H. and Macfarlane, G.T. (1997). Role of intestinal bacteria in nutrient metabolism. JPEN J Parenter Enteral Nutr *21*, 357-65.

Dear, K.L., Elia, M., and Hunter, J.O. (2005). Do interventions which reduce colonic bacterial fermentation improve symptoms of irritable bowel syndrome? Dig Dis Sci *50*, 758-66.

Deplancke, B., Hristova, K.R., Oakley, H.A., McCracken, V.J., Aminov, R., Mackie, R.I., and Gaskins, H.R. (2000). Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. Appl Environ Microbiol *66*, 2166-74.

Dickinson, R.J., Varian, S.A., Axon, A.T., and Cooke, E.M. (1980). Increased incidence of faecal coliforms with *in vitro* adhesive and invasive properties in patients with ulcerative colitis. Gut *21*, 787-92.

Dore, J., Morvan, B., Rieu-Lesme, F., Goderel, I., Gouet, P., and Pochart, P. (1995). Most probable number enumeration of H2-utilizing acetogenic bacteria from the digestive tract of animals and man. FEMS Microbiol Lett *130*, 7-12.

Drasar, B. S. The Host and His Gut Flora: Bacteria. Manuel, P. D. Walker-Smith J. A. and Tomkins A. Infections of the Gastrointestinal Tract. 1-10. 86. Longman Group Limited.

Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S., and Flint, H.J. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. Int J Syst Evol Microbiol *52*, 1615-20.

Dzierzewicz, Z., Cwalina, B., Weglarz, L., Wisniowska, B., and Szczerba, J. (2004a). Susceptibility of Desulfovibrio desulfuricans intestinal strains to sulfasalazine and its biotransformation products. Med Sci Monit *10*, BR185-90.

Dzierzewicz, Z., Cwalina, B., Weglarz, L., Wisniowska, B., and Szczerba, J. (2004b). Susceptibility of Desulfovibrio desulfuricans intestinal strains to sulfasalazine and its biotransformation products. Med Sci Monit *10*, BR185-90.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A. (2005). Diversity of the human intestinal microbial flora. Science *308*, 1635-8.

El Oufir, L., Flourie, B., Bruley des Varannes, S., Barry, J.L., Cloarec, D., Bornet, F., and Galmiche, J.P. (1996). Relations between transit time, fermentation products, and hydrogen consuming flora in healthy humans. Gut *38*, 870-7.

Espejo, R.T., Feijoo, C.G., Romero, J., and Vasquez, M. (1998). PAGE analysis of the heteroduplexes formed between PCR-amplified 16S rRNA genes: estimation of sequence similarity and rDNA complexity. Microbiology *144 (Pt 6)*, 1611-7.

Fabia, R., Ar'Rajab, A., Johansson, M.L., Andersson, R., Willen, R., Jeppsson, B., Molin, G., and Bengmark, S. (1993). Impairment of bacterial flora in human ulcerative colitis and experimental colitis in the rat. Digestion *54*, 248-55.

Fagarasan, S. and Honjo, T. (2004). Regulation of IgA synthesis at mucosal surfaces. Curr Opin Immunol *16* , 277-83.

Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K., and Honjo, T. (2001). *In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature *413*, 639-43.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., and Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424-7.

Fagarasan, S. and Honjo, T. (2003). Intestinal IgA synthesis: regulation of front-line body defences. Nat Rev Immunol *3*, 62-72.

Falciglia, G.A. and Norton, P.A. (1994). Evidence for a genetic influence on preference for some foods. J Am Diet Assoc *94*, 154-8.

Fang, Y., Wu, W. H., Pepper, J. L., Larsen, J. L., Marras, S. A., and Nelson, E. A. (2002). Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples. J Clin Microbiol *40*, 287-91

Favier, C.F., Vaughan, E.E., De Vos, W.M., and Akkermans, A.D. (2002). Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol *68*, 219-26.

Fite, A., Macfarlane, G.T., Cummings, J.H., Hopkins, M.J., Kong, S.C., Furrie, E., and Macfarlane, S. (2004). Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. Gut *53*, 523-9.

Flatz, G., Czeizel, A., Metneki, J., Flatz, S.D., Kuhnau, W., and Jahn, D. (1985). Pulmonary hydrogen and methane excretion following ingestion of an unabsorbable carbohydrate: a study of twins. J Pediatr Gastroenterol Nutr *4*, 936-41.

Fleet Pharmaceuticals. URL accessed July 2005. http://www.phosphosoda.com/

Florin, T.H. and Woods, H.J. (1995). Inhibition of methanogenesis by human bile. Gut *37*, 418-21.

Florin, T.H., Zhu, G., Kirk, K.M., and Martin, N.G. (2000). Shared and unique environmental factors determine the ecology of methanogens in humans and rats. Am J Gastroenterol *95*, 2872-9.

Fontana, C., Vignolo, G., and Cocconcelli, P.S. (2005). PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. J Microbiol Methods

Franchimont, D., Vermeire, S., El Housni, H., Pierik, M., Van Steen, K., Gustot, T., Quertinmont, E., Abramowicz, M., Van Gossum, A., Deviere, J., and Rutgeerts, P. (2004). Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut *53*, 987-92.

Friedrich, M.W. (2002). Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. J Bacteriol *184*, 278-89.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., and Rossi, P. (2002). Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ Microbiol *4*, 634-43.

Furrie, E., Macfarlane, S., Kennedy, A., Cummings, J.H., Walsh, S.V., O'neil, D.A., and Macfarlane, G.T. (2005a). Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut *54*, 242-9.

Furrie, E., Macfarlane, S., Thomson, G., and Macfarlane, G.T. (2005b). Toll-like receptors-2, -3, and –4 expression patterns on human colon and their regulation by mucosal-associated bacteria. Immunology *115*, 565-74.

Galvez, J., Rodriguez-Cabezas, M.E., and Zarzuelo, A. (2005). Effects of dietary fiber on inflammatory bowel disease. Mol Nutr Food Res *49*, 601-8.

Garcia-Lafuente, A., Antolin, M., Guarner, F., Crespo, E., and Malagelada, J.R. (2001). Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut *48*, 503-7.

Gassull, M.A. (2004). Review article: the role of nutrition in the treatment of inflammatory bowel disease. Aliment Pharmacol Ther *20 Suppl 4*, 79-83.

Gaurner, F., and Malagelada J.-R. (2003). Gut flora in health and disease. Lancet *360*, 512-9.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J., and Madara, J.L. (2001). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol *167*, 1882-5.

Geypens, B., Claus, D., Evenepoel, P., Hiele, M., Maes, B., Peeters, M., Rutgeerts, P., and Ghoos, Y. (1997). Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. Gut *41*, 70-6.

Giaffer, M.H., Holdworth, C.D., and Duerden, B.I. (1991). The assessment of faecal flora in patients with inflammatory bowel disease by a simplified bacteriolocial technique. J Med Microbiol *35*, 238-43.

Gibson, G.R., Macfarlane, G.T., and Cummings, J.H. (1988). Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. J Appl Bacteriol *65*, 103-11.

Gibson, G.R., Macfarlane, S., and Macfarlane, G.T. (1993a). Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. FEMS Microb Ecol *12*, 117-25.

Gibson, G.R., Macfarlane, G.T., and Cummings, J.H. (1993b). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut *34*, 437-9.

Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., Poggioli, G., Miglioli, M., and Campieri, M. (2000). Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology *119*, 305-9.

Grossart, H.P., Levold, F., Allgaier, M., Simon, M., and Brinkhoff, T. (2005). Marine diatom species harbour distinct bacterial communities. Environ Microbiol *7*, 860-73.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature *389*, 737-42.

Haines, A., Metz, G., Dilawari, J., Blendis, L., and Wiggins, H. (1977). Breath-methane in patients with cancer of the large bowel. Lancet *2*, 481-3.

Halfvarson, J., Bodin, L., Tysk, C., Lindberg, E., and Jarnerot, G. (2003). Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology *124*, 1767-73.

Hampe, J., Cuthbert, A., Croucher, P.J., Mirza, M.M., Mascheretti, S., Fisher, S., Frenzel, H., King, K., Hasselmeyer, A., MacPherson, A.J., Bridger, S., van Deventer, S., Forbes, A., Nikolaus, S., Lennard-Jones, J.E., Foelsch, U.R., Krawczak, M., Lewis, C., Schreiber, S., and Mathew, C.G. (2001). Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet *357*, 1925-8.

Hao, W.L. and Lee, Y.K. (2004). Microflora of the gastrointestinal tract: a review. Methods Mol Biol *268*, 491-502.

Harper, P.H., Lee, E.C., Kettlewell, M.G., Bennett, M.K., and Jewell, D.P. (1985). Role of the faecal stream in the maintenance of Crohn's colitis. Gut *26*, 279-84.

Hartley, M.G., Hudson, M.J., Swarbrick, E.T., Hill, M.J., Gent, A.E., Hellier, M.D., and Grace, R.H. (1992). The rectal mucosa-associated microflora in patients with ulcerative colitis. J Med Microbiol *36*, 96-103.

Hebden, J.M., Blackshaw, E., D'Amato, M., Perkins, A.C., and Spiller, R.C. (2002). Abnormalities of GI transit in bloated irritable bowel syndrome: effect of bran on transit and symptoms. Am J Gastroenterol *97*, 2315-20.

Heidelberg, J.F., Seshadri, R., Haveman, S.A., Hemme, C.L., Paulsen, I.T., Kolonay, J.F., Eisen, J.A., Ward, N., Methe, B., Brinkac, L.M., Daugherty, S.C., Deboy, R.T., Dodson, R.J., Durkin, A.S., Madupu, R., Nelson, W.C., Sullivan, S.A., Fouts, D., Haft, D.H., Selengut, J., Peterson, J.D., Davidsen, T.M., Zafar, N.,

Zhou, L., Radune, D., Dimitrov, G., Hance, M., Tran, K., Khouri, H., Gill, J., Utterback, T.R., Feldblyum, T.V., Wall, J.D., Voordouw, G., and Fraser, C.M. (2004). The genome sequence of the anaerobic, sulfate-reducing bacterium Desulfovibrio vulgaris Hildenborough. Nat Biotechnol *22*, 554-9.

Heilig, H.G., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D., and de Vos, W.M. (2002). Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol *68*, 114-23.

Holdeman, L.V., Good, I.J., and Moore, W.E. (1976). Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol *31*, 359-75.

Hooper, L.V. and Gordon, J.I. (2001). Commensal host-bacterial relationships in the gut. Science *292*, 1115-8.

Hooper, L.V., Midtvedt, T., and Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr *22*, 283-307.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. Science *291*, 881-4.

Hooper, L.V., Xu, J., Falk, P.G., Midtvedt, T., and Gordon, J.I. (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc Natl Acad Sci U S A *96*, 9833-8.

Hopkins, M.J., Sharp, R., and Macfarlane, G.T. (2001). Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut *48*, 198-205.

Hudson, M.J., Tomkins, A.M., Wiggins, H.S., and Drasar, B.S. (1993). Breath methane excretion and intestinal methanogenesis in children and adults in rural Nigeria. Scand J Gastroenterol *28*, 993-8.

Hughes, M.S., Beck, L.A., and Skuce, R.A. (1994). Identification and elimination of DNA sequences in Taq DNA polymerase. J Clin Microbiol *32*, 2007-8.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M., and Thomas, G. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature *411*, 599-603.

Huijsdens, X.W., Linskens, R.K., Mak, M., Meuwissen, S.G., Vandenbroucke-Grauls, C.M., and Savelkoul, P.H. (2002). Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J Clin Microbiol *40*, 4423-7.

Iwasaki, A. and Kelsall, B.L. (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper

type 2 cells. J Exp Med *190*, 229-39.

Iwasaki, A. and Kelsall, B.L. (2001). Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. J Immunol *166*, 4884-90.

Jang, M.H., Kweon, M.N., Iwatani, K., Yamamoto, M., Terahara, K., Sasakawa, C., Suzuki, T., Nochi, T., Yokota, Y., Rennert, P.D., Hiroi, T., Tamagawa, H., Iijima, H., Kunisawa, J., Yuki, Y., and Kiyono, H. (2004). Intestinal villous M cells: an antigen entry site in the mucosal epithelium. Proc Natl Acad Sci U S A *101*, 6110-5.

Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. (2001). Endotoxin-induced maturation of MyD88-deficient dendritic cells. J Immunol *166*, 5688-94.

Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., and Conway, S. (2004). Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nat Immunol *5*, 104-12.

Kennedy, R.J., Kirk, S.J., and Gardiner, K.R. (2002). Mucosal barrier function and the commensal flora. Gut *50*, 441-2.

King, T.S., Elia, M., and Hunter, J.O. (1998). Abnormal colonic fermentation in irritable bowel syndrome. Lancet *352*, 1187-9.

Kisand, V., and Wilkner, J. (2003). Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J Microbiol Methods *54*, 183-91.

Koide, A., Yamaguchi, T., Odaka, T., Koyama, H., Tsuyuguchi, T., Kitahara, H., Ohto, M., and Saisho, H. (2000). Quantitative analysis of bowel gas using plain abdominal radiograph in patients with irritable bowel syndrome. Am J Gastroenterol *95*, 1735-41.

Kowalchuk, G.A., Gerards, S., and Woldendorp, J.W. (1997a). Detection and characterization of fungal infections of Ammophila arenaria (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18s rDNA. Appl Environ Microbiol *63*, 3858-65.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M., and Woldendorp, J.W. (1997b). Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol *63*, 1489-97.

Krinos, C.M., Coyne, M.J., Weinacht, K.G., Tzianabos, A.O., Kasper, D.L., and Comstock, L.E. (2001). Extensive surface diversity of a commensal microorganism by multiple DNA inversions. Nature *414*, 555-8.

Lajoie, S.F., Bank, S., Miller, T.L., and Wolin, M.J. (1988). Acetate production from hydrogen and. Appl Environ Microbiol *54*, 2723-7.

Landers, C.J., Cohavy, O., Misra, R., Yang, H., Lin, Y.C., Braun, J., and Targan, S.R. (2002). Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. Gastroenterology *123*, 689-99.

Langlands, S.J., Hopkins, M.J., Coleman, N., and Cummings, J.H. (2004). Prebiotics carbohydrates modify the mucosa associated microflora of the human large bowel. Gut *53*, 1610-16.

Lee, J.S., Heo, G.Y., Lee, J.W., Oh, Y.J., Park, J.A., Park, Y.H., Pyun, Y.R., and Ahn, J.S. (2005). Analysis of kimchi microflora using denaturing gradient gel electrophoresis. Int J Food Microbiol *102*, 143-50.

Levine, A., Karban, A., Eliakim, R., Shaoul, R., Reif, S., Pacht, A., Wardi, J., Yakir, B., and Silver, E.L. (2005). A polymorphism in the TNF-alpha promoter gene is associated with pediatric onset and colonic location of Crohn's disease. Am J Gastroenterol *100*, 407-13.

Levine, J., Ellis, C.J., Furne, J.K., Springfield, J., and Levitt, M.D. (1998). Fecal hydrogen sulfide production in ulcerative colitis. Am J Gastroenterol *93*, 83-7.

Levitt, M.D.G.G.R.a.C.S.U. (1995). Gas Metabolism in the Large Intestine. In Human Colonic Bacteria. Role in Nutrition, Physiology, and Pathology. G.R.a.M.G.T. Gibson, ed. (CRC Press, Inc), pp. 131-154.

Levy, J. (2000). The effects of antibiotic use on gastrointestinal function. Am J Gastroenterol *95*, S8-10.

Li, M., Gong, J., Cottrill, M., Yu, H., de Lange, C., Burton, J., and Topp, E. (2003). Evaluation of QIAamp DNA Stool Mini Kit for ecological studies of gut microbiota. J Microbiol Methods *54*, 13-20.

Maarit Niemi, R., Heiskanen, I., Wallenius, K., and Lindstrom, K. (2001). Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J Microbiol Methods *45*, 155-65.

Macdonald, T.T. and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. Science *307*, 1920-5.

Macfarlane, G.T. and Macfarlane, S. (1997). Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand J Gastroenterol Suppl *222*, 3-9.

Mackay, I.M. (2004). Real-time PCR in the microbiology laboratory. Clin Microbiol Infect *10*, 190-212.

Mackie, R.I., Sghir, A., and Gaskins, H.R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr *69*, 1035S-1045S.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-6.

Magee, E.A., Richardson, C.J., Hughes, R., and Cummings, J.H. (2000). Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans. Am J Clin Nutr *72*, 1488-94.

Mai, V., Katki, H.A., Harmsen, H., Gallaher, D., Schatzkin, A., Baer, D.J., and Clevidence, B. (2004). Effects of a controlled diet and black tea drinking on the fecal microflora composition and the fecal bile acid profile of human volunteers in a double-blinded randomized feeding study. J Nutr *134*, 473-8.

Malinen, E., Kassinen, A., Rinttila, T., and Palva, A. (2003). Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiology *149*, 269-77.

Malinen, E., Rinttila, T., Kajander, K., Matto, J., Kassinen, A., Krogius, L., Saarela, M., Korpela, R., and Palva, A. (2005). Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol *100*, 373-82.

Marteau, P., Lepage, P., Mangin, I., Suau, A., Dore, J., Pochart, P., and Seksik, P. (2004). Review article: gut flora and inflammatory bowel disease. Aliment Pharmacol Ther *20 Suppl 4*, 18-23.

Martino, T.K., Hernandez, J.M., Beldarrain, T., and Melo, L. (1998). Identification of bacteria in water for pharmaceutical use. Rev Latinoam Microbiol *40*, 142-50.

Matto, J., Maunuksela, L., Kajander, K., Palva, A., Korpela, R., Kassinen, A., and Saarela, M. (2005). Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. FEMS Immunol Med Microbiol *43*, 213-22.

Matzinger, P. (1998). An innate sense of danger. Semin Immunol *10*, 399-415.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science *296*, 301-5.

Maxwell, P.R., Rink, E., Kumar, D., and Mendall, M.A. (2002). Antibiotics increase functional abdominal symptoms. Am J Gastroenterol *97*, 104-8.

McCaig, A.E., Phillips, C.J., Stephen, J.R., Kowalchuk, G.A., Harvey, S.M., Herbert, R.A., Embley, T.M., and Prosser, J.I. (1999). Nitrogen cycling and community structure of proteobacterial beta-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. Appl Environ Microbiol *65*, 213-20.

McCracken, V.J., Simpson, J.M., Mackie, R.I., and Gaskins, H.R. (2001). Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J Nutr *131*, 1862-70.

McOrist, A.L., Jackson, M., and Bird, A.R. (2002). A comparison of five methods for extraction of bacterial DNA from human faecal samples. J Microbiol Methods *50*, 131-9.

Metchnikoff, E. (1907). The prolongation of life. Optimistic Studies (London, United

Kingdom: William Heinemann).

Metchnikoff, E. (1908). The nature of man. Studies of optimistic philosophy. (London, United Kingdom: William Heinemann).

Miller, T.L. and Wolin, M.J. (1983). Stability of Methanobrevibacter smithii populations in the microbial flora excreted from the human large bowel. Appl Environ Microbiol *45*, 317-8.

Miller, T.L. and Wolin, M.J. (1985). Methanosphaera stadtmaniae gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Arch Microbiol *141*, 116-22.

Miller, T.L., Wolin, M.J., de Macario, E.C., and Macario, A.J. (1982). Isolation of Methanobrevibacter smithii from human feces. Appl Environ Microbiol *43*, 227-32.

Monstein, H.J., Tiveljung, A., Kraft, C.H., Borch, K., and Jonasson, J. (2000). Profiling of bacterial flora in gastric biopsies from patients with Helicobacter pylori-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. J Med Microbiol *49*, 817-22.

Moore, J., Babidge, W., Millard, S., and Roediger, W. (1998). Colonic luminal hydrogen sulfide is not elevated in ulcerative colitis. Dig Dis Sci *43*, 162-5.

Moore, W.E. and Holdeman, L.V. (1974). Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol *27*, 961-79.

Moore, W.E. and Moore, L.H. (1995). Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol *61*, 3202-7.

Morotomi, M., Guillem, J.G., Pocsidio, J., LoGerfo, P., Treat, M., Forde, K.A., Weinstein, I.B., Watanabe, I., and Mutai, M. (1989). Effect of polyethylene glycol-electrolyte lavage solution on intestinal microflora. Appl Environ Microbiol *55*, 1026-8.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol *2*, 317-22.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek *73*, 127-41.

Myers, R.M., Fischer, S.G., Lerman, L.S., and Maniatis, T. (1985). Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Res *13*, 3131-45.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology *148*, 257-66.

Nagler-Anderson, C. (2001). Man the barrier! Strategic defences in the intestinal mucosa. Nat Rev Immunol *1*, 59-67.

Neish, A.S., Gewirtz, A.T., Zeng, H., Young, A.N., Hobert, M.E., Karmali, V., Rao, A.S., and Madara, J.L. (2000). Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science *289*, 1560-3.

Nelson, K.E., zinder, S.H., Hance, I., Burr, P., Odongo, D., Wasawo, D., Odenyo, A., and Bishop, R. (2003). Phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract: insights into an unexplored niche. Environ Microbiol *5*, 1212-20.

Neut, C., Bulois, P., Desreumaux, P., Membre, J.M., Lederman, E., Gambiez, L., Cortot, A., Quandalle, P., van Kruiningen, H., and Colombel, J.F. (2002). Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. Am J Gastroenterol *97*, 939-46.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., Littman, D.R., and Reinecker, H.C. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science *307*, 254-8.

Nobaek, S., Johansson, M.L., Molin, G., Ahrne, S., and Jeppsson, B. (2000). Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. Am J Gastroenterol *95*, 1231-8.

Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., and Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J Bacteriol *178*, 5636-43.

O'Keefe, S.J., Kidd, M., Espitalier-Noel, G., and Owira, P. (1999). Rarity of colon cancer in Africans is associated with low animal product consumption, not fiber. Am J Gastroenterol *94*, 1373-80.

Obana, N., Takahashi, S., Kinouchi, Y., Negoro, K., Takagi, S., Hiwatashi, N., and Shimosegawa, T. (2002). Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, CD14. Scand J Gastroenterol *37*, 699-704.

Ohge, H., Furne, J.K., Springfield, J., Rothenberger, D.A., Madoff, R.D., and Levitt, M.D. (2005). Association between fecal hydrogen sulfide production and pouchitis. Dis Colon Rectum *48*, 469-75.

Osborne, C.A., Galic, M., Sangwan, P., and Janssen, P.H. (2005). PCR-generated artefact from 16S rRNA gene-specific primers. FEMS Microbiol Lett *248*, 183-7.

Ott, S.J., Musfeldt, M., Wenderoth, D.F., Hampe, J., Brant, O., Folsch, U.R., Timmis, K.N., and Schreiber, S. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut *53*, 685-93.

Otte, J.M., Cario, E., and Podolsky, D.K. (2004). Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology *126*, 1054-70.

Parry, V. A matter of life and death. URL accessed July 2005. http://www.guardian.co.uk/genes/article/0,2763,1254975,00.html

Passos, M.C., Serra, J., Azpiroz, F., Tremolaterra, F., and Malagelada, J.R. (2005). Impaired reflex control of intestinal gas transit in patients with abdominal bloating. Gut *54*, 344-8.

Peck, H.D. (1962). Symposium on metabolism of inorganic compounds. V. Comparative metabolism of inorganic sulfur compounds in microorganisms. Bacteriol Rev *26*, 67-94.

Peled, Y., Gilat, T., Liberman, E., and Bujanover, Y. (1985). The development of methane production in childhood and adolescence. J Pediatr Gastroenterol Nutr *4*, 575-9.

Peled, Y., Weinberg, D., Hallak, A., and Gilat, T. (1987). Factors affecting methane production in humans. Gastrointestinal diseases and alterations of colonic flora. Dig Dis Sci *32*, 267-71.

Peltonen, R., Nenonen, M., Helve, T., Hanninen, O., Toivanen, P., and Eerola, E. (1997). Faecal microbial flora and disease activity in rheumatoid arthritis during a vegan diet. Br J Rheumatol *36*, 64-8.

Picton, R., Eggo, M.C., Merrill, G.A., Langman, M.J., and Singh, S. (2002). Mucosal protection against sulphide: importance of the enzyme rhodanese. Gut *50*, 201-5.

Pimentel, M., Mayer, A.G., Park, S., Chow, E.J., Hasan, A., and Kong, Y. (2003). Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Dig Dis Sci *48*, 86-92.

Pique, J.M., Pallares, M., Cuso, E., Vilar-Bonet, J., and Gassull, M.A. (1984). Methane production and colon cancer. Gastroenterology *87*, 601-5.

Pitcher, M.C., Beatty, E.R., and Cummings, J.H. (2000). The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. Gut *46*, 64-72.

Pitcher, M.C. and Cummings, J.H. (1996). Hydrogen sulphide: a bacterial toxin in ulcerative colitis? Gut *39*, 1-4.

Pitcher, M.C.L., Beatty, E.R., and Cummings, J.H. (1995). Salicylates inhibit bacterial sulphide production within the coloinc lumen in ulcerative colitis. Gut *37*, A15

Pitcher, M.C.L., Gibson, G.R., Neale, G., and Cummings, J.H. (1994). Gentamcin kills multiple drug-resistant sulfate-reducing bacteria in patients with ulcerative colitis. Gastroenterology *106*, A753

Polz, M.F., and Cavanaugh, C.M. (1998). Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol *64*, 3724-30.

Porrini, M., Santangelo, A., Crovetti, R., Riso, P., Testolin, G., and Blundell, J.E. (1997). Weight, protein, fat, and timing of preloads affect food intake. Physiol Behav *62*, 563-70.

Prescott, L.M., Harley, J.P., and Klein, D.A. (1999). Microbiology (USA: McGraw Hill).

Qiu, X., Wu, L., Huang, H., McDonel, P.E., Palumbo, A.V., Tiedje, J.M., and Zhou, J. (2001). Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl Environ Microbiol *67*, 880-7.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell *118*, 229-41.

Rath, H.C., Schultz, M., Freitag, R., Dieleman, L.A., Li, F., Linde, H.J., Scholmerich, J., and Sartor, R.B. (2001). Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. Infect Immun *69*, 2277-85.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol *2*, 361-7.

Rhee, S.H., Keates, A.C., Moyer, M.P., and Pothoulakis, C. (2004). MEK is a key modulator for TLR5-induced interleukin-8 and MIP3alpha gene expression in non-transformed human colonic epithelial cells. J Biol Chem *279*, 25179-88.

Roche Molecular Biochemicals (2000). Absolute Quantification with External Standards. Technical Note No. LC 11/2000.

Roediger, W.E., Duncan, A., Kapaniris, O., and Millard, S. (1993). Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. Gastroenterology *104*, 802-9.

Roediger, W.E., Moore, J., and Babidge, W. (1997). Colonic sulfide in pathogenesis and treatment of ulcerative colitis. Dig Dis Sci *42*, 1571-9.

Rogosa, M. (1969). Acidaminococcus gen. n., Acidaminococcus fermentans sp. n., anaerobic gram-negative diplococci using amino acids as the sole energy source for growth. J Bacteriol *98*, 756-66.

Rook, G.A. and Brunet, L.R. (2005). Microbes, immunoregulation, and the gut. Gut *54*, 317-20.

Rutili, A., Brusa, T., Canzi, E., and Ferrari, A. (1993). Human intestinal methanogens and lactulose administration. New Microbiol *16*, 99-104.

Rutili, A., Canzi, E., Brusa, T., and Ferrari, A. (1996). Intestinal methanogenic bacteria

in children of different ages. New Microbiol *19*, 227-43.

Salvioli, B., Serra, J., Azpiroz, F., Lorenzo, C., Aguade, S., Castell, J., and Malagelada, J.R. (2005). Origin of gas retention and symptoms in patients with bloating. Gastroenterology *128*, 574-9.

Sansonetti, P.J. (2004). War and peace at mucosal surfaces. Nat Rev Immunol *4*, 953-64.

Sartor, R.B. (2004). Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology *126*, 1620-33.

Satokari, R.M., Vaughan, E.E., Akkermans, A.D., Saarela, M., and De Vos, W.M. (2001). Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal bifidobacterium populations in a prebiotic and probiotic feeding trial. Syst Appl Microbiol *24*, 227-31.

Schabereiter-Gurtner, C., Maca, S., Kaminsky, S., Rolleke, S., Lubitz, W., and Barisani-Asenbauer, T. (2002). Investigation of an anaerobic microbial community associated with a corneal ulcer by denaturing gradient gel electrophoresis and 16S rDNA sequence analysis. Diagn Microbiol Infect Dis *43*, 193-9.

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. Gut *35*, S35-8.

Schillinger, J. and Du Vall Knorr, S. (2004). Drinking-water quality and issues associated with water vending machines in the city of Los Angeles. J Environ Health *66*, 25-31, 43; quiz 45-6.

Schottelius, M. (1902). Die Bedeutung der Darmbakterien fur die Ehrnahrung. II. Archives of Hygiene *42*, 48-70.

Schreiber, S., Hanpe, J., Nikolaus, S., and Foelsch, U.R. (2004). Review article: exploration of the genetic aetiology of inflammatory bowel disease--implications for diagnosis and therapy. Aliment Pharmacol Ther *20 Suppl 4*, 1-8.

Segal, I., Walker, A.R., Lord, S., and Cummings, J.H. (1988). Breath methane and large bowel cancer risk in contrasting African populations. Gut *29*, 608-13.

Seksik, P., Rigottier-Gois, L., Gramet, G., Sutren, M., Pochart, P., Marteau, P., Jian, R., and Dore, J. (2003). Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut *52*, 237-42.

Sheffield, V.C., Cox, D.R., Lerman, L.S., and Myers, R.M. (1989). Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci U S A *86* , 232-6.

Shimoda, M., Inoue, Y., Azuma, N., and Kanno, C. (1999). Natural polyreactive immunoglobulin A antibodies produced in mouse Peyer's patches. Immunology

97, 9-17.

Simpson, J.M., McCracken, V.J., White, B.A., Gaskins, H.R., and Mackie, R.I. (1999). Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J Microbiol Methods *36*, 167-79.

Siqueira, J.F. Jr, Rocas, I.N., and Rosado, A.S. (2004). Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. Oral Microbiol Immunol *19*, 363-70.

Soares, A.C., Lederman, H.M., Fagundes-Neto, U., and de Morais, M.B. (2005). Breath methane associated with slow colonic transit time in children with chronic constipation. J Clin Gastroenterol *39*, 512-5.

Stappenbeck, T.S., Hooper, L.V., and Gordon, J.I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci U S A *99*, 15451-5.

Stark, P.L. and Lee, A. (1982). The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol *15*, 189-203.

Stephen, A.M. and Cummings, J.H. (1980). The microbial contribution to human faecal mass. J Med Microbiol *13*, 45-56.

Stille, W.a.T.H.G. (1984). Adenylylsulfate reductase in some new sulfate-reducing bacteria. Arch Microbiol *137*, 145-150.

Strocchi, A., Ellis, C.J., and Levitt, M.D. (1993). Use of metabolic inhibitors to study H2 consumption by human feces: evidence for a pathway other than methanogenesis and sulfate reduction. J Lab Clin Med *121*, 320-7.

Strocchi, A., Furne, J., Ellis, C., and Levitt, M.D. (1994). Methanogens outcompete sulphate reducing bacteria for H2 in the human colon. Gut *35*, 1098-101.

Stubner, S. (2002). Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreen detection. J Microbiol Methods *50*, 155-64.

Suzuki, M.T., Taylor, L.T., and DeLong, E.F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl Environ Microbiol *66*, 4605-14.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M., and Lochs, H. (2002). Mucosal flora in inflammatory bowel disease. Gastroenterology *122*, 44-54.

Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L.P., and Lochs, H. (2005). Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. J Clin Microbiol *43*, 3380-9

Takai, K. and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol *66*, 5066-72.

Talham, G.L., Jiang, H.Q., Bos, N.A., and Cebra, J.J. (1999). Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect Immun *67*, 1992-2000.

Tannock, G.W., Munro, K., Bibiloni, R., Simon, M.A., Hargreaves, P., Gopal, P., Harmsen, H., and Welling, G. (2004). Impact of consumption of oligosaccharide-containing biscuits on the fecal microbiota of humans. Appl Environ Microbiol *70*, 2129-36.

Tannock, G.W. (1999). Medical Improtance of the Normal Microflora (The Netherlands: Kluwer Academic Publishers).

Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernandez-Sueiro, J.L., Balish, E., and Hammer, R.E. (1994). The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med *180*, 2359-64.

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M.T., Engele, M., Sieling, P.A., Barnes, P.F., Rollinghoff, M., Bolcskei, P.L., Wagner, M., Akira, S., Norgard, M.V., Belisle, J.T., Godowski, P.J., Bloom, B.R., and Modlin, R.L. (2001). Induction of direct antimicrobial activity through mammalian toll-like receptors. Science *291*, 1544-7.

Thompson, J.R., Marcelino, L.A., and Polz, M.F. (2002). Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res *30*, 2083-8.

Thompson, W.G., Longstreth, G.F., Drossman, D.A., Heaton, K.W., Irvine, E.J., and Muller-Lissner, S.A. (1999). Functional bowel disorders and functional abdominal pain. Gut *45 Suppl 2*, II43-7.

Toivanen, P., Vaahtovuo, J., and Eerola, E. (2001). Influence of major histocompatibility complex on bacterial composition of fecal flora. Infect Immun *69*, 2372-7.

Turunen, U.M., Farkkila, M.A., Hakala, K., Seppala, K., Sivonen, A., Ogren, M., Vuoristo, M., Valtonen, V.V., and Miettinen, T.A. (1998). Long-term treatment of ulcerative colitis with ciprofloxacin: a prospective, double-blind, placebo-controlled study. Gastroenterology *115*, 1072-8.

Van de Merwe, J.P., Schroder, A.M., Wensinck, F., and Hazenberg, M.P. (1988). The obligate anaerobic faecal flora of patients with Crohn's disease and their first-degree relatives. Scand J Gastroenterol *23*, 1125-31.

Van de Merwe, J.P., Stegeman, J.H., and Hazenberg, M.P. (1983). The resident faecal flora is determined by genetic characteristics of the host. Implications for Crohn's disease? Antonie Van Leeuwenhoek *49*, 119-24.

van den Bogaard, A.E., Weidema, W.F., van Boven, C.P., and van der Waay, D. (1986). Recolonization and colonization resistance of the large bowel after three methods of preoperative preparation of the gastrointestinal tract for elective colorectal surgery. J Hyg (Lond) *97*, 49-59.

van den Bree, M.B., Eaves, L.J., and Dwyer, J.T. (1999). Genetic and environmental influences on eating patterns of twins aged >/=50 y. Am J Clin Nutr *70*, 456-65.

Vernia, P., Gnaedinger, A., Hauck, W., and Breuer, R.I. (1988). Organic anions and the diarrhea of inflammatory bowel disease. Dig Dis Sci *33*, 1353-8.

Viala, J., Sansonetti, P., and Philpott, D.J. (2004). Nods and 'intracellular' innate immunity. C R Biol *327*, 551-5.

Wang, L.H., Fang, X.C., and Pan, G.Z. (2004). Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. Gut *53*, 1096-101.

Watanabe, K., Teramoto, M., Futamata, H., and Harayama, S. (1998). Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl Environ Microbiol *64*, 4396-402.

Weaver, G.A., Krause, J.A., Miller, T.L., and Wolin, M.J. (1986). Incidence of methanogenic bacteria in a sigmoidoscopy population: an association of methanogenic bacteria and diverticulosis. Gut *27*, 698-704.

Weinacht, K.G., Roche, H., Krinos, C.M., Coyne, M.J., Parkhill, J., and Comstock, L.E. (2004). Tyrosine site-specific recombinases mediate DNA inversions affecting the expression of outer surface proteins of Bacteroides fragilis. Mol Microbiol *53*, 1319-30.

Woese, C.R. (1987). Bacterial evolution. Microbiol Rev *51*, 221-71.

Wold, A.E., Mestecky, J., Tomana, M., Kobata, A., Ohbayashi, H., Endo, T., and Eden, C.S. (1990). Secretory immunoglobulin A carries oligosaccharide receptors for Escherichia coli type 1 fimbrial lectin. Infect Immun *58*, 3073-7.

Woodmansey, E.J., McMurdo, M.E., Macfarlane, G.T., and Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl Environ Microbiol *70*, 6113-22.

Wostmann, B.S., Larkin, C., Moriarty, A., and Bruckner-Kardoss, E. (1983). Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci *33*, 46-50.

Zhang, C., Liu, Z.K., and Yu, P.W. (2004a). Effects of bile reflux and intragastric microflora changes on lesions of remnant gastric mucosa after gastric operation. World J Gastroenterol *10*, 1537-9.

Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A., and Ghosh, S. (2004b). A toll-like receptor that prevents infection by uropathogenic

bacteria. Science *303*, 1522-6.

Zoetendal, E.G., Akkermans, A.D., and De Vos, W.M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol *64*, 3854-9.

Zoetendal, E.G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A.D., and de Vos, W.M. (2002). Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol *68*, 3401-7.

Zoetendal, E.G., Akkermans A.D., Akkermans-van Vliet W.M., de Visser J.A., de Vos W.M. (2001). The Host Genotype Affects the Bacterial Community in the Human Gastronintestinal Tract. Microb Ecol Health and Dis *13*, 129-34.