IN VIVO UTILISATION OF FRUCTOOLIGOSACCHARIDES BY SHEEP FAECAL BIFIDOBACTERIA AND *IN VITRO* ANTAGONISTIC EFFECTS AGAINST INTESTINAL PATHOGENS

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ABSTRACT

The application of pro-, pre, and synbiotics has been studied primarily in humans and some other monogastric animals. Very few studies have been made to determine their effects on the ruminant intestinal microflora. This project tested whether or not four commercial food-grade oligo- and polysaccharides (three FOS products and one polysaccharide control, Arabinogalactan) could modify the hindgut microflora of sheep towards a more salutary community in which the health-promoting bacterial groups bifidobacteria and lactobacilli predominate, whereas the potential intestinal pathogens and putrefactive bacteria *E. coli* and sulphite reducing clostridia are suppressed.

A fructo-6-phosphate phosphokatolase (F6PPK) enzyme-based identification protocol was developed and optimised for identifying and large-scale screening of presumptive bifidobacteria isolates from gut contents or faecal samples.

An *in vivo* experiment was then carried out to determine the bifidogenic effect (promotion of bifidobacteria by prebiotics) and the associated antimicrobial effect (suppression of potential pathogens due to the increase in the populations of bifidobacteria) of the four oligo- and polysaccharides on sheep hindgut microflora. Twelve fistulated sheep were managed in a balanced, two Latin square, cross-over design experiment, which was run in 5 consecutive periods, with each of 5 treatments (Arabinogalactan, Fibruline, Raftilose, Yacon, and an "acidified saline" carbohydrate-free control) administered to two sheep in each period. Each period consisted of a 1 week of stabilisation to the pelleted diet (no oligo- and polysaccharides), followed by 14 days of daily abomasal supplementation of oligo- and polysaccharide/acidified saline, followed by about 12 days of normal pelleted diet. In each period, sheep faecal bifidobacteria, lactobacilli, *E. coli/ Enterobacteriaceae*, sulphite reducing clostridia, and total anaerobes were enumerated on the day -4 of the

"stabilisation" period; days 3 & 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions. Raftilose, Yacon, and Fibruline all exerted significant bifidogenic effects on sheep faecal bifidobacteria after 9 days of daily dosing. Raftilose produced the greatest stimulation of bifidobacteria, reaching counts of approximately 10^7 CFU/g of faeces. No significant changes in the populations of bifidobacteria were observed in Arabinogalactan-treated sheep. Raftilose and Yacon significantly increased the number of lactobacilli, reaching approximately 10^7 CFU/g of faeces, after 9 days of daily dosing. With Fibruline, the lactobacilli increase after 9 days of administration was not significant. Arabinogalactan did not elevate the populations of lactobacilli. All four carbohydrate treatments significantly increased the number of total anaerobes to approximately 10^7 to 10^8 CFU/g of faeces after 9 days of daily dosing. Supplementation of the test oligo- and polysaccharides had no significant effect on the other determined groups of gut microflora: sulphite reducing clostridia and *E. coli/Enterobacteriaceae*. There were no significant changes in sheep faecal pH and dry matter content with the four treatments.

Further *in vitro* antagonistic experiments were carried out to determine whether or not the isolated sheep faecal bifidobacteria inhibited the growth of potential intestinal pathogens in fermentation broth containing the bifidogenic FOS. One hundred and seventeen bifidobacterial isolates from sheep faeces, were screened for their capacity to utilise different oligo- and polysaccharides. Eighteen of these, with strong fermentation patterns, were selected for further study. In the first preliminary experiment, the 18 isolates plus 2 reference cultures of bifidobacteria were divided into 5 groups of 4 strains each. The 5 groups of bifidobacteria were compared for their *in vitro* antagonistic activities against *E. coli* in Peptone Yeast Extract broth containing Yacon, Raftilose, or Fibruline as primary carbon sources. Two groups, exerting 100% antagonistic effects against *E. coli* after 48-hour anaerobic co-culture at 37°C, were selected for further examination. The eight individual strains in these two groups were tested individually for their antagonistic activities against *E. coli* in

PY broth containing Yacon or Raftilose. Fibruline was eliminated due to its low antagonistic activity by bifidobacteria. Six isolates, showed 100% inhibitory effects against *E. coli*, which made them particularly promising for use as probiotics. The pH of the fermented broths showed a clear negative correlation with the rank transformed or angular transformed inhibition rate of *E. coli*. One bifidobacterial strain, P5-Po4-37, was subsequently investigated for its *in vitro* antagonistic activity against *E. coli* by determination of bacterial growth kinetics over 60 hours anaerobic incubation at 37°C in PY broth containing Raftilose or Yacon. In this experiment, two different concentrations of bifidobacteria, 10^7 to 10^8 CFU/mL and 10^3 to 10^4 CFU/mL, were incubated with 10^4 to 10^5 CFU/mL of *E. coli*. After 30 and 48-60 hours of incubation, the growth of *E. coli* was completely inhibited by both the higher and lower concentrations of bifidobacteria, respectively. The presence of *E. coli* did not affect the growth of bifidobacteria. With the higher inoculum level, the populations of bifidobacteria increased by only approximately 1.30 $\log_{10}$ cycles; whereas with the lower inoculum level, the populations of bifidobacteria increased by approximately 5.62 $\log_{10}$ cycles, to attain the same maximum viable counts at approximately 10^9 CFU/mL. Fermentation products were analysed in Raftilose containing PY fermented broth. The inhibitory activity of strain P5-Po4-37 was associated with the production of acetic and lactic acids. Strain P5-Po4-37 also exerted strong antagonistic activities against *Clostridium perfringens*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella* Dublin, and *Salmonella* Menston. These findings indicated that Yacon and Raftilose potentiate an organic acid mediated inhibitory action of bifidobacterial strain P5-Po4-37 against the test potential intestinal pathogens. To date, this demonstration of inhibitory activities has been convincingly made only in *in vitro* studies. The combination of Yacon or Raftilose and bifidobacterial strain P5-Po4-37 may exert a promising synbiotic effect on sheep hindgut microflora, which will be investigated *in vivo* in the near future.

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LIST OF ABBREVIATIONS

AG	Arabinogalactan
ATCC	American Type Culture Collection
BL medium	Glucose Blood-Liver medium
CFU	Colony forming unit
DAPI	4',6-diamidino-2-phenylindole
DM	Dry matter
DMCC	Direct microscopic clump count
DP	Degree of polymerization
DSM	Deutsche Sammlung von Mikroorganismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EMP	The Embden-Meyerhof Pathway
F6PPK	Fructose-6-phosphate phosphoketolase
FAB	Fastidious Anaerobe Broth
FISH	Fluorescent *in situ* hybridisation
FOS	Fructooligosaccharides
G+C	Guanine-plus-cytosine
GC	Gas Chromatography
GDH	Glutamate dehydrogenase
GGT	Gamma glutamyl transferase
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
HMG-CoA	Hydroxymethylglutaryl - Coenzyme A
IgA	Immunoglobulin A
IL	Interleukin
K_a	Dissociation constant
LAB	Lactic Acid Bacteria

LPS	Lipopolysaccharide
mL	Millilitres
MRSS	de Man, Rogosa, Sharpe agar with sorbic acid
NDOs	Non-digestible oligosaccharides
NZRM	New Zealand Reference Culture Collection, Medical Section
O/A	Omasum/Abomasum
OF test	Oxidation-fermentation test
PFGE	Pulse field gel electrophoresis
PG	Peptidoglycan
PP	Pellagra-preventing factor
PRA	Phenol Red Agar Base
PY	Peptone Yeast Extract
R/R	Rumen/Reticulum
RB	Raffinose-Bifidobacterium medium
RCM	Reinforced Clostridial Medium
REML	Residual maximum likelihood (or: Restricted maximum likelihood)
SCFA	Short chain fatty acids
SRC	Sulphite reducing clostridia
TGGE	Temperature gradient gel electrophoresis
TNFα	Tumour necrosis factor α
TPY medium	Tryptone Phytone Yeast medium
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSC	Egg-yolk-free Tryptose Sulphite Cycloserine agar
TTC	2,3,5-triphenyltetrazolium chloride
VFA	Volatile fatty acids
VLDL	very-low-density lipoproteins

Chapter 1

LITERATURE REVIEW — NUTRITIONAL MODULATION OF GUT MICROECOLOGY

1.1 The gastrointestinal microflora of mammals

1.1.1 The digestive system of mammals

The digestive system of mammals consists of the mouth, oesophagus, stomach, small intestine, caecum, colon and rectum. The small intestine can be divided into duodenum, jejunum and ileum. The caecum, colon and rectum constitute the large intestine (MacFarlane and Cummings, 1991). The stomach may be simple or complex. The animals which possess one simple stomach are known as monogastrics (**Figure 1.1**). These include humans, pigs, chickens, and rats. Ruminants, such as cows, sheep, and goats, possess a four-chambered stomach, comprised of the rumen, reticulum, omasum and abomasum (**Figure 1.2**).

1.1.1.1 Monogastric digestive physiology

The digestive process converts foods or feedstuffs into chemical compounds which can be absorbed into the bloodstream to be used as nutrients or to generate energy for the body.

Digestion begins in the mouth with the teeth, which grind the food into small particles. The tongue with taste buds, a powerful muscular organ, detects the

"taste sensations" of sour, sweet, bitter and salty in molecules of food dissolved in saliva, moves the food around during mastication, and initiates swallowing by moving the food to the pharynx. Saliva, a watery fluid, not only lubricates chewing and swallowing, but also begins the process of digestion with salivary amylase, which breaks down starch into disaccharides and dextrins. Saliva also contains the antimicrobial enzyme lysozyme and Immunoglobulin A (IgA) antibodies.

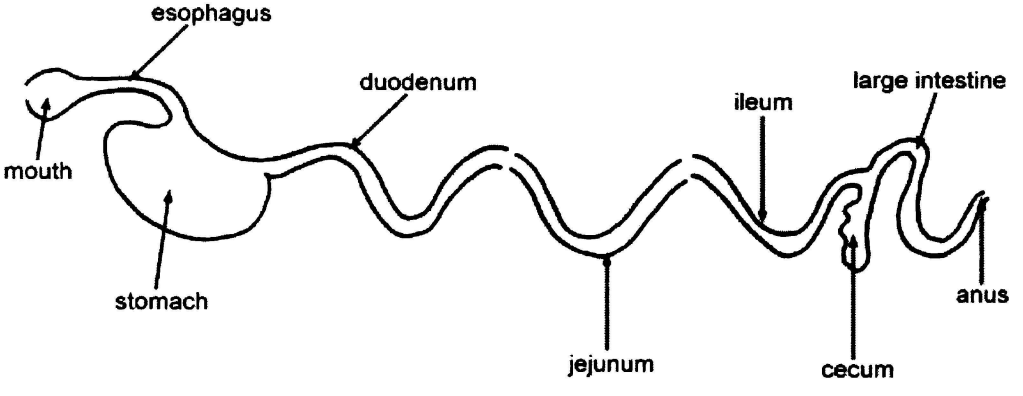


Figure 1.1 Digestive tract of the monogastric mammal (University of Kentucky, 2002a, reproduced with kind permission)

The pharynx, which is a mass of muscles and tissues, transports food into the oesophagus. The oesophagus, which connects the pharynx above with the stomach below, moves the swallowed food to the stomach by peristalsis.

The stomach stores the swallowed food and liquid, and churns its contents together with digestive juice produced by the stomach wall into a chyme. Protein digestion is initiated here with the breakdown of proteins to polypeptides; this is aided by the enzyme *pepsin* and hydrochloric acid. Absorption of small molecules such as water, aspirin and alcohol occurs from the stomach. The stomach also produces an intrinsic factor important for vitamin B12 absorption in

the small intestine. After several hours the stomach empties its contents slowly into the small intestine *via* the pyloric sphincter.

The small intestine is divided into three regions, duodenum, jejunum and ileum. Most chemical digestion of food occurs in the duodenum with the aid of secretions from the gut wall and from two other digestive organs – the pancreas and liver. The pancreas produces a digestive juice that contains a wide array of enzymes to break down the carbohydrate, fat, and protein in food. The liver produces yet another digestive juice – bile, which is stored between meals in the gallbladder. At mealtimes, it is squeezed out of the gallbladder into the bile ducts to reach the small intestine and mixed with the fat in food. The bile acids emulsify the fat into the watery contents of the small intestine. After the fat is emulsified, it is digested by enzymes from the pancreas and the lining of the small intestine. Finally, all of the digested molecules of food like amino acids, fatty acids and monosaccharides, as well as water and minerals from the diet, are absorbed from the cavity of the small intestine. Most absorbed materials cross the mucosa into the blood and are carried off in the bloodstream to other parts of the body for storage or further chemical change. Some digestion and some absorption occur in the jejunum, but most absorption occurs in the ileum, the lower small intestine. The enzymes in the small intestine are listed in **Table 1.1**.

All the indigestible foods, such as fibre, as well as a substantial part of the intestinal secretions, are propelled into the large intestine (colon), which harbours a large number of bacteria. These bacteria, which are commonly referred to as the intestinal microflora, have an enormous fermenting capacity. They degrade and ferment indigestible fibre ingredients. The end products of bacterial fermentation are absorbed and thus contribute to the total nutritional value of the ingested foods (Cummings and MacFarlane, 1991). The large intestine also functions in water absorption, production of some B vitamins and vitamin K synthesis, and waste storage.

Table 1.1 Enzymes involved in the digestion in the small intestine (modified from University of Kentucky, 2002a)

Enzyme	Function	Source
Trypsin (peptides → amino acids)	Digest proteins	Secreted from pancreas
Chymotrypsin		
Carboxypeptidases		
Pancreatic amylases (plant starches → compound sugars)	Digest carbohydrates	Secreted from pancreas
Pancreatic lipases	Digest lipids	
Disaccharidases	Digest carbohydrates	Secreted from small intestine wall
Dipeptidases	Digest peptides	

At the end of the process, the material that collects at the end of the digestive system is either undigested or simply cannot be digested regardless of mechanical and chemical actions. This is faecal material. Faeces also contain bacteria and other microorganisms that may begin to multiply in the large intestine. There may also be dead cells that might have sloughed off from the luminal (inner) walls of the digestive system during cell rejuvenation. Water is also present and is necessary to avoid constipation in the animal (vanLoon, 1976).

1.1.1.2 Ruminant digestive physiology

For the most part, the digestive system of ruminants is very similar to that of other mammals, but the stomach is considerably different from the "monogastric" condition. The ruminant stomach is comprised of four compartments called the rumen, reticulum, omasum and abomasum. This complex stomach enables the ruminants to digest substances that are considered indigestible by humans and

other monogastric species. These substances include all feeds which may contain cellulose. Cellulose is a carbohydrate polymer that is extremely insoluble and is somewhat resistant to chemical breakdown (Findlay, 1998). Both ruminants and monogastrics lack the cellulases that are needed for the breakdown or hydrolysis of cellulosic compounds into individual glucose molecules. However, many microorganisms found in the rumen possess these cellulases allowing them to digest cellulose (Campbell, 1999). The rumen is the first compartment of the ruminant stomach. It is virtually non-functional at birth, but rapidly activates when calves or other young ruminants are fed solid feeds including grain, hay and grass (**Table 1.2**).

Table 1.2 Ratio of Rumen/Reticulum volume to Omasum/Abomasum volume according to age (University of Kentucky, 2002b, reproduced with kind permission)

Age	R/R : O/A
Birth	1 : 3
6 months	4 : 1
1 year	8-10 : 1

The rumen churns the food particles with its liquid contents and also serves as a "fermentation vat" for the digestion of the food particles by a large population of microorganisms, which are present there (**Table 1.3**). Fermentation in the rumen produces volatile fatty acids such as acetic acid, propionic acid and butyric acid. Volatile fatty acids are generally absorbed directly into the bloodstream through the rumen wall to provide nutrient and energy requirements for the host (University of Alberta, 1998). Fermentation products that pass out of the rumen into the lower digestive tract include microbial protein, B-complex vitamins, and

vitamin K. Microbial fermentation also results in large amounts of gaseous methane and carbon dioxide, which are relieved by a process called eructation or belching.

Table 1.3 Microorganisms in the rumen (University of Kentucky, 2002b, reproduced with kind permission)

Protozoa	100,000 per gram of fluid
Bacteria	100 million per gram of fluid
Fungi	

The second chamber of the ruminant stomach is the reticulum which functions in conjunction with the rumen. The reticulum participates in moving the fibrous feeds up the oesophagus. This process is called regurgitation or rumination. (vanLoon, 1976). Rumination is the process whereby semi-liquid material is regurgitated up the oesophagus to the mouth, where it undergoes remastication or a second chewing before being swallowed again in the form of a bolus (Pond *et al.*, 1995). Rumination usually takes place at times in which the animal is at rest. The reticulum is also called the "hardware" stomach since it also functions in sorting ingested foreign objects from the digesta. Ingested foreign objects in domestic ruminants most commonly include nails, bailing twine and stones (University of Alberta, 1998).

In young or newborn calves, lambs or fawns, a groove termed the oesophageal groove extends from the oesophagus to the omasum allowing the direct delivery of milk into the abomasum. This groove remains functional only until the calf or young animal is weaned onto solid food (University of Alberta, 1998). Though its function is not clearly understood, the omasum or third chamber consists of a

body region and a canal region. The body region is composed of a multitude of muscular leaves that project from the greater curvature. These leaves act in increasing the absorptive capacity for water and fatty acids. The canal region of the omasum is "located on the lesser curvature" and functions to connect the reticulum to the abomasum (Cunningham and James, 1997).

The abomasum, or the "true stomach", has the same functions as a nonruminant stomach (University of Albert, 1998). The abomasum secretes hydrochloric acid and enzymes such as *pepsin*, that begin the chemical digestion of proteins to peptides and amino acids (vanLoon, 1976). The abomasum also consists of a multitude of folds that increase secretory capacity (University of Alberta, 1998).

Later, when the food passes into the intestine, absorption of amino acids, lipids, and carbohydrates takes place as in monogastic animals.

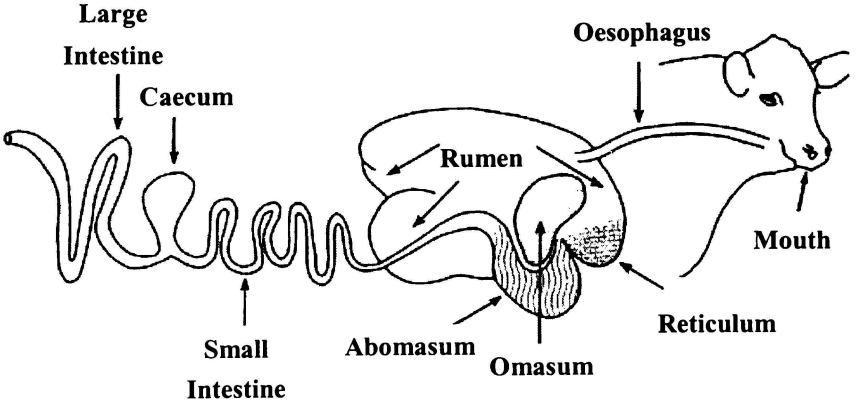


Figure 1.2 Digestive tract of the ruminant (Oklahoma State University, 2002, reproduced with kind permission)

1.1.2 The gastrointestinal microflora of mammals

The gastrointestinal tract (GIT) of mammals harbours a complex microbial community comprising about 10^{14} microorganisms consisting of more than 400 different species of bacteria (Moore and Holdeman, 1974). These microorganisms, which are present in the complex ecological niche of the GIT, can be found in the lumen where they are either attached to the feed particles or existing freely in the fluid. They may also be found in association with the mucous epithelium, or in the bottom of the crypts (Savage, 1986). These microorganisms live in a stable relationship with the host.

The distribution of microorganisms within gastrointestinal tracts varies according to animal species. In the human GIT variability exists in bacterial numbers and populations between the mouth, stomach, small intestine and colon. In the mouth, saliva is the main habitat of bacteria and has been reported to contain 10^4 to 10^9 organisms/mL of contents. (O'Sullivan, 1996). After the more or less neutral pH of the oral cavity, the low pH of the stomach (ranging from 2.5 to 3.5) is lethal to most microbes. The total bacterial counts in the stomach are therefore below 10^3 organisms/mL of contents and are dominated by Gram-positive bacteria such as streptococci and lactobacilli and by yeasts (**Figure 1.3**). Due to the aggressive bile and pancreatic juices and the short transit time, the duodenum also represents a hostile environment and contains relatively low numbers of transient bacteria. Along the length of the small intestine, the flow rate of the digesta is somewhat reduced, the bile is diluted, the pH becomes more neutral and the oxygen tension drops rapidly, all of which result in the gradual increase in the microbial population (up to 10^8 organisms/mL of contents) in the jejunum and ileum, initially mainly facultative anaerobic species, but in the ileum strict anaerobes are also present (**Figure 1.3**) (Bhat *et al.*, 1989; Nielsen *et al.*, 1994; Hill, 1995). Once past the ileum, the intestinal populations of microbes increase dramatically. The strict anaerobes outnumber the facultative anaerobes

in the lumen by 100 to 1000-fold, and total bacterial counts may exceed 10^{11} organisms/mL of contents (**Figure 1.3**). The microflora in the large intestine are fairly stable. These bacteria fall into three categories: *1)* organisms which are almost always present in large numbers, for example *Bacteroides* and *Bifidobacterium* spp.; *2)* organisms which are part of the resident flora but which are normally present in small numbers, for example members of the *Enterobacteriaceae*, *Lactobacillus*, and *Streptococcus* spp.; and *3)* organisms present in small numbers which originate from other regions of the body, for example *Staphylococcus* spp., or from the environment, for example *Bacillus* spp. (O'Sullivan, 1996).

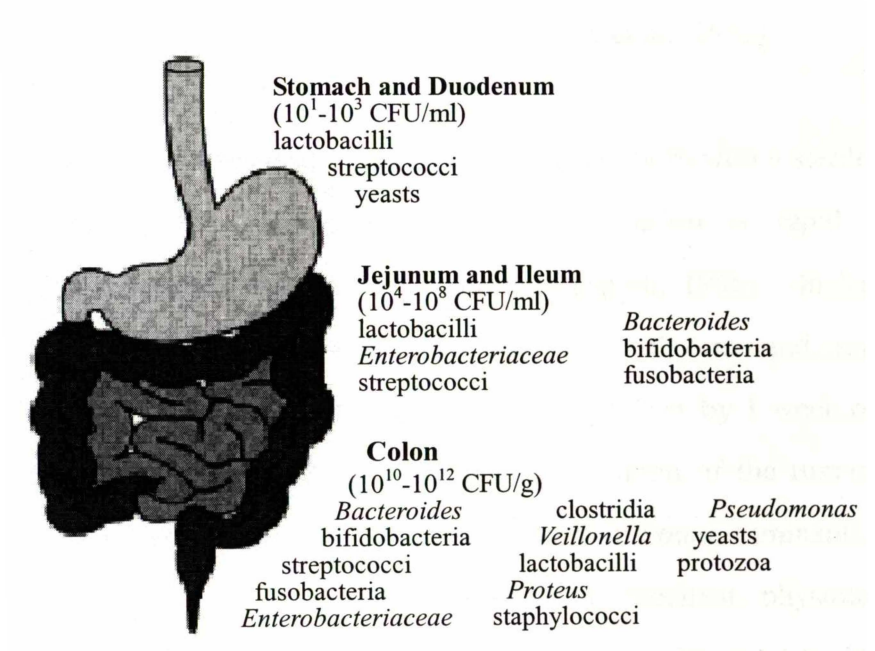


Figure 1.3 Microbial colonisation of the human gastro-intestinal tract (Holzapfel *et al.*, 1998, reproduced with kind permission from author and Elsevier)

The GIT of newborns is inoculated primarily by organisms from the birth canal of the mother and the environment. At first facultative anaerobic strains such as *Escherichia coli* and *Streptococcus* spp. exist in the highest numbers (Rotimi and

Duerden, 1981; Stark and Lee, 1982). These bacteria may subsequently create a highly reduced environment that allows the growth of strictly anaerobic species. Differences in the gastrointestinal flora of breast-fed and bottle-fed infants exist and in breast-fed infants there is a sharp increase in the numbers of *Bifidobacterium* spp. with a concomitant decrease of *E. coli* and *Streptococcus* spp. (Braun, 1981; Benno *et al.*, 1984; Modler *et al.*, 1990; Yoshita *et al.*, 1991). In contrast, there is a more complex flora in bottle-fed infants with relatively high numbers of *Bacteroides*, *Clostridium* and *Streptococcus* spp. *Bifidobacterium* spp. are present but do not predominate. After weaning, a conversion to the normal adult flora occurs. Generally, *Streptococcus* spp. and *E. coli* populations decrease and by the second year of life the intestinal microflora are similar to those of the adult (Bullen *et al.*, 1977; Mevissen-Verhage *et al.*, 1985).

Like other young mammals (Savage, 1977), ruminants are born with a sterile GIT (Cushnie *et al.*, 1981). However, bacterial colonisation is rapid, with *Escherichia coli* detectable from 24 hours onwards (Smith, 1965). In healthy animals, lactobacilli quickly colonise the gut, displacing coliforms and reaching numbers of 10^7 to10^9/mL of contents throughout the intestines by 1 week of age (Smith, 1965; Karney *et al.*, 1986). Microbial colonisation of the rumen, the second stage in the establishment of an intestinal flora in young ruminants, also occurs rapidly. The rumen of a ruminant is a excellent physiological environment (anaerobic – H_2 0.2%, O_2 0.5%, N_2 7%, CO_2 65.5% and CH_4 26.8%; 38 – 41°C; low redox potential – -250 to -450 mV; well buffered by saliva – HCO_3^- and PO_4^{3-}; pH 4.6 to 7.0; osmotic pressure 260 – 340 mOsmol) for the development of microbes. A large population of strictly anaerobic bacteria are present after 48 hours (Ziolecki and Briggs, 1961; Fonty *et al.*, 1987). Cellulolytic and methanogenic bacteria can be found at the age of three days in the reticulorumen (Anderson *et al.*, 1987). At the age of one to three weeks cellulolytic and lactate-fermenting bacteria and coliforms are present in the microflora (Bryant *et al.*, 1958; Ziolecki and Briggs, 1961). Lactate-fermenting

bacteria decrease after this period, and at the age of 9 to 13 weeks the ruminal flora of the ruminant are similar to those of an adult ruminant.

As indicated above, the rumen is not fully functional at an early age. Milk bypasses the rumen, moving directly *via* the oesophageal groove to the omasum and abomasum, and then into the small intestine (Hungate, 1966a). Rumen microflora have no great effect on food digestion at this stage. As the ruminant begins to consume solid feed, the microbial populations in the rumen increase and it begins to be colonised by a microflora containing obligately anaerobic bacteria, fungi and protozoa, whose metabolic activities are essential for the digestion of plant structural materials in the ruminant's diet. A profuse collection of bacteria is also present in the hindgut (caecum and colon) of ruminants. However, the effect of these intestinal microbes on the well-being of the host is more speculative (Tannock, 2000).

1.1.3 Role of the gastrointestinal microflora in the host

The established normal microflora influence host health and well-being. On the basis of their interactions with the host and their metabolic activities, the different bacteria can be further categorised into those that are perceived as being beneficial and those that have the potential of detrimentally influencing the health of the host (Gibson and Roberfroid, 1995).

1.1.3.1 Barrier effect

The stable flora which develop in the intestine are an important contact surface between the environment and the host providing a barrier against harmful components of the diet as well as against pathogenic bacteria (Benno and Mitsuoka, 1986; Grönlund *et al.*, 2000; Kirjavainen *et al.*, 2001). The best evidence for this protective effect of the gut flora comes from the observation that

germ-free animals are more susceptible to disease than corresponding conventional animals with a complete intestinal flora. For example, it requires 10^6 cells of *Salmonella* Enteritidis to kill a conventional mouse, whereas a germ-free mouse can be killed with only 10 cells (Collins and Carter, 1978). This effect is also confirmed by the finding that faecal organisms from adult chickens, when fed to newly hatched chicks, prevented colonisation of the gut by *Salmonella* Infantis (Nurmi and Rantala, 1973).

Freter (1974) has analysed a number of parameters related to mechanisms controlling the intestinal colonisation. One of the mechanisms of the "barrier effect" may be related to intestinal colonisation by the indigenous flora, which prevent colonisation by the invader through effective competition for essential nutrients or attachment sites on the epithelium. Another mechanism to prevent colonisation by exogenous pathogens is production of bacteriocidal or bacteriostatic agents.

1.1.3.2 Intestinal bacteria as immunomodulators

The bacterial cell wall consists of two major components: peptidoglycan (PG) and lipopolysaccharide (LPS). Both types of macromolecules are continuously released in small amounts during cell proliferation and cell death. PG and, in particular, LPS have been shown to be activators of the host immune system. They are capable of stimulating mononuclear phagocytes and endothelial cells to release immune modulators such as tumour necrosis factor α (TNFα), members of the interleukin family (IL-1, IL-6, IL-8, and IL-12), interferon α, reduced oxygen species and lipids (Nathan, 1987). The production of small amounts of PG and LPS is believed to be advantageous to the host immune system. Small amounts of LPS in the bloodstream induce moderate fever, a generalised immune stimulation and enhanced microbial killing (Rietschel and Brade, 1992). In addition, small amounts of LPS are able to induce polyclonal stimulation of

B-cells to differentiate, proliferate and secrete immunoglobulins and to enhance the cytotoxicity and phagocytosis of macrophages (Chatterjee *et al.*, 1992). It has also been shown that the lymphoid tissue of germ-free mice is only poorly developed (Schwab, 1977). Furthermore, C3H/HeJ mice which, due to a genetic defect, do not respond to LPS are highly susceptible to bacterial infections (Jenny *et al.*, 1977). Therefore, small amounts of LPS or PG derived from the intestinal flora have been postulated to represent bacteria-derived "vitamins", which might be indispensable for the development, maintenance and proper functioning of the immune system and, thus, for normal health. On the other hand, the massive release into the bloodstream of LPS and PG during a severe bacterial infection results in pathophysiological reactions due to an overstimulation of the immune system (Nogare, 1991).

1.1.3.3 Metabolic activities of the microflora of the large intestine

In recent years, attention has been focused on the metabolic activities of the microflora of the large intestine and in particular on the physiological effects of end-products of these activities (Smith and Bryant, 1979; Cummings and MacFarlane, 1991; Szylit and Andrieux, 1993; Gibson and Roberfroid, 1995). The major growth substrates for gut bacteria are of dietary origin and consist of foodstuffs that have not been absorbed in the upper GIT. These are carbohydrate-based materials such as resistant starches, dietary fibre, oligosaccharides, and other nonabsorbed sugars. There is also a quantitatively lower contribution from endogenous nitrogen-based materials such as mucins, sloughed epithelial cells, bacterial lysis products, proteins, peptides, and amino acids. Some dietary and endogenous lipids may also reach the large intestine in a metabolisable form. The main types of fermentation that are carried out in the large intestine are saccharolytic, proteolytic, and lipolytic.

The principal end products of saccharolytic fermentation in the colon are short chain fatty acids (SCFA), i.e. acetate, propionate and butyrate. A variety of other metabolites are also produced, including electron sink products such as lactate, pyruvate, ethanol, hydrogen and succinate (**Table 1.4**). These substances are formed to maintain the redox balance during fermentation. Electron sink products also act as fermentation intermediates because they are further metabolised to SCFA by other species. Most of the SCFA formed by intestinal bacteria are absorbed, and systematically metabolised, thereby contributing towards host energy gain (Cummings, 1995).

Unlike saccharolytic metabolism, in which the end products are benign and may even be of some benefit to the host, those from proteolysis are toxic. These include ammonia, phenols, indoles, and amines (MacFarlane and MacFarlane, 1995).

The gut flora contribute to lipid metabolism of the host in two different ways: firstly, bacterial lipases can breakdown dietary and endogenous lipids, however, other enzymes can hydrogenate the free fatty acids. The apparent digestibility of fats may thus be decreased since hydrogenated fatty acids are less absorbable than unsaturated ones. Secondly, bacteria can deconjugate bile acids and modify cholesterol metabolism. The primary bile acids are deconjugated by the gut flora, especially by lactobacilli (Gilliland and Speck, 1977), to less soluble and absorbable secondary products (Eyssen and van Eldere, 1984). The secondary bile acids are partly converted to tertiary bile products by the gut flora and hepatic enzymes and they may be toxic to the host. The gut flora are also involved in the cholesterol metabolism, because bile acids are synthesised from cholesterol. Evidence from some animal species suggests that the gut flora decrease the body pool of cholesterol by catabolism making it less absorbable (van Eldere and Eyssen, 1984).

Table 1.4 Predominant products of carbohydrate metabolism in the human colon (Gibson, 1999, reproduced with kind permission)

End product	Bacterial group involved	Metabolic fate
Acetate	*Bacteroides*, bifidobacteria, eubacteria, lactobacilli, clostridia, ruminococci, peptococci, *Veillonella*, peptostreptococci, propionibacteria, fusobacteria, *Butyrivibrio*	Metabolised in muscle, kidney, heart and brain.
Propionate	*Bacteroides*, propionibacteria, *Veillonella*	Cleared by the liver; possible gluceogenic precursor; suppresses cholesterol synthesis.
Butyrate	clostridia, fusobacteria, *Butyrivibrio*, eubacteria, peptostreptococci	Metabolised by the colonic epithelium; regulator of cell growth and differentiation.
Ethanol, Succinate, Lactate, Pyruvate	*Bacteroides*, bifidobacteria, lactobacilli, eubacteria, peptostreptococci, clostridia, ruminococci, *Actinomycetes*, enterococci, fusobacteria	Absorbed electron sink products; further fermented to short-chain fatty acids.
Hydrogen	clostridia, ruminococci, fusobacteria	Partially excreted in breath; metabolised by hydrogenotrophic bacteria.

In addition to the above interactions with the host, the intestinal microflora have further beneficial effects by synthesising B complex vitamins (Mitsuoka, 1982), by stimulating bowel motility (Shortt, 1997), by conferring resistance to gastroenteritis, by increasing lactose tolerance (Collins and Gibson, 1999), by participating in detoxification of some toxic substances (Barnsley, 1975; Rowland and Grasso, 1975), and by reducing tumorogenesis (Collins and Gibson, 1999).

On the other hand, some of the intestinal microflora are considered as possible pathogens, causing infections, inducing autogenous disorders, or producing some potentially harmful substances that may be involved in the formation of intestinal cancers (Ochi and Miyairi, 1943; Hill *et al.*, 1971; Goddard *et al.*, 1975; Reddy *et al.*, 1977).

In ruminant animals, intensive investigation has revealed that the conversion of celluloses, hemicellulose, starch, proteins and other plant polymers into fatty acids and other nutrients involves the integrated activities of a highly complex mixed population of bacteria, fungi and protozoa in the rumen (Hungate, 1966b; Hobson, 1988). However, the role of microflora in the large intestine of ruminants has to date been little studied. This hindgut microflora may possible have a role, at least in part, of protection against infection by pathogens such as *Salmonella* spp. and *Clostridium* spp.

1.1.4 Factors affecting the balance of intestinal microflora

In a healthy state, the beneficial flora which become established in the gut appear to be relatively stable; pathogenic bacteria are present in low numbers and the quantity of toxic metabolites seems insufficient to act detrimentally on the host. However, the composition of intestinal flora can be altered by many endogenous and exogenous factors (Holzapfel *et al.*, 1998), such as changes in physiological conditions of the host (aging, stress, health status, and environment), composition of the diet, and environmental circumstances (climate, contamination with pathogens, and use of medicines), all of which may shift the balance of the intestinal flora away from potentially beneficial or health promoting bacteria such as lactobacilli and bifidobacteria, and towards a predominance of potentially harmful or pathogenic microorganisms, such as sulphite-reducing clostridia and certain *Bacteroides* spp. Predominance of these latter populations may further result in a number of clinical disorders, including cancer, inflammatory disease,

and ulcerative colitis, in addition to making the host more susceptible to infections by transient enteropathogens like *Salmonella*, *Campylobacter*, *Escherichia coli* and *Listeria* spp.

To solve these medical problems, antibiotics have usually been used, which have indeed proved to be very effective in some cases such as decreasing diarrhoea in, and promoting growth of, farm animals (Armstrong, 1984; Armstrong, 1986; Parker and Armstrong, 1987). However, some side-effects of antibiotic-therapy, such as occurrence of *per os* antibiotic treatment associated enteritis or diarrhoea, derangement of indigenous beneficial flora (Bohnhoff *et al.*, 1954; Freter, 1955; Freter, 1956; Smith and Tucker, 1975; Bartlett, 1992), and the development of resistant strains of harmful bacteria (Keen *et al.*, 1999), may interfere with the use of antibiotics (Hedges and Linton, 1988; Linton *et al.*, 1988) and decrease their efficiency.

For reasons outlined above there is wide interest in replacing antibiotics with more natural feed additives and there is considerable benefit to be gained by the host in maintaining a good microflora community structure through increased predominance of bacteria such as lactobacilli and bifidobacteria. To achieve these aims the use of probiotics, prebiotics, and synbiotics may all be feasible.

1.1.5 Molecular methods in gut microbial ecology

As discussed previously, the intestinal tract of mammals harbours an extremely complex, diverse and dynamic microbial ecosystem, usually referred to as the normal flora. This normal flora, mainly obligately anaerobic bacteria, inhabiting distal ileum and the colon, play an important role in host health through colonisation resistance and/or bacterial antagonism against the establishment of pathogenic microorganisms in the gut, and might stimulate immune responses against potentially pathogenic bacteria (Franks *et al.*, 1998). In a healthy state,

the normal flora appear to be relatively stable. However, the composition of intestinal flora can be altered by many endogenous and exogenous factors, such as changes in physiological conditions of the host (aging, stress, health status, and environment), composition of the diet, and environmental circumstances (climate, contamination with pathogens, and use of medicines), all of which may shift the balance of the intestinal flora and influence its effectiveness in colonisation resistance, antimicrobial action, and immunomodulation. This suggests there is a requirement to modulate the flora towards a balanced and healthy microflora by application of effective dietary strategies such as the use of pro, pre and synbiotics. Since a complete and in-depth understanding of intestinal microflora is a paramount part to the gut flora nutritional modulation studies, it is necessary to have accurate means to determine the biodiversity and dynamics of the intestinal flora, evaluate its functional impacts on host health, monitor any changes of the flora in response to dietary modulation, and track probiotics in the gut.

The gut microflora have largely been investigated by anaerobic culture techniques; faecal samples usually contain 10^{10} to 10^{11} CFU per g of (Holdeman *et al.*, 1976; Savage, 1977; Finegold *et al.*, 1983). The predominant genera in the large intestine are reported to be *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium*, and *Fusobacterium* (Suau, *et al.*, 1999). Despite these intensive investigations, however, there is much concern that culture-based methods provide an incomplete picture of the diversity of the predominant organisms of the gut flora. The reason for this concern is that anaerobic selective media used to determine the composition of gut microflora are not truly specific or are too selective for certain bacteria. Many bacteria which have fastidious growth conditions within the host, or which have interactions with other gut microorganisms, are difficult to culture or are unculturable (Ward *et al.*, 1992; Nelson and George, 1995; Wilson and Blitchington, 1996). Other limitations of cultivation include the stress imposed by cultivation procedures and the necessity of maintaining strictly anaerobic conditions. Moreover,

culture-based methodology is laborious, time-consuming, and prone to statistical and experimental error (Langendijk, *et al.*, 1995; Zoetendal, *et al.*, 1998). Evidence has accumulated indicating that culturability of bacteria in the gastrointestinal tract varies from 10 to 50%. In other words 50 to 90% of the organisms in the total human microflora have not been cultivated (McFarlene and Gibson, 1994; Langendijk, *et al.*, 1995; Wilson and Blitchington, 1996). Consequently, the development of an accurate culture-independent strategy is absolutely essential for assessing the gut microflora diversity and for monitoring the bacterial changes. Various culture independent molecular identification techniques based on the molecular detection of 16S rRNA or its encoding gene have been developed and introduced into gut microbial ecology to monitor the composition and dynamics of gut microflora and provide an objective evaluation of gut flora modulation studies.

1.1.5.1 Temperature gradient gel electrophoresis analysis of 16S rRNA from human faecal samples

Zoetendal *et al.* (1998) suggested that the combination of cloning and temperature gradient gel electrophoresis (TGGE) analysis of the V6 to V8 amplicons of faecal 16S rRNA and rDNA would be a powerful tool for analysing complex microbial communities in faecal samples and for studying the effects of dietary modulation such as the use of probiotics and/or prebiotics, the use of antibiotics, as well as the influence of the genetic background of the host, on the stability and composition of the dominant microbial community. In their studies, TGGE of faecal 16S rDNA amplicons from 16 individuals showed different profiles, with some common bands. Faecal samples from two individuals were also monitored over time and showed remarkably stable profiles over a period of at least 6 months. These results demonstrated that TGGE patterns were unique for each individual, i. e. each healthy person has his or her own unique faecal flora, and that the dominant active flora are stable over time. The reasons for this uniqueness are

likely to be found in host factors. The possibility of missing some important, hitherto unknown bacteria during the investigation was also suggested.

1.1.5.2 Comparative analysis of cloned 16S rRNA gene (rDNA) sequences to derive a detailed phylogenetic inventory of the human gut flora

The bacterial diversity (both cultivated and noncultivated bacteria) of a faecal sample from an adult-male was analysed by using comparative analysis of cloned 16S rRNA gene (rDNA) sequences (Suau *et al.*, 1999). Two hundred and eighty-four clones obtained from 10-cycle PCR were classified into 82 molecular species (at least 98% similarity). However, only 24% of the molecular species recovered corresponded to the described organisms, in which 95% of the clones belonged to three phylogenetic groups: the *Bacteroides* group, the *Clostridium coccoides* group, and the *Clostridium leptum* subgroup. The remaining clones were distributed among a variety of phylogenetic clusters. The majority of generated rDNA sequences (76%) which did not correspond to known organisms derived from hitherto unknown species within this human gut microflora. Total bacterial counts simultaneously enumerated anaerobically on a nonselective medium corresponded to 21% of bacteria counted through 4',6-diamidino-2-phenylindole (DAPI) staining and 32% of those counted by oligonucleotide probe hybridization, indicating the culturability of total anaerobes in the gastrointestinal tract was quite limited. This direct analysis of genes encoding 16S rRNA has been proved useful in facilitating the assessment of both cultivated and noncultivated microorganisms.

1.1.5.3 16S rRNA-targeted oligonucleotide probes and quantitative dot blot hybridisation technique

A new oligonucleotide probe S-G-Clept-1240-a-A-18 was designed, validated, and used with a set of five 16S rRNA-targeted oligonucleotide probes with the quantitative dot blot hybridization technique to investigate the population structure of the predominant phylogenetic groups of the human adult faecal microflora (Sghir *et al.*, 2000). Application of the six probes to faecal samples from 27 human adults showed an average of 70% of all bacterial 16S rRNA detected by the bacterial domain probes: total *Bacteroides* group-specific probe accounted for 37% ± 16% of the total rRNA; the enteric group probe accounted for less than 1%; *Clostridium leptum* subgroup and *Clostridium coccoides* group-specific probes accounted for 16% ± 7% and 14% ± 6%, respectively; low guanine-plus-cytosine (G+C) content Gram-positive organisms, including those of the genera *Lactobacillus*, *Streptococcus*, and *Enterococcus*, accounted for up to 1% of the total 16S rRNA; and the *Bifidobacterium* group represented less than 1% of the total. The occurrence of low levels of enteric group rRNA and *Bifidobacterium* group rRNA can be explained as the suboptimal physicochemical conditions in faecal matter, such as loss of contact with the mucosa or depletion of their preferred energy substrates would yield lower rRNA contents since the ecological niche of organisms in these two groups is the caecum. The minor contribution of lactobacilli in the total colon flora is due to a low supply in the colon of complex nutritional requirements such as amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acid esters, and fermentable carbohydrates for growth of lactobacilli which were degraded and absorbed in the small intestine. The methodology in this study also provides a direct quantification of the microbial community and identification of major microbial species without culture-based enumeration and identification.

1.1.5.4 Fluorescence *in situ* hybridization technique with specific 16S rRNA-targeted probes

A more direct method for identifying specific bacteria within the intestinal microflora at the single cell level is fluorescent *in situ* hybridisation (FISH). This technique relies on the development of specific oligonucleotide probes which are labelled with sensitive fluorescent tags that enable target bacteria to be visualised by fluorescent microscopy (O'Sullivan, 1999).

Three genus-specific 16S rRNA-targeted hybridisation probes were developed and validated by Langendijk *et al.* (1995). One of these probes (Bif164) which is complementary to a site in variable region V2 of the 16S rRNA unique to the genus *Bifidobacterium* was further used to determine the population of *Bifidobacterium* spp. in human faecal samples by FISH technique. Conventional plating method plus colony hybridisation test were used for enumeration of bifidobacteria in the faecal samples as a culture-based technique control. Total anaerobes in the faeces were also enumerated by conventional plating method, by epifluorescence microscopy of DAPI-stained cells and by the direct microscopic clump count (DMCC) method. The results indicated that there were no significant differences between the *Bifidobacterium* cultural counts and the microscopic *Bifidobacterium* counts with probe Bif164, which suggested that all bifidobacteria in faeces were culturable. On average, bifidobacteria accounted for 6.9% ± 3.3% of the total culturable counts. However, the total culturable anaerobes in the faeces were approximately 15% of the DAPI total counts and 37% of the DMCC total counts, which indicated that the culturability of total anaerobes was quite low. On the basis of DAPI total counts which are closer to real values compared with the DMCC counts, the contribution of bifidobacteria to the total intestinal microflora was only on average 0.8% ± 0.4%, i.e. was overestimated by almost 10-fold when cultural methods were used as the sole

method for enumeration. This result is similar to the proportion of bifidobacteria (less than 1% of the total rRNA) obtained by Sghir *et al.* (2000).

Franks *et al.* (1998) also designed, validated and used six 16S rRNA-targeted oligonucleotide probes to quantify predominant groups of anaerobic bacteria in human faecal samples and to explore the normal biological fluctuations of the these populations within and between the different individuals. In their initial FISH experiments, these new probes were used to enumerate faecal bacteria in nine volunteers. The results showed that the combination of the two *Bacteroides*-specific probes Bfra602-Bdis656 detected a mean of 5.4×10^{10} cells per g (dry weight) of faeces; the *Clostridium coccoides-Eubacterium rectale* group-specific probe Erec482 detected a mean of 7.2×10^{10} cells per g (dry weight) of faeces; the *Clostridium histolyticum*, *Clostridium lituseburense*, and *Streptococcus-Lactococcus* group-specific probes Chis150, Clit135, and Strc493 detected only numbers of cells ranging from 1×10^{7} to 7×10^{8} per g (dry weight) of faeces, which occasionally reached, or could not reach, a reliable quantification. These probes, Chis150, Clit135, and Strc493, are therefore not useful for enumerating the target groups in faecal samples from healthy volunteers by FISH and so were not included in the population dynamics study. However, these probes can be applied to describe the full biodiversity of the gut flora by demonstrating the low numbers of these groups and may also be important in investigations of unbalanced gut flora from people suffering from gut disorders or disturbances caused by antibiotic treatment of the more dominant pathogens in the gut flora. Three of the newly designed probes Bfra602, Bdis656 and Erec482 and three additional probes Bact338 for the total bacterial counts, Bifl64 for the *Bifidobacterium*, and Lowgc2P for the uncultivated Low G+C #2 group, Gram-positive bacteria within the *C. leptum* subgroup were used in further FISH experiments to study the faecal flora composition of the nine volunteers over a period of 8 months. The combination of these probes was able to detect at least two-thirds of the faecal flora. On average, the Bfra602-Bdis656 probe

combination detected 5.7×10^{10} cells per g, or 20% of the total population, identifying them as *Bacteroides*; Erec482 detected 8.1×10^{10} cells per g, or 29% of the total population; together, they accounted for almost half of the flora. The Lowgc2P probe detected 3.3×10^{10} cells per g, or 12% of the total population. Bif164 detected only 3% (9.4×10^{9} cells per g) of the population. The 3% *Bifidobacterium* appears higher than the proportion (less than 1% of the total rRNA) obtained by Sghir *et al.* (2000) and the $0.8\% \pm 0.4\%$ of the DAPI total counts obtained by Langendijk *et al.* (1995). The discrepancy of the results in the studies can partly be explained by the use of a different sequence region of the 16S rRNA targeted probes. The results in this study also indicated that biological variations within the normal faecal populations occurred, which should be deducted when evaluating the effects of agents modulating the flora.

The above studies detected the major groups of anaerobic bacteria in human faecal samples using the FISH technique with specific 16S rRNA-targeted probes. FISH techniques were also used for analysing and comparing the differences of intestinal flora development in breast-fed and formula-fed infants (Harmsen *et al.*, 2000). This study revealed an initial colonisation in all infants with a diverse and adult-like flora. This flora then changed rapidly under the influence of diet. In the breast-fed newborn infants the flora became dominated by bifidobacteria. This is possibly caused by selective agents (bifidogenic factors) that are present in human milk. The minor components in the breast-fed infant gut were mainly lactobacilli and streptococci. In contrast, formula-fed infants developed a more diverse flora, consisting of similar populations of bifidobacteria and *Bacteroides*, and minor components staphylococci, *Escherichia coli*, and clostridia.

The application of FISH with the previously described probes provides a more accurate and additional information on the biodiversity and dynamics of the normal human faecal flora and succession of bacterial strains after the initial colonisation of the neonatal gut in a culture independent manner. While this is a

definite improvement over conventional plating methods, there are still limitations with the FISH technique (Franks *et al.*, 1998). Probe permeation of the Gram-positive cell wall remains problematic, although permeabilisation protocols were optimised for the Gram-positive target groups. In addition, the probes are only designed on the basis of currently available 16S rRNA sequences and not for unknown gut bacteria. Future studies will concentrate on the development of more genus-specific probes for detection of other major subpopulations of bacteria in human faeces. For detecting low number of organisms and for refinements at a species level, alternative molecular methods such as PCR will be required (Franks *et al.*, 1998).

A more detailed introduction to the molecular methods for analysis of the intestinal microflora has been provided by O'Sullivan (1999). These methods have been applied and the results indicate that higher estimates of the intestinal microflora were achieved by using the molecular quantitative methods than by culture-based enumeration (Langendijk *et al.*, 1995; Suau *et al.*, 1999). However, even though the various molecular methods described in these studies detect a greater proportion of the normal flora, there is still a fraction (30 to 76%) that remains unknown (Franks *et al.*, 1998; Suau *et al.*, 1999; Sghir *et al.*, 2000). These findings indicated that current knowledge about the gut microbial ecosystem is extremely limited and incomplete. Future studies must concentrate on the development of more accurate culture-independent determination techniques for isolating these unknown populations of bacteria in human faeces. This work will then present a complete picture of the composition and dynamics of the gut microbial ecosystem and monitor the changes of the flora in response to the health-promoting dietary modulation, thereby helping in design of various health-promoting dietary modulation strategies.

1.2 Nutritional modulation of the gastrointestinal microecology

1.2.1 Probiotics

The term probiotic means "for life" in Greek. It was first used by Lilly and Stillwell (1965), who described a growth-promoting effect on an organism by another organism when they were co-cultured. Sperti (1971) referred to tissue extracts that stimulated microbial growth as "probiotics". It was Parker (1974) who first used the term probiotic in the sense that it is used today. He defined probiotics as "organisms and substances which contribute to intestinal microbial balance". However, the word substances in this definition also included antibiotics. Fuller (1989) refined Parker's definition of probiotics and gave the most widely used and accepted version so far as follows: *live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance.* This definition emphasises the importance of live cells as an essential component of an effective probiotic and removes the confusion created by the use of the word "substances".

In fact, probiotics have been used for as long as people have eaten fermented foods. However, it was Elie Metchnikoff who first advocated that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract (Metchnikoff, 1907). He suggested that the long life of Bulgarian peasants resulted from their regular consumption of large quantities of fermented milk products, in which Lactic Acid Bacteria (LAB) carried out carbohydrate fermentation. These products, when consumed, positively influenced the microflora of the colon by preventing the growth of putrefactive microorganisms and decreasing their toxic effects. A number of scientific progressions then ensued to reach the current situation whereby many probiotic strains exist and are

in widespread use. Presently, probiotic products are commercially available either as fermented food commodities or in lyophilised form, both as supplements and as pharmaceutical preparations, representing a major and still growing segment of the world functional food market. This market is estimated to be worth a total value of US$ 10 to 20 million (Holzapfel and Schillinger, 2002). Such probiotic strains have been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which form part of the normal human intestinal microflora or mucosal microflora. In the probiotic approach, these ingested bacteria are selected to survive gastrointestinal transit, arrive viable, and contribute positively to the activity of the intestinal microflora, and therefore, to the health of the host. Thus, for organisms to achieve probiotic status, they must fulfill a series of criteria.

1.2.1.1 Criteria for selection of probiotic strains

Three categories of key criteria have been defined as desirable for probiotic bacteria (Havenaar *et al.*, 1992) and are briefly discussed below.

Microbiological characteristics

There are a number of properties that a bacterium must possess to make it desirable as a probiotic (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995). The bacterium should be capable of surviving the defense system located in the upper GIT of the host such as saliva, gastric and bile juice, capable of proliferating in and/or adhering to the intestinal epithelium, and of metabolising in the gut environment, thereby exerting beneficial effects by altering the dynamics of the resident microflora towards metabolic processes. They must not be pathogenic, toxic, mutagenic, or carcinogenic to the host. They must not produce undesirable side-effects when ingested, and in the case of food animals, not produce toxic residues in the carcass. Whilst this seems unproblematic for

commercial strains with some "safety record", further approval of new strains may require back-up by sound scientific data. A scheme proposed by Donohue and Salminen (1996) for safety assessment takes into account intrinsic properties of a strain, its metabolic products, toxicity, mucosal effects, dose-response effects, clinical assessment and epidemiological studies. Furthermore, the bacterium should be of host origin, i.e. isolated from the same species as its intended host, be genetically stable, and have no plasmid transfer mechanism. Finally, for an organism to be suitable as a probiotic, it should be easily reproducible and capable of remaining viable for long periods under processing, storage and field conditions, while maintaining the good sensory properties of the product.

Technological properties

A probiotic strain cultivated on an industrial scale should be adapted to a suitable carrier or fermentable substrate (e.g. milk). The final product should have an acceptable shelf-life and sensory attributes such as colour, taste, aroma and texture. Probiotic strains claimed to be present in a product should remain viable in sufficiently high numbers and retain metabolic activity during storage and use (Holzapfel *et al.*, 1998). It is essential that a probiotic product sold with any health claims meets the criterion of a minimum populations of 10^6 CFU/mL at the expiry date, however, the minimum therapeutic dose per day is suggested to be 10^8 to10^9 cells (Kurmann and Rasic, 1991).

Functional aspects

An effective probiotic should also be a strain, which is capable of exerting a beneficial effect on the host animal. A number of beneficial roles for probiotic strains have been reported or theorised, including some important functional effects on the "normal" (healthy) intestinal microbial compostion and therapeutic effects on intestinal disorders (**Figure 1.4**). Well-designed *in vitro* assays and

human or animal studies are essential to demonstrate probiotic activities of a certain strain, and to pre-select probiotic strains. Such pre-selection tests include resistance to bile and acid, adhesion to the intestinal mucosa, effects on immunocompetent cells, antibacterial activities, and antimutagenicity. Furthermore, the proof of efficacy in hosts should be tested by at least two independent clinical intervention studies. The studies should preferentially be published in peer-reviewed journals (Guarner and Schaafsma, 1998).

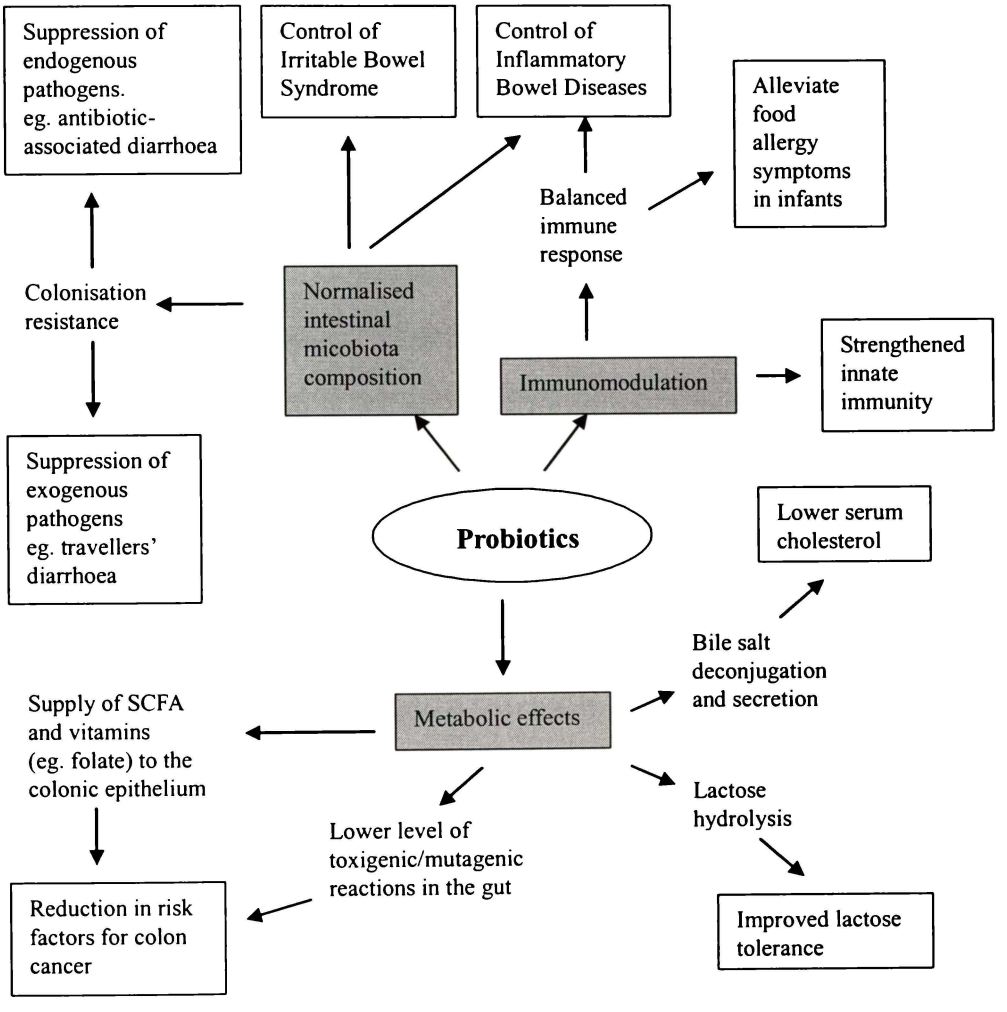


Figure 1.4 Proposed health benefits stemming from probiotic consumption (Saarela *et al.*, 2002, reproduced with kind permission from author and Elsevier)

1.2.1.2 Probiotic cultures

Many microorganisms have been used or considered for use as probiotics. A probiotic preparation may contain one or several different strains of microorganisms. The most typical active components of probiotic products are strains of LAB, particularly *Lactobacillus* spp. and *Bifidobacterium* spp. (**Table 1.5**).

Table 1.5 Bacterial species primarily used as probiotic cultures (Shah, 2001 reproduced with kind permission)

Species	Strains
Lactobacillus acidophilus	La2, La5 (also known as La1), Johnsonii (La1; also known as Lj1), NCFM, DDS-1, SBT-2062
L. bulgaricus	Lb12
L. lactis	La1
L. plantarum	299v, Lp01
L. rhamnosus	GG, GR-1, 271, LB21
L. reuteri	SD 2112 (also known as MM2)
L. casei	Shirota, Immunitass, 744, 01
L. paracasei	CRL 431
L. fermentum	RC-14
L. helveticus	B02
Bifidobacterium adolescentis	—
B. longum	B536, SBT-2928
B. breve	Yakult
B. bifidus	Bb-11
B. essensis	Danone, (Bio Activia)
B. lactis	Bb-02
B. infantis	Shirota, Immunitass, 744, 01
B. laterosporus	CRL 431
B. subtilis	—

Lactobacilli have the longest history of use and are still the most common ingredients of probiotics. This choice of probiotic bacteria seems appropriate, because the GIT of humans and animals under the optimal functional conditions are usually rich in lactobacilli. Bifidobacteria are now nearly as common as lactobacilli in probiotic preparations because their populations, found inhabiting the human GIT, are larger than those of lactobacilli (Tannock, 1997). Additionally, bifidobacteria are found principally in the large intestine while lactobacilli inhabit mainly the distal end of the small intestine (Tomomatsu, 1994). The beneficial effects of *Lactobacillus* and *Bifidobacterium* have been discussed for decades. Bacteria in these two genera resist gastric acid, bile salts and pancreatic enzymes, adhere to intestinal mucosa, and readily colonise the intestinal tract. They are considered as part of the normal intestinal microflora or the mucosal microflora and are relatively harmless. They have been demonstrated to inhibit the *in vitro* growth of many enteric pathogens including *Salmonella* Typhimurium, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens,* and *Clostridium difficile* (Rolfe, 2000). In addition, they have been used in both humans and animals to contribute some important functional effects to "normal" (healthy) intestinal microbial composition and to treat a broad range of gastrointestinal disorders (**Figure 1.4**).

1.2.2 Bifidobacteria used as probiotics

1.2.2.1 Discovery and Taxonomy

Poupard *et al.* (1973), Modler *et al.* (1990), and Ballongue (1993), in their review articles, have explored the history of discovery and taxonomy of bifidobacteria. The discovery of bifidobacteria dates back to the beginning of the twentieth century. It was Tissier, in 1900, who first observed and isolated a bacterium with curved and bifurcated X- or Y-shaped rod-like cells from the faeces of newborn infants and named it *Bacillus bifidus communis* (currently known as

Bifidobacterium bifidum) based on the morphology of the bacterium. In 1924, Orla-Jensen suggested that bifidobacteria be classified as a separate genus, the genus *Bifidobacterium*. However, this suggestion remained in doubt as these bacteria were still thought to be included in the genus *Lactobacillus* because of their morphological and fermentative similarities. De Vries and Stouthamer (1967) then demonstrated the presence of fructose-6-phosphate phosphoketolase (F6PPK) in these bacteria and the absence of aldolase and glucose-6-phosphatase dehydrogenase, two enzymes found in the lactobacilli, thereby proving that these bacteria could not be included in the genus *Lactobacillus*. At nearly the same time, the advent of molecular taxonomy showed that the percentage of G+C in the DNA of *Bifidobacterium* differed from that of *Lactobacillus*, *Corynebacterium* and *Propionibacterium* (Sebald *et al.*, 1965). Based on these findings, *Bifidobacterium* has been convincingly established as a unique genus and this genus belongs to the family *Actinomycetaceae* (Rogosa, 1974; Scardovi, 1986). Currently, there are 29 described species (Bahaka *et al.*, 1993), 10 of which colonise the human large intestine (Pot *et al.*, 1994).

1.2.2.2 Occurrence

Bifidobacteria occur in the intestines of birds and mammals including humans and of honey bees. Some species are also found in sewage and human clinical materials (Scardovi, 1986). *Bifidobacterium* spp. can be isolated from faeces of humans and other animals at all stages of life. They are the largest group in human infants, particularly in those that are breast fed, reaching a level of up to 95% of the faecal flora; but become the third largest group in adults, constituting, on average, 25% of the total flora; and then decrease significantly in the elderly with the increase in the faecal populations of coliforms, enterococci, lactobacilli and *Clostridium perfringens* (Modler *et al.*, 1990; Ballongue, 1993; Gibson *et al.*, 1994; Hoover, 1999; Tamime, 1999). This change may be the reason why the elderly are susceptible to enteric infections. The proportions of the various

species of the genus *Bifidobacterium* also vary with age (Ballongue, 1993). The most common species in human infants are *Bifidobacterium infantis*, *B. breve* and *B. longum*. In adults, *B. adolescentis* and *B. longum* are well represented, whereas *B. infantis* and *B. breve* are not present.

1.2.2.3 Cultural and physiological characteristics

Bifidobacteria are rods of various shapes (short, regular, thin cells with pointed ends, coccoidal regular cells, and long cells with slight bends or swollen extremities or with a large variety of forms of branching) and are often arranged in "V" or "palisade" patterns or "star-like" aggregates. They are Gram-positive, nonmotile and nonspore-forming (Scardovi, 1986). Cells often stain irregularly. This reveals a frequently irregular distribution of chromatin which often accumulates in the bifurcations or lumps (Ballongue, 1993). The composition of the culture medium may influence the cell morphology of bifidobacteria (Glick *et al.*, 1960; Kojima *et al.*, 1970; Husain *et al.*, 1972). These bacteria show more highly branched shapes in nutritionally deficient media than when the bacteria are grown in favourable media.

The colony morphology of bifidobacteria varies on different agar media. In general, the colonies are smooth, convex, with entire edges, cream to white in colour, glistening and of soft consistency (Scardovi, 1986).

Bifidobacteria are anaerobic microorganisms. However, the sensitivity to oxygen is different among different strains and species. Some are obligate anaerobes while others tolerate oxygen but only in the presence of elevated levels of carbon dioxide (Scardovi, 1986).

The optimum temperature for human species of bifidobacteria is 36-38°C, and for other animal species is slightly higher, about 41-43°C and may even reach 46.5°C.

(Ballongue, 1993). The optimum pH for initial growth of bifidobacteria is 6.5-7.0; no growth occurs below 5.0 or above 8.0 (Scardovi, 1986).

It is notable that various species of the genus *Bifidobacterium*, and even different strains of the same species have different nutritive requirements. Most species are unable to develop in a totally synthetic medium and require complex biological substances such as bovine casein digestate, lactoserum from bovine milk, porcine gastric mucin or yeast extract (Poch and Bezkorovainy, 1988; Petschow and Talbott, 1990). The growth of bifidobacteria is also stimulated by some carbohydrates such as *N*-acetylglucosamine found in human milk, lactulose in processed milk products, raffinose, stachyose, oligo- and polysaccharides of inulin with a degree of polymerisation of between 2 and 35 and the tri- to pentasaccharides of dextran (Hidaka *et al.*, 1986; Modler *et al.*, 1990). These carbohydrates usually resist degradation by the enzymes in the digestive tract and can be metabolised selectively by bifidobacteria in the large intestine of humans and animals. All these specific growth factors required for the development of the bifidobacteria in the gut are described as being "bifidogenic factors".

Most strains of bifidobacteria are able to utilise ammonium salts as their sole source of nitrogen. However, some species such as *B. suis*, *B. magnum*, *B. choerinum* and *B. cuniculi* will not grow without organic nitrogen (Scardovi, 1986).

1.2.2.4 Carbohydrate metabolism

Bifidobacteria are saccharoclastic microorganisms. Glucose is degraded exclusively and characteristically in the genus *Bifidobacterium* by the fructose-6-phosphate shunt in which fructose-6-phosphate phosphoketolase (F6PPK – EC 4.1.2.22) cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate (Scardovi and Trovatelli, 1965). End products, acetic and

lactic acid, are formed theoretically in the molar ratio of 3:2 through the sequential action of transaldolase (EC 2.2.1.2), transketolase (EC 2.2.1.1), xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) and enzymes of the Embden-Meyerhof Pathway (EMP) acting on glyceraldehyde-3-phosphate (**Figure 1.5**). However, the theoretical ratio of acetate/lactate (3:2) is scarcely ever found in growing cultures of bifidobacteria.

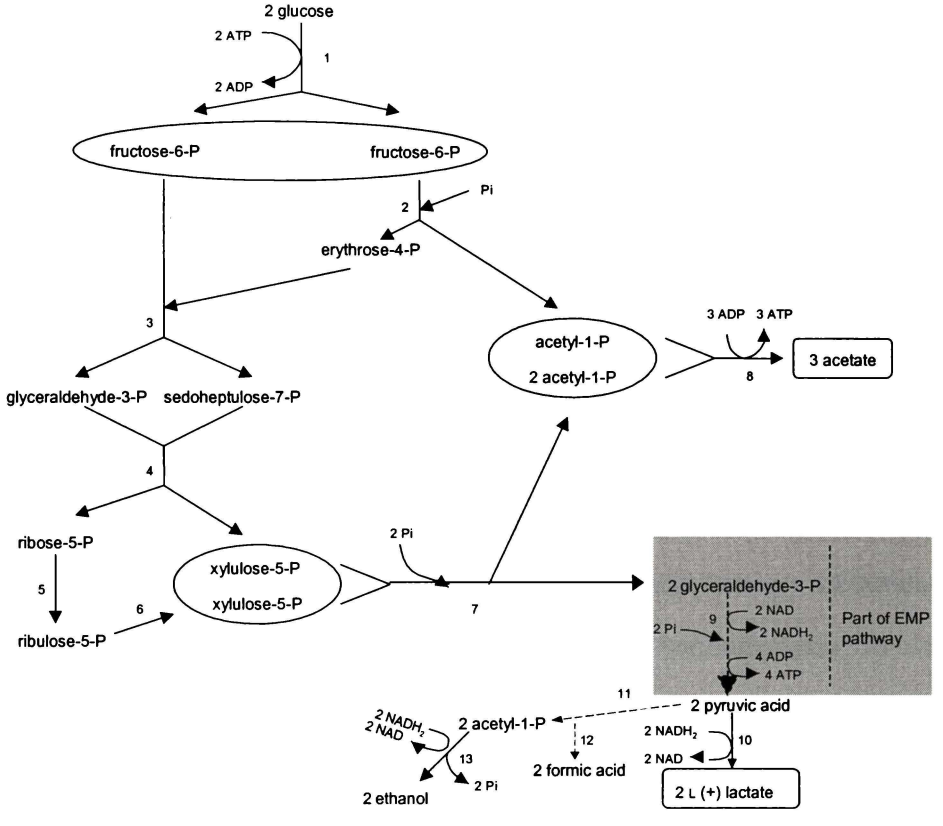


Figure 1.5 Metabolic pathway of *Bifidobacterium* spp. (modified from Ballongue, 1993). 1 = hexokinase and glucose-6-phosphate isomerase; 2 = fructose-6-phosphate phosphoketolase; 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphoketolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase; 11 = phosphoroclastic enzyme; 12 = formate dehydrogenase (EC 1.2.1.2); 13 = alcohol dehydrogenase (EC 1.1.1.1).

Additional acetic and formic acid may be formed through a cleavage of pyruvate by a phosphoroclastic enzyme. Ethanol is subsequently produced by the reduction of acetyl phosphate. Small amounts of succinic acid are also produced by some strains (Scardovi, 1986). Carbon dioxide, butyric and propionic acid are not produced (Kurmann, 1983). Glucose-6-phosphate dehydrogenase and aldolase are claimed to be absent or not detectable, thus ruling out the operation of the hexose monophosphate shunt and the glycolytic system (De Vries *et al.*, 1967).

1.2.2.5 Classical approaches for detection and identification of bifidobacteria

Phenotypic characterisation is still a valuable approach for detecting and identifying the different types of bifidobacteria in the gut of humans and animals although a number of molecular typing methods have been introduced. It is, however, necessary to develop suitable media which selectively encourage the growth of bifidobacteria from faecal samples. A large variety of media have been devised for isolating, enumerating and culturing bifidobacteria from natural habitats. Some are complex culture media such as Glucose Blood-Liver (BL) medium (Teraguchi *et al.*, 1978) and Tryptone Phytone Yeast medium (TPY medium) (Scardovi, 1986), some are semisynthetic culture media, and some are entirely synthetic. These media are efficient for the maintenance of pure strains but are less effective for isolating them from complex flora since these culture media also permit the growth of other genera in addition to bifidobacteria. Therefore, antibiotics (Kanamycin: Matteuzzi *et al.*, 1983; Polymyxin: Ushijima *et al.*, 1985; Nalidixic acid, polymyxin B and kanamycin: Munoa and Pares, 1988) or some other ingredients such as 2,3,5-triphenyltetrazolium chloride (TTC), iodoacetic acid, linoleate, lithium chloride, sodium azide, sodium propionate, sorbic acid (Beerens, 1990) or even bifidogenic factors (*trans*-galactosylated oligosaccharides: Sonoike *et al.*, 1986; Raffinose: Hartemink *et al.*, 1996) have

been used to improve selectivity. In addition, some substances such as cysteine, cystine, ascorbic acid, or sodium sulfite are also added to lower the redox potential (Chang *et al.*, 1983; Rasic and Kurmann, 1983). However, the physiological requirements of bifidobacteria are extremely varied; it is difficult to develop a single selective medium appropriate for all species. Presently, preference should be given to substrates that permit satisfactory growth of as many strains of bifidobacteria as possible (Scardovi, 1986).

Following plating of diluted faecal material onto selective media for detection and isolation of bifidobacteria and anaerobic incubation of plates at 37°C, characteristic colonies are subcultured for further identification. Culture and physiological characteristics are examined to rule out the morphologically unrelated genera that grow on the bifidobacterial selective media. However, some genera, such as *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium* and *Actinomyces*, which share the "pseudobifid" morphology of bifidobacteria (Scardovi and Trovatelli, 1965), are hard to differentiate morphologically from bifidobacteria.

1.2.2.6 Enzymes used for differentiation of bifidobacteria

F6PPK is the characteristic key enzyme of the "bifid shunt". This enzyme, which is virtually specific to the genus *Bifidobacterium*, is apparently absent in anaerobic Gram-positive bacteria of "pseudobifid" morphology, i.e. *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium* and *Actinomyces* (Scardovi and Trovatelli, 1965). The demonstration of F6PPK in cellular extracts is therefore the most direct and reliable characteristic for assigning an organism to the genus *Bifidobacterium* (Scardovi, 1986), although this enzyme has recently been detected in the closely related genus *Gardnerella* found in the human genital/urinary tract where it causes vaginitis (Gavini *et al.*, 1996). The two genera can be differentiated on the basis of ecological source,

growth characteristics and cell morphology. The presence of F6PPK can be tested with fructose-6-phosphate as substrate. The formation of acetyl phosphate from fructose-6-phosphate is evidenced by the reddish violet colour formed by the ferric chelate of its hydroxamate (Scardovi, 1986).

1.2.2.7 Probiotic function of bifidobacteria

Bifidobacteria have long been recognized as suitable bacteria for use in probiotic cultures because of their nutritive and therapeutic properties for humans and animals. These properties have been clearly defined based on numerous studies: suppression of the potential pathogens and putrefactive bacteria, maintenance and improvement of normal intestinal conditions, prevention and/or treatment of enteric infections, alleviation of constipation in the elderly, suppression of some cancers, stimulation of the immune system, enhancement of lactose digestion, reduction in serum cholesterol levels, and production of certain nutritional substances. The production of various bacterial metabolites such as organic acids, particularly acetic and lactic acids or bacteriocins (antagonistic effects), or competition for nutrients and adhesion sites on the epithelial cells or on food particles (competitive exclusion), are the modes of action for bifidobacteria in some of these probiotic effects.

Antibacterial action

There have been a variety of reports indicating that bifdobacteria are effective for inhibiting the growth of many potential pathogens and putrefactive bacteria both *in vivo* and *in vitro*. These include clostridia, *E. coli*, *Salmonella*, *Listeria*, *Shigella*, *Bacillus cereus*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Vibrio cholerae*, and the pathogenic yeast, *Candida albicans* (O'Sullivan and Kullen, 1998; Hoover, 1999). These microorganisms contribute to the production of various noxious compounds and detrimental enzymes in the colon. The noxious

compounds include ammonia, which has toxic effects on the liver and brain, amines, nitrosoamines, indole and skatole, secondary bile acids, and oestrogens, some of which are carcinogens, suspected carcinogens, or cancer promoters, phenols and cresols which are cancer promoters and aglycones which were presumably formerly conjugated with sugars and are mutagenic factors (Mitsuoka, 1982; Mitsuoka, 1990). Some of these noxious compounds result from specific enzyme activities such as ammonia formed as a result of urease, amines from decarboxylase, *N*-nitroso compounds from azoreductase, nitrite from *N*-nitrosation, indole from tryptophanase, and oestrogen from *N*-nitro reductase (Mitsuoka, 1982). In general, the concentrations of these noxious compounds and detrimental enzymes are low in the colon. However, their toxic effects cannot be ignored.

Bifidobacteria produce acetic and lactic acids from saccharolytic fermentation (Scardovi, 1986). The production of these acids lowers gut pH, which consequently inhibits the growth of the potential pathogens and putrefactive bacteria, thereby reducing the toxic metabolites and detrimental enzymes produced by these undesirable bacteria (Rasic, 1983). Acetic acid has a stronger antimicrobial activity than lactic acid at the same pH and it is produced in greater quantities by *Bifidobacterium* (Rasic and Kurmann, 1983). This may explain a greater bacteriostatic effect of bifidobacteria compared to certain other bacteria in yogurt, which produce only lactic acid (Symons, 1997). This difference may be related to the dissociation constants (K_a), and the undissociated acid concentration at a given pH of the particular acid (Modler *et al.*, 1990; Tamime, 1999).

Bifidobacteria may also produce certain antimicrobial metabolites such as bacteriocins (Meghrous *et al.*, 1990), which are the subject of another hypothesis advanced to explain the antagonistic effects. The production of bacteriocins by bifidobacteria has been screened by Meghrous *et al.* (1990) in 13 strains. The antimicrobial substance detected is of a protein type, heat-stable, active at pH

values ranging from 2 to 10, and is active against Gram-positive species including some strains of clostridia.

In addition to any possible antagonistic effects, competition for nutrients and for binding sites on the epithelial surfaces may be extremely important in providing protection against pathogens and putrefactive bacteria (Ballongue, 1993). The adhesion of bifidobacteria to epithelial cells permits bifidobacteria to maintain stable growth and prevent the colonisation of the intestine by pathogens and putrefactive bacteria. Various strains of bifidobacteria have been shown to be capable of adhering to HT-29, Caco-2, and HT29-MTX cell lines (Bernet *et al.*, 1993; Crociani *et al.*, 1995; Gopal *et al.*, 2001). These studies suggested that adherence of bifidobacteria to these cell lines was mediated by a bifidobacteria-produced proteinacious adhesion factor (Bernet *et al.*, 1993; Crociani *et al.*, 1995) or a synergistic action of lactic acid and proteinaceous substances (Gopal *et al.*, 2001). Certain strains of bifidobacteria have been shown to compete with enterotoxigenic *E. coli* for the binding of gliotetraosylceramide, a neutral sphiingoglycolipid present on the intestinal brush border membrane, through a high molecular weight, proteinaceous compound excreted by the bifidobacteria (Fujiwara *et al.*, 1997).

Maintenance/improvement of normal intestinal conditions

Regular consumption of bifidobacteria can maintain and even improve normal intestinal conditions of the host. Tohyama *et al.* (1982) observed that when rats were inoculated with trans-galactosylated oligosaccharides and *B. breve*, activities of some detrimental enzymes such as nitroreductase, urea amidohydrolase (EC 3.5.1.5), and L-lysine decarboxylase (EC 4.1.1.18) were significantly reduced. The production of some noxious compounds such as indole and cadaverine in the intestine decreased. There was also a significant drop in indican, indole-3-acetate, *p*-cresol, piperidine and cadaverine in the urine. Normal liver function was

maintained, which might be due to suppression of Gram-negative microorganisms and subsequent lower production of toxic metabolites. These findings demonstrate that bifidobacteria regulate the metabolic activities of other intestinal microorganisms, thereby improving intestinal conditions. In the case of extremely premature babies, the administration of lyophilised *B. breve* or *B. longum* soon after birth, was found to be effective in the promotion of formation of a bifidobacterial flora (Akiyama *et al.*, 1994a; Akiyama *et al.*, 1994b). In addition, regular supplementation of the infant diet with bifidobacteria can be used to maintain normal intestinal conditions of infants (Hoover, 1999). The ingestion of milk fermented with *B. longum* is successful in regularising digestive transit in pregnant women, reducing abdominal ballooning, diarrhea-type phenomena, or constipation (Ballongue, 1993).

Prevention and/or treatment of enteric infections

The ingestion of bifidobacteria not only has beneficial effects on the maintenance of normal intestinal conditions in healthy subjects, but also plays a therapeutic role in patients suffering from enteric infections. The intact intestinal epithelium and the intestinal mucosa, together with the adherent normal flora, represent a barrier against invasion of pathogenic bacteria from dietary and environmental sources. This barrier is normally stable, protecting the host and providing normal intestinal function. However, antibiotic therapies, radiation treatment, some medical procedures and many other exogenous and endogenous factors such as microflora interactions, climate, diet, stress, frequent travel and aging may disrupt this barrier through disturbance of the normal microflora, epithelia cells or the intestinal mucosa. The consequent increase in permeability predisposes to a variety of enteric infections such as antibiotic-associated diarrhoea, rotavirus gastroenteritis, and *Campylobacter*-induced enteritis (Modler *et al.*, 1990; Duffy *et al.*, 1999). Colombel *et al.* (1987) reported that Bifidus yoghurt containing *B. longum* alleviated the symptoms of erythromycin-induced enteric disorders. In

infants suffering from rotavirus-induced diarrhoea, feeding *B. bifidum* and *Streptococcus thermophilus* was demonstrated to reduce significantly the risk of diarrhoea and shedding of rotavirus (Saavedra *et al.*, 1994). Tojo *et al.* (1987) studied the effects of *B. breve* on *Campylobacter*-induced enteritis and the results showed that *B. breve* is useful for eradicating *C. jejuni* from stools and for restoring the normal intestinal flora in *Campylobacter*-induced enteritis when 3×10^9 CFU of *B. breve* is administered daily, together with anti-diarrhoeal medication.

Alleviation of constipation in the elderly

Constipation is a significant problem for many elderly people, who also have a lower proportion of bifidobacteria in the gut. Bifidobacteria have been reported to exert a significant effect on improving stool frequency of aged people with irregularity of stools. After the administration of Bifidus milk (milk containing *B. longum* and *L. acidophilus*), the stool frequency increased significantly, and the number of *Bifidobacterium* in the faeces of the test subjects also increased (Yaeshima, 1996).

Anticarcinogenic and antitumorigenic effects

Putrefactive bacteria contribute to the production of many potential carcinogens in the colon such as *N*-nitroso compounds, phenolic products of tyrosine and tryptophan, and metabolites of biliary steroids, and the production of some detrimental enzymes such as azoreductases which can reduce both azo and aromatic nitrogen compounds to form *N, N-diphenylnitroso* compounds which are also potential carcinogens (Modler *et al.*, 1990). Both *B. longum* and *B. infantis* have been proved to have antitumorigenic effects in the mouse (Kohwi *et al.*, 1978; Mitzutani and Mitsuoka, 1979). Reddy and Rivenson (1993) also found that lyophilised cultures of *B. longum* reduced carcinogenesis in rats, which

had been induced by the food mutagen, 2-amino-3-methylimidazo[4,5-f]quinoline. A possible explanation for the protective effects of bifidobacteria might include the ability of these organisms to partially degrade the potential carcinogens, to inactivate the enzymes involved in carcinogen synthesis, or to inhibit the growth of the putrefactive bacteria which produce the carcinogens or detrimental enzymes (Modler *et al.*, 1990; O'Sullivan and Kullen, 1998).

Stimulation of the immune system

The influence of bifidobacteria on the immune system of host has been studied by Yamazaki *et al.* (1982, 1985, and 1991) who investigated various aspects of the immunological responses in *B. longum* monoassociated mice which were treated by intragastric intubation with *B. longum* for a period of 16 weeks. *B. longum*-specific IgA appeared in the bile, intestinal tissue and contents, and serum, after an 8-12 week induction period. Total IgA was observed in body fluids and tissues after the first week and the level increased gradually and reached the same level as that of flora-bearing mice after 4-6 weeks. They also demonstrated that a cell-mediated immune response was induced by the addition of a protein fraction derived from *B. longum.* This imparted resistance to the toxic activity of *E. coli* even at dosages of 10^{10} CFU/mouse. Additionally, they found anticholera toxin IgA antibodies were produced and developed to an augmented level after oral co-administration of cholera toxin and *B. longum* to germ-free mice. Bifidobacteria may also be capable of inhibiting bacterial translocations due to their reinforcement of the immune barriers (Faure *et al.*, 1982; Yamazaki *et al.*, 1985). The underlying mechanisms of immunomodulation are not yet well understood. There are indications that specific cell wall components or surface layers may be involved (See Section 1.1.3.2).

Suppression of lactose intolerance

It has been estimated that nearly 25% of Americans suffer from the symptoms of lactose maldigestion because of a congenital deficiency of the enzyme β-galactosidase which is involved in the digestion and absorption of lactose (Scrimshaw and Murray, 1988). Bifidobacteria have been shown to contribute to the improvement of lactose tolerance, reducing the symptoms of lactose malabsorption (Martini *et al.*, 1991; Jiang *et al.*, 1996). This may be due to the distinct β-galactosidase activities of the corresponding bifidobacterial strains (Jiang *et al.*, 1996), which have been observed to be correlated with the alleviation of lactose intolerance. Lactase-positive strains of bacteria (e.g., *Lactobacillus*, *Bifidobacterium* and *Streptococcus*) are commonly added to pasteurised dairy products to increase the digestibility of lactose present in the products (Kim and Gilliland, 1983; Gilliland and Kim, 1984; Pettoello *et al.*, 1989). There are two probable mechanisms by which the addition of these bacteria is beneficial. One is the reduction of lactose in the dairy product through fermentation, and the other is the replication of the probiotics in the gastrointestinal tract, which release lactase.

Anticholesterolemic effects

The administration of fermented milk containing 10^9 bifidobacteria/mL resulted in a reduction in the total cholesterol level in hypercholesterolemic human subjects (Homma *et al.*, 1967; Homma, 1988). *In vitro*, bifidobacteria were shown to have an effect on the activity of hydroxymethylglutaryl - Coenzyme A reductase (HMG-CoA reductase, Homma, 1988), which is involved in the synthesis of cholesterol (Mann and Spoerry, 1974). Further studies are necessary to demonstrate the specific involvement of bifidobacteria in cholesterol reduction.

Production of nutritional substances

Some *Bifidobacterium* strains of human origin are claimed to be associated with the synthesis of certain vitamins. These are thiamine (B1), riboflavine (B2), pyridoxine (B6), folic acid (B9), cyanocobalamine (B12), and nicotinic acid (pellagra-preventing factor, PP) (Deguchi *et al.*, 1985). Bifidobacteria also function in the production of amino acids such as L-alanine, L-valine, L-aspartic acid and L-threonine; and in the production of L(+)-lactic acid which can be easily metabolised by infants. In contrast, D(-)-lactic acid, produced by *Lactobacillus*, can cause metabolic acidosis in infants during the first year (Rasic, 1983).

1.2.2.8 Possible pathogenicity

Not all bifidobacteria can be considered as GRAS (generally regarded as safe) for use in foods. *B. dentium* appears to have the highest pathogenic potential. Most strains of *B. dentium* have been isolated from human dental caries, human abscesses and human clinical material (Scardovi, 1986). However, members of this species are not considered highly infectious or virulent in comparison to many common bacterial pathogens (Hoover, 1999).

1.2.2.9 Limitation of bifidobacteria as probiotics

It has been suggested that for bacteria to act as probiotics they must arrive in the intestines alive and in sufficient number (10^6 CFU/g or mL) (Samona and Robinson, 1991) to have an effect *per se*, or adhere or implant and multiply. Thus for the bifidobacteria to have a probiotic effect, they must resist the acidic conditions of the stomach and action of bile salts in the small intestine, survive intestinal transit and reach the colon in significant numbers. However, in some cases the outcome of such selection has been questioned. The storage and delivery of viable bifidobacteria in food products is still difficult due to the high

sensitivity of these microorganisms to low pH, oxygen, heat and cold (Hoover, 1999). Modler and Villa-Garcia (1993) have ever reported that bifidobacteria did not survive in several yoghurt products in North America, due to highly acidic conditions.

Another approach that overcomes the limitations of probiotics is to use prebiotics, which are non-viable entities and function to promote the proliferation of indigenous *Bifidobacterium* spp. in the intestine (Gibson *et al.*, 1994; Gibson and Roberfroid, 1995).

1.2.3 Prebiotics

The term prebiotic was defined, by Gibson and Roberfroid (1995), as "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon*". Gibson (2001) further elaborated this definition, in his article "Prebiotics for Improved Gut Health", as: *A prebiotic substrate is selectively utilised by beneficial components of the indigenous gut flora, such as bifidobacteria and/or lactobacilli; but does not promote growth of potential pathogens, such as toxin producing clostridia, proteolytic Bacteroides, and toxigenic Escherichia coli. In this manner, a "healthier" microflora composition is obtained, in which the bifidobacteria and/or lactobacilli become predominant in the intestine and exert possible health-promoting effects.*

Regarding prebiotics for stimulating the growth of bifiodobacteria, earliest studies centered on the effects of human milk on gut bacteria and have been extensively reviewed (Modler, 1994; Collins and Gibson, 1999; Hoover, 1999; Gibson, 2001). These authors indicated that different gut microflora compositions occur in breast-fed infants and formula-fed infants. The breast-fed infants have bifidobacteria as the numerically predominant genus, whereas formula-fed infants

have a more complex, adult-like gut flora with clostridia, *Bacteroides*, bifidobacteria and streptococci as the prevalent genera. This difference is attributed to the different compositions of the two milks: human breast milk contains certain glycoproteins which contain oligosaccharides, including glucose, galactose, fructose oligomers, and *N*-acetylglucosamine. These components may be specific growth factors for bifidobacteria. Corresponding fractions in bovine milk used in infant formula appear to have no stimulatory effect on the growth of bifidobacteria. Consequently, a higher proportion of bifidobacteria occurs in breast-fed infants, and this is associated with a lower risk of gastrointestinal infection compared to formula-fed infants.

Today, many food ingredients have been shown to have bifidogenic properties (stimulating the growth of bifidobacteria in the gut). These ingredients are commercially produced worldwide and incorporated into beverages, infant milk powders, confectionery, and dairy desserts (Playne and Crittenden, 1996). Furthermore, the market for new or improved prebiotic products is expanding rapidly, which demands that a set of criteria is developed to define appropriate prebiotics for use in foods.

1.2.3.1 Criteria for selection of prebiotic substrates

For a food ingredient to be classified as a prebiotic, it must: 1) be neither hydrolysed, nor absorbed in the upper part of the GIT; 2) be selectively fermented by potentially beneficial bacteria in the colon, such as bifidobacteria; 3) alter the composition of the colonic microflora towards a healthier state; and 4) induce beneficial luminal/systemic effects within the host (Gibson and Roberfroid, 1995; Fooks *et al.*, 1999; Gibson, 2001).

A number of non-digestible oligosaccharides (NDOs) have now been developed, which fulfil the above criteria of prebiotics, that is, reaching the colon intact, and

producing some prebiotic effects on the host (Mizota, 1996; O'Sullivan, 1996; Fooks *et al.*, 1999). The NDOs include: fructooligosaccharides (FOS), glucooligosaccharides, galactooligosaccharides, transgalactooligosaccharides, isomaltooligosaccharides, xylooligosaccharides, and soybean oligosaccharides. Among these, FOS has been the most thoroughly investigated.

1.2.3.2 Fructooligosaccharides used as prebiotics

Definition and chemical structures

Fructooligosaccharides (FOS) or oligofructose is the generic name for all nondigestible oligosaccharides composed mainly of fructose (Roberfroid and Slavin, 2000). From the point of view of chemical nomenclature, they are oligomers and polymers of D-fructose joined by β (2→ 1) linkages and terminating with a sucrose molecule. Those with degrees of polymerisation (DP) varying from 2 to 20 are referred to as FOS or oligofructose; longer-chained polysaccharides, having a DP up to 60, are commonly known as Inulin (Modler, 1994; Gibson, 1999). There are two slightly different chemical structures in commercially available FOS derived from inulin. These are $G_{py}F_n$ oligomers, i.e. [β-D-Fruf(2→ 1)]$_n$-β-D-Fruf(2↔1)-α-D-Glcp, and $F_{py}F_n$ oligomers, i.e. [β-D-Fruf-(2→ 1)]$_n$-D-Frup (Skinner, 2001). In addition to inulin-derived FOS, FOS can be obtained by enzymatic treatment of sucrose and this product is commonly known as "Neosugar™" or "Nutraflora™". This is a mixture of $G_{py}F_n$ oligomers, including 1-Kestose (DP3), Nystose (DP4), and 1^F-β-D-fructofuranosylnystose (DP5), and also contains unreacted sucrose, glucose and fructose (Modler, 1994; Grizard and Barthomeuf, 1999).

Natural sources and mode of production

There are many natural sources of FOS such as chicory roots, Jerusalem artichoke, dahlia, onion, garlic, leek, wheat, triticale, and rye, in which FOS serves as an energy storage carbohydrate (Modler, 1994; Grizard and Barthomeuf, 1999). Jerusalem artichoke and Chicory are two species currently used by the food industry to produce inulin (Grizard and Barthomeuf, 1999). The amount of FOS and their DP vary greatly between sources and seasons. Storage also influences the FOS profile, with some depolymerisation of the FOS components occurring during some storage conditions. This suggests that freshly harvested plants should be processed immediately if longer-chained FOS is desired. On the contrary, these plants can be stored for longer periods of time to obtain shorter-chained FOS (Modler, 1994). Inulin and oligofructose are usually obtained from Jerusalem artichoke and Chicory by direct plant extraction or by controlled partial enzymatic hydrolysis of natural long-chain inulin with inulinase, such as endoinulinase (E.C.3.2.1.7), which depolymerises the fructan polymers to yield FOS of different DP (Modler, 1994; Grizard and Barthomeuf, 1999; Prapulla *et al.*, 2000; Roberfroid and Slavin, 2000). Commercially, inulin is mostly marketed by Orafti (previously named Raffinerie Tirlemontoise S. A., Tienen, Belgium) and Cosucra (Momalle, Belgium) under the trade names "Raftiline" and "Fibruline", respectively. The partial enzymatic hydrolysate of inulin, namely oligofructose, is marketed by Orafti as "Raftilose" in a variety of purities, either as a powder or in syrup form (Playne and Crittenden, 1996; Grizard and Barthomeuf, 1999).

Another mode of production to yield FOS is enzymatic synthesis. Short-chained FOS, "Neosugar™ and Nutraflora™", are obtained in this way. They can be produced by transfer of a fructose molecule (F) from sucrose (GF) to the terminal end of another sucrose molecule to yield GF_2 (1-Kestose), GF_3 (1-Nystose) and GF_4 (1^F-β-fructofuranosylnystose) by the action of β-fructofuranosidase

(E.C.3.2.1.26) or β-fructosyltransferase (E.C.2.4.1.9). Sucrose is an inexpensive substrate and one that serves as a fructose donor (Modler, 1994; Prapulla *et al.*, 2000). β-fructofuranosidase can be derived from the fungi *Aspergillus japonicus* (Chen and Liu, 1996), *Aspergillus niger* (Hidaka *et al.*, 1988), *Penicillium frequentans* (Usami *et al.*, 1991), and the bacterium *Arthrobacter* sp. K1 (Fujita *et al.*, 1990). β-fructosyltransferase can be derived from the fungi *Aspergillus phoenicis* (Balken *et al.*, 1991), *Fusarium oxysporum* (Patel *et al.*, 1994), *Penicillium rugulosum* (Barthomeuf and Pourrat, 1995), and *Aureobasidium pullulans* (Jung *et al.*, 1987). Commercially, these sugars are produced by Meiji Seika Kaisha Co. Ltd. (Tokyo, Japan), Beghin-Meiji Industries (Paris, France), Golden Technologies Co. Inc. (Westminster, USA), and Cheil Foods and Chemicals Inc. (Seoul, Korea) as "Meioligo", "Actilight", "NutraFlora", and "Oligo-sugar", respectively (Playne and Crittenden, 1996; Grizard and Barthomeuf, 1999).

The main drawback of enzymatic synthesis of Neosugar is the low conversion yield (55-60%) due to the liberation of glucose that acts as a competitive inhibitor. Use of mixed-enzyme systems has been studied as a means of eliminating glucose end-product repression and improving the conversion yield. In this system, high FOS yields are achieved by the action of β-fructofuranosidase or β-fructosyltransferase with simultaneous oxidation of glucose by the action of glucose oxidase (Yun and Song, 1993; Yun *et al.*, 1994). The enzyme glucose oxidase is very effective in allowing continuous conversion to FOS by complete removal of glucose. The mixed-enzyme systems could therefore be applied to large-scale production of highly concentrated FOS.

Novel FOS can be produced by using different monosaccharides and disaccharides as acceptors of the fructosyl residue in place of sucrose (Fujita *et al.*, 1994). For example, 2-*O*-β-D-fructofuranosyl-α-L-sorbo pyranoside is formed with L-sorbose as the acceptor, 3-*O*-β-D-fructofuranosyl-D-galactopyranose is

formed with D-galactose as the acceptor, and 4-*O*-β-D-fructofuranosyl-L-arabinopyranose is formed with L-arabinose as the acceptor.

Physicochemical properties

Some physicochemical properties of FOS are similar to those of other NDOs, which have been extensively reviewed by Crittenden and Playne (1996), Playne and Crittenden (1996), Prapulla *et al.* (2000), and Roberfroid and Slavin (2000). Briefly, they are water soluble and mildly sweet, typically 0.3 to 0.6 times as sweet as sucrose. The sweetness is dependent on the chemical structure and molecular mass of the oligosaccharides present, and the levels of mono- and disaccharides in the mixture. It decreases with longer chain length. Oligo- and polysaccharides with an average DP higher than 10 do not taste sweet. The sweetness values of FOS, relative to a 10% solution of sucrose at 100% sweetness are, 31, 22, and 16% for 1-Kestose, Nystose, and 1^F-β-D-fructofuranosylnystose, respectively. Their relatively low sweetness makes their use as bulking agents desirable.

Increased viscosity is obtained from higher molecular weight oligosaccharides compared with mono- and disaccharides. This improves the "mouth feel" of food products. For example, inulin, used as a texture modifier, provides a creamy mouth feel in processed foods.

Oligosaccharides also provide a high moisture-retaining capacity, acting as a humectant and preventing excessive drying; yet maintaining a low water activity, which is important in controlling microbial growth.

In addition, FOS has higher thermal stability than sucrose. They can be used to control the amount of browning due to Maillard reactions in heat-processed foods. They are highly stable at the normal pH range of food (pH 4.0-7.0) and are stable

at refrigerated conditions for over a year. They behave as cryoprotectants and can be used to alter the freezing temperature of frozen foods.

FOS is "generally recognised as safe". An extensive toxicological evaluation has previously shown no deleterious effect of FOS in traditional toxicity tests (Clevenger *et al.*, 1988).

Physiological properties

Nondigestibility in the upper part of the digestive tract. The concept of nondigestible oligosaccharides originates from the observation that the anomeric C atom (C_1 or C_2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bonds nondigestible by the hydrolytic activity of the human digestive enzymes. In FOS, it is the β configuration of the anomeric C_2 in their fructose monomers that makes FOS resistant to the hydrolysis by human digestive enzymes in the upper part of the digestive tract, such as α-(1→ 4)-glucosidase, α-(1→ 6)-glucosidase, sucrase, and α-amylase, which are mostly specific for α osidic linkages (Roberfroid and Slavin, 2000). This property has been demonstrated in human ileostomy models, which are often used to quantify the small-intestinal excretion of carbohydrates (Cummings and Englyst, 1991). In such a model, 86-88% of the ingested inulin and oligofructose are recovered in the ileostomy effluent (Bach Knudsen and Hessov, 1995; Ellegärd *et al.*, 1997). In hydrogen breath tests, FOS is also shown not to be absorbed in the small intestine (Rumessen *et al.*, 1990). In addition, Oku *et al.* (1984) demonstrated the nondigestibility of neosugar in rat pancreatic homogenates and small intestinal mucosa homogenates.

Fermentability in the large intestine. A number of studies have demonstrated that FOS is a good *in vitro* carbon source utilised by some isolated intestinal bacteria; or *in vivo*, escapes the enzyme hydrolysis in the upper GIT, and reaches

the large intestine without change to their structure, thereby becoming potential substrates for fermentation by the microflora, especially by the bifidobacteria in the lower gut. Hidaka *et al.* (1986) reported that a range of bifidobacteria, but not *B. bifidum*, could utilise neosugars. In addition, other enteric bacteria, such as the *Bacteroides fragilis* group, *Peptostreptococcus* spp., and *Klebsiella pneumoniae*, were also able to grow with neosugars as their carbon source. With FOS as the sole carbon and energy source and with a glucose control, higher specific growth rates for some *Bifidobacterium* spp. were observed, whereas, clostridia and *Bacteroides* preferred glucose to FOS, which suggested that FOS would be preferentially utilised by bifidobacteria on reaching the colon (Gibson and Wang, 1994a). Further confirmation has been made using a chemostat continuous culture system which mimicked the physical and nutritional characteristics of the proximal and distal colon (Gibson and Wang, 1994b). In a single stage continuous culture of human faecal bacteria, FOS, in comparison to sucrose and inulin, preferentially enriched bifidobacteria. This bifidogenic effect of FOS was also confirmed in a three-stage continuous culture model of large intestine. Wang and Gibson (1993) also demonstrated that FOS and inulin selectively stimulated growth of bifidobacteria in mixed faecal bacteria, whilst maintaining populations of potential pathogens (*Escherichia coli* and clostridia) at relatively low levels. Moreover, *in vitro* competition experiments, by Wang and Gibson (1993), and Gibson and Wang (1994c), indicated that *B. infantis* grew well and exerted an inhibitory effect towards *E. coli* and *C. perfringens* in co-culture of these three bacteria when oligofructose was the sole carbohydrate substrate. These results show that bifidobacteria are able to exert more than one mechanism of inhibition. These findings were subsequently confirmed *in vivo* in healthy human volunteers, in whom the proportion of bifidobacteria was significantly increased from 8.8 to 9.5 $\log_{10}$ CFU/g of stool and from 9.2 to 10.1 $\log_{10}$ CFU/g of stool, after two-week ingestion of 15 g/day of inulin and oligofructose, respectively, whereas *Bacteroides*, clostridia, and fusobacteria decreased when

subjects were fed oligofructose, and Gram-positive cocci decreased when subjects were fed inulin. Total bacterial counts were unchanged (Gibson *et al.*, 1995).

The high selectivity of oligofructose for bifidobacteria is due to the production of cell associated β-fructosidases (inulinases) by bifidobacteria which hydrolyse fructose moieties from the non-reducing end of the inulin molecule or from certain sugars displaying a fructose unit at the terminal β (2→ 1) position (Wang, 1993; Bouhnik *et al.*, 1996; O'Sullivan, 1996). However, production of this enzyme was found to be catabolite repressed by monosaccharides and short-chain FOS (O'Sullivan, 1996).

Acetic and lactic acid are the major products of FOS breakdown by bifidobacteria in the gut and these influence many aspects of bowel function such as inhibition of the growth of other bacteria (See Section 1.2.2.7), improvement of the metabolic absorption of Ca, Mg and Fe ions (Scharrer and Lutz, 1990; Lutz and Scharrer, 1991; Rémésy *et al.*, 1993; Delzenne and Roberfroid, 1994; Ohta *et al.*, 1994), and increase in faecal excretion of nitrogen (Gibson *et al.*, 1995). Gaseous hydrogen and carbon dioxide, and bacterial cell mass are also products of the fermentation of FOS. Roberfroid *et al.* (1993) calculated that colonic fermentation of 1 mol of hexosyl equivalent from oligofructose produces 40% SCFAs, 15% lactate, 5% CO_2, and up to 40% bacterial cell mass composed mainly of bifidobacteria. Finally, FOS is metabolised completely and is not excreted in faeces or urine samples (Alles *et al.*, 1996; Molis *et al.*, 1996).

Production of the gases hydrogen and carbon dioxide are the major clinical disincentive against the consumption of FOS, as they cause some unwanted symptoms, such as eructation, flatulence, bloating, abdominal pain, borborygmi (rumbling of gas in the intestine), stomach and gut cramps, and diarrhoea (Cummings *et al.*, 2001). Some studies of FOS at doses of 15 to 30 g/day showed dose-related increases in gas associated side effects (Stone-Dorshow and

Levitt, 1987; Bornet, 1994; Briet *et al.*, 1995). This is due to the use of high doses of FOS that exceed the capacity of the bifidobacteria to ferment these sugars (Hammer *et al.*, 1989). Rao (2001), in his low-dose oligofructose study, showed that consumption of 5 g of oligofructose daily is sufficient for exerting the prebiotic effects. Brighenti *et al.* (1995) also suggested that molecules with longer chain lengths are fermented more slowly and with less net hydrogen production than equal doses of short chained carbohydrates.

FOS has also been demonstrated to increase the bacterial cell mass when utilised by bifidobacteria, and thus stimulate gut peristalsis by the increased bowel content, which produces a laxative effect in the bowel (Cummings, 1994; Gibson *et al.*, 1995). Along with increase in faecal output, there is a significant increase in the faecal excretion of nitrogen.

Health benefits

As mentioned above, FOS resists enzymatic digestion in the upper GIT, and enters the large intestine without change to its structure, where it is selectively fermented by bifidobacteria. This property of FOS is known as the "bifidogenic effect". Consequently, FOS is claimed to be a "bifidogenic factor" which is defined as *a compound that is either partially or not degraded by the digestive enzymes in the upper GIT of host and is preferentially utilised by bifidobacteria in the large intestine as carbon and energy sources* (Mizota, 1996; O'Sullivan, 1996).

By stimulating bifidobacteria, FOS may have the following indirect health benefits on host health, which can be attributed to increased populations of bifidobacteria in the large intestine (See Section 1.2.2.7), and demonstrated in a variety of *in vitro* and *in vivo* studies: reduction of detrimental bacteria (Bailey *et al.*, 1991; Wang and Gibson, 1993; Gibson *et al.*, 1995; Buddington *et al.*, 1996); reduction of toxic metabolites and detrimental enzymes (Buddington *et al.*, 1996);

prevention of pathogenic and autogenous diarrhoea (Hidaka *et al.*, 1986); alleviation of constipation (Kleessen *et al.*, 1997); anticancer effect (Koo and Rao, 1991; Reddy *et al.*, 1997; Pierre *et al.*, 1997; Taper *et al.*, 1997; Rowland *et al.*, 1998; Reddy, 1999); improvement of lipid and glucose metabolisms such as reduction of fasting or postprandial glycemia and insulinemia (Yamashita *et al.*, 1984; Kok *et al.*, 1996a); reduction of the hepatic synthesis of triglycerides, which results in a decrease in the concentration of plasma very-low-density lipoproteins (VLDL), and in turn the hypotriglyceridemic effect (Fiordaliso *et al.*, 1995; Kok *et al.*, 1996b), and reduction of serum cholesterol (Yamashita *et al.*, 1984; Fiordaliso *et al.*, 1995; Davidson *et al.*, 1998); and reduction of the renal nitrogen excretion (Delzenne *et al.*, 1995; Younes *et al.*, 1995; Younes *et al.*, 1996).

In addition, FOS also exerts some direct health benefits on the host:

Improvement in the bioavailability of essential minerals such as Ca, Mg and Fe (Delzenne *et al.*, 1995; Ohta *et al.*, 1995a; Ohta *et al.*, 1995b; Coudray *et al.*, 1997; Ohta *et al.*, 1998). The hypotheses for the enhancing effect of FOS on mineral absorption have been reviewed by Roberfroid and Slavin (2000), who highlighted several mechanisms which are the osmotic effects, acidification of the colonic content due to fermentation and production of SCFA, formation of soluble calcium and magnesium salts of these acids, and hypertrophy of the colon wall.

Low cariogenic properties. These properties allow FOS to be used for tooth decay prevention (Ikeda *et al.*, 1990). Nystose has a low cariogenic factor. It cannot be used as a substrate for water-insoluble glucan formation which is involved in plaque formation and dental cariogenesis (Ikeda *et al.*, 1990).

Low energy food ingredients (Roberfroid *et al.*, 1993; Molis *et al.*, 1996). As FOS is not digested and absorbed in the small intestine, it has no caloric value in the traditional sense (Roberfroid and Slavin, 2000). After reaching the large

intestine, the selective fermentation of FOS products by colonic bifidobacteria results in the loss of energy for the host in the following ways: 1) the growth of bifidobacteria releases part of the energy as heat; 2) the growth of bifidobacteria produces SCFA and gas. The SCFA (acetate, propionate and butyrate) are in turn absorbed by the large intestine, or mainly used by the liver, muscle and adipose tissues as fuel (Bornet, 1994; Delzenne *et al.*, 1995). As a result, ingestion of FOS contributes lower caloric value to the host than that of a digestible carbohydrate (Roberfroid and Slavin, 2000). For example, the caloric value of a fructosyl unit of inulin was found to be approximately 25-35% that of a digested molecule of hexose (Roberfroid *et al.*, 1993; Delzenne and Roberfroid, 1994). Consequently, FOS can be used as a fat replacer or in other lower energy food products, especially those for potential therapeutic use in obese patients. (Roberfroid *et al.*, 1993).

1.2.4 Synbiotics

In addition to probiotics and prebiotics mentioned above, another approach, on which future research will focus, is the combination of both probiotics and prebiotics in a synbiotic, which may be defined as *a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare* (Gibson and Roberfroid, 1995). As indicated in this definition, the use of synbiotics may offer the dual/synergistic benefits of both approaches, whilst the use of a selective substrate (prebiotic) may help long term persistence of growth and/or activities of both the exogenous live microorganisms (probiotics) and endogenous bacteria (Roberfroid, 1998; Gibson, 2001). An in-depth analysis of data has been reviewed by Roberfroid (1998), who highlighted in his article that, six volunteers who received the synbiotic-type product still retained a

significantly ($P < 0.01$) higher number of bifidobacteria after stopping the consumption of the synbiotic product for 2 weeks than those receiving the probiotic alone. This could be attributed to either better colonic colonisation by the probiotic bifidobacteria or a prebiotic effect on endogenous bifidobacteria.

In addition to giving a more efficient bifidogenic effect, a synbiotic could be more efficient in some of other health-promoting effects than either a probiotic or a prebiotic alone (Roberfroid, 1998). This hypothesis was supported by Rowland *et al.* (1998) who treated carcinogen-induced rats for 12 weeks with a synbiotic product which contained both chicory inulin (5%) and *Bifidobacterium longum* 25 (approximately 10^8 CFU/g), and with either chicory inulin (5%) or *Bifidobacterium longum* 25 (approximately 10^8 CFU/g) alone, as a prebiotic and probiotic control, respectively. The results demonstrated that the synbiotic product provided the most efficient treatment which reduced the total number of aberrant crypt foci, the number of foci with one to three aberrant crypts, and the number of foci with four or more aberrant crypts, by 74%, 80% and 59%, respectively. In contrast, inulin reduced the total number of aberrant crypt foci, and the number of foci with one to three aberrant crypts, by 29% and 41%, respectively; *Bifidobacterium longum* 25 reduced the total number of aberrant crypt foci, and the number of foci with one to three aberrant crypts, by 21% and 26%, respectively. Neither inulin nor *Bifidobacterium longum* 25 had any effect on the number of foci with four or more aberrant crypts. In addition to these supporting data, further research is still required to fully validate the hypotheses relating to probiotic and prebiotic synergism occurring in synbiotics.

1.3 Overall aim, objectives, and outline of this project

1.3.1 Overall aim of the project

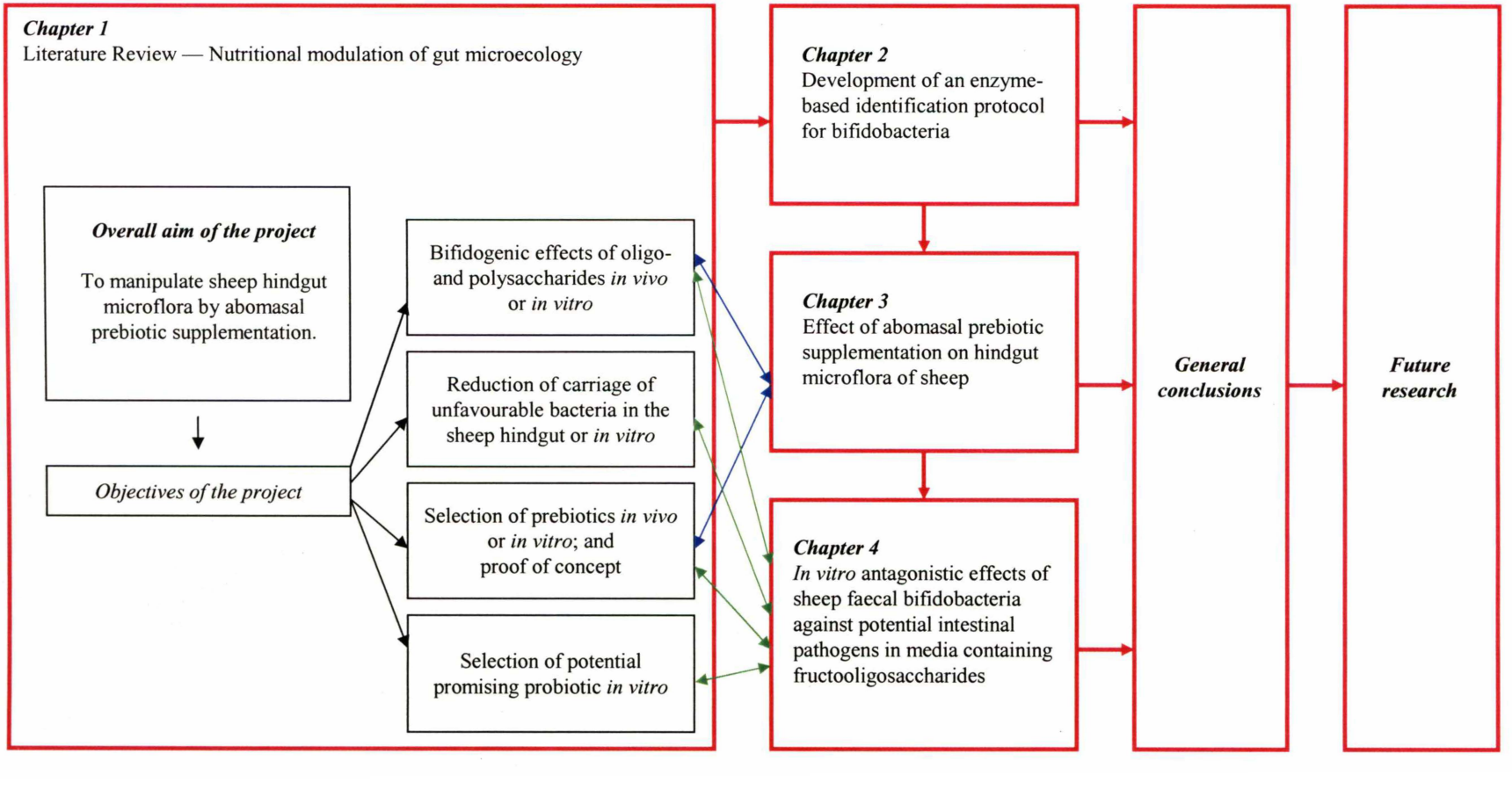
As indicated above, the application of pro-, pre, and synbiotics has been studied primarily in humans and some other monogastric animals. Very few studies have been made to determine their effects on the ruminant intestinal microflora. The overall aim of this project was to test whether or not four commercial food-grade oligo- and polysaccharides (three FOS products and one polysaccharide control, Arabinogalactan) could modify the hindgut microflora in a sheep model towards a more salutary community in which the health-promoting bacterial groups lactobacilli and bifidobacteria predominate, whereas the potential intestinal pathogens and putrefactive bacteria *E. coli* and sulphite-reducing clostridia are suppressed. This work is of value to New Zealand agricultural (meat) industry by potentially reducing the risk of carcass contamination with enteric bacteria, and also through improvement of animal health, well-being, and digestion. The animal health application is likely to be of greatest benefit in calf rearing as an effective method of reducing scouring.

1.3.2 Objectives and outline of this project

This thesis forms a part of a research project on Prebiotic Effect of Fructooligosaccharide on Ruminant Hindgut Microflora (FOS-project). The project was FRST funded and carried out at Food Safety Group, AgResearch Limited, AgResearch MIRINZ Centre; and at the Chemistry Department of the University of Waikato. This thesis describes three experiments carried out at the Food Safety Group of AgResearch MIRINZ Centre (**Figure 1.6**). The objectives and results of each experiment have been written in separate chapters.

Chapter 1 describes the conceptual basis and background for undertaking the research, and the aim of this project. **Chapter 2** describes the standardisation and optimisation of the Scardovi methodology for determination of fructo-6-phosphate phosphokatolase (F6PPK) activity in bifidobacteria. A F6PPK enzyme-based identification protocol was thus developed for identifying and large-scale screening of presumptive bifidobacteria isolates from gut contents or faecal samples. **Chapter 3** describes the effect of abomasal prebiotic supplementation on intestinal microflora of sheep. Four commercial oligo- and polysaccharide products were used in the study. These were Fibruline® LC, Raftilose® P95, Yacon, and Arabinogalactan. Fibruline® LC, Raftilose® P95, and Yacon, all belonging to FOS group, showed great bifidogenic effectiveness and also selectively stimulated lactobacilli in the hindgut microflora of sheep, and so offer promise as prebiotics for manipulation of ruminant intestinal bacteria. **Chapter 4** describes *in vitro* antagonistic effects of sheep faecal bifidobacteria against potential intestinal pathogens in media containing fructooligosaccharides. Raftilose and Yacon both selectively stimulate the growth of sheep faecal bifidobacteria *in vitro* and also potentiate the inhibitory action of bifidobacteria against the test potential intestinal pathogens. Finally, **chapter 5** contains the summary discussion and concluding remarks of entire project. Some suggestions for future research are also given.

Figure 1.6 Outline of the thesis



Chapter 2

DEVELOPMENT OF AN ENZYME-BASED IDENTIFICATION PROTOCOL FOR BIFIDOBACTERIA

2.1 Introduction

Bifidobacteria are non-motile, Gram-positive anaerobic bacteria, which normally inhabit the large intestines of humans and animals (Scardovi, 1986). These organisms are recognised as possessing various health-promoting attributes as outlined in chapter 1 (See Section 1.2.2.7, Chapter 1). For these reasons, there is currently growing interest in using bifidobacteria as probiotics and in the use of prebiotics that enhance proliferation of resident bifidobacteria within the gut (Hoover, 1999).

For bifidobacteria to have a probiotic effect or be stimulated to give a prebiotic effect, they must reach the colon in sufficient number or multiply to a significant number in the gut (Modler *et al.*, 1990; Holzapfel *et al.*, 1998; Stanton *et al.*, 2001). A variety of methods have been used to enumerate bifidobacteria for evaluating the efficiency of *live* bifidobacteria used as probiotics or their stimulation following prebiotic treatment. These include plate count methods, enzymatic methods or molecular methods (Hartemink *et al.*, 1996; Rada, 1997; Burton, 2000; Roy, 2001; Requena *et al.*, 2002). Of these, plate count methods using various selective media (See Section 1.2.2.5, Chapter 1) are still commonly used. Considering that the selectivity achieved for detecting these organisms is only partial and other genera could also show characteristic growth on the

selective media, further confirmation tests are required, which rapidly and reliably identify and screen presumptive bifidobacterial isolates.

According to Bergey's Manual of Systematic Bacteriology (Scardovi, 1986), the most direct and reliable characteristic for assigning an organism to the genus *Bifidobacterium* is based on the demonstration of the enzyme fructose-6-phosphate phosphoketolase (F6PPK – EC 4.1.2.22) in cellular extracts. F6PPK is the characteristic intracellular enzyme of bifidobacterial carbohydrate metabolism, "the bifidus shunt" (See Section 1.2.2.4, Chapter 1). This enzyme, which is virtually specific to the genus *Bifidobacterium*, is absent in the anaerobic bacteria that could be morphologically confused with the bifidobacteria, namely: *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium*, and some members of the Actinomycetaceae (Scardovi and Trovatelli, 1965).

In "the bifidus shunt", which has been described in Chapter 1 (See Section 1.2.2.4, Chapter 1), F6PPK cleaves fructose-6-phosphate into acetyl-1-phosphate and erythrose-4-phosphate. The presence of F6PPK can be ascertained using fructose-6-phosphate as a substrate. The acyl part of the acetyl-1-phosphate thus produced can be converted into hydroxamic acid by reaction with hydroxylamine (Bibiloni, *et al.*, 2000). The hydroxamic acid then forms a reddish violet colour, the ferric chelate of its hydroxamate, upon reaction with trivalent iron (Bibiloni, *et al.*, 2000; Scardovi, 1986).

In this study, we investigated the standardisation and optimisation of the Scardovi methodology for determination of F6PPK activity (Scardovi, 1986) with respect to the growth phase, sonication time, and enzyme reaction time and temperature. The use of a non-ionic detergent Triton X-100 as an alternative to sonication for releasing F6PPK from cells was also evaluated.

2.2 Materials and Methods

2.2.1 General methods

Bacterial anaerobic growth. The growth conditions throughout the study for all strains of bifidobacteria were in an anaerobic system (Forma Scientific Inc., USA) with 5% CO_2, 10% H_2 and 85% N_2 gas atmosphere.

Bacterial growth measurement. Bacterial growth was monitored by the measurement of the optical density using a visible spectrophotometer (Pharmacia LKB·Novaspec II, Amersham Biosciences, Buckinghamshire, England) at 600nm (OD_{600}).

Centrifugation. Centrifugation was carried out using a SORVALL® RC-5C Plus Superspeed Centrifuge (Sorvall Products, USA) at 14,000 × g for 10 minutes at 4°C.

Tip-sonication. Tip-sonication was carried out using a Micro Ultrasonic Cell Disrupter (Kontes) at 50 W in an ice bath.

Phase contrast microscopy. Phase contrast microscopy was carried out using an Olympus BX60F5 microscope (Olympus Optical Co. Ltd., Japan) to assess the extent of cell disruption by tip-sonication.

Shaking-incubation. Shaking-incubation (100/min, Julabo SW1, Germany) was carried out at 37°C for the F6PPK enzyme reaction after the chemical treatment of cells to release the enzyme F6PPK.

2.2.2 Bacteria and growth conditions

Bifidobacterial strains *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, *B. animalis* DSM 20 104, and *B. boum* DSM 20 432 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. These strains were resuscitated in Reinforced Clostridial Medium (RCM, Merck, Darmstadt, Germany) in accordance with the instructions for "Opening of Ampoules and Rehydration of Dried Cultures" (DSMZ, Braunschweig, Germany), which were received with the freeze-dried vials. Colonies were checked for purity with Gram stain, cell morphology, colony characteristics, oxidase test, catalase test and API rapid *ID* 32A test (Biomérieux-department API, France). Then the cultures were transferred into the maintenance medium (15% Glycerol in Tryptic Soy Broth (TSB, Bacto™, Difco, USA)) and stored frozen at -85°C for preservation. Working cultures were prepared on RCM agar slants and stored at 2-8°C for use within a month. Weekly working cultures were then transferred from slant cultures onto RCM agar plates. The growth conditions throughout the resuscitation and cultivation for all strains of bifidobacteria were under anaerobic conditions at 37°C for 72 hours.

2.2.3 Chemicals

0.05M *phosphate buffer pH 6.50 plus cysteine 500mg/L.* 685 mL of 0.05M $NaH_2PO_4 \cdot H_2O$ (6.90 g/L, AnalaR®, BDH, England) and 315 mL of 0.05M $Na_2HPO_4 \cdot 12H_2O$ (17.91 g/L, AnalaR®, BDH, England) were mixed together to give a 0.05M phosphate buffer at pH 6.50. L-cysteine (0.50 g, Aldrich, USA) was then dissolved in 1000 mL of this buffer.

Fructose-6-phosphate (80 mg/mL). Fructose-6-phosphate (4 g, disodium salt, 99%, Sigma, USA) was dissolved in distilled water (50 mL). This solution was

then filter-sterilised and stored at 4°C for up to 1 month.

A solution containing sodium fluoride (6 mg/mL) and sodium iodoacetate (10 mg/mL). NaF (0.06 g, Sigma, USA) and ICH_2CO_2Na (0.10 g, Sigma, USA) were dissolved in 10 mL of distilled water.

13.9% (w/v) hydroxylamine hydrochloride, freshly neutralised with sodium hydroxide to pH 6.5. Hydroxylamine HCl (1.39 g, Sigma, USA) was dissolved in 10 mL of distilled water and then adjusted to pH 6.5 with 5M NaOH. This solution was made freshly on the day of enzyme assays.

15% (w/v) trichloroacetic acid. Trichloroacetic acid (15 g, AnalaR®, BDH, England) was dissolved in 100 mL of distilled water.

4M hydrochloric acid. Commercial HCl solution (~ 7M, ConvoL® hydrochloric acid, BDH, England) was diluted and made up to 250 mL with distilled water.

5% (w/v) ferric chloride hexahydrate in 0.1M hydrochloric acid. $FeCl_3 \cdot 6H_2O$ (5 g, Panreac, Barcelona) was dissolved in 0.1M HCl (100 mL).

Unless stated otherwise, all reagents above were made up in volumetric flasks with distilled water and stored at 4°C in the dark.

2.2.4 Growth measurement of Bifidobacterium spp.

Determination of growth curves of Bifidobacterium spp. in Reinforced Clostridial Medium (1). One well-isolated colony of each strain of bifidobacteria tested was transferred, in duplicate, into fresh RCM broth (10mL) from weekly working cultures, and grown under anaerobic conditions at 37°C. Growth was monitored

by the measurement of the optical density at 600nm (OD_{600}) every 12 hours until the growth ceased. The exponential growth phase for each strain was recorded.

Preparation of stock bacterial cultures. One well-isolated colony of each strain of bifidobacteria tested was transferred, in duplicate, into fresh RCM broth (10mL) from weekly working cultures, and grown under anaerobic conditions at 37°C until the end of the exponential growth phase. These broth cultures were stored at 2-8°C for use as stock bacterial cultures.

Determination of growth curves of Bifidobacterium spp. in Reinforced Clostridial Medium (2). Stock bacterial culture (0.5 mL) of each strain was transferred, in duplicate, into fresh RCM broth (10 mL), and grown under anaerobic conditions at 37°C. Growth was monitored every 4 hours as described previously. The exponential growth phase for each strain was recorded.

2.2.5 Preparation of cell-free extracts

Preparation of stock bacterial suspensions. Stock bacterial culture (0.5 mL) of each strain was transferred, in duplicate, into fresh RCM broth (10 mL), and grown under anaerobic conditions at 37°C until the end of the exponential growth phase. Cells from each strain were then harvested by centrifugation at 14,000 × *g* for 10 minutes at 4°C. The pellets were washed twice with 0.05M phosphate buffer pH 6.5 plus cysteine (500mg/L) and re-suspended in 1.0 mL of the same buffer. These cell suspensions were stored at 2-8°C for use as stock bacterial suspensions. Stock bacterial suspensions from each strain were prepared freshly on the day of enzyme assays.

Preparation of cell-free extracts. Stock bacterial suspension (0.5 mL) from each strain was disrupted by tip-sonication at 50 W power in an ice bath. Sonicates were examined every 30 seconds under the phase contrast microscope to assess

the extent of cell disruption. Crude extracts were obtained when cells were disrupted completely. The whole procedures were carried out in quadruplicate. Two of the 0.5 mL volumes of the crude extracts of each strain were combined together and enzyme activity assays were performed on 1.0 mL volumes as described below.

2.2.6 Detection of F6PPK activity

To each 1.0 mL of crude extract of each strain were added 0.25 mL each of fructose-6-phosphate (80 mg/mL) and a solution containing NaF (6 mg/mL) and Na-iodoacetate (10 mg/mL). The mixtures were incubated at 37°C for 30 minutes. The reaction was then stopped by adding 1.5 mL of 13.9% (w/v) hydroxylamine HCl, freshly neutralised with NaOH to pH 6.5. After 10 minutes at room temperature, 1.0 mL each of 15 % (w/v) trichloroacetic acid and 4M HCl were added. Colour development was then achieved by adding 1.0 mL of 5% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.1M HCl. A reddish violet colour was considered positive in comparison with a control, in which the reaction mixture lacked the fructose-6-phosphate substrate, and which gave a yellow colour on addition of trivalent iron. For ambiguous results, if the colour observed was darker than the control it was considered as weakly positive. The whole procedure was carried out in duplicate.

2.2.7 The pre-treatment of cells with Triton X-100

The pre-treatment of cells was also carried out using Triton X-100 (BDH, England) as an alternative to sonication for releasing F6PPK from cells. In this method, stock bacterial suspension (1.0 mL) from each strain was mixed with Triton X-100 (0.1 mL, 4% v/v) solution. The mixtures were then immediately assayed for enzyme activity as described in Section 2.2.6 except that shaking-incubation was used during the enzyme reaction. The whole procedure was carried out in duplicate.

2.2.8 Comparison of static-incubation and shaking-incubation for enzyme reaction in B. pseudolongum subsp. globosum DSM 20 092

Stock bacterial suspension (1.0 mL) from *B. pseudolongum* subsp. *globosum* DSM 20 092 was mixed with Triton X-100 (0.1 mL, 4% v/v) solution, fructose-6-phosphate (0.25 mL, 80 mg/mL), and a solution (0.25 mL) containing NaF (6 mg/mL) and Na-iodoacetate (10 mg/mL). The mixtures were subject to static-incubation or shaking-incubation at 37°C for 30 minutes. The reaction suspensions were then assayed for enzyme activity as described in Section 2.2.6. The whole procedure was carried out in duplicate.

2.2.9 Optimisation of the F6PPK enzyme reaction time and temperature

F6PPK activity in *B. animalis* DSM 20 104 was also determined, in duplicate, as described in the Section 2.2.6 with three different enzyme reaction times (30 min, 45 min and 60 min) and three different enzyme reaction temperatures (30°C, 37°C and 42°C), respectively. The intensity of the colour formed was recorded by reading absorbance at 505 nm.

2.3 Results

2.3.1 Growth measurement of Bifidobacterium spp.

One well-isolated colony of each strain of bifidobacteria tested was transferred, in duplicate, into fresh RCM broth (10mL) from weekly working cultures, and grown under anaerobic conditions at 37°C. Growth was monitored by the

measurement of the optical density at 600nm (OD_{600}) every 12 hours until the growth ceased (**Figure 2.1**).

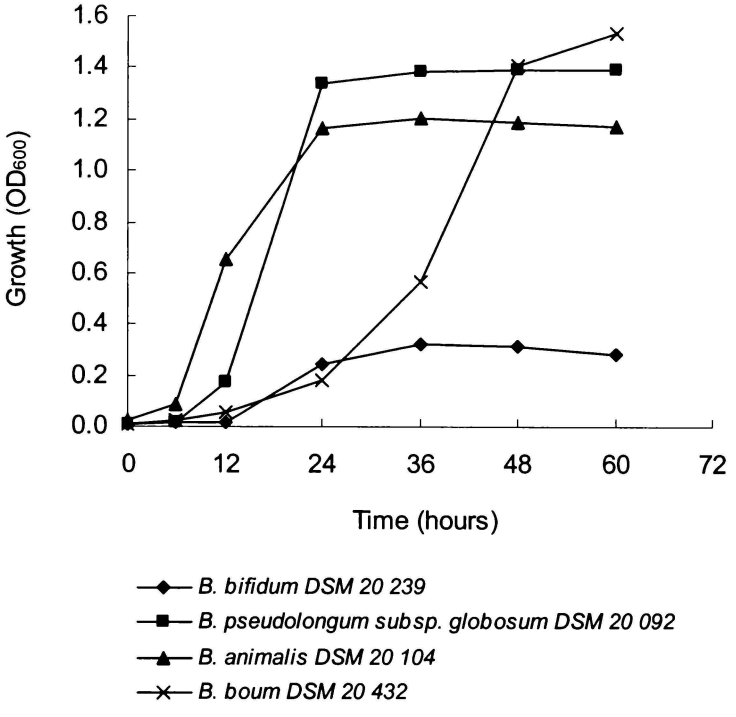


Figure 2.1 Growth curves (1) of four bifidobacterial strains in Reinforced Clostridial Medium. One well-isolated colony for each bifidobacterial strain was transferred into 10 mL of fresh RCM broth. Data represent means of duplicate determinations.

The exponential growth phases for *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, *B. animalis* DSM 20 104, and *B. boum* DSM 20 432 were 12-24 hours, 12-24 hours, 6-24 hours, and 24-48 hours, respectively (**Figure 2.1**). Compared to the other species, *B. bifidum* DSM 20 239 grew more slowly and reached lower maximum numbers. At the end of exponential growth phases, *B. boum* DSM 20 432 reached the highest absorbance (OD_{600}) indicating greatest maximum cell number among the four strains which was 1.407, the

absorbance of *B. pseudolongum* subsp. *globosum* DSM 20 092 was 1.338, the absorbance of *B. animalis* DSM 20 104 was 1.166, and *B. bifidum* DSM 20 239 reached the lowest absorbance (OD_{600}) which was 0.243.

5% inocula of these broth cultures incubated to the end of the exponential phases were then transferred to fresh RCM broth (10mL) and the growth was measured again (**Figure 2.2**).

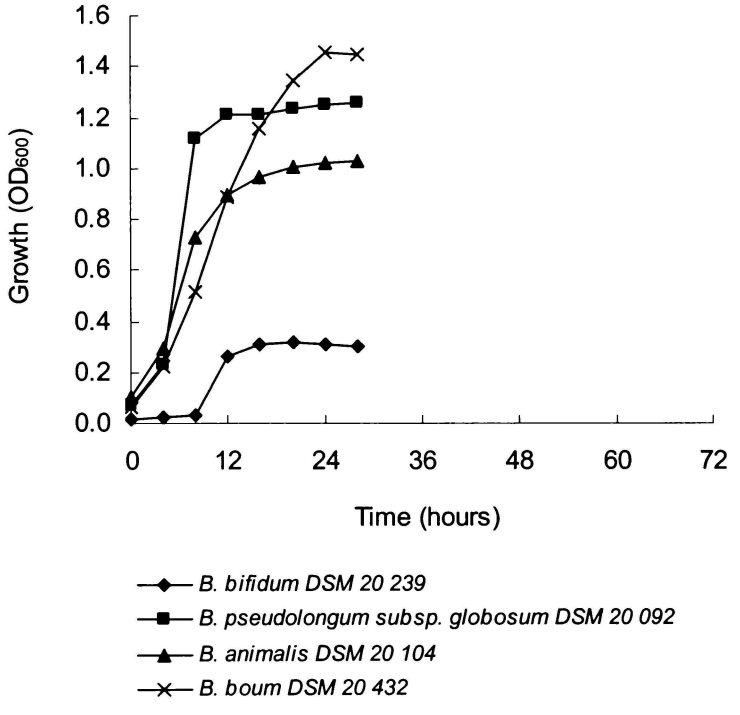


Figure 2.2 Growth curves (2) of four bifidobacterial strains in Reinforced Clostridial Medium. 5% inocula for each bifidobacterial strain were transferred to fresh RCM broth. Data represent means of duplicate determinations.

The exponential growth phases for *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, *B. animalis* DSM 20 104, and *B. boum* DSM 20 432 were 8-12 hours, 4-8 hours, 4-8 hours, and 4-20 hours, respectively.

Once again, compared to the other species, *B. bifidum* DSM 20 239 grew more slowly and reached lower maximum numbers. At the end of exponential growth phases, *B. boum* DSM 20 432 reached the highest absorbance (OD_{600}) indicating greatest maximum cell number among the four strains which was 1.350, the absorbance of *B. pseudolongum* subsp. *globosum* DSM 20 092 was 1.120, the absorbance of *B. animalis* DSM 20 104 was 0.731, and *B. bifidum* DSM 20 239 reached the lowest absorbance (OD_{600}) which was 0.265.

As shown in these two graphs, shorter lag phases and exponential growth phases were achieved after transferring the bifidobacterial cultures twice, which would speed up the F6PPK test process.

2.3.2 Optimisation of the sonication time

The optimum sonication time for F6PPK release from the test strains was evaluated using phase contrast microscopy. In this method, bifidobacterial cells were disrupted by tip-sonication at a 50 W power in an ice bath. Sonicates were then examined every 30 seconds under the phase contrast microscope until the cells were disrupted completely. Sonication times for F6PPK release differed between strains: the optimum sonication time was four 30-s periods for *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, and *B. animalis* DSM 20 104, and five 30-s periods for *B. boum* DSM 20 432. This difference is probably due to the different cell wall murein structures among the test strains (Scardovi, 1986).

2.3.3 Influence of tip-sonication and Triton X-100 treatment on the determination of F6PPK in the four bifidobacterial strains

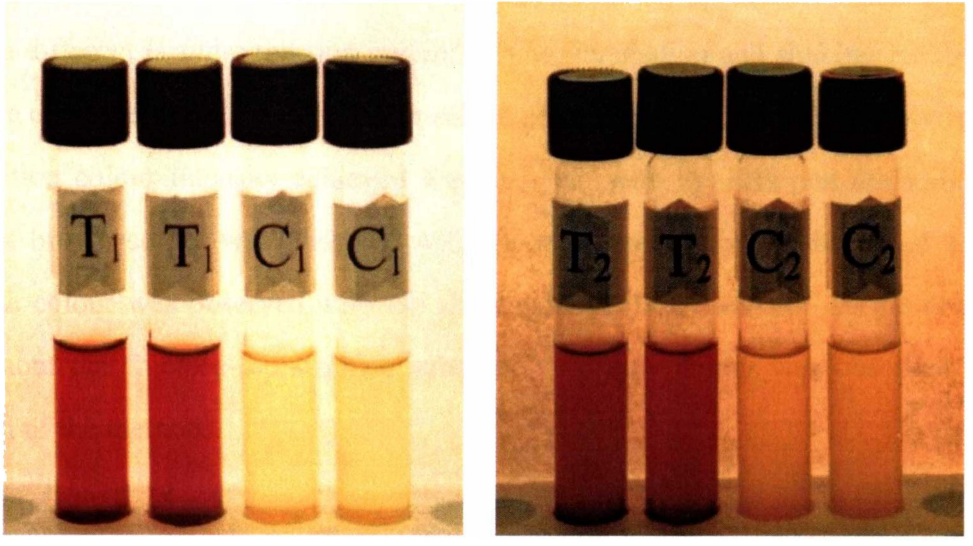
The influence of tip-sonication and Triton X-100 treatment on the determination of F6PPK activity was compared in the four bifidobacterial strains (**Table 2.1**).

Table 2.1 Influences of tip-sonication and Triton X-100 treatment on the determination of F6PPK in the four bifidobacterial strains. Results are presented from duplicate determinations.

Strains	Tip-sonication		Triton X-100	
	Treatment	**Control**	**Treatment**	**Control**
B. bifidum DSM 20 239	Yellow brown Clear	Bright yellow Clear	Light reddish brown Turbid	Bright Yellow Turbid
B. pseudolongum subsp. *globosum* DSM 20 092	Port wine Clear	Bright yellow Clear	Port wine Turbid	Bright Yellow Turbid
B. animalis DSM 20 104	Port wine Clear	Bright yellow Clear	Port wine Turbid	Bright Yellow Turbid
B. boum DSM 20 432	Dark yellow Clear	Bright yellow Clear	Port wine Turbid	Bright Yellow Turbid

Different degrees of enzyme reaction colour development and transparency of the enzyme reaction suspensions were achieved by both of the cell pre-treatment methods with the four strains tested. Stronger colour developed with strain *B. pseudolongum* subsp. *globosum* and strain *B. animalis* than with the other two test strains when tip-sonication was used for cell disruption. Improvement in colour was observed in strain *B. boum* and strain *B. bifidum* through the use of Triton X-100 to release F6PPK. No colour developed in the controls without the fructose-6-phosphate substrate, which remained yellow, the original colour of the $FeCl_3$ solution added. The enzyme reaction suspension was clear with the use of tip-sonication, but turbid with the use of Triton X-100. This difference is due to the different mode of action on cells between these two methods for cell disruption (Tzannis, 1991; Sigma-Aldrich, 2002).

Figure 2.3 shows the influence of tip-sonication and Triton X-100 treatment for enzyme release on enzyme reaction colour development in strain *B. animalis*.



Tip-sonication Triton X-100

Figure 2.3 Influences of tip-sonication (T1) and Triton X-100 treatment (T2) on enzyme reaction colour development in strain *B. animalis* DSM 20 104. No colour development was detected in the control tubes without the fructose-6-phosphate substrate (C1, C2). Results are presented from duplicate determinations.

In **Figure 2.3**, a port wine colour is displayed in both of tip-sonication (T1) and Triton X-100 treatment (T2), which is considered as positive enzyme reaction. No colour development is displayed in the control tubes without the fructose-6-phosphate substrate (C1 and C2), which remain yellow, the original colour of the $FeCl_3$ solution added. In addition, the solutions appear clear when they have been treated with tip-sonication (T1 and C1), but turbid with Triton X-100 treatment (T2 and C2).

2.3.4 Comparison of static-incubation and shaking-incubation for enzyme reaction in B. pseudolongum subsp. globosum DSM 20 092

Two different enzyme reaction methods: static-incubation and shaking-incubation were compared in *B. pseudolongum* subsp. *globosum* DSM 20 092. The enzyme reaction colour intensity achieved was different with the different methods. A light brown colour was observed with the static-incubation method, and a port wine colour was observed with the shaking-incubation method. In the control, without the fructose-6-phosphate substrate, a yellow colour was observed with both of the methods.

2.3.5 Influences of enzyme reaction time and temperature on F6PPK activity in B. animalis DSM 20 104

Influences of enzyme reaction time and temperature on F6PPK activity were studied in *B. animalis* DSM 20 104. The optimum enzyme reaction time was considered as 45 minutes although higher enzymatic activity was yielded after 60-minute enzyme reaction (**Figure 2.4**). The reason for taking 45 minutes as the optimum enzyme reaction time is that a more rapid detection method, with which to identify bifidobacteria is necessary; and an appreciable enzymatic activity has been achieved after a 45-min enzymatic reaction time. The optimum enzyme reaction temperature was found to be 37°C (**Figure 2.5**).

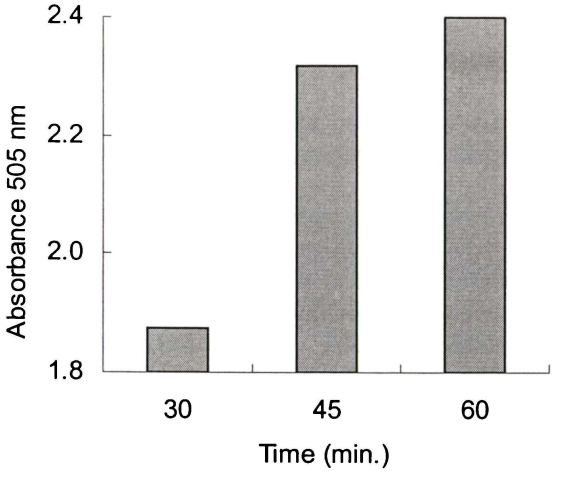


Figure 2.4 Influence of enzyme reaction time on F6PPK activity in *B. animalis* DSM 20 104. Data represent means of duplicate determinations.

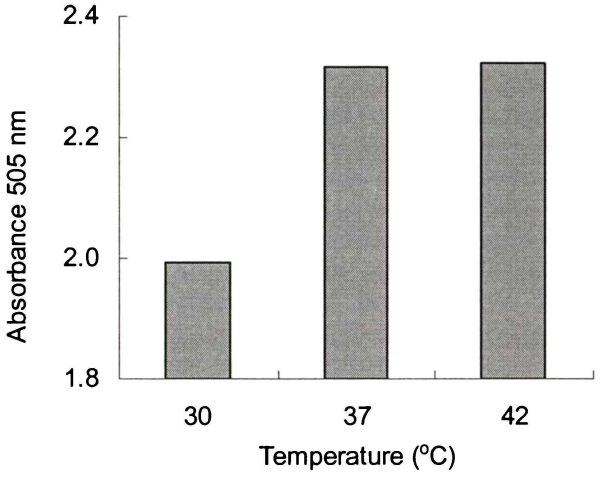


Figure 2.5 Influence of enzyme reaction temperature on F6PPK activity in *B. animalis* DSM 20 104. Data represent means of duplicate determinations.

2.4 Discussion

With growing interest in using bifidobacteria as probiotics and in the use of prebiotics to stimulate the growth of bifidobacteria within the gut, a rapid, reliable and relatively inexpensive test, with which to detect bifidobacteria in the gut or faecal samples, has become essential. Today, classic approaches such as examination of phenotypic characterisation combined with various enzymatic and molecular techniques such as the use of DNA probes, pulse field gel electrophoresis (PFGE), and fluorescent *in situ* hybridisation (FISH) have been developed to detect and identify bifidobacteria in the intestine or faecal samples (O'Sullivan and Kullen, 1998). Among these methods, demonstration of F6PPK activity is still the most direct and reliable characteristic for assigning an organism to the genus *Bifidobacterium* (Scardovi, 1986), although this enzyme has been recently detected in some strains of *Gardnerella vaginalis* found in the human genital tract/urinary tract (Gavini *et al.*, 1996). Additionally, the reagents used for the F6PPK test are relatively inexpensive and the test procedures are simple and rapid. Based on all these attributes, the colourimetric determination of F6PPK activity is deemed to be preferable for rapid identification, and, in particular, for large-scale screening of presumptive bifidobacterial cultures isolated from the gut contents or faecal samples.

As discussed previously, F6PPK is the characteristic enzyme of bifidobacterial carbohydrate metabolism, "the bifidus shunt". This enzyme, which is virtually specific to the genus *Bifidobacterium*, is absent in the anaerobic bacteria that could be morphologically confused with the bifidobacteria, namely: *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium*, and some members of the Actinomycetaceae (Scardovi and Trovatelli, 1965). F6PPK in the "the bifidus shunt" cleaves fructose-6-phosphate into acetyl-1-phosphate and erythrose-4-phosphate (Scardovi and Trovatelli, 1965). In the colourimetric test,

the presence of F6PPK can be tested with fructose-6-phosphate as a substrate. The formation of acetyl phosphate from fructose-6-phosphate is evidenced by the reddish violet colour formed by the ferric chelate of its hydroxamate (Scardovi, 1986).

In order to obtain more sensitivity in F6PPK activity detection and a more rapid test procedure, several modifications have been made to the Scardovi methodology (Scardovi, 1986) in this study.

Growth curves of the four bifidobacterial strains tested were measured in this study to determine their exponential growth phases and the maximum cell numbers, as F6PPK activity in cells harvested at the end of this growth phase may reach its highest level and be easiest to detect. Bibiloni *et al.* (2000) studied the relationship between F6PPK activity and bacterial biomass. The experimental results demonstrated a direct linear relationship increase between F6PPK activity and cell biomass for all the test strains. In addition, the degree of enzymatic activity showed variations between species, and even between strains of the same species. Consequently, for demonstration of F6PPK activity in strains of *Bifidobacterium* spp., the exponential growth phases of those strains grown in some specific media should be determined first. In this study, **Figure 2.1** and **Figure 2.2** both showed that there were differences in exponential growth phases for the different strains tested and also differences in maximum cell numbers attained in RCM broth. Consequently, different degrees of enzyme reaction colour development were observed from the different strains tested (**Table 2.1**). However, it was not determine whether the different colour intensity was due to the different cell masses or the different strains tested. The bifidobacterial cultures were transferred twice in this study so that shorter lag phases and exponential growth phases could be achieved which would speed up the F6PPK test process.

Tip-sonication is a mechanical cell disruption method in which the cell is disrupted with high frequency sound that is produced electronically and transferred through a metallic tip into an appropriately concentrated cellular suspension. In this process, the cell wall is physically broken, releasing all intracellular components into the surrounding medium. Because of the very high costs of equipment, it is only used on a laboratory scale for lysis of cells with less resistant cell walls, such as bacteria and fungi (Tzannis, 1991). The optimum sonication time for different strains tested was determined in this study, which showed different sonication times suited different strains. This may be due to different cell wall murein structures for different strains which resist cell disruption (Scardovi, 1986). The cells were treated by tip-sonication for 30 seconds in an ice bath, treatment was then stopped and the tip was allowed to cool for 30 seconds in the ice before the next 30 second burst of sonication. This procedure was adopted for the protection of enzymes, proteins, or other intracellular components from denaturation by heat generated by the device. The degree of cell disruption was examined under phase contrast microscope after every 30 seconds of sonication until the cells were disrupted completely, but not longer, as this would increase the risk of heat denaturation.

Tip sonication as a method for cell disruption has several disadvantages: the high costs of the device; the heat generated which causes thermal denaturation of enzymes and other intracellular components; and, in addition, it is time-consuming; and noisy. Triton X-100 was therefore used as an alternative to sonication for releasing F6PPK from cells.

Triton X-100 is a non-ionic surfactant that is widely used for extracting intracellular components from microorganisms by rendering their cell membrane permeable under non-denaturing conditions (Tzannis, 1991; Sigma-Aldrich, 2002). Both tip-sonication and Triton X-100 permeabilisation yielded positive results for F6PPK with all four bifidobacterial strains examined. However, a more definite

positive result was obtained for strain *B. boum* and strain *B. bifidum* when using Triton X-100 to release F6PPK (**Table 2.1**). This indicated that a higher efficiency of enzyme release and lower enzyme denaturisation was achieved by the use of Triton X-100 than by tip-sonication. Overall, the use of Triton X-100 is the more convenient, rapid, and effective enzyme release technique for identifying, in particular, for mass screening, presumptive bifidobacterial isolates in comparison with tip sonication.

A shaking-incubation method is recommended with the use of Triton X-100 for cell permeabilization, as the cells in some bifidobacterial strains such as *B. pseudolongum* subsp. *globosum* DSM 20 092 readily settle out of suspension during static-incubation, which may influence the efficiency of Triton X-100 to release the enzyme.

The optimum enzyme reaction time and temperature were determined (**Figure 2.4** and **Figure 2.5**), which was 45 minutes at 37°C. Although higher enzymatic activity was yielded after 60-minutes enzyme reaction or at 42°C, the reason for selecting 45 minutes at 37°C as the optimum enzyme reaction time and temperature is a compromise allowing a more rapid assay of F6PPK; but still allowing appreciable enzymatic activity.

In summary, a F6PPK enzyme-based identification protocol has been developed for the identification and large-scale screening of presumptive bifidobacterial isolates from the gut contents or faecal samples. In this protocol, the detection of F6PPK activity was optimised, for a more rapid and sensitive enzyme reaction, which was achieved by using cells at the end of the exponential growth phase, by using Triton X-100 for F6PPK release to enhance colour development and test sensitivity, by using shaking-incubation for the enzyme reaction, and by using an enzyme reaction at 37°C for 45 minutes.

Chapter 3

EFFECT OF ABOMASAL PREBIOTIC SUPPLEMENTATION ON INTESTINAL MICROFLORA OF SHEEP

3.1 Introduction

The GIT of humans and animals harbours a complex microbial community comprising about 10^{14} microorganisms consisting of more than 400 different species of bacteria (Moore and Holdmman, 1974). These microorganisms live in a stable relationship with the host, playing a major role in host health and well-being (See Section 1.1.2 and 1.1.3 in Chapter 1). Some of these microorganisms are perceived as being beneficial such as bifidobacteria and lactobacilli, whereas others are considered as possible pathogens (*E. coli*, *Salmonella*, *Listeria* and *Shigella*) or putrefactive bacteria (*Clostridium perfringens*). There is, therefore, some interest in the manipulation of the composition of the gut microflora towards a potentially more advantageous community, that is, an increase in numbers and activities of the beneficial bacteria. A widely used approach to achieve this is the use of prebiotics. A prebiotic is *a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve host health* (Gibson and Roberfroid, 1995). NDOs, in particular, FOS have been the most thoroughly investigated food ingredients for prebiotic use. FOS, *in vivo*, escape digestion in the upper gastrointestinal tract and reach the large intestine without change to their structure, thereby becoming selective substrates for fermentation by LAB, especially, by bifidobacteria in the

lower gut. This selective stimulation of the growth of bifidobacteria by FOS is known as the "bifidogenic effect" of prebiotics. Increased populations of indigenous bifidobacteria in the gut, by virtue of their antagonistic effects, in turn, suppress the growth and activities of the potential intestinal pathogens and putrefactive bacteria (See Section 1.2.2.7, Chapter 1). To date, this "bifidogenic" effect and the associated antimicrobial effect by FOS have been mainly studied in humans and some other monogastric animals. Very few studies have been made to determine these effects on the ruminant intestinal microflora.

Ruminants are differentiated from monogastric animals on the basis of their specific four-compartment stomachs, which include rumen, reticulum, omasum and abomasum. Among them, the abomasum is the fourth and the final acid digestion stomach. This stomach is most like that of a monogastric, whereas the rumen and reticulum, the first two parts of the stomach, contain a prolific, specialised and delicately balanced ecosystem of microbes that enable the ruminant animal to digest, by rumen fermentation, dietary fibre normally unavailable to most monogastric animals. Introducing FOS directly into the digestive system of ruminant would most likely result in the rumen fermentation of FOS and the failure for FOS to enter the hindgut without change to their structure.

Consequently, an abomasal supplementation model with a fistula attached to the sheep abomasum was used in this study to allow direct post-rumino-reticular administration of FOS to investigate the bifidogenic and antimicrobial effects of FOS on the hindgut microflora of ruminants. Four commercial food-grade oligo- and polysaccharides (three FOS products and one polysaccharide control, Arabinogalactan) were tested to determine whether these commercially available products can be of use in manipulating hindgut microflora of sheep towards a potentially more advantageous community in which the health-promoting bacterial groups such as LAB, in particular, bifidobacteria are stimulated to

assume predominance, whereas potential intestinal pathogens and putrefactive bacteria such as *E. coli* and *Clostridium perfringens* are suppressed to relatively low levels. This work is of value to New Zealand agricultural (meat) industry by affording an opportunity to reduce the risk of carcass contamination with undesirable bacteria that reside in the sheep hindgut, and that may during slaughter and dressing be accidentally transferred to a human food stuff, namely, meat.

3.2 Materials and Methods

3.2.1 Pre-trial animal selection and acclimatisation

3.2.1.1 Selection panel for animals

Twenty-two healthy, non-pregnant, female sheep, one year of age, were obtained from AgResearch farms (AgResearch Ltd., Hamilton, New Zealand). Male sheep were excluded from this study because of possible urinary tract complications such as obstructive urethral calculi during long-term indoor penning. Animal ethics approvals were obtained from the AgResearch Ruakura Animal Ethics Committee (MZLHJ 002/01 AEC 3843, Animal Ethics Committee, AgResearch, New Zealand) and the Animal Ethics Committee of the University of Waikato (Protocol 492, Animal Ethics Committee, the University of Waikato, New Zealand).

The 22 sheep were selected on the basis of clinical, haematological and biochemical health, which were assessed by analysis of haemoglobin concentration and indicators of liver function such as plasma glutamate dehydrogenase (GDH), gamma glutamyl transferase (GGT), bilirubin, and total protein and albumin (Agri-Quality Animal Health Laboratory, Ruakura, Hamilton,

New Zealand; Anderson *et al.*, 1981). Pregnancy scanning was also conducted to ensure no animals entering the trial were pregnant (Agri-Quality Animal Health Laboratory, Ruakura, Hamilton, New Zealand).

The sheep were not in full fleece and were shorn about a month to eight weeks before commencement of the trial. They were also ear tagged for individual identification for data recording during the trial. The numbers used were 115, 137, 434, 459, 9603, 9610, 9616, 9641, 9644, 9649, 9660, 9661, 9671, 9706, 9715, 9719, 9752, 9776, 9824, 9832, 9861, and 9865.

3.2.1.2 Acclimatisation of animals to holding facilities and sampling

The 22 sheep were individually penned in the Growth and Physiology Unit's sheep housing barn (Ruakura, Hamilton, New Zealand) to be acclimatised to the holding facilities and the sampling procedures.

The sheep were introduced to a dried, pelleted, lucerne-based diet, which was balanced for essential minerals and vitamins required for the healthy maintenance of sheep, and comprised 82.5% lucerne, 12% maize, 4% molasses, 0.5% calcium phosphate, 0.8% salt and 0.2% Mineral and Vitamin Premix (Camtech Nutrition Ltd., Hamilton, New Zealand). As copper accumulation might be a problem with indoor, housed sheep, the Mineral and Vitamin Premix used in the diet was copper free. The pellet diet was fed once daily in the morning at a rate (650-690 g per sheep) that allowed maintenance of normal body weight of the sheep. They were also fed a handful of lucerne chaff with the pellets and a handful of meadow hay every afternoon during the acclimatisation period and also during the main trials, for maintenance of normal rumen function.

The sheep were also trained to defecate on demand to give fresh samples which were necessary for immediate microbiological and chemical assays during the main trials. The method for faecal sampling involved inserting a gloved

lubricated (paraffin oil, BDH, Poole, England) finger gently into the anus of the sheep to stimulate the rectal wall just inside the anus. This procedure usually resulted in a good-sized defecation. A prompt word and a reward of food were given immediately on production of a sample to encourage a consistent and timely delivery of samples. If the sheep failed to produce a sample on the first stimulation, further attempts, but no more than three were made at hourly intervals. The faecal sample training was conducted each day of the two week acclimatisation period.

The sheep that did not acclimatise to the housing conditions and diet were excluded from the trial. Finally, twelve candidates were selected for abomasal fistulation by a veterinarian on the basis of their clinical, haematological and biochemical health conditions determined and their acclimatisation to the holding facilities and faecal sampling procedure. These twelve prospective trial sheep were then vaccinated by the veterinarian against clostridial (blood poisoning) infections (Agri-Quality Animal Health Laboratory, Ruakura, Hamilton, New Zealand), and prepared for abomasal fistulation surgery.

3.2.2 Abomasal Fistulation Surgery

3.2.2.1 Fistula Design

The fistula (**Figure 3.1**) was designed and constructed by Tony Day (Registered Veterinarian, Tony Day Veterinarian Ltd., Hamilton, New Zealand).

It was made of flexible silastic tubing which was chosen for its low irritational properties in comparison to more rigid tubing.

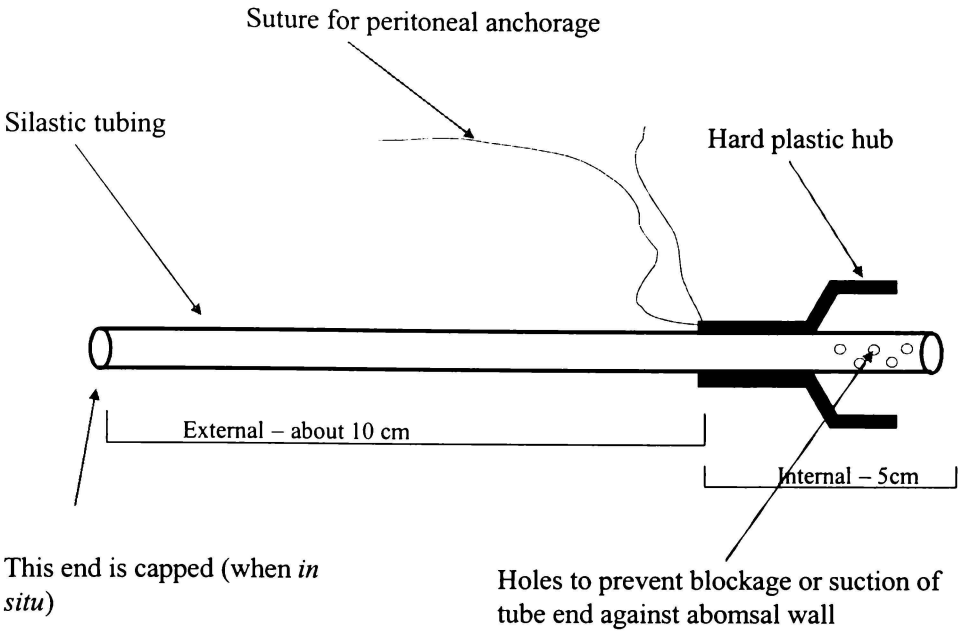


Figure 3.1 Fistula design model applied for the main experiment

The tubing was about 10.0 mm O.D. and 7.0 mm I.D. There were multiple holes (about 0.5-1.0 cm diameter) in the tubing wall from 3.0-5.0 cm from the internal end of the tubing. These holes were designed to reduce the possibility of the tube becoming blocked by fibrous abomasal contents. A plastic flange, about 3.0 cm in diameter, was also attached to the internal end. This flange, together with the attached silastic tubing, were situated inside the abomasal fundus (the main body of the fourth stomach of sheep), and sutured to the abomasal wall from the external surface of the abomasal wall. This provided an attachment site for the fistula, and protected the tissue and fistula. A purse-string suture was used to close the abomasal wall around the fistula tubing where it exited from the abomasal wall. The other end (external) of the fistula tubing that was sited outside the abdominal wall was capped. The capping was designed to seal the fistula against leakage of abomasal contents and entry of air or other foreign substances, and also allowed attachment of syringes for administration of test substances and back sampling of abomasal contents.

3.2.2.2 Abomasal fistulation surgery

The twelve selected trial sheep were taken to the surgical facility (MIRINZ Physiology Shed, Ruakura, Hamilton, New Zealand) and deprived of food and water overnight. Then they were operated on for abomasal fistulation by Tony Day. Postoperative care included antibiotic injection with 4 mL of Bomacillin (Procaine penicillin, 300 mg/mL, BOMAC Laboratories Ltd., Manukau City, Auckland, New Zealand) immediately after operation and then daily for three days. Observations of appetite, behaviour, body temperature, and wound condition were recorded daily until the fistula wounds in all 12 sheep had healed (as determined by Tony Day). Postoperative faecal microflora were monitored to ensure the test faecal bacteria returned to normal.

3.2.3 Main Experiment — Abomasal Supplementation

3.2.3.1 Experimental design

The experiment was a balanced, two Latin square, cross-over design.

Of the 12 sheep fistulated, the ten healthiest sheep were used in the experiment. The remaining 2 sheep were managed so as to be ready replacements for up to two sheep in the experiment, should any become unsuitable for whatever reason during the course of the experiment.

There were 5 treatments: a) Control (pH 2.23 acidified saline), b) Raftilose® P95, c) Fibruline® LC, d) Arabinogalactan, and e) Yacon. The 5 treatments are indicated as "a" to "e" in the body of **Table 3.1**.

The experiment was run in 5 consecutive periods, to allow every sheep to experience all 5 treatments ("cross over") and also to allow two sheep to be used per treatment per period.

Each period consisted of a 1 week of stabilisation to the pelleted diet (no treatment), followed by 14 days of treatment supplementation *via* daily infusions into the abomasum and faecal sampling, followed by a period of basic, pelleted diet with faecal collection for about 12 days after infusions ceased.

Table 3.1 Experimental design for abomasal prebiotic supplementation

Animal	Period 1	Period 2	Period 3	Period 4	Period 5
1	a	b	e	c	d
2	b	c	a	d	e
3	c	d	b	e	a
4	d	e	c	a	b
5	e	a	d	b	c
6	d	c	e	b	a
7	e	d	a	c	b
8	a	e	b	d	c
9	b	a	c	e	d
10	c	b	d	a	e
11 (follows 3)	c	d	b	e	a
12 (follows 5)	e	a	d	b	c

3.2.3.2 Experimental procedures

Sheep were fed a pelleted, lucerne-based diet (as described in Section 3.2.1.2) daily in the morning throughout the main experiment. The feed level of the pelleted lucerne-based diet was controlled to maintain body weight. Lucerne chaff and meadow hay were also fed to encourage normal rumination and rumen health.

Each experimental period began with a week stabilisation to the pelleted diet (no treatment). Basic husbandry of sheep was maintained during this period and faecal samples were taken from each sheep as described in Section 3.2.1.2 on day -4. The collected faecal samples from each sheep were divided into four parts. One part was put into a stomacher bag, stored near (but not on) ice in a chilly bin, and transferred straight to the microbiology laboratory for microbiological analysis within four hours. The second part of the faecal samples was put into plastic containers with airtight lids, and stored on ice until transferred to the chemistry laboratory for immediate pH determination. The third part was put into foil caps, weighed, and an immediate oven dry matter content analysis was performed. The fourth part was put into glass containers with airtight lids, and held on ice before freeze-drying for analysis of residual oligosaccharides, which was carried out at the Chemistry Department of The University of Waikato.

After 1 week, treatment supplementation was infused into the abomasum daily for 14 days. For administration of treatment infusions, each sheep was lightly restrained while on its feet. About 5-10 mL of abomasal contents were first drawn from the fistula using a 60-mL syringe. Then, the infusions were administered into the fistula using another 60-mL syringe. Finally, the abomasal contents initially drawn were infused back into the fistula to clear the fistula tubing. All sheep were checked as to their physiological responses to the infusions every hour for the first 8 hours, then every 3 hours for the next 16 hours,

after the first administration in case there was any adverse response such as prolific diarrhoea or signs of colic. The faecal samples were taken from each sheep on days 3 and 9 of this period for microbiological and chemical assays. The administration of treatment supplementation and the faecal sampling were performed before feeding the pelleted diet and lucerne chaff.

After 14 days, the daily treatment infusion ceased, followed with a further period of basic pelleted diet until near-pretrial faecal microbiology was demonstrated. Faecal samples were taken from each sheep on days 15, 16, 19 & 26 during this period for microbiological and chemical assays.

The sheep remained in the pens with basic husbandry for 1-2 weeks before the start of the next experimental period. The protocol was repeated as described previously for subsequent experimental periods until the fifth i.e. last period was completed, and all animals experienced each of the 5 treatments. The animals were then returned to the farm until required for use in subsequent experiments.

3.2.3.3 Prebiotic supplements

Four commercially available, food-grade oligo- and polysaccharides were examined in the experiments for their prebiotic effects on hind gut microflora. These were Fibruline® LC (COSUCRA SA, Fontenoy, Belgium), Raftilose® P95 (Orafti s. a., Oreye, Belgium), Yacon (N.Z. Biotechnology Ltd., Hamilton, New Zealand) and Arabinogalactan (AG, Larex® AC 9, Larex Inc. St. Paul, Minnesota, USA). In this study, Fibruline® LC, Raftilose® P95 and Yacon were collectively termed FOS (See FOS definition in Section 1.2.3.2 of Chapter 1). AG was used as a polysaccharide control.

Fibruline® LC (hereafter referred to as Fibruline) is a whitish, neutral tasting, non-sweet powder. It is hygroscopic, and water dispersible with agitation. It is

obtained from chicory root inulin by removal of the short chain oligomers and has a minimum DP of 20. This compositional characteristics give a high fibre content (min. 98.5%), mixed with a maximum 1.0% glucose, fructose and sucrose, and 0.5% ash. Structurally, it is a chain of fructofuranosyl residues terminating in a glucopyranosyl residue and is non-reducing (Skinner, 2001).

Raftilose® P95 (hereafter referred to as Raftilose) is a whitish, slightly sweet, free flowing powder. It is water dispersible with agitation. It is obtained from chicory inulin by partial enzymatic hydrolysis, and consists of oligosaccharides of DP 2 to 6. Structurally, it is short chains of fructofuranosyl units terminating in either fructose (furanose or pyranose) or glucopyranose. It mainly consists of inulobiose, inulotriose, inulotetraose, and inulopentaose which are reducing (Skinner, 2001).

Yacon (actually a dried extract of Yacon) is a yellow free flowing powder. It is hygroscopic, and water dispersible with agitation. It is derived from the tuberous root of the yacon plant (*Smallanthus sonchifolius,* Poepp. and Endl.). Structurally, it consists of short chains (with DP 2 to 10) of fructofuranosyl units terminating in glucopyranosyl and is non-reducing (Goto *et al.*, 1995; Skinner, 2001).

Arabinogalactan (AG) is a pale brown free flowing powder. It is a high molecular weight (MW, around 20,000 Daltons) polysaccharide consisting of chains of galactopyranose residues with very large numbers of branches containing galactopyranose and arabinose residues (Skinner, 2001). It is extracted from harvested Western Larch trees (*Larix occidentalis*). Its molecular structure and solution conformation result in several unique features including the highest aqueous solubility and the lowest viscosity of any commercially available, natural polysaccharide; broad range pH and ionic stability, and a narrow molecular weight distribution with low polydispersity.

The above four oligo- and polysaccharides were selected for the animal experiments on grounds of varying DP (chain length), structure of the chain (the type of units present and the way the units are linked together), and in the terminal sugar structure which makes the chain either reducing or non-reducing. All of these features could affect the way in which intestinal microflora respond to the carbohydrates.

3.2.3.4 Dose of supplements

Seven gram of each supplement were administered daily to each sheep in a particular group during each supplementation period.

3.2.3.5 Composition of supplement infusions

Each supplement infusion was made as an acidified solution at pH 2.23 and with an osmolality of about 258.83 mOsmol. The pH value was chosen to match the normal abomasal pH of sheep, which had been determined in the twelve prospective trial sheep to average 2.23. HCl was used in making the infusions because it is the acid secreted by the stomach lining, so would be stable in the sheep abomasum. The stability of the four carbohydrates in pH 2.5 HCl solution at 37°C (mimicking the abomasal environment) had been previously determined by Skinner (2001). The ratio of dilute acid to each carbohydrate, and resulting final volume to be infused into the abomasum, were balanced to ensure osmolality of the infused solutions was similar to that of normal abomasal contents. This had been determined in the twelve, prospective trial sheep to give an average osmolality of 258.83 mOsmol. This would ensure the sheep remained free of osmotic diarrhoea. The carbohydrate treatments had varying amounts of monosaccharides and disaccharides in them; these are more osmotically active than polysaccharides; therefore the final volumes of infused solutions varied slightly between treatments. However, the volume of each infused solution did

not exceed a maximum volume of 150 mL to ensure the infusion did not exceed the volume capacity of the abomasum. The capacity had been previously determined in abattoir slaughtered sheep (fasted) and sheep fed pasture up to slaughter in a separate, ethically approved, terminal experiment by Clyde Daly and Chris Mudford, (AEC 3771, Animal Ethics Committee, AgResearch, New Zealand). This indicated that abomasal volume ranged from about 50 mL (fasted) to about 735 mL (fully fed). The control animals received the mean treatment volume of pH 2.23 acidified saline solutions only.

pH 2.23 HCl solution. Commercial HCl solution (~ 7M, ConvoL® hydrochloric acid, BDH, England) was diluted with UHQ water to the required volume, adjusted to pH 2.23, and stored at 4°C in the glass containers.

pH 2.23 acidified saline control. 7.5972 g of NaCl was dissolved and made up to 1000 mL in a volumetric flask with pH 2.23 HCl solution. This pH 2.23 acidified saline control was stored at 4°C in the glass containers. 70 mL of this control solution was administered to each sheep in the control group daily during the "treatment supplementation" period.

Fibruline infusion. 6.1362 g of NaCl was dissolved and made up to 1000 mL in a volumetric flask with pH 2.23 HCl solution. This was Fibruline pH 2.23 acidified saline, which was stored at 4°C in the glass containers. 7 g of Fibruline was dissolved in 63.00 mL of the Fibruline acidified saline to give the final Fibruline infusion. This Fibruline infusion was administered to each sheep in the requisite group daily during the "treatment supplementation" period.

Raftilose infusion. 7 g of Raftilose was dissolved in 52.17 mL of pH 2.23 HCl solution. This Raftilose infusion was administered to each sheep in the requisite group daily during the "treatment supplementation" period.

Yacon infusion. 28.0 mL of 1M HCl solution was diluted and made up to 1000 mL in a volumetric flask with UHQ water. This was 0.0280M HCl, which was stored at 4°C in the glass containers. 7 g of Yacon was dissolved in 120.27 mL of 0.0280M HCl to give the final Yacon infusion. This Yacon infusion was administered to each sheep in the requisite group daily during the "treatment supplementation" period.

Arabinogalactan infusion. 38.4 mL of 1M HCl solution was diluted and made up to 1000 mL in a volumetric flask with UHQ water. This was 0.0384M HCl, which was stored at 4°C in the glass containers. 7 g of Arabinogalactan was dissolved in 37.59 mL of 0.0384M HCl to give the final Arabinogalactan infusion. This Arabinogalactan infusion was administered to each sheep in the requisite group daily during the "treatment supplementation" period.

All the above infusions were made freshly on each day of abomasal supplementation to ensure minimal pre-infusion acid hydrolysis of the carbohydrates, and also to reduce the opportunity for the growth of bacterial contaminants.

3.2.4 Microbiological assays

A faecal sample from each sheep was collected, put into a stomacher bag, stored near (but not on) ice in a chilly bin, and transferred directly to the microbiology laboratory for detection and enumeration of sheep faecal bifidobacteria, lactobacilli, *E.coli/Enterobacteriaceae*, sulphite reducing clostridia, and total anaerobes.

3.2.4.1 Reference cultures used during microbiological assays

B. pseudolongum subsp. g*lobosum* DSM 20092 was purchased from DSMZ, Braunschweig, Germany. *Lactobacillus viridescens* R 61, *Klebsiella pneumoniae* R 54, *Enterobacter aerogenes* R 62, *and Clostridium perfringens* R 70 were preserved in our laboratory (MIRINZ Protect Culture Collection, 1996). *Escherichia coli* NZRM 916 was purchased from the Communicable Disease Center of ESR, Porirua, New Zealand. *Salmonella* Dublin ATCC 15480 was purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, USA.

These bacteria were used as the positive controls for media quality control during the microbiological assays.

3.2.4.2 Culture media used during microbiological assays

Raffinose-Bifidobacterium agar (RB, Hartemink *et al.*, 1996), de Man, Rogosa, Sharpe agar with sorbic acid (MRSS, Avery and Bell, 1991), Chromocult® Coliform Agar (Merck, Darmstadt, Germany), Egg-yolk-free Tryptose Sulphite Cycloserine agar (TSC agar, Avery and Cook, 1991), and Reinforced Clostridial Medium (RCM, Merck, Darmstadt, Germany) were used in the microbiological assays for detection and enumeration of sheep faecal bifidobacteria, lactobacilli, *E.coli/Enterobacteriaceae*, sulphite reducing clostridia, and total anaerobes, respectively.

All the quality control procedures were carried out for RB, MRSS, and TSC agar using a blank control and *B. pseudolongum* subsp. g*lobosum* DSM 20092, *Lactobacillus viridescens* R 61, *and Clostridium perfringens* R 70 respectively as the positive controls.

Escherichia coli NZRM 916 (dark-blue to violet colonies), *Klebsiella pneumoniae* R 54 (salmon to red colonies), *Enterobacter aerogenes* R 62 (salmon to red colonies), *and Salmonella* Dublin ATCC 15480 (white colonies) were used as positive controls for quality control of Chromocult® Coliform Agar. *B. pseudolongum* subsp. g*lobosum* DSM 20092 and *Clostridium perfringens* R 70 were also used as positive controls for quality control of RCM agar.

3.2.4.3 Preparation of serial ten-fold dilutions of faecal samples

Five gram of each sample was weighed out in a stomacher bag and diluted with 45 mL of Maximum Recovery Diluent (DIFCO, Becton Dickinson, USA) to give a ten-fold dilution. This ten-fold dilution was homogenised using a Colworth stomacher (Seward Stomacher® 400, Seward, England) for two minutes on the normal speed setting. Then 0.5 mL of the homogenate was serially diluted to appropriate ten-fold dilutions in 4.5 mL of Maximum Recovery Diluent.

3.2.4.4 Method for enumeration of sheep faecal bifidobacteria

One hundred microlitre volumes of the appropriate dilutions were spread over the surface of duplicate dried RB agar plates. All the spread plates were then incubated anaerobically (Anaero*Gen*, Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 72 hrs. All the yellow-green colonies with yellow opaque halos and precipitation zones around the colonies appearing on RB agar plates were counted on the duplicate plates of the highest countable dilution (30-300CFU). However, instead of simply being counted, these colonies were differentiated by their colony appearance including form, size, surface, elevation, edge, and consistency; and the proportion of each colony type relative to the total counts was calculated. Then one typical colony from each different colony type was selected at random using a needle and streaked onto non-selective medium (RCM agar plates) to obtain pure cultures. Gram staining and microscopic examination were also performed. All

Gram-positive, curved, bifurcated V, X, Y-shaped or club-shaped rods with typical colony morphology on RCM agar plates (smooth, convex, entire edges, cream to white, glistening and of soft consistency) were considered as presumptive positive bifidobacteria. These presumptive positive bifidobacterial isolates were further identified to genus level using a series of confirmation tests, which were the catalase test, the oxidase test, and the optimised F6PPK test (Chapter 2). Positive bifidobacterial reactions should be catalase test (-), oxidase test (-), and F6PPK test (+) (Scardovi, 1986). Finally, the number of bifidobacteria was expressed as the number of CFU/gram of sample.

3.2.4.5 Method for enumeration of sheep faecal lactobacilli

One hundred microlitre volumes of the appropriate dilutions were spread over the surface of duplicate dried MRSS agar plates. All the spread plates were then incubated in a 10% CO_2 incubator (Heraeus® HERAcell® gas addition incubator, Kendro, D-63450 Hanau, Germany) at 37°C for 72 hrs. All the colonies appearing on the duplicate plates of the highest countable dilution (30-300CFU) were counted and differentiated by their colony appearance. The proportion of each colony type relative to the total counts was then calculated. One typical colony from each different colony type was selected at random using a needle and streaked onto non-selective medium (MRS Agar plates, Merck, Darmstadt, Germany) to obtain pure cultures. Gram staining and microscopic examination were also performed. All Gram-positive, long and slender, sometimes bent, or short, often coryneform coccobacilli, non-sporing rods with typical colony morphology on MRS (2-5 mm, convex, entire, opaque, and without pigment) were considered as presumptive positive lactobacilli. These presumptive positive lactobacilli isolates were further identified to genus level using a series of confirmation tests, which were catalase test, oxidase test, oxidation-fermentation (OF) test, motility test, and growth in MRS Broth (Merck, Darmstadt, Germany) at pH 5.0. Positive lactobacilli reactions should be catalase test (-), oxidase test

(-), OF test (F), non-motile, and good growth in MRS broth at pH 5.0 (Avery and Bell, 1991; Hartemink, 1999). Finally, the number of lactobacilli was expressed as the number of CFU/gram of sample.

3.2.4.6 Method for enumeration of sheep faecal *E. coli/Enterobacteriaceae*

One hundred microlitre volumes of the appropriate dilutions were spread over the surface of duplicate dried Chromocult® Coliform Agar plates. All the spread plates were then incubated aerobically at 37°C for 24 hours. All the dark-blue to violet colonies were counted as *E.coli*; all the salmon to red colonies plus dark-blue to violet colonies were counted as total coliforms; and all the total coliform colonies plus colourless colonies and light-blue to turquoise colonies (other *Enterobacteriaceae*) were counted as *Enterobacteriaceae*. This method does not require confirmatory tests to be performed. Finally, the numbers of presumptive *E. coli* and *Enterobacteriaceae* were expressed as the number of CFU/gram of sample.

3.2.4.7 Method for enumeration of sheep faecal sulphite reducing clostridia

TSC overlay agar was melted, cooled and held at 45 to 50°C. One hundred microlitre volumes of the appropriate dilutions were spread over the surface of duplicate dried TSC agar plates. Inoculums were allowed to soak into the agar for 15 minutes. Then each of the spread plates was overlaid with 10 mL of molten TSC agar tempered to between 45 and 50°C, and then allowed to set. All the inoculated plates were inverted and incubated anaerobically (Anaero*Gen*, Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for no more than 20 hours. All the black colonies were counted as presumptive sulphite reducing clostridia. This method does not require confirmatory tests to be performed. Finally, the numbers of presumptive sulphite reducing clostridia were expressed as the number of CFU/gram of sample.

3.2.4.8 Method for enumeration of sheep faecal total anaerobes

One hundred microlitre volumes of the appropriate dilutions were spread over the surface of duplicate dried RCM agar plates. All the spread plates were incubated anaerobically (Anaero*Gen*, Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 72 hours. All the colonies present were counted as total anaerobes. Finally, the number of total anaerobes was expressed as the number of CFU/gram of sample.

3.2.5 Chemical assays

3.2.5.1 pH determination of sheep faecal samples

The methodology for determination of sheep faecal pH in this study was modified from AOAC Official Methods of Analysis (1990) for measuring the pH of peat. The procedures were as follows: 1.0-1.4 g of each faecal sample was weighed and put into a plastic container with airtight lid. 16.67 g of UHQ water per gram of faeces was added to each container. The container with the mixture inside was then held in ice for 1.5 hours. After 1.5 hours, the faecal lumps were crushed with a spatula to give a homogeneous faecal suspension. The pH of this faecal homogenate was then read using a pH meter (Metrohm 686 Titroprocessor, Series 04, Herisau, Switzerland). Determinations were carried out in duplicate.

3.2.5.2 Determination of sheep faecal dry matter

Approximately 2 g of faecal pellets from each sheep were put into a foil cap, weighed accurately, uncovered, and put into a 90°C of oven until constant weight (about 30 hours) was attained. The oven-dried faecal pellets were then removed from oven, covered tightly, cooled, and reweighed. Determinations were carried out in duplicate. The results was expressed as

$$\% \text{ Dry matter} = \frac{\text{dry weight of oven-dried sheep pellets (g)}}{\text{wet weight of sheep pellets (g)}} \times 100\%$$

3.2.6 Data analysis

The trial was designed as a balanced Latin square plus two extra animals, but, because one animal died, the balance was lost. This meant that Residual Maximum Likelihood (REML) Variance Components Analysis was required to combine the between and within animal information in the most efficient manner. The model fitted the five treatments as a fixed effect with *sheep* and *period* as random effects. Results were expressed as means and average standard errors of differences between the treatment means for each time and changes between times. Wald test statistics were used to test the significance of the differences in changes between the treatments at each time interval. In case of a significant difference ($P < 0.05$) between treatments, group means were compared in pairs with Student's t-test. The statistical analysis package GenStat, version 6.1.0.20 (Lawes Agricultural Trust, supplied by VSN International Ltd., Oxford, UK) was used to perform the statistical analysis with the exception of Student's t-test which was carried out by Microsoft® Excel 2002 (Microsoft Corporation).

3.3 Results

3.3.1 Pre-trial animal selection and acclimatisation

Twelve sheep were selected for abomasal fistulation by a veterinarian on the basis of the clinical, haematological and biochemical health conditions determined, pregnancy scanning results, and acclimatisation to the holding facilities and faecal

sample production. These sheep were numbered as 115, 434, 459, 9603, 9641, 9660, 9661, 9706, 9715, 9719, 9752, and 9861 (**Table 3.2**).

Table 3.2 Surgery selection on basis of faecal sampling training record and animal health. Selected animals are indicated in bold.

Order	Sheep No.	Days of faeces sampling	Total Days faecal sample collected	Pregnancy scanning	Physiology and health conditions
1	9671	9	3		Sensitive
2	**9706**	**9**	**4**		
3	**9752**	**9**	**4**		
4	**115**	**9**	**7**		
5	137	9	6		Limping slightly an front left leg
6	9832	6	3		Poor Blood Results
7	9824	6	5	Pregnant	
8	**9715**	**9**	**6**		
9	**9603**	**9**	**6**		
10	**459**	**9**	**8**		
11	9865	6	1		Poor Blood Results
12	**434**	**9**	**9**		
13	**9661**	**3**	**3**		
14	9649	3	3		Reluctant to give a sample
15	**9861**	**3**	**3**		
16	9644	3	3		Hit her nose against bars - bleeding
17	9610	3	3		Reluctant to give a sample
18	**9660**	**3**	**3**		
19	**9719**	**3**	**3**		
20	**9641**	**3**	**3**		
21	9776	3	?		
22	9616				Failed to acclimatise to crates

The other ten sheep were excluded from the trial because of one or more of the following reasons: reluctance of giving faecal samples (sheep 9671, 9832, 9865, 9649, 9610, and 9776); poor blood results (sheep 9832 and 9865); failure to

acclimatise to crates (sheep 9671, 9644, and 9616); poor health conditions (sheep 137); and being pregnant (sheep 9824) (**Table 3.2**).

3.3.2 *Effect of abomasal prebiotic supplementation on sheep faecal bifidobacteria*

The effect of 2-week abomasal prebiotic supplementation on sheep faecal bifidobacteria was monitored (**Figure 3.2**).

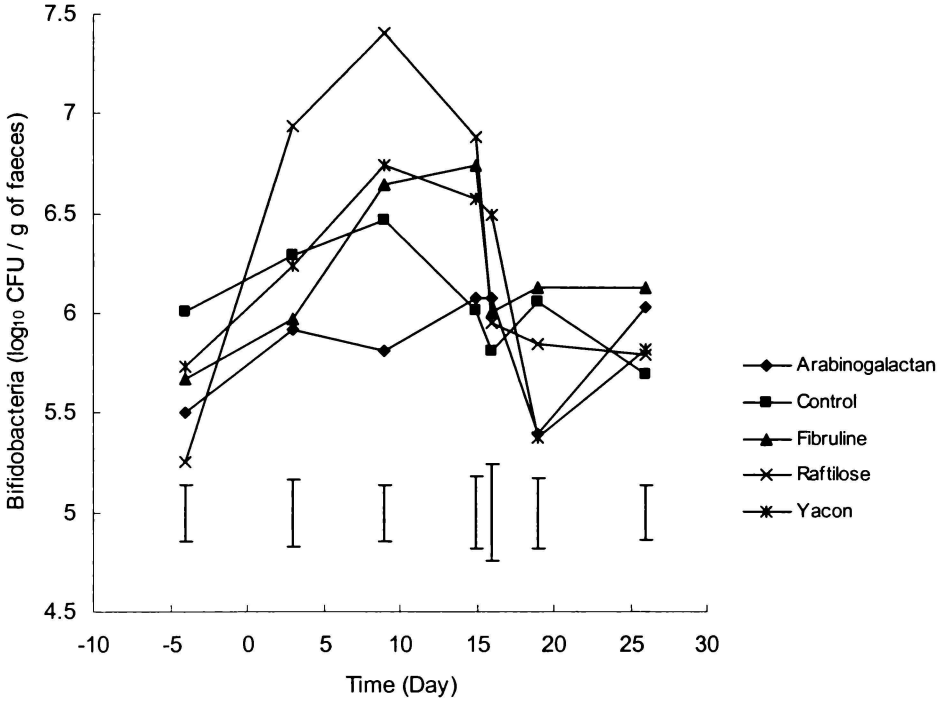


Figure 3.2 Effect of abomasal prebiotic supplementation on sheep faecal bifidobacteria. Data represent means and average standard errors of differences.

Faecal samples were collected for detection and enumeration of bifidobacteria on day -4 during the 1-week "stabilisation" period to the pelleted diet (no treatment), days 3 and 9 during the 2-week "treatment supplementation" period, and days 15,

16, 19 & 26 after the daily supplementation ceased. Results are expressed as means and average standard errors of differences shown as error bars under the curve of logarithm of the number of sheep faecal bifidobacteria *v.* time. Yacon, Fibruline and Raftilose all significantly increased the number of bifidobacteria after 9 days of daily dosing in comparison with the acidified saline control. Among them, Yacon significantly increased the number of bifidobacteria by 0.990 $\log_{10}$ CFU/g of faeces ($P < 0.05$), Fibruline by 0.925 $\log_{10}$ CFU/g of faeces ($P = 0.05$), and Rafilose by 2.128 $\log_{10}$ CFU/g of faeces ($P < 0.01$). Raftilose not only produced the highest increases in, but also the highest counts (2.57×10^7 CFU/g) of bifidobacteria on Day 9 in comparison with all the other treatments ($P < 0.01$). With Fibruline, the number of bifidobacteria continued to increase and reached the maximum counts on day 15, i.e. one day after supplementation ceased, which might be due to the longer chain structure of Fibruline, which made it a more difficult fermentation substrate for bifidobacteria. No significant differences were observed between Yacon and Fibruline. No significant changes were observed in AG compared with the acidified saline control. Finally, bifidobacteria began to decrease after day 9 in Yacon and Raftilose, and began to decrease after day 15 in Fibruline. Significant differences between the treatments in changes in sheep faecal bifidobacteria are shown in **Table 3.3**.

3.3.3 Effect of abomasal prebiotic supplementation on sheep faecal lactobacilli

The effect of 2-week abomasal prebiotic supplementation on sheep faecal lactobacilli was monitored on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.3**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of logarithm of the number of sheep faecal lactobacilli *v.* time.

Table 3.3 Significant differences between treatments on basis of changes between times in bacterial populations

Bacteria	Time intervals	Control *v.* AG	Control *v.* Fibruline	Control *v.* Raftilose	Control *v.* Yacon	AG *v.* Fibruline	AG *v.* Raftilose	AG *v.* Yacon	Fibruline *v.* Raftilose	Fibruline *v.* Yacon	Raftilose *v.* Yacon
Bifidobacteria	Day -4 ~ Day 3			0.000					0.000		0.002
	Day -4 ~ Day 9		0.050	0.000	0.031				0.000		0.001
	Day -4 ~ Day 15		0.040	0.001							
Lactobacilli	Day -4 ~ Day 9			0.015	0.041						
Total anaerobes	Day -4 ~ Day 3			0.001	0.001		0.043	0.040	0.027	0.025	
	Day -4 ~ Day 9	0.049*	0.034	0.001	0.001						
	Day -4~ Day 15		0.006**	0.000	0.001		0.043				
	Day 15 ~ Day 16			0.001	0.004						
SRC	Day 16 ~ Day 19		0.010								
Enterobacteriaceae	Day 9 ~ Day 15		0.047								

*$P < 0.05$
**$P < 0.01$
P values were calculated from Student's t-test between treatments for comparing the prebiotic effects on the changes between times in bacterial populations.

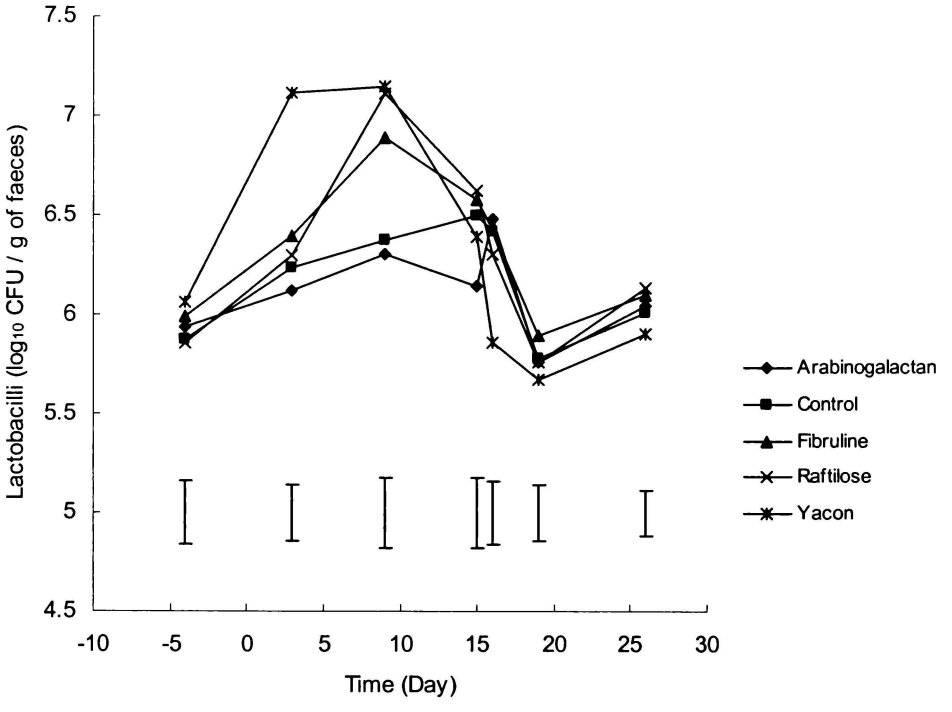


Figure 3.3 Effect of abomasal prebiotic supplementation on sheep faecal lactobacilli. Data represent means and average standard errors of differences.

As shown in **Figure 3.3**, both Yacon and Raftilose significantly increased the number of lactobacilli after 9 days of daily dosing in comparison with the acidified saline control. Yacon significantly increased the number of lactobacilli by 1.138 $\log_{10}$ CFU/g of faeces ($P < 0.05$), reaching the highest counts of lactobacilli at 1.44×10^7 CFU/g of faeces, while Raftilose significantly increased the number of lactobacilli by 1.286 $\log_{10}$ CFU/g of faeces ($P < 0.05$), with counts reaching 1.33×10^7 CFU/g of faeces. No significant differences were observed between Yacon and Raftilose. Fibruline also increased the number of lactobacilli by 0.774 $\log_{10}$ CFU/g of faeces after 9 days of daily dosing, but did not, however, reach a significant level in comparison with the acidified saline control. No significant changes were observed in AG compared with the acidified saline control. Finally, lactobacilli began to rapidly decrease from day

9 to day 19 and then increased back to initial counts in Yacon, Raftilose, and Fibruline. Significant differences between the treatments in changes in sheep faecal lactobacilli are shown in **Table 3.3**.

3.3.4 Effect of abomasal prebiotic supplementation on sheep faecal total anaerobes

The effect of 2-week abomasal prebiotic supplementation on sheep faecal total anaerobes was monitored on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.4**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of logarithm of the number of sheep faecal total anaerobes *v.* time. The four carbohydrate treatments all significantly increased the number of total anaerobes in comparison with the acidified saline control. Yacon, Raftilose, and AG significantly increased total anaerobes after 9 days of daily dosing by 1.257 $\log_{10}$ CFU/g of faeces ($P < 0.01$), 1.249 $\log_{10}$ CFU/g of faeces ($P < 0.01$), and 0.830 $\log_{10}$ CFU/g of faeces ($P < 0.05$); reaching maximum numbers of total anaerobes of 2.47×10^8 CFU/g of faeces, 2.24×10^8 CFU/g of faeces, and 6.68×10^7 CFU/g of faeces, respectively. Fibruline significantly increased total anaerobes on day 15, i.e. one day after daily dosing ceased, by 0.935 $\log_{10}$ CFU/g of faeces ($P < 0.01$), reaching a maximum of 9.16×10^7 CFU/g of faeces. No significant differences were observed between Yacon and Raftilose, or between Fibruline and AG. However, Yacon and Raftilose both increased the total anaerobes significantly more than Fibruline and AG ($P < 0.05$). Total anaerobes began to decrease significantly from day 15 to day 16 in Yacon and Raftilose ($P < 0.01$), to rapidly decrease from day 15 to day 19 in Fibruline, and to rapidly decrease from day 9 to day 19 in AG. Significant differences between the treatments in changes in sheep faecal total anaerobes are shown in **Table 3.3**.

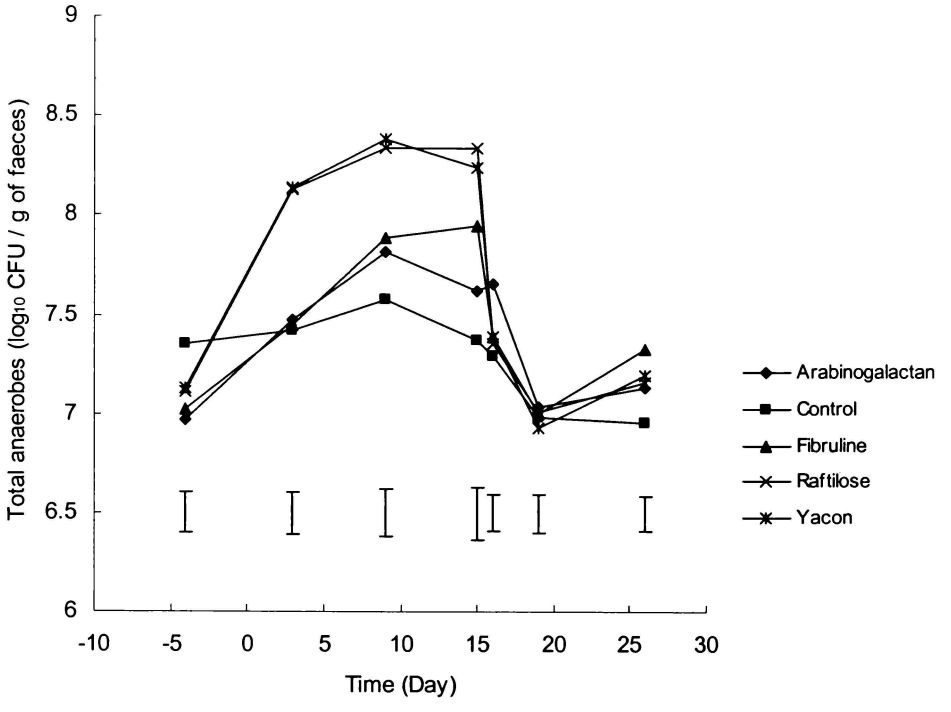


Figure 3.4 Effect of abomasal prebiotic supplementation on sheep faecal total anaerobes. Data represent means and average standard errors of differences.

3.3.5 Effect of abomasal prebiotic supplementation on sheep faecal sulphite reducing clostridia

The effect of 2-week abomasal prebiotic supplementation on sheep faecal sulphite reducing clostridia (SRC) was monitered on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.5**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of logarithm of the number of sheep faecal SRC *v.* time.

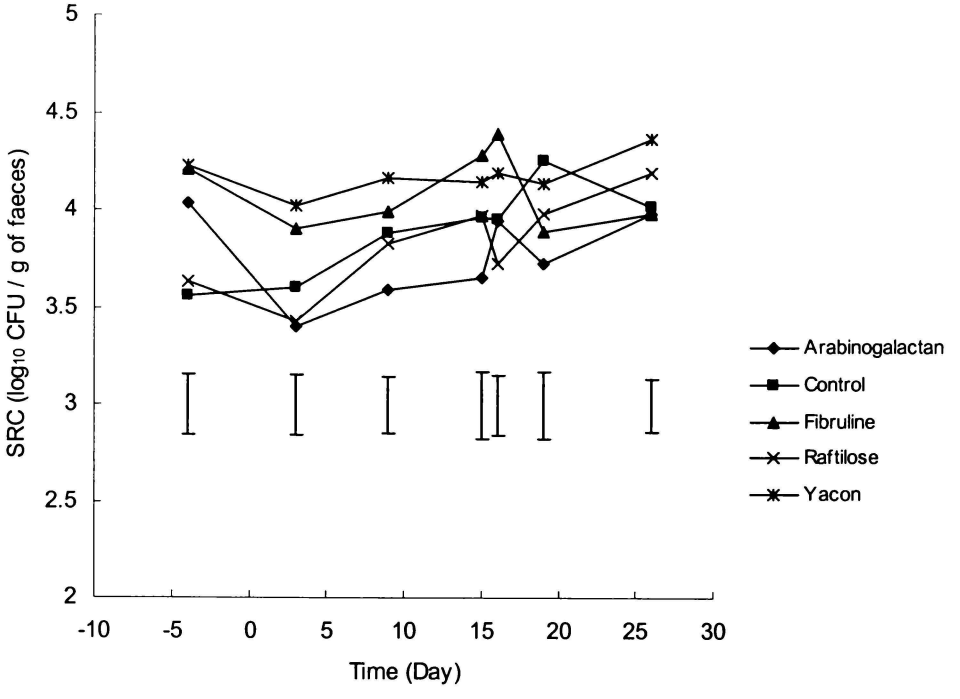


Figure 3.5 Effect of abomasal prebiotic supplementation on sheep faecal sulphite reducing clostridia. Data represent means and average standard errors of differences.

As shown in **Figure 3.5**, there were no significant differences in the changes of the number of SRC during the "treatment supplementation" period between the five treatments. In summary, Yacon slightly decreased SRC during day -4 to day 19. With AG, SRC showed a tendency over time to decrease to lower numbers than the initial counts. With Fibruline, SRC decreased from day -4 to day 9, then increased from day 9 to day 16, and then decreased significantly ($P < 0.05$) in comparison with the acidified saline control from day 16 to day 19, to a lower number than the initial counts. With Raftilose, SRC decreased from day -4 to day 3, but then showed a tendency to increase. SRC showed a tendency to increase over time in the acidified saline control. Significant differences between the treatments in changes in sheep faecal SRC are shown in **Table 3.3**.

3.3.6 *Effect of abomasal prebiotic supplementation on sheep faecal E. coli/Enterobacteriaceae*

The effects of 2-week abomasal prebiotic supplementation on sheep faecal *E. coli/Enterobacteriaceae* were monitored on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.6** and **Figure 3.7**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of logarithm of the number of sheep faecal *E. coli/Enterobacteriaceae v.* time.

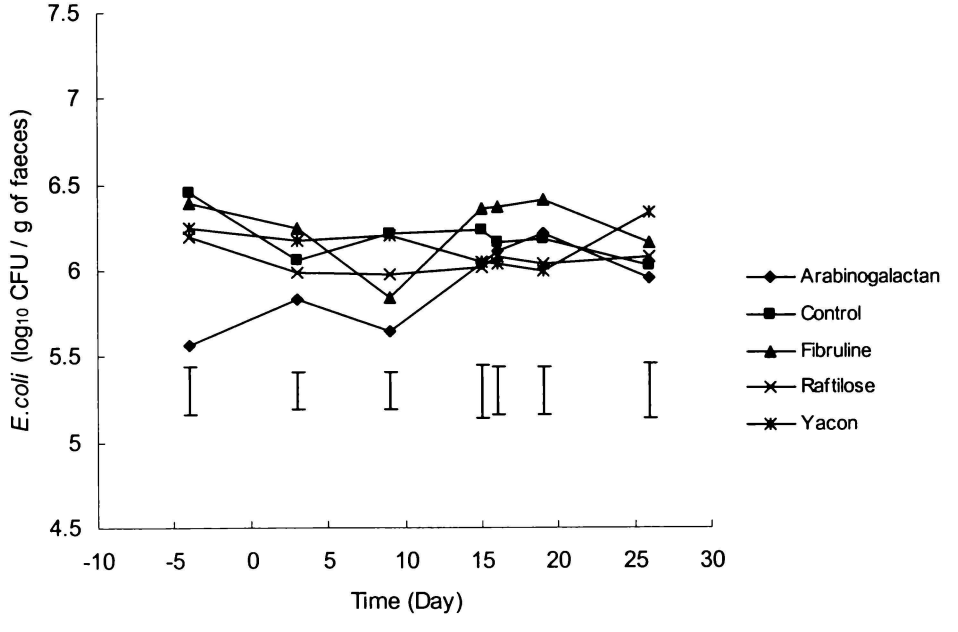


Figure 3.6 Effect of abomasal prebiotic supplementation on sheep faecal *E. coli*. Data represent means and average standard errors of differences.

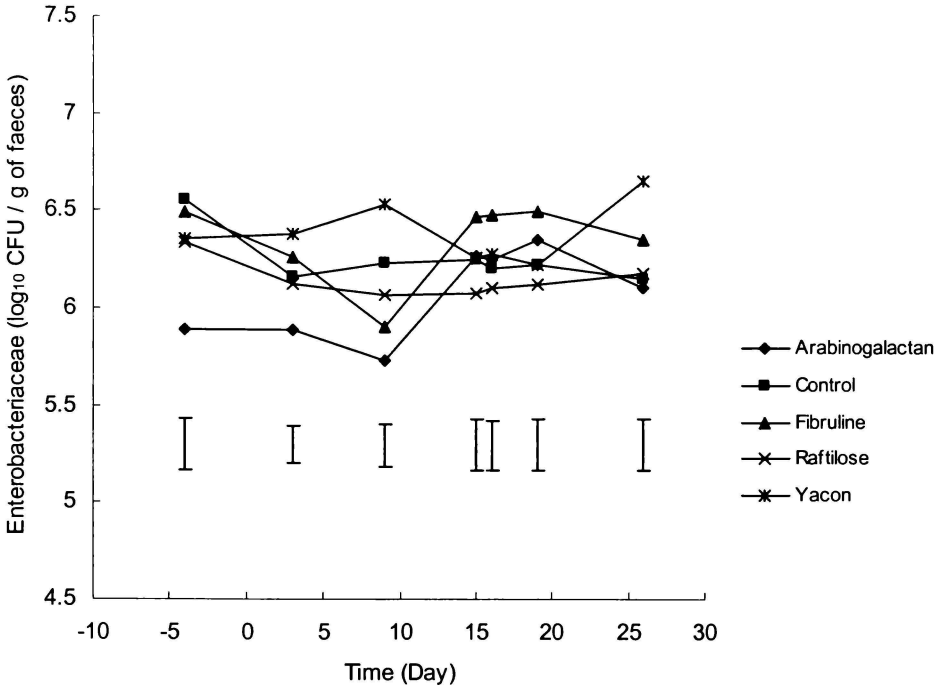


Figure 3.7 Effect of abomasal prebiotic supplementation on sheep faecal *Enterobacteriaceae*. Data represent means and average standard errors of differences.

As shown in **Figure 3.6** and **Figure 3.7**, there were no significant differences in the changes of the number of *E. coli*/*Enterobacteriaceae* during the "treatment supplementation" period between the five treatments. In summary, Yacon slightly decreased *E. coli* from day -4 to day 19. Both *E. coli* and *Enterobacteriaceae* showed a tendency to decrease in Raftilose over time to a lower number than the initial counts. With Fibruline, *E. coli* and *Enterobacteriaceae* decreased rapidly from day -4 to day 9 by 0.542 and 0.572 $\log_{10}$ CFU/g of faeces, respectively, but did not reach a significant level in comparison to that of the acidified saline control. Then, between day 9 and day 15, *E. coli* increased rapidly and *Enterobacteriaceae* increased rapidly and significantly ($P < 0.05$) back to near their initial counts. With AG, both *E. coli* and *Enterobacteriaceae* showed a tendency to increase over time. With the

acidified saline control, *E. coli* and *Enterobacteriaceae* showed a tendency to decrease to lower numbers than the initial counts over the monitoring period. Significant differences between the treatments in changes in sheep faecal *Enterobacteriaceae* are shown in **Table 3.3**.

3.3.7 *Effect of abomasal prebiotic supplementation on sheep faecal pH*

The effects of 2-week abomasal prebiotic supplementation on sheep faecal pH were monitored on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.8**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of pH *v.* time.

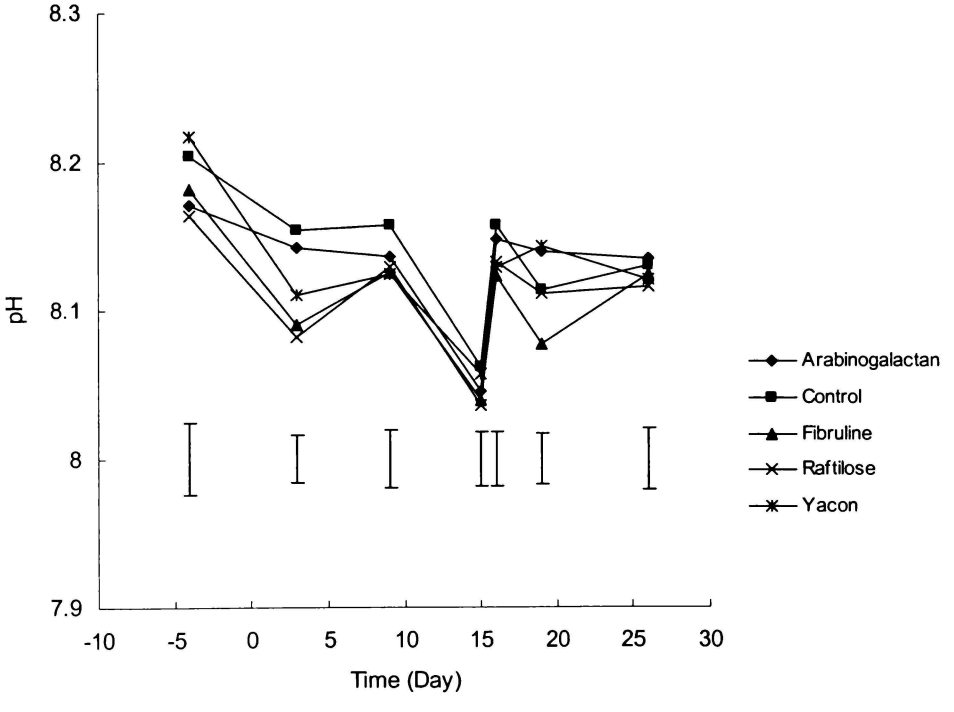


Figure 3.8 Effect of abomasal prebiotic supplementation on sheep faecal pH. Data represent means and average standard errors of differences.

As shown in **Figure 3.8**, there were no significant differences in the changes of sheep faecal pH between the five treatments. Data showed a tendency to decrease from Day -4 to Day 15 and then increased rapidly again from Day 15 to Day 16 in all the five treatments.

3.3.8 Effect of abomasal prebiotic supplementation on sheep faecal dry matter

The effects of 2-week abomasal prebiotic supplementation on sheep faecal dry matter (DM) were monitored on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions (**Figure 3.9**). Results are expressed as means and average standard errors of differences shown as error bars under the curve of DM *v.* time.

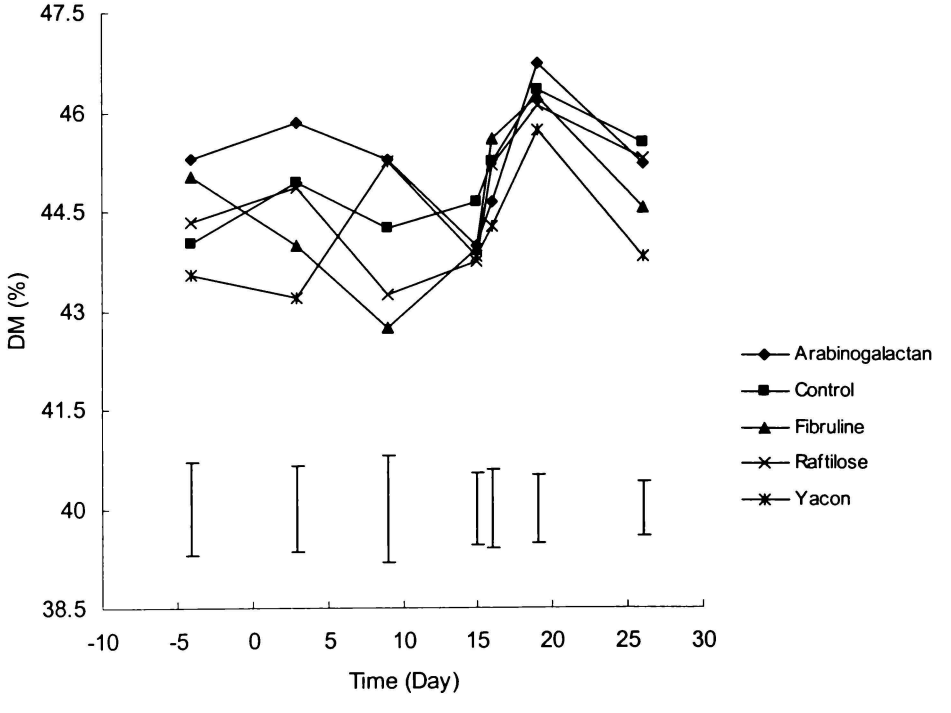


Figure 3.9 Effect of abomasal prebiotic supplementation on sheep faecal dry matter. Data represent means and average standard errors of differences.

As shown in **Figure 3.9**, there were no significant differences in changes in sheep faecal DM between the five treatments.

3.4 Discussion

Four commercial food-grade oligo- and polysaccharides were investigated for their bifidogenic and the associated antimicrobial effects on sheep hindgut microflora. Twelve fistulated sheep were managed in a balanced, two Latin square, cross-over design experiment, which was run in 5 consecutive periods, with each of 5 treatments (Arabinogalactan, Fibruline, Raftilose, Yacon, and "acidified saline" as a control) administered to two sheep in each period. Each period consisted of a 1 week of stabilisation to the pelleted diet (no treatments), followed by 14 days of daily abomasal treatment supplementation, and followed by about 12-day rest period on normal pelleted diet. In each period, sheep faecal bifidobacteria, lactobacilli, *E. coli/Enterobacteriaceae*, SRC, and total anaerobes were enumerated on day -4 of the "stabilisation" period; days 3 and 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions.

The effect of abomasal prebiotic supplementation on sheep faecal bifidobacteria was shown in **Figure 3.2**. Raftilose, Yacon, and Fibruline all significantly increased the number of bifidobacteria after 9 days of daily dosing. Among them, Raftilose produced the highest increase in sheep faecal bifidobacteria of 2.128 log CFU/g, reaching the highest observed counts of bifidobacteria of approximately 10^7 CFU/g. As mentioned in Section 3.2.3.3, Raftilose, Yacon, and Fibruline all belong to FOS group. They were selected for use in this study based on their varying degrees of polymerisation (chain length), structure of the chain (the type of units present and the way the units are linked together), and in the terminal sugar structure which makes the chain either reducing or

non-reducing. In this study, these FOS all showed significant bifidogenic effects on sheep faecal bifidobacteria after 9 days of daily dosing, which made them promising bifidogenic factors for use in ruminants. The time required to exert the bifidogenic effect appears to be 9 days. Similar studies on the bifidogenic effect of FOS were also carried out by Kleessen and Blaut (2000), who determined the number of bifidobacteria in oligofructose-treated rats and observed the bifidogenic effect on a comparable level to the present study. Gibson *et al.* (1995) found that oligofructose (DP 4) significantly increased bifidobacteria populations in healthy volunteers in comparison to the sucrose control group. Bielecka *et al.* (2002) found incorporation of oligofructose into diets resulted in significant increases of the faecal bifidobacteria of 1.6 log cfu/g in comparison with the control group.

However, even though the "bifidogenic effect" exerted by prebiotics has been widely studied and has been proved both *in vitro* and *in vivo*, the mechanisms by which the population of bifidobacteria becomes elevated in the large intestinal still remain unclear. The physiological relevance of the "bifidogenic effect" is also difficult to evaluate. The establishment, maintenance, restoration, and enhancement of intestinal microflora by prebiotics in humans and animals have yet to be investigated by employing a range of *in vitro* models. Overall, the science of prebiotics is still in its infancy. More *in vivo* clinical evidence is required to demonstrate that the use of prebiotics has the potential to positively influence human health and is efficacious in the prevention and treatment of human diseases. The selection of more slowly fermentable carbohydrates as prebiotics should also be considered as these candidate compounds are likely to reach the distal colon. This approach obviates the rapid and complete utilisation of readily fermentable substrates in the proximal colon leading to primarily protein metabolism in the distal gut which may produce toxins and increase the cancer risk.

Subsequently, bifidobacteria began to decrease after the abomasal supplementation with these FOS ceased, which was in agreement with the studies by Rao (2001), who observed that, after consumption of oligofructose for 11 days ceased, elevated bifidobacteria numbers fell rapidly to almost pre-trial levels. This suggests that long-term administration of FOS is required to sustain the bifidogenic effect. Considering that FOS are composed of natural monosaccharide units that are hydrolysed and fermented by the endogenous bacteria of the colonic microflora to produce SCFA, prolonged use is not of toxicological concern, in other words, FOS are "generally recognised as safe" (GRAS) (Roberfroid and Slavin, 2000). Traditional toxicity tests have also showed the absence of any deleterious effects with FOS usage (Clevenger *et al.*, 1988). Therefore, no side-effects will result from long-term FOS administration. Development and application of a delivery system will need to be taken into consideration in the near future for passing the FOS intact through the biochemically aggressive rumen environment.

No significant changes of the populations of bifidobacteria were observed in AG-treated sheep, which demonstrated that AG cannot be used as a bifidogenic factor in sheep.

The effect of abomasal prebiotic supplementation on sheep faecal lactobacilli was shown in **Figure 3.3**. Raftilose and Yacon significantly increased the number of lactobacilli, by 1.286 and 1.138 log units, respectively, reaching approximately 10^7 CFU/g of faeces, after 9 days of daily dosing; Fibruline increased lactobacilli after 9 days of administration, but did not differ significantly from the control; AG did not elevate the populations of lactobacilli. Lactobacilli began to decrease after abomasal supplementation with these treatments ceased. Lactobacilli are also widely used probiotic bacteria which exert various health effects on human and animals (See Section 1.2.1.2, Chapter 1). Therefore, these bacteria were also used as indicator bacteria in the present study for determination of the prebiotic

effect of the test oligo- and polysaccharides. However, compared with bifidobacteria, lower populations of lactobacilli are found inhabiting the human GIT (Tannock, 1997). In addition, bifidobacteria are found principally in the large intestine while lactobacilli inhabit mainly the upper gut (Tomomatsu, 1994).

All four carbohydrate treatments increased the number of total anaerobes to approximately 10^7 to 10^8 CFU/g of faeces after 9 days of daily dosing (**Figure 3.4**). Total anaerobes began to decrease after the abomasal supplementation with these carbohydrates ceased. The promotion of total anaerobes by the test carbohydrates may be due to the increase in the number of bifidobacteria and lactobacilli, both of which are relatively predominant genera in the gut of humans and animals (See Section 1.1.2 & 1.2.1.2 of Chapter 1).

Supplementation with the test oligo- and polysaccharides had no significant effect on the other groups of gut microflora: sulphite reducing clostridia and *E. coli/Enterobacteriaceae* (**Figure 4.5** - **Figure 4.7**). Clostridia and *E. coli/Enterobacteriaceae* were used as indicator bacteria as they are normal gut inhabitants of mammals but are also potential pathogens. Their potential pathogenicity cannot be neglected even if the host is healthy. Therefore, the suppression and elimination of these bacteria are required to improve the food safety status of meat. However, in the present study, the populations of sulphite reducing clostridia and *E. coli/Enterobacteriaceae* remained at the pre-trial level. Other studies report different results, both positive and negative effects. For example, Bielecka *et al.* (2002) studied the influence of oligofructose on gut microecology of Wistar rats and found that a 14-day supplementation of 5% (w/w) oligofructose in the diet had almost no effect on coliforms, spores of aerobic bacteria, spores of anaerobic bacteria, and total mesophilic bacteria. This result was comparable with that of our study. Rao (2001), in his low-dose oligofructose study, also observed that consumption of oligofructose, 5 g/d, for 11 days resulted in approximately one log cycle increase in bifidobacteria, whereas

the coliforms and the group of total aerobes remained at the same levels during treatment with oligofructose. Contrary to these results, the studies by Gibson *et al.* (1995) demonstrated that *Bacteroides*, clostridia and fusobacteria decreased along with the increase in the populations of bifidobacteria in groups fed 15 g/d oligofructose. Apajalahti *et al.* (2002) also noticed that stimulation of bifidobacteria and suppression of clostridia occurred in the cecum of mice fed 10% (w/w) inulin.

It is necessary to consider in the present study what the required dosage is to exert a significant bifidogenic effect and the associated antimicrobial effect. Gibson (2001), in his online article "Prebiotics for Gut Health", states that at least 4 g/d, but more preferably 8 g/d of FOS, would be needed to significantly elevate bifidobacteria in the human gut. He also showed data from a volunteer trial carried out at the University of Reading, where shortbread containing 7 g/d FOS was fed to human subjects exerted a profound effect on bifidobacteria as compared to a no FOS added placebo. As to the associated antimicrobial effect, Rao (2001) suggested that the reduction in the potential pathogenic bacteria was associated with the increase of bifidobacteria resulting from the use of a higher dose (15 g/d or 10% (w/w)) of oligofructose which was fermented by certain groups of bacteria (such as bifidobacteria) to produce a higher concentrations of SCFA and lactic acid, these being important factors inhibiting the growth of some potential pathogens and putrefactive bacteria. Unfortunately, high doses of FOS may cause intestinal discomfort or even diarrhoea due to their osmotic effect, high fermentation rate and production of gases (Roberfroid and Slavin, 2000). Therefore, an acceptable dose of FOS is not only related to the bifidogenic effect and the associated antimicrobial effect achieved, but also takes the osmotic effect into account in case any unexpected intestinal discomfort occurs. Seven grams of each test oligo- and polysaccharide was administered in the present study as the selected dose. Three of these significantly increased the populations of bifidobacteria in the sheep hindgut. Each treatment infusion was adjusted to

match the normal abomasal pH (2.23) and osmolality (258.83 mOsmol) of sheep to ensure the sheep were not affected with osmotic diarrhoea. The effect of abomasal prebiotic supplementation on sheep faecal DM was also determined (**Figure 3.9**), which showed no significant differences in sheep faecal DM between the five treatments including the no carbohydrate control. Consequently, 7 g/d FOS can be regarded as an acceptable dose for sheep with respect to the desirable bifidogenic effect and the undesirable osmotic effect. However, no significant changes in sulphite reducing clostridia and *E. coli*/*Enterobacteriaceae* were observed. Further *in vivo* studies with higher doses of the bifidogenic FOS will be carried out to determine the relationship between the dosages of FOS supplements and the antimicrobial effect on sheep faecal pathogens.

There were no significant changes in sheep faecal pH were observed in the four carbohydrate treatments in comparison with the acidified saline control (**Figure 3.8**). Similar results were obtained by Alles *et al.* (1996), who evaluated the fate of two different doses of FOS (5 and 15 g/d) in the intestine of healthy men. No changes in the total concentration of SCFA in faeces or faecal pH were observed after 7 days of supplementation with the FOSs. These authors suggested that SCFA are mainly produced in the proximal colon where the amount of fermentable substrates is highest. Rapid absorption by the colonic mucosa leads to a lower concentration in the faeces, therefore, no changes in faecal SCFA or pH can be expected to be found. Incorporation of longer chain FOS may increase fermentation in the distal colon, which could be considered in the future studies.

In summary the test FOS, Raftilose, Yacon and Fibruline, all showed great bifidogenic effectiveness and also selectively stimulated lactobacilli in the hindgut microflora of sheep, and thus offer promise as prebiotics for manipulation of ruminant intestinal bacteria. Among them, Yacon is New Zealand made and will be of value to New Zealand animal nutrition and the meat industry with the demonstrated prebiotic effects on manipulation of ruminant hindgut microflora.

Further *in vitro* antagonistic experiments must be carried out to determine whether or not the isolated sheep faecal bifidobacteria inhibit the growth of potential intestinal pathogens in fermentation broth containing these selected FOS.

Chapter 4

IN VITRO ANTAGONISTIC EFFECTS OF SHEEP FAECAL BIFIDOBACTERIA AGAINST POTENTIAL INTESTINAL PATHOGENS IN MEDIA CONTAINING FRUCTO-OLIGOSACCHARIDES

4.1 Introduction

As reviewed in Chapter 1, pro-, pre-, and synbiotics can be used to manipulate the composition of the gut microflora towards a potentially more advantageous community in which the health-promoting bacterial groups such as LAB, in particular, where bifidobacteria are predominant. A variety of beneficial health effects are in turn contributed to the host due to the increased populations of exogenous and/or indigenous bifidobacteria in the large intestine such as suppression of the potential pathogens and putrefactive bacteria, maintenance and improvement of normal intestinal conditions, prevention and/or treatment of enteric infections, alleviation of constipation in the elderly, suppression of some cancers, stimulation of the immune system, enhancement of lactose digestion, reduction in serum cholesterol levels, and production of certain nutritional substances. Since effective pro-, pre-, or synbiotics are capable of exerting such beneficial effects on humans and other animal species, the search for new probiotic strains and effective prebiotic substrates is accelerating within the food industry. Consequently, well-designed *in vitro* assays and human or animal studies are required to demonstrate the probiotic activity of a certain strain and/or

prebiotic activity of a certain substrate for the screening of bacterial strains and/or substrates for use as probiotics and/or prebiotics. In general, *in vivo* studies for this end are difficult due to the highly complex ecosystem within the large intestine, whereas *in vitro* investigations provide a relatively easy way to obtain, under well-controlled conditions, the first line of evidence for some antimicrobial activities of a probiotic and/or a prebiotic and point to mechanisms that may occur *in vivo*. Therefore, *in vitro* experiments should be conducted with subsequent proof of efficacy in hosts sought from *in vivo* studies only if convincing demonstrations of activity are first made *in vitro*.

To date fructooligosaccharides (FOS) have been by far the most thoroughly investigated and developed prebiotic products because of their nondigestibility in the upper GIT, selective fermentability in the large intestine, and demonstrated beneficial health effects exerted on the host. In particular, FOS are good *in vitro* carbon sources utilised by some intestinal bacteria; *in vivo* they escape the enzymic hydrolysis in the upper GIT and reach the large intestine without change to their structure, thereby, becoming potential substrates for fermentation by elements of the microflora, especially the bifidobacteria in the lower gut (See Section 1.2.3.2, Chapter 1). By stimulating bifidobacteria, FOS may produce a variety of health benefits in humans and animals, which are associated with the increased population of indigenous bifidobacteria in the large intestine. One important health benefit is the antibacterial effect of bifidobacteria, potentiated by FOS, on potential intestinal pathogens and putrefactive bacteria. This may be attributed to the production of acetic and lactic acids, other antimicrobial metabolites, competition for nutrients, competition for binding sites on the epithelial surfaces, or a combination of these factors. The capacity of bifidobacteria to inhibit the growth of potential intestinal pathogens and putrefactive bacteria and the relevant mechanisms differ and depend on the properties and activities of the bacterial strains involved, their population size achieved by fermentation of the substrates, and environmental conditions (Fuller,

1989; Raibaud, 1992; Vandenbergh, 1993). In this study, a number of bifidobacterial strains isolated from sheep faeces were assessed for their capacity to inhibit the growth of potential intestinal pathogens when grown in media containing several different fructooligosaccharides. An *in vitro* approach was undertaken at this stage to select strains with effective inhibitory activity against potential intestinal pathogens and to identify those substrates with bifidogenic effects. The mechanisms involved in the inhibitory effect of selected bifidobacterial strains were also investigated.

4.2 Materials and Methods

4.2.1 Pure culture studies

4.2.1.1 *In vitro* utilisation of oligo- and polysaccharides by potential intestinal pathogens

Bacteria

The seven strains of potential intestinal pathogens, that were investigated in this study, *Clostridium perfringens* R70, *Enterobacter aerogenes* R62, *Enterococcus faecalis* R65, *Klebsiella pneumoniae* R54, and *Salmonella* Menston R71, were preserved in our laboratory (MIRINZ Protect Culture Collection, AgResearch, Hamilton, New Zealand, 1996); *Escherichia coli* NZRM 916 was purchased from the Communicable Disease Center of ESR, Porirua, New Zealand; *Salmonella* Dublin ATCC 15480 was purchased from the American Type Culture Collection, Rockville, Maryland, USA. Tryptic Soy Agar (TSA, DIFCO, Becton Dickinson, USA) was used to prepare the stock bacterial cultures of these strains which were incubated aerobically on TSA at 37°C for 24 hours, with the exception of

Clostridium perfringens R70 which was cultivated on RCM agar plates and incubated anaerobically at 37°C for 48 hours.

Oligo- and polysaccharides

Fibrulose (COSUCRA SA, Fontenoy, Belgium), Fibruline® LC (COSUCRA SA, Fontenoy, Belgium), Raftilose® P95 (Orafti s. a., Oreye, Belgium), and Arabinogalactan (AG, Larex® AC 9, Larex Inc. St. Paul, Minnesota, USA) were used in this study.

Fibrulose is obtained from chicory inulin by partial enzymatic hydrolysis, and consists of oligosaccharides of DP 2 to 5. Structurally, it is short chains of fructofuranosyl units terminating in glucopyranosyl and is non-reducing. It mainly consists of sucrose, 1-Kestose, nystose, and fructosylnystose (Skinner, 2001). The structure and the length of the chain of Fibrulose are similar to that of Yacon (See Section 3.2.3.3, Chapter 3) which replaced Fibrulose in subsequent trials.

Fibruline® LC (hereafter referred to as Fibruline) is obtained from chicory root inulin by removal of the short chains oligomers and has a minimum DP of 20. Structurally, it is a chain of fructofuranosyl residues terminating in a glucopyranosyl residue and is non-reducing (Skinner, 2001).

Raftilose® P95 (hereafter referred to as Raftilose) is obtained from chicory inulin by partial enzymatic hydrolysis, and consists of oligosaccharides of DP 2 to 6. Structurally, it is short chains of fructofuranosyl units terminating in either fructose (furanose or pyranose) or glucopyranose. It mainly consists of inulobiose, inulotriose, inulotetraose, and inulopentaose which are reducing (Skinner, 2001).

Arabinogalactan (AG) is a high molecular weight (around 20,000 Daltons) polysaccharide consisting of chains of galactopyranose residues with very large numbers of branches containing galactopyranose and arabinose residues (Skinner, 2001).

In this study, 10 g of each test oligo- and polysaccharide were dissolved in 100 mL of distilled water, giving a concentration of 10% (w/v) solution. These solutions were sterilised by filtration (for Fibrulose and Raftilose) through a 0.45 μm cellulose ester membrane (Millipore S. A. Molsheim, France) or autoclaved (for Fibruline and AG) at 10 lb/inch^2 (115.2°C) for 10 minutes.

Fermentation experiments

Phenol Red Agar (PRA) Test. The basal medium was Phenol Red Agar Base (PRA, DIFCO, Detroit, USA). Test medium was prepared by aseptically adding 1.0 mL of each of 10% sterile sugar solution to 9.0 mL of molten PRA base which was cooled at 50°C, to give a 1.0% (w/v) final concentration of each substrate in each test medium. The final pH of the medium was 7.4 ± 0.2. Each test strain, with the exception of *Clostridium perfringens* R70, was stab-inoculated and streaked upon the surface of the slant of one tube of each test medium and one tube of basal medium as a negative control. Incubation was carried out at 37°C aerobically for 24 hours. The fermentation tubes were examined for a colour change from red to yellow (acid formation) and the presence of gas bubbles. The experiment was carried out in triplicate. *Clostridium perfringens* R70, an obligate anaerobe, was subjected to the comparable Fastidious Anaerobe Broth (FAB) test described below.

Fastidious Anaerobe Broth (FAB) Test. The basal medium was Fastidious Anaerobe Broth (FAB, LAB M, Lancashire, UK). Test medium was prepared by aseptically adding 1.0 mL of each of 10% sterile sugar solution to 9.0 mL of FAB to give a 1.0% (w/v) final concentration of each sugar in each test medium. The

final pH of the medium was adjusted to 7.2 ± 0.2. One loopful (1μL) of *Clostridium* perfringens R70 was then inoculated into one tube of each test medium, and one of basal medium served as a negative control. Incubation was carried out anaerobically at 37°C until adequate growth was obtained.
Bromo-thymol blue (0.04%, pH 6.0-7.6, BDH, Poole, England) was added to each tube to determine the acid formation which showed as a colour change from blue to yellow. The experiment was carried out in triplicate.

4.2.1.2 *In vitro* utilisation of oligo- and polysaccharides by bifidobacteria

Test strains

One hundred and seventeen bifidobacterial strains isolated from sheep faeces were differentiated from other bacteria grown on RB using the following phenotypic characteristics: Gram reaction, cell morphology, colony characteristics, catalase test and oxidase test and identified to the genus level on the basis of the presence of F6PPK (see **Appendix 1**). These isolates were preserved at -85°C in the maintenance medium (15% Glycerol in TSB). Working cultures were prepared by first resuscitating the preserved cultures on RCM agar plates at 37°C for 48 hours under anaerobic conditions. The designations and origins of these strains are detailed in **Appendix 1**.

The following reference strains were also used in this study: *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, *B. animalis* DSM 20 104, and *B. boum* DSM 20 432.

Basal media

The basal media was modified from Raffinose Bifidobacterium (RB) medium (Hartemink *et al.*, 1996), by omitting raffinose from the medium. It consisted of:

Bacto® agar (DIFCO, 18.0 g/L); sodium caseinate (Acros Organics, 5.0 g/L); yeast extract (DIFCO, 5.0 g/L); lithium chloride (BDH, AnalaR®, 3.0 g/L); sodium propionate (Acros Organics, 15.0 g/L); L-cysteine.HCl (SERVA, 0.5 g/L); sodium thioglycollate (Sigma, 0.5 g/L); bromocresolpurple (BDH, 0.15 g/L); and salts-solution (40 mL/L). The salts solution contained: $MgSO_4$ (0.2 g/L); $CaCl_2$ (0.2 g/L); K_2HPO_4 (1.0 g/L); KH_2PO_4 (1.0 g/L); $NaHCO_3$ (10.0 g/L) and NaCl (2.0 g/L). The final pH of the medium was adjusted to 6.7 ± 0.1, using 4N NaOH or HCl. The media were then autoclaved at 121°C for 20 minutes.

Preparation of oligo- and polysaccharide solutions

Five grams of Yacon, Fibruline, Raftilose, and AG were each dissolved in 50 mL of distilled water, to give a 10% solution of each substrate. The pH of each solution was then adjusted to 6.7 ± 0.1. A 10% Raffinose solution was also made as described above as a positive control. These solutions were sterilised by filtration (for Yacon, Raftilose, and Raffinose) or autoclaved (for Fibruline and AG) at 10 lb/inch^2 (115.2°C) for 10 minutes.

Preparation of oligo- and polysaccharide fermentation media

Six bottles each containing 450 mL of basal media were melted and cooled to 50°C. Each pre-sterilised substrate solution was also put into a 50°C water bath to pre-warm. Then 50 mL of each substrate solution was added aseptically to 450 mL of each basal media to give 1% (w/v) Yacon, Fibruline, Raftilose, AG, and Raffinose in each substrate fermentation medium. Fifty mL of distilled water was added aseptically into 450 mL of basal media to serve as a negative control. The mixtures were swirled gently, and then poured immediately into the sterile Petri dishes.

Fermentation experiments

A cross-stripe of agar was cut aseptically out of each sugar fermentation agar plate to give four separate parts to each plate. Each isolate culture or reference culture, freshly prepared on RCM agar plates, was subcultured onto each substrate fermentation media as a point inoculum, or a small "X" mark. The inoculated plates were incubated anaerobically at 37°C for 48 hours. Growth and various degrees of colour changes of the agar were recorded as the fermentation results.

4.2.2 Mixed culture studies

4.2.2.1 Selection of bifidobacteria

Eighteen representative bifidobacterial strains were selected from the 117 isolates (**Appendix 1**) on the basis of six different colony types and four different cell morphologies observed when grown on RB agar plates (data not shown), strong colour development in F6PPK tests, animal sources from the *in vivo* tests, i. e. the sheep from which they were isolated; and which treatment was administered before the bifidobacterial strains were isolated, and their *in vitro* oligo- and polysaccharide fermentation capability.

Two reference strains *B. pseudolongum* subsp. *globosum* DSM 20 092 and *B. animalis* DSM 20 104 were also used in this study.

These strains were subcultured twice onto RCM agar plates and incubated anaerobically at 37°C for 48 hours. The colony morphologies of these strains on RCM agar plates were recorded. Then 1 loopful (1 µl) of each bifidobacterial RCM agar culture were subcultured onto one fresh RCM agar slope. The inoculated slopes were incubated anaerobically at 37°C for 48 hours, and used as stock bacterial cultures in mixed culture studies.

4.2.2.2 Mixed cultures of bifidobacteria

The 20 test strains of bifidobacteria (See Section 4.2.2.1) were further divided into 5 groups on the basis of the colony morphologies on RCM agar plates. Each group included 4 strains (**Table 4.1**). These 5 groups of bifidobacteria were investigated for their antagonistic effects against *E. coli*.

Table 4.1 Mixtures of bifidobacteria used in mixed culture studies

Mixtures of Bifidobacteria	Colony morphologies on RCM agar plates	Strains in each mixture
1	0.5-1.0 mm, **white**, convex, circular	*B. pseudolongum* subsp. *globosum* DSM 20 092; P3-Pre1-45S; *P5-Po2-23; *P5-Po4-37
2	0.5-1.0 mm, **cream to white**, convex, circular	*B. animalis* DSM 20 104; P2-Po2-1; P2-Po2-96; P3-In3-15
3	0.5-1.0 mm, cream to white, **flat to raised**, circular	P1-pre1-60; P3-In3-22; P4-Pre1-17; P4-In3-46
4	0.5-1.0 mm, cream to white, **raised to convex**, circular	P2-Po2-10; P2-Po2-30a; P3-Po3-31; P5-Po3-22
5	0.5-2.0 mm, cream to white, convex, circular, **glistening**	P2-Po2-76; P2-Po2-80; P3-Pre1-68; P5-In2-42

* Isolates from morphological group 5.

4.2.2.3 Escherichia coli

One single colony of *E. coli* was subcultured onto a TSA slope from a TSA plate culture and incubated aerobically at 37°C for 18 hours. Then 1 loopful (1 µl) of *E. coli* TSA slope culture was subcultured onto another fresh TSA slope and incubated aerobically at 37°C for a further 18 hours. The latter slope culture was then used as a stock bacterial culture in mixed culture studies.

4.2.2.4 Other potential intestinal pathogens

Enterobacter aerogenes R62, *Salmonella* Dublin ATCC 15480, *Salmonella* Menston R71, *Klebsiella pneumoniae* R54, *Enterococcus faecalis* R65, and *Clostridium perfringens* R70 were also used in mixed culture studies. The preparation of stock bacterial culture of these strains with the exception of *Clostridium perfringens* R70 was as described in section 4.2.2.3. *Clostridium perfringens* R70 was subcultured twice onto RCM agar plates and incubated anaerobically at 37°C for 48 hours. Then 1 loopful (1 μl) of *Clostridium perfringens* RCM agar plate culture was transferred onto a RCA slope and incubated anaerobically at 37°C for 48 hours, and used as a stock bacterial culture in mixed culture studies.

4.2.2.5 Preparation of bacterial stock suspensions

The initial counts for each bifidobacteria in fermentation broth were expected to be between 10^6 to 10^8 CFU/mL. To achieve this number, viable counts of the prepared stock cell suspension had been 2.0×10^7 to 2.0×10^9 CFU/mL for a 5% inoculum size. A 48-hour RCM agar slope stock culture for each bifidobacteria was washed off with 4.5 mL of Maximum Recovery Diluent (DIFCO, Becton Dickinson, USA). These initial suspensions were considered to be 10^0 dilutions, which were homogenised with a MS1 minishaker (1 KA® Works (Asia) Sdn. Bhd., Malaysia) for approximately 1 minute and serially diluted to ten-fold dilutions with 4.5 mL of Maximum Recovery Diluent. One hundred microlitre volumes of the appropriate dilutions for each bifidobacterial strain were spread over the surface of duplicate dried RB agar plates. All the spread RB agar plates were then incubated anaerobically (Anaero*Gen*, Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 48 hrs. All the colonies developing were counted on the duplicate plates of the highest countable dilution (30-300CFU). The results were expressed as the viable counts (CFU/mL) of the

10^0 dilutions. These 10^0 dilutions were used as stock cell suspensions directly, or diluted to appropriate dilutions before use to give the required initial viable counts.

The initial counts for *E. coli* and other potential intestinal pathogens in fermentation broth were expected to be approximately 10^5 CFU/mL. To achieve this number, viable counts of the prepared stock cell suspensions had been approximately 2.0×10^6 CFU/mL for a 5% inoculum size. The preparation of these bacterial stock suspensions has been described previously (Section 4.2.2.5) with Chromocult® Coliform Agar being used for enumeration of *E. coli* 916, *Enterobacter aerogenes* R62, *Salmonella* Dublin ATCC 15480, *Salmonella* Menston R71, and *Klebsiella pneumoniae* R54; KF-streptococcus agar (DIFCO, Detroit, USA) being used for enumeration of *Enterococcus faecalis* R65; and TSC for enumeration of *Clostridium perfringens* R70.

All these bacterial stock suspensions were freshly prepared on the day of each mixed culture study, and were put in an anaerobic chamber (Forma Scientific Inc., USA) with 5% CO_2, 10% H_2 and 85% N_2 gas atmosphere before use in the mixed culture studies.

4.2.2.6 Basal media

Peptone Yeast Extract (PY) basal broth (Wang and Gibson, 1993) was used for each mixed culture experiment in this study. It was prepared in double strength, consisting of: Bacto® Proteose peptone (DIFCO, 10.0 g/L), Tryptone peptone (DIFCO, 10.0 g/L), Bacto™ Yeast extract (DIFCO, 20.0 g/L), "Lab-Lemco" Powder (OXOID, 20.0 g/L), Resazurin (BDH, 0.002 g/L), L-cysteine.HCl (SERVA, 1.0 g/L), and salts-solution (80mL/L). The salts solution contained: $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.2 g/L), K_2HPO_4 (1.0 g/L), KH_2PO_4 (1.0 g/L), $NaHCO_3$ (10.0 g/L) and NaCl (2.0 g/L). The L-cysteine.HCl was added after all the other

ingredients had been mixed together and boiled to achieve dissolution. The final pH of the medium was adjusted to 7.0 ± 0.2, using 4N NaOH or HCl. The media were autoclaved at 121°C for 20 minutes.

4.2.2.7 Preparation of fructooligosaccharide solutions

Four percent of Yacon, Fibruline, and Raftilose were prepared and sterilised as described in Section 4.2.1.2.

4.2.2.8 Preparation of fermentation media

For co-culture of E. coli and mixtures of bifidobacteria: 5.0 mL aliquots of the autoclaved PY (double strength) broth were dispensed into sterile Hungate tubes. Then 2.5 mL of the respective 4% trial substrate solution were added into each of these tubes. For the no carbohydrate control, 2.5 mL of sterile distilled water were added to a sterile Hungate tube instead of a substrate solution.

For co-culture of E. coli (or other potential intestinal pathogens) and individual strain of bifidobacteria: 5.0 mL aliquots of the autoclaved PY (double strength) broth were dispensed into sterile Hungate tubes. Then 2.5 mL of the respective 4% trial substrate solution and 1.5 mL of sterile distilled water were added into each of these tubes.

For monoculture of E. coli (or other potential intestinal pathogens): 5.0 mL aliquots of the autoclaved PY (double strength) broth were dispensed into sterile Hungate tubes. Then 2.5 mL of the respective 4% trial substrate solution and 2.0 mL of sterile distilled water were added into each of these tubes. For the no carbohydrate control, 4.5 mL of sterile distilled water were added to a sterile Hungate tube instead of a substrate solution.

For determination of bacterial growth kinetics in co-culture of E. coli and bifidobacterial strain P5-Po4-37: 100 mL aliquots of the autoclaved PY (double strength) broth were dispensed into 250 mL sterile Schott bottles. Then 50 mL of the respective 4% trial substrate solution and 30 mL of sterile distilled water were added to each bottle. For monoculture of *E. coli* or bifidobacteria, an extra 10 mL of sterile distilled water were added to each bottle.

All the above PY fermentation broths prepared were fully reduced under anaerobic conditions before being inoculated with bacteria.

4.2.2.9 Co-culture of *E. coli* and mixtures of bifidobacteria

Each of the 5 mixtures of bifidobacteria and *E. coli* were simultaneously inoculated and co-cultured in PY fermentation broth containing either Yacon, Raftilose, or Fibruline, and also simultaneously inoculated and co-cultured in PY basal broth which served as a no carbohydrate control. The inoculum size was 5%, namely, 0.5 mL of bacterial stock suspension for each strain in each mixture of bifidobacteria and *E. coli* were inoculated into 7.5 ml of each substrate fermentation broth and also into 7.5 ml of PY basal no carbohydrate control broth. *E. coli* was also monocultured in each of the substrate fermentation broths and PY basal no carbohydrate control broth to serve as a pure culture control. The initial counts of the mixtures of bifidobacteria ranged from 10^7 to10^8 CFU/mL of fermentation broth. The initial counts of *E. coli* were 1.01×10^5 CFU/mL of fermentation broth. All the inoculated tubes were incubated anaerobically at 37°C for 48 hours. After 48-hour fermentation, *E. coli* was enumerated in each fermentation tube as described in Section 4.2.2.5. The pH of the fermented broth in each tube was also determined (PHM 210 Standard pH Meter, MeterLab™, Radiometer Analytical SAS, Lyon, France). The experiment was carried out in triplicate.

4.2.2.10 Co-culture of *E. coli* and individual strains of bifidobacteria

Each of the 8 individual strains from mixture 1 and mixture 5 (**Table 4.1**) of bifidobacteria and *E. coli* were simultaneously inoculated and co-cultured in PY fermentation broth containing Yacon or Raftilose. The initial counts of each bifidobacterial strain ranged from 10^6 to10^7 CFU/mL of fermentation broth. The initial counts of *E. coli* were 3.55×10^5 CFU/mL of fermentation broth. The procedures were carried out as described in section 4.2.2.9. The experiment was carried out in triplicate.

4.2.2.11 Determination of bacterial growth kinetics in co-culture of *E. coli* and bifidobacterial strain P5-Po4-37

Strain P5-Po4-37 was selected from the 8 individual bifidobacterial strains tested because its maximum biomass was reached and 100% inhibition of *E. coli* obtained. Two different concentrations of stock cell suspension of bifidobacterial strain P5-Po4-37 were prepared to give initial counts of this strain of 10^7 to10^8 (higher) or 10^3 to10^4 (lower) CFU/mL of fermentation broth, respectively. The initial counts of *E. coli* in fermentation broth were 10^4 to10^5 CFU/mL. 10 mL of each concentration of the bifidobacterial strain and 10 mL of *E. coli* were simultaneously inoculated and co-cultured in 180 mL of PY fermentation broth containing Yacon or Raftilose. 10 mL of each different concentration of the bifidobacterial strain or 10 mL of *E. coli* were also monocultured in 190 mL of PY fermentation broth containing Yacon or Raftilose to serve as pure culture controls. All the inoculated broths were incubated anaerobically at 37°C for up to 60 hours. 10 mL of fermented broth were removed after 0, 6, 9, 12, 24, 30, 36, 48, 54, 60 hours incubation. 5 mL of each sample were used for the enumeration of *E. coli* and bifidobacteria, and measurement of pH of the fermented broth. The other 5 mL of each sample was kept at -35°C for later fermentation product analysis. The procedures for

enumeration of *E. coli* and bifidobacteria were performed as described in section 4.2.2.5. The experiment was carried out in triplicate.

4.2.2.12 Co-culture of bifidobacterial strain P5-Po4-37 and other pathogens

Each of the other 6 potential intestinal pathogens (Section 4.2.2.4) and bifidobacterial strain P5-Po4-37 were simultaneously inoculated and co-cultured in PY fermentation broth containing Yacon or Raftilose. The initial counts for each pathogen were approximately 10^5 CFU/mL of fermentation broth. The initial counts of bifidobacterial strain P5-Po4-37 were 5.70×10^7 CFU/mL of fermentation broth. Each pathogen was also monocultured in PY fermentation broth containing Yacon or Raftilose as pure culture controls. All the inoculated broths were incubated anaerobically at 37°C for 48 hours. After 48-hour fermentation, viable counts for each pathogen were determined as described in Section 4.2.2.5. The pH of the fermented broth in each tube was measured. The experiment was carried out in triplicate.

4.2.2.13 Chemical analysis

In the co-culture studies of *E. coli* and bifidobacterial strain P5-Po4-37 in Raftilose-containing PY broth (Section 4.2.2.11): fermented broths were also collected periodically for determining the production of acetate, propionate, butyrate, lactate, succinate and total volatile fatty acids (VFA). The concentration (mmol/L) of these fermentation products were determined by gas chromatography (GC) of their ether or chloroform extracts as described in the Anaerobe Laboratory Manual (Holdeman *et al.*, 1977). Total VFA was the sum of volatile fatty acids identified (acetic, propionic, butyric, isobutyric, iso-valeric, and caproic acids). Triplicate samples were used for each determination.

4.2.2.14 Data analysis

Inhibition rate (%) of *E. coli* (or other pathogen) by bifidobacteria was calculated as:

$$\% \text{ inhibition} = \frac{Mono - Mix}{Mono} \times 100\% \quad \text{(Fan } et\ al.\text{, 1988)}$$

This expression can be re-written in terms of logarithm transformed counts as:

$$\% \text{ inhibition} = \left(1 - 10^{\left(\log_{10} Mix - \log_{10} Mono\right)}\right) \times 100\%$$

Mono: viable counts of *E. coli* after 48-hour monoculture of *E. coli* (or other pathogen);

Mix: viable counts of *E. coli* (or other pathogen) after 48-hour co-culture of *E. coli* (or other pathogen) and bifidobacteria;

Viable counts of *E. coli* (or other pathogen) were expressed as the number of CFU/mL of fermented broth.

In the co-culture studies of E. coli and mixtures of bifidobacteria (Section 4.2.2.9): the inhibition rate (%) of *E. coli* by each mixture of bifidobacteria in each substrate was calculated to select the mixture of bifidobacteria which exerts the maximum inhibitory effect on *E. coli* and to select the substrate which most significantly potentiates the inhibition effect. The significance of the differences between treatments on basis of the inhibition rate (%) and pH of the fermented broth was assessed by analysis of variance which was performed by GenStat, version 6.1.0.20 (Lawes Agricultural Trust, supplied by VSN International Ltd., Oxford, UK). The inhibition rate (%) was rank transformed to meet the assumptions required for the analysis of variance. In the case of a significant difference ($P < 0.05$) between treatments, group means were compared in pairs

using Student's t-test, which was carried out by Microsoft® Excel 2002 (Microsoft Corporation). The correlation between the pH of the fermented broth and the rank transformed % inhibition of *E. coli* was also analysed using Microsoft® Excel 2002.

In the co-culture studies of E. coli and individual strains of bifidobacteria (Section 4.2.2.10): The correlation between the pH of the fermented broth and the angular transformed % inhibition of *E. coli* was analysed using Microsoft® Excel 2002.

4.3 Results

4.3.1 In vitro utilisation of oligo- and polysaccharides by potential intestinal pathogens

Seven potential intestinal pathogens were investigated for their capacity for *in vitro* utilisation of Fibrulose, Raftilose, AG, and Fibruline. As shown in **Table 4.2**, all the test strains grew well and utilised the test substrates with the exception of *Clostridium perfringens* R70 which did not utilise any of the test substrates. Among the strains, *Enterobacter aerogenes* R62, *Escherichia coli* NZRM 916, *Klebsiella pneumoniae* R54, *Salmonella* Dublin ATCC15480, and *Salmonella* Menston R71 also produced various amounts of gas.

Table 4.2 *In vitro* fermentation of oligo- and polysaccharides by potential intestinal pathogens. Data were recorded from 3 tests. "+" represents positive fermentation in the 3 tests; "-" represents no fermentation observed in any of the tests, "N/A" represents no growth or no test available.

Bacteria	Fibrulose	Raftilose	AG	Fibruline	Control
Clostridium perfringens R70	-	-	-	N/A	-
Enterobacter aerogenes R62	+	+	+	+	-
Enterococcus faecalis R65	+	+	+	+	-
Escherichia coli NZRM 916	+	+	+	+	-
Klebsiella pneumoniae R54	+	+	+	+	-
Salmonella Dublin ATCC 15480	+	+	+	+	-
Salmonella Menston R71	+	+	+	+	-

4.3.2 *In vitro utilisation of oligo- and polysaccharides by bifidobacteria*

The 117 bifidobacterial strains, isolated from sheep faeces, and 4 reference strains of bifidobacteria were screened for their capacity for utilisation of Yacon, Raftilose, AG, and Fibruline (**Appendix 1**). Growth and colour change of agar exhibited various degrees of fermentation which were arranged into 5 levels: "-" ~ "3+" (as detailed in **Appendix 1**). Yacon was utilised by 119 of the 121 strains tested, varying from weak substrate utilisation (+/-) by 1 strains, moderate fermentability (1+) by 47 strains, to marked fermentation activity (2+ ~ 3+) by 71 strains. Raftilose was utilised by 91 of the 121 strains tested, varying from weak substrate utilisation (+/-) by 26 strains, moderate fermentability (1+) by 35 strains, to marked fermentation activity (2+ ~ 3+) by 30 strains. Fibruline was utilised

by only 18 of the 121 strains, varying from weak substrate utilisation (+/-) by 8 strains, moderate fermentability (1+) by 8 strains, to marked fermentation activity (2+ ~ 3+) by 2 strains. AG was utilised by only 24 of the 121 strains, varying from weak substrate utilisation (+/-) by 22 strains to moderate fermentability (1+) by 2 strains. No fermentation reactions were observed on the basal media. These fermentation patterns demonstrated that Yacon exhibits the strongest fermentability among the test substrates, which is comparable to that of raffinose; followed by Raftilose which also shows strong fermentability. Neither Fibruline nor AG shows any obvious fermentation patterns with these test strains. These results also indicated that the capacity for different strains to utilise the test substrates differed. Strong fermentation capacity for the test substrates as well as typical growth and physiological characteristics of bifidobacteria were used as criteria for the further selection of bifidobacterial test strains for mixed culture studies.

4.3.3 Inhibition of in vitro growth of E. coli by mixtures of bifidobacteria

The mixed cultures of bifidobacteria were evaluated with respect to their capabilities for inhibiting the *in vitro* growth of *E. coli*. Each mixture of bifidobacteria and *E. coli* were co-inoculated in the PY fermentation broth containing either Yacon, Raftilose, Fibruline, or no carbohydrate control. *E. coli* was also monocultured in each of the substrate fermentation broths and PY basal no carbohydrate control broth as a pure culture control. The viable counts of *E. coli* when either co-cultured with bifidobacteria or monocultured alone were determined after a 48-hour anaerobic incubation at 37°C. The inhibition rate of *E. coli* by each mixture of bifidobacteria in each substrate fermentation or basal broth was then calculated and rank transformed for comparison of the inhibitory capabilities among the 5 mixtures.

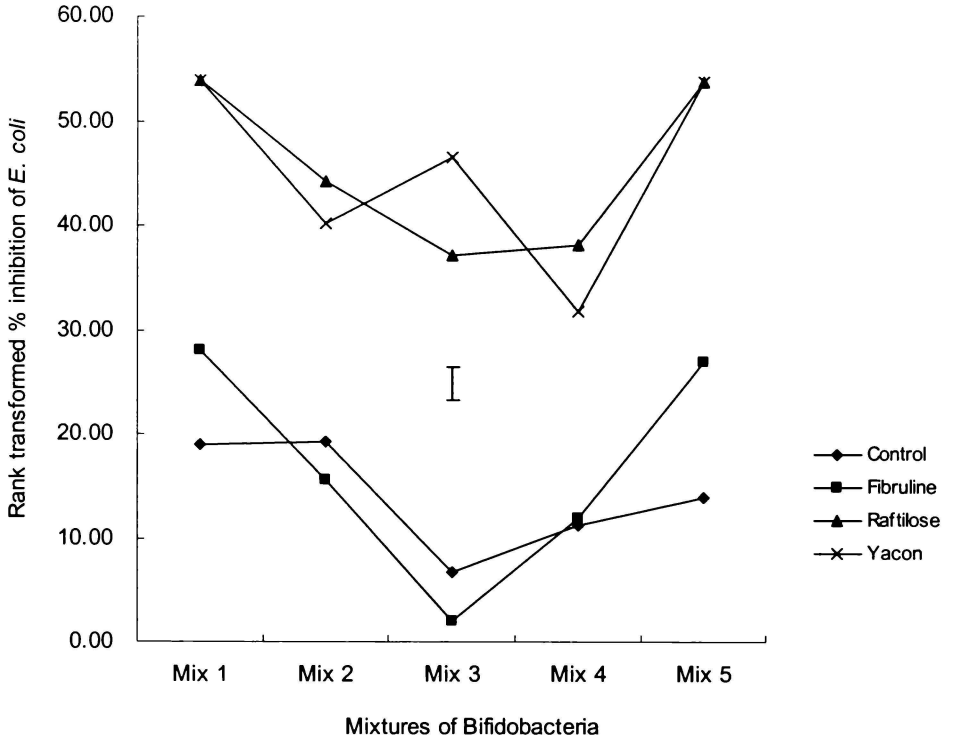


Figure 4.1 Inhibition of i*n vitro* growth of *E. coli* by mixtures of bifidobacteria (shown as Mix 1 to 5). Data represent means of triplicate determinations of rank transformed % inhibition of *E. coli* and standard error of differences of means shown as an error bar.

As is evident from **Figure 4.1**, two of the five mixtures, mixtures 1 and 5, significantly inhibited the growth of *E. coli* ($P < 0.001$), compared with the other 3 mixtures. The inhibition of *E. coli* on Raftilose and Yacon were significantly higher than that on Fibruline and control ($P < 0.001$); for this reason Fibruline was excluded from subsequent experiments.

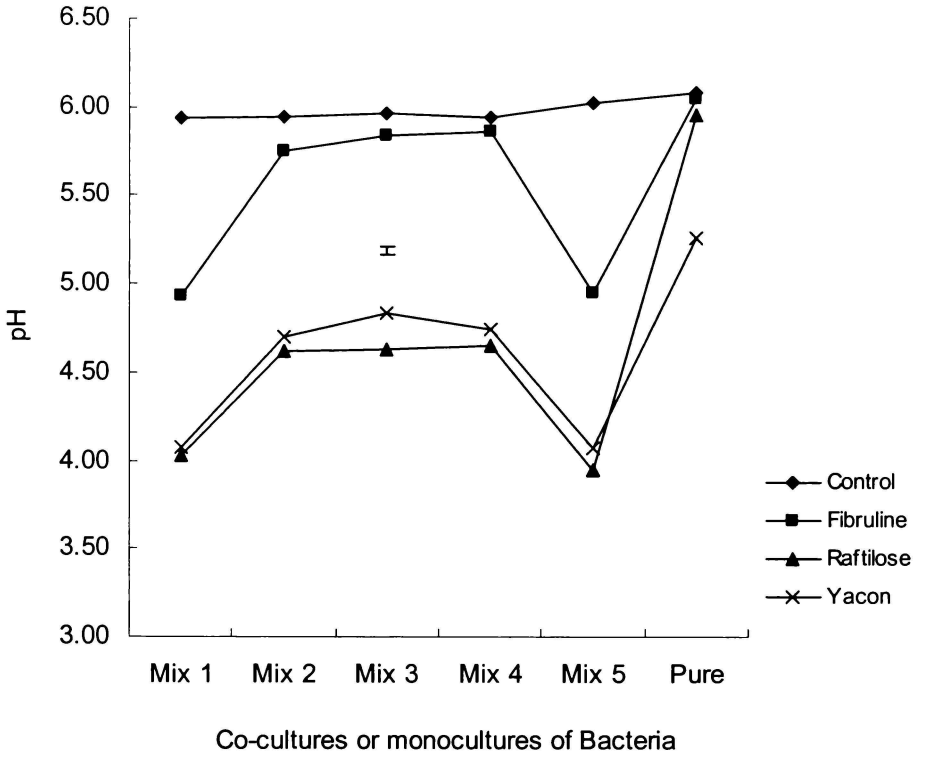


Figure 4.2 Final pH in fermented broths after 48-hour co-cultures of *E. coli* and mixtures of bifidobacteria (shown as Mix 1 to Mix 5) or monocultures of *E. coli* (shown as Pure). Data represent means of triplicate determinations of pH and standard error of differences of means shown as an error bar.

The pH of the fermented broth was also determined after 48-hour co-culture of *E. coli* and each mixture of bifidobacteria and monocultures of *E. coli* in Raftilose, Yacon, Fibruline, or PY basal broth (**Figure 4.2**). The final pH in co-cultures of *E. coli* and bifidobacteria mixture 1 and mixture 5 decreased significantly ($P < 0.001$) compared with the other 3 co-cultures and the monocultures of *E. coli*. The pH of the fermented broth with Raftilose and Yacon as primary carbon sources were significantly lower ($P < 0.001$) than that with Fibruline as carbon source and that of PY basal broth.

The correlation between pH of the fermented broth and the rank transformed % inhibition of *E. coli* was analysed. A clear negative correlation ($R^2 = 0.9037$) is evident in **Figure 4.3**.

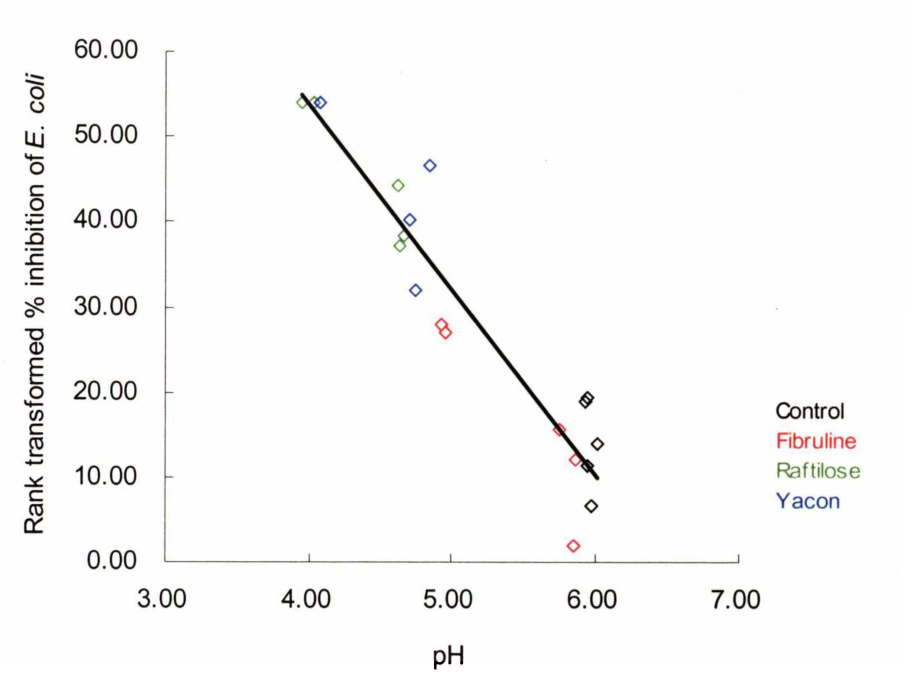


Figure 4.3 Correlation between the means of rank transformed % inhibition of *E. coli* and pH of fermented broth after 48-hour fermentation in co-cultures of *E. coli* and mixtures of bifidobacteria. Each point represents a mean of triplicate determinations. $R^2 = 0.9037$.

4.3.4 *Inhibition of in vitro growth of E. coli by individual strains of bifidobacteria*

Eight individual bifidobacterial strains from mixture 1 and mixture 5 were assessed and compared with respect to their inhibitory activities against *E. coli* by calculation of the inhibition rate of *E. coli* by each strain of bifidobacteria in PY broths containing Raftilose and Yacon (**Table 4.3**).

Table 4.3 Inhibition of *in vitro* growth of *E. coli* by individual strains of bifidobacteria. Data represent means of triplicate determinations of % inhibition of *E. coli* with individual standard errors of means and final pH in the co-cultures with standard error of differences of means.

Strains of bifidobacteria	% inhibition of *E. coli*		Final pH in co-cultures	
	Raftilose	Yacon	Raftilose	Yacon
B. pseudolongum subsp. *globosum* DSM 20 092	66.45 ± 2.61	68.93 ± 3.32	4.91	4.79
P3-Pre1-45S	97.64 ± 0.51	98.27 ± 0.89	4.48	4.58
P2-Po2-76	100	100	3.96	4.12
P2-Po2-80	100	100	3.95	4.13
P3-Pre1-68	100	100	3.97	4.12
P5-In2-42	100	100	3.95	4.12
P5-Po2-23	100	100	3.96	4.13
P5-Po4-37	100	100	3.95	4.12
Standard error of differences of means			0.011	

In general, all strains from bifidobacteria mixtures 1 and 5 were antagonistic to *E. coli*, ranging from 66.45 to 100% inhibition. Among them, six strains completely inhibited the growth of *E. coli*, reaching a nominal 100% inhibition rate. As regards the two substrates investigated, there were no significant differences between Raftilose and Yacon in their inhibition of *E. coli* by the test strains of bifidobacteria.

The pH of the fermented broth in each tube was also determined after 48-hour co-culture of *E. coli* and each strain of bifidobacteria in Raftilose and Yacon (**Table 4.3**). Compared with the initial pH (7.0 ± 0.2), the final pH in the co-cultures of *E. coli* and individual bifidobacterial strains decreased to below 5.0.

With the six most inhibitory strains co-cultured with *E. coli*, the final pH decreased to lower levels ranging from 3.95 to 3.97 in Raftilose and ranging from 4.12 to 4.13 in Yacon. In the monocultures of *E. coli* the pH remained at 5.86 during the fermentation in Raftilose and at 5.21 in Yacon.

The correlation between pH of fermented broth and the angular transformed % inhibition of *E. coli* was analysed. A clear negative correlation ($R^2 = 0.8271$) is evident in **Figure 4.4**.

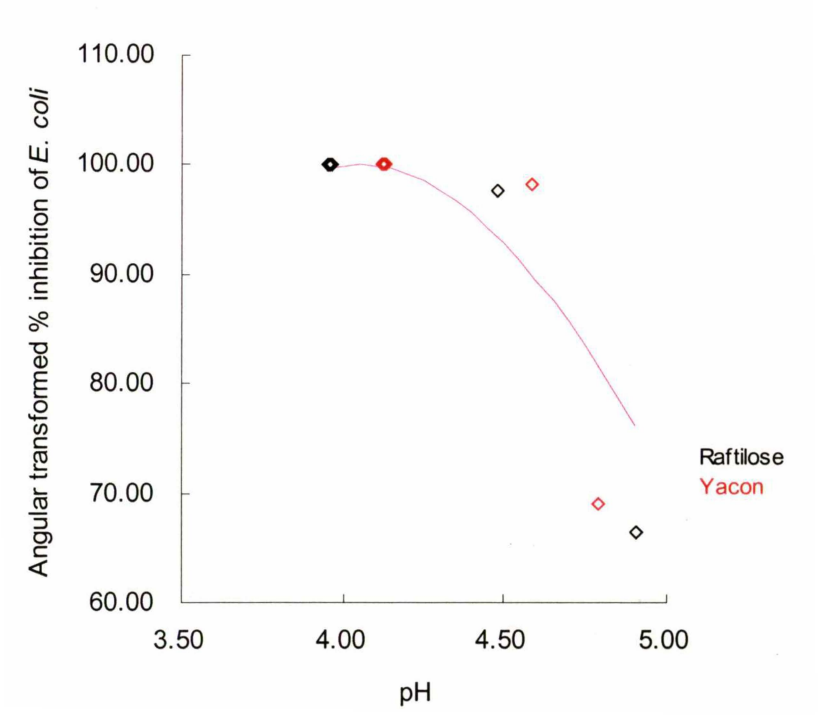


Figure 4.4 Correlation between angular transformed means of % inhibition of *E. coli* and means of pH of fermented broth after 48-hour fermentation in co-cultures of *E. coli* and individual strains of bifidobacteria. Each point represents a mean of triplicate determinations. $R^2 = 0.8271$.

4.3.5 Bacterial growth kinetics in co-culture of *E. coli* and bifidobacterial strain P5-Po4-37

Each of two different concentrations of cells of bifidobacterial strain P5-Po4-37 and *E. coli* were co-cultured anaerobically in PY fermentation broth containing Raftilose and Yacon for 60 hours at 37°C. Each of the above strains was also monocultured in PY fermentation broth containing Raftilose and Yacon, as pure culture controls. Growth curves of pure cultures of bifidobacterial strain P5-Po4-37 and *E. coli* when monocultured as well as the growth curves of the microorganisms when co-cultured in PY fermentation broth containing Raftilose are shown in **Figure 4.5 – 4.9**.

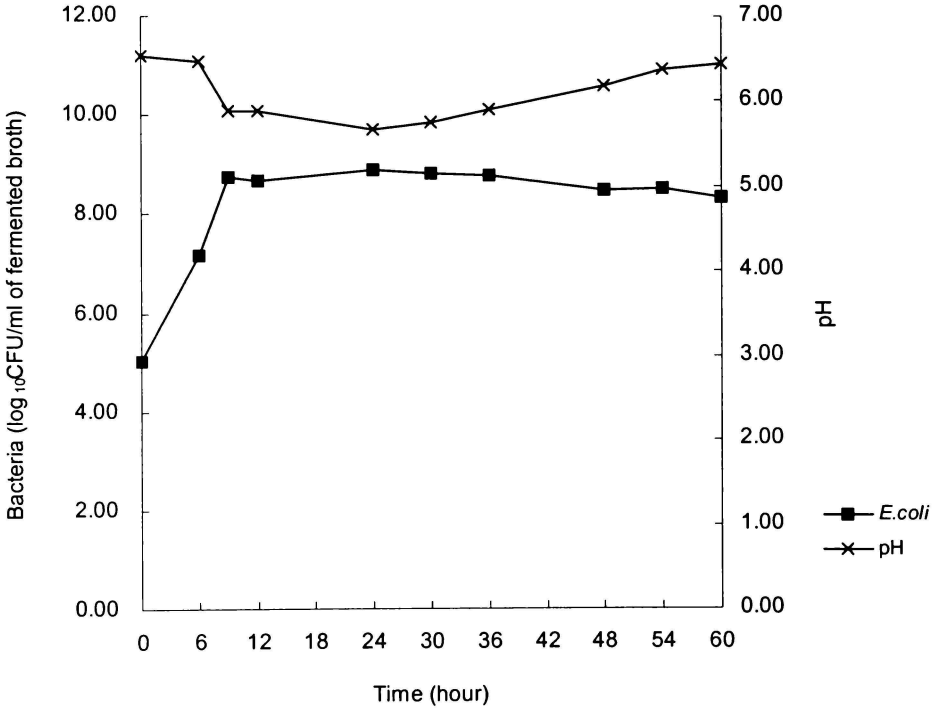


Figure 4.5 Growth curve of *E. coli* and pH of fermented broth during 60-hour fermentation in a monoculture of *E. coli* in Raftilose-containing PY Broth. Data represent means of triplicate determinations.

In the initial stages of fermentation (0 ~ 12 hours), *E. coli* grew well in the monoculture (**Figure 4.5**), the co-culture with the higher concentration of bifidobacteria (**Figure 4.6**), and the co-culture with the lower concentration of bifidobacteria (**Figure 4.7**), reaching the stationary phases after 9 hours, and remaining at that high number (approximately 10^8 CFU/mL) until 12 hours.

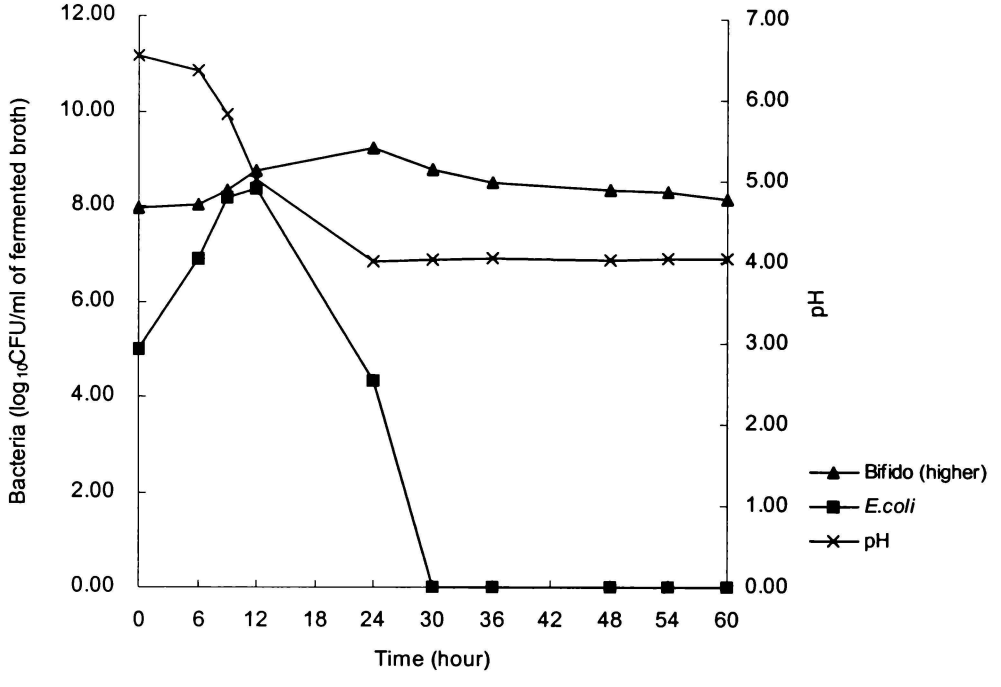


Figure 4.6 Growth curves of bacteria (Bifido = bifidobacteria) and pH of fermented broth during 60-hour fermentation in a co-culture of *E. coli* and a higher concentration of bifidobacteria in Raftilose-containing PY Broth. Data represent means of triplicate determinations.

After 12 hours, differences were observed under the different conditions: *E. coli* remained at the stationary phase up to 60-hour fermentation when monocultured in the PY fermentation broth containing Raftilose (**Figure 4.5**), but the number of *E. coli* began to decrease markedly at the beginning of the stationary phase and

reached zero (no *E. coli* detected) after 30 hours when co-cultured with the higher concentration of bifidobacteria (**Figure 4.6**). *E. coli* remained at a high level until 30-hour fermentation, then began to decrease, and reached zero after 60 hours when co-cultured with the lower concentration of bifidobacteria (**Figure 4.7**).

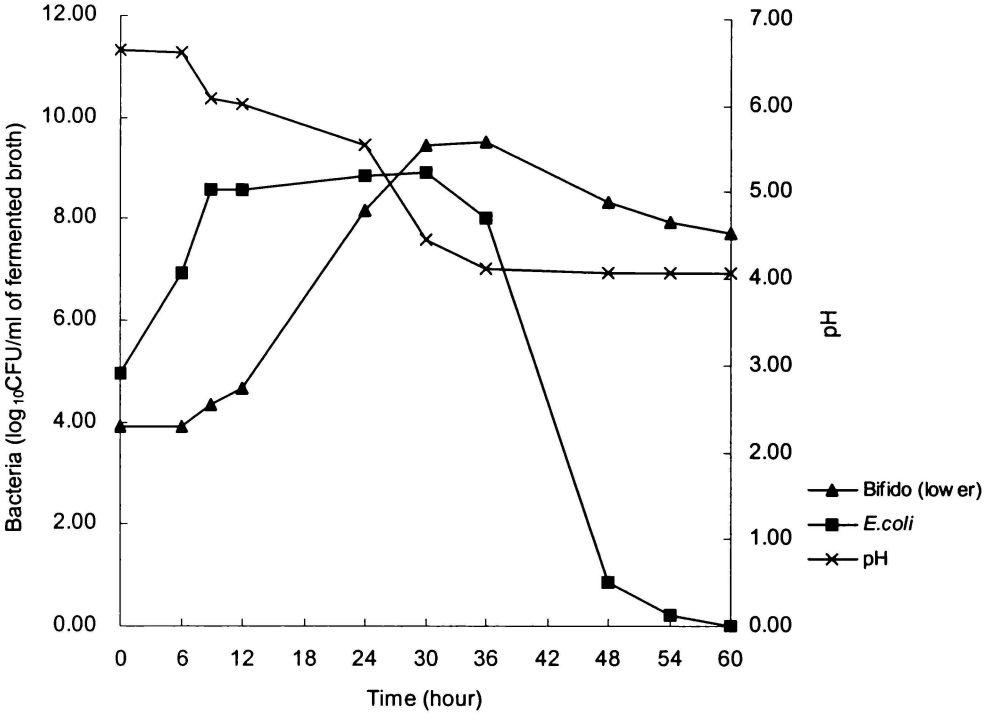


Figure 4.7 Growth curves of bacteria (Bifido = bifidobacteria) and pH of fermented broth during 60-hour fermentation in a co-culture of *E. coli* and a lower concentration of bifidobacteria in Raftilose-containing PY Broth. Data represent means of triplicate determinations.

The growth of bifidobacteria in the co-cultures (**Figure 4.6** and **Figure 4.7**) was similar to that in the monocultures (**Figure 4.8** and **Figure 4.9**), which indicated that the growth of bifidobacteria was not inhibited by the presence of *E. coli*. It should be noted that with the higher inoculum level (**Figure 4.6** and **Figure 4.8**), the number of bifidobacteria increased by approximately 1.30 $\log_{10}$ cycles,

reaching the maximum (approximately 10^9 CFU/mL) after 24-hour fermentation, and then began to decrease from 24 up to 60 hours. With the lower inoculum level (**Figure 4.7** and **Figure 4.9**), the numbers of bifidobacteria in the co-culture and the monoculture increased by 5.63 and 5.61 $\log_{10}$ cycles, reaching the stationary phases after 30 and 24 hours, respectively, and then remained at a high number (10^9 to 10^{10} CFU/mL) until 36 hours.

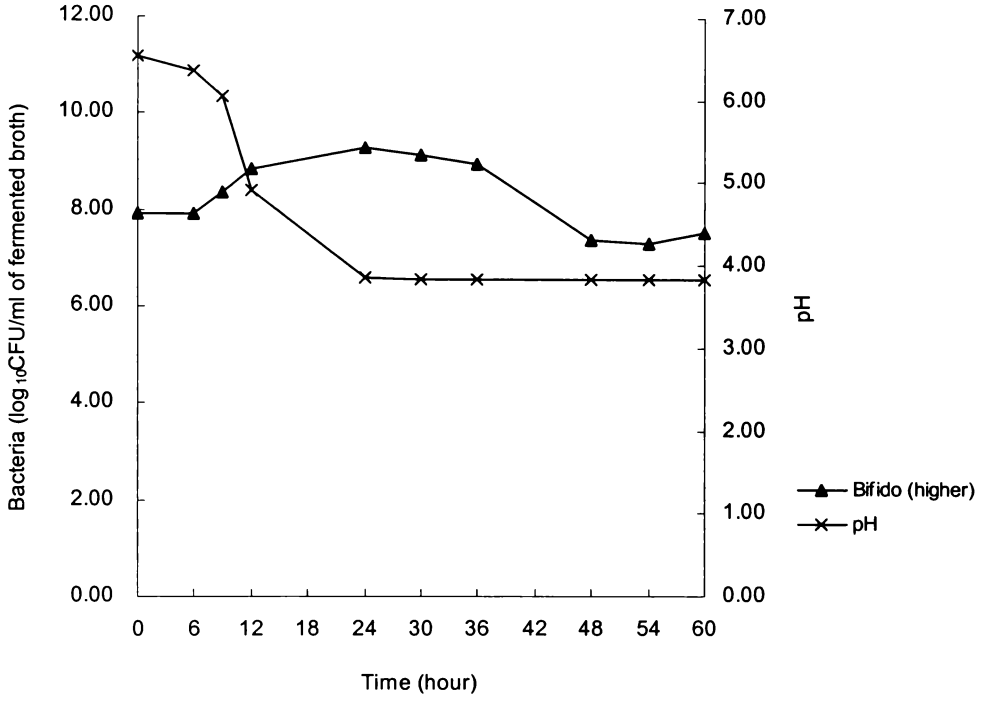


Figure 4.8 Growth curve of bifidobacteria (Bifido) and pH of fermented broth during 60-hour fermentation in a monoculture of a higher concentration of bifidobacteria in Raftilose-containing PY Broth. Data represent means of triplicate determinations.

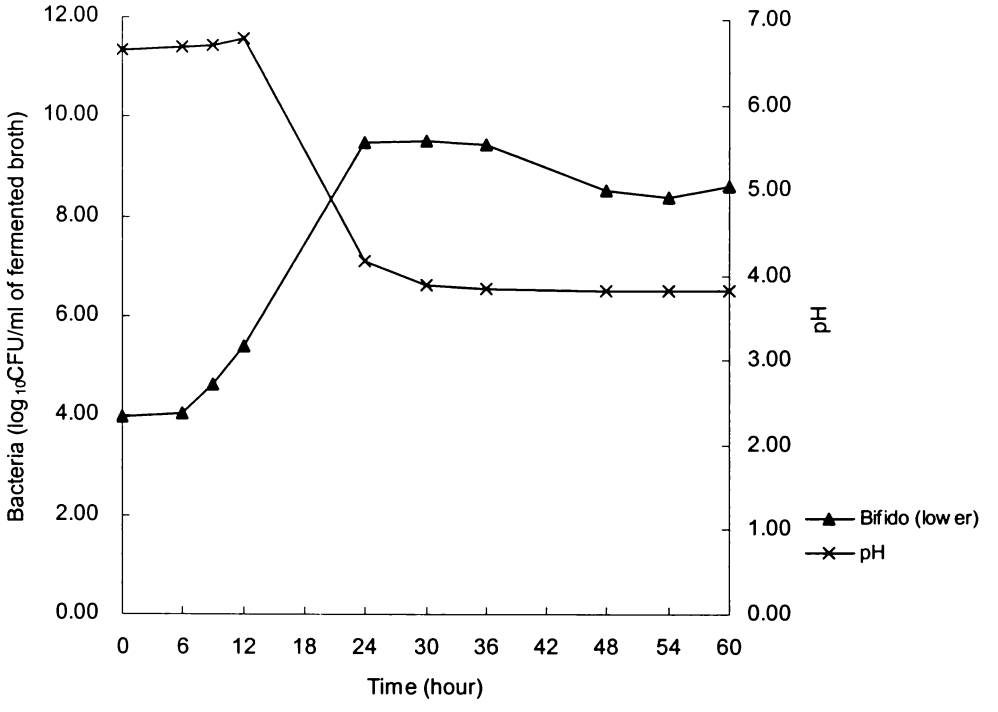


Figure 4.9 Growth curve of bifidobacteria (Bifido) and pH of fermented broth during 60-hour fermentation in a monoculture of a lower concentration of bifidobacteria in Raftilose-containing PY Broth. Data represent means of triplicate determinations.

The pH of the fermented broth was also measured (**Figure 4.5 - 4.9**). The initial value in all fermentation tubes was the same (7.0 ± 0.2). The pH decreased to approximately 4.0 after 24-hour fermentation by the co-culture of *E. coli* and the higher concentration of bifidobacteria (**Figure 4.6**); but decreased to approximately 4.1 only after 36-hour fermentation by the co-culture of *E. coli* and the lower concentration of bifidobacteria (**Figure 4.7**). In the monocultures the pH decreased to below 4.0 after 24 and 30-hour fermentation by the higher (**Figure 4.8**) and lower (**Figure 4.9**) concentration of bifidobacteria, respectively. The pH remained above 5.5 during the fermentation by an *E. coli* monoculture (**Figure 4.5**).

Fermentation product analysis showed that acetic and lactic acids were the most important products produced during the process of fermentation (**Figure 4.10** and **Figure 4.11**). As shown in **Figure 4.10**, high concentrations of acetic and lactic acids were achieved after 24-hour fermentation by the co-culture of *E. coli* and the higher concentration of bifidobacteria, reaching 74.83 and 57.05 mmol/L, respectively. During fermentation by the co-culture of *E. coli* and the lower concentration of bifidobacteria, high concentrations of acetic and lactic acids were achieved after 36 hours, reaching the maximum concentrations at 81.20 and 59.99 mmol/L, after 54 and 60 hours, respectively (**Figure 4.11**).

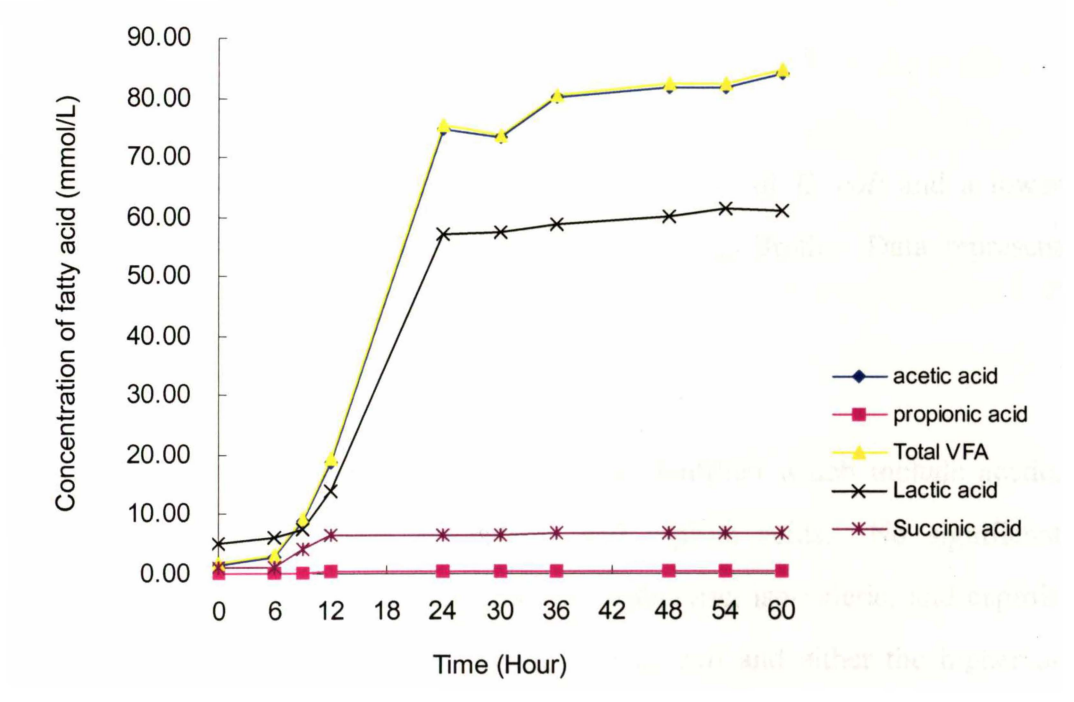


Figure 4.10 Fatty acid production from the co-culture of *E. coli* and a higher concentration of bifidobacteria in Raftilose-containing Broth. Data represent means of triplicate determinations.

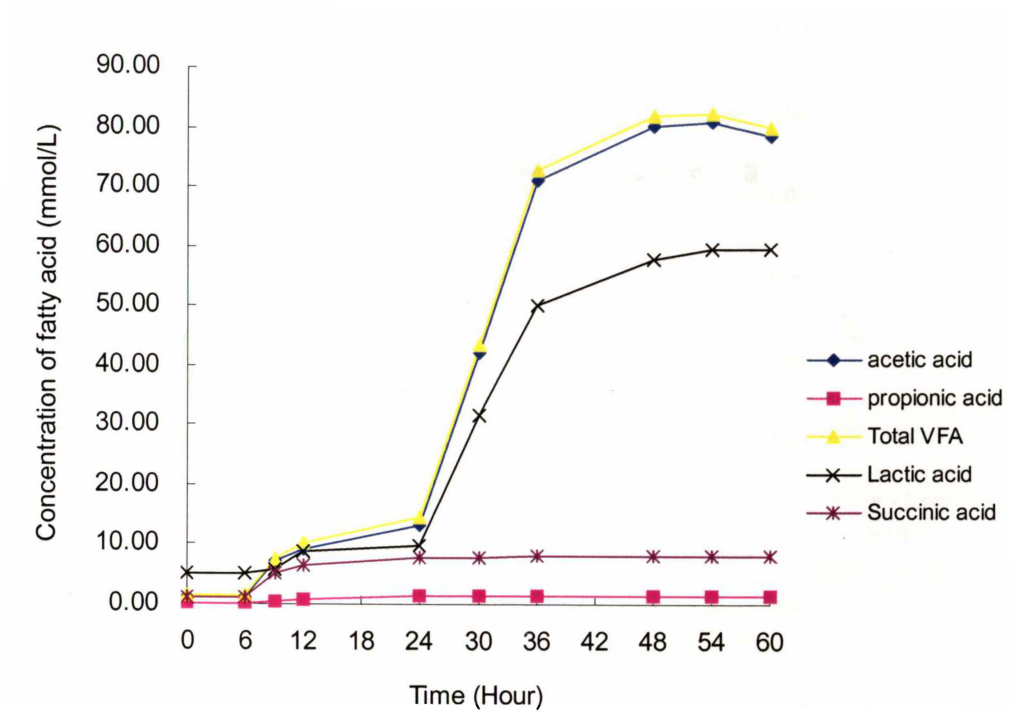


Figure 4.11 Fatty acid production from the co-culture of *E. coli* and a lower concentration of bifidobacteria in Raftilose-containing Broth. Data represent means of triplicate determinations.

Total VFA was the sum of volatile fatty acids identified which include acetic, propionic, butyric, isobutyric, iso-valeric, and caproic acids. No significant changes were observed in propionic, butyric, isobutyric, iso-valeric, and caproic acids during fermentation by the co-culture of *E. coli* and either the higher or lower concentration of bifidobacteria. Therefore, the concentration of total VFA achieved was mainly due to the production of acetic acid. This is shown in **Figure 4.10** and **Figure 4.11**.

Similar results were observed in the co-culture of *E. coli* and either the higher concentration or the lower concentration of bifidobacteria in Yacon-containing fermentation broth (**Figure 4.12 – 4.16**).

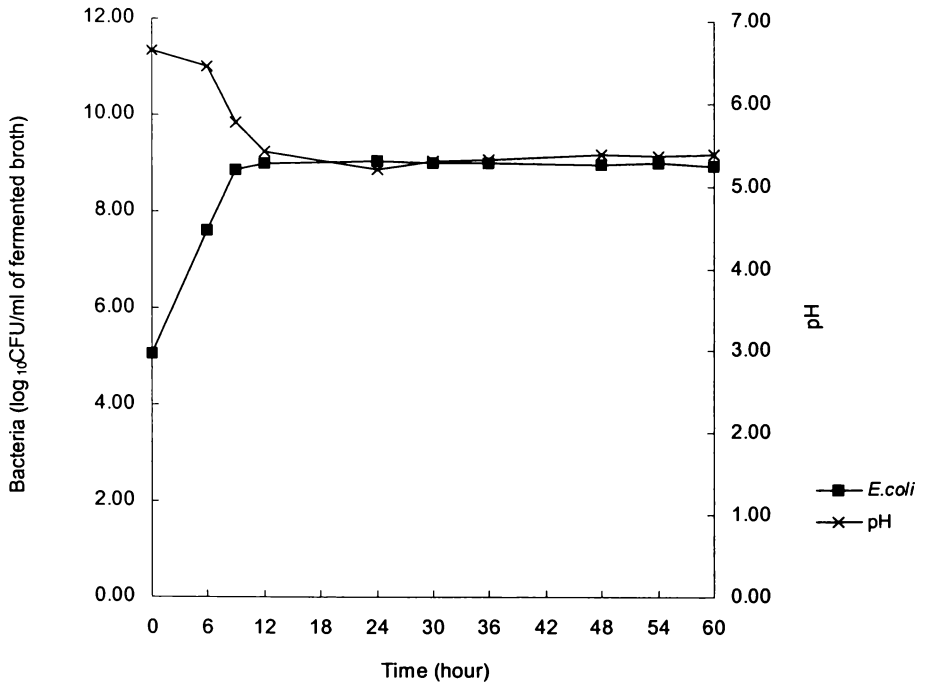


Figure 4.12 Growth curve of *E. coli* and pH of fermented broth during 60-hour fermentation in a monoculture of *E. coli* in Yacon-containing PY Broth. Data represent means of triplicate determinations.

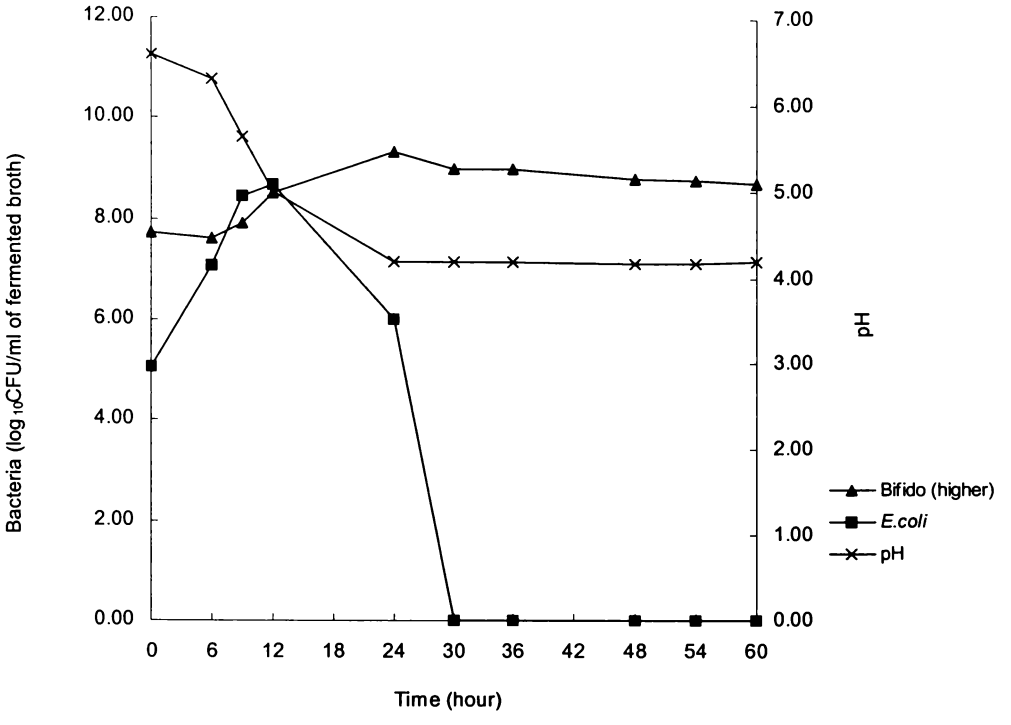


Figure 4.13 Growth curves of bacteria (Bifido = bifidobacteria) and pH of fermented broth during 60-hour fermentation in a co-culture of *E. coli* and a higher concentration of bifidobacteria in Yacon-containing PY Broth. Data represent means of triplicate determinations.

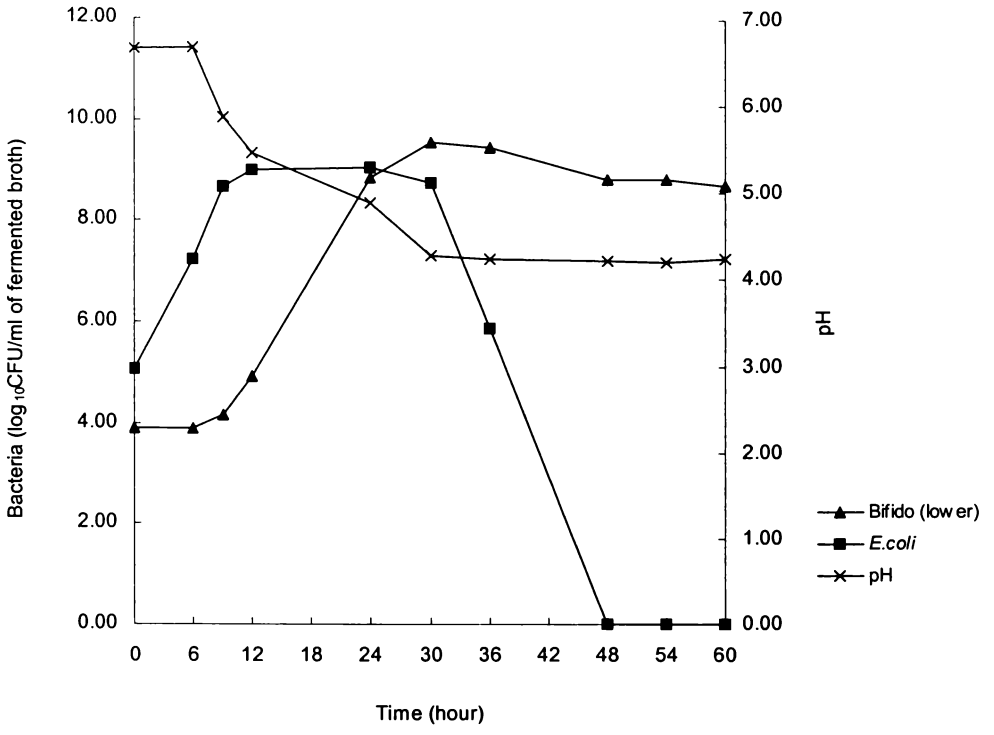


Figure 4.14 Growth curves of bacteria (Bifido = bifidobacteria) and pH of fermented broth during 60-hour fermentation in a co-culture of *E. coli* and a lower concentration of bifidobacteria in Yacon-containing PY Broth. Data represent means of triplicate determinations.

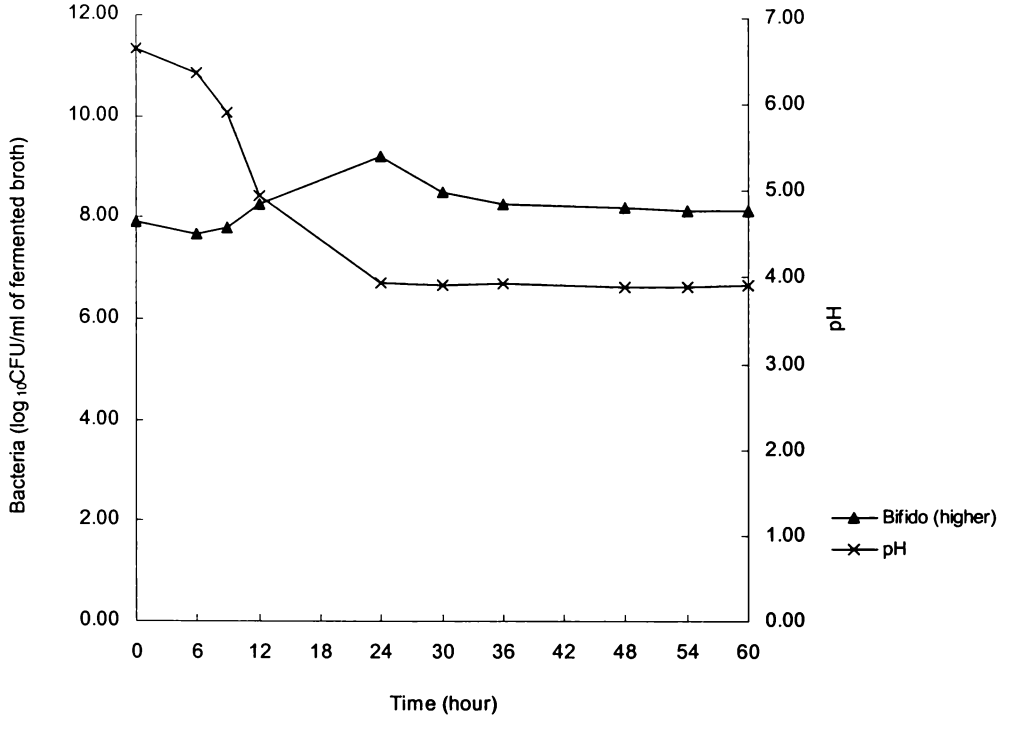


Figure 4.15 Growth curve of bifidobacteria (Bifido) and pH of fermented broth during 60-hour fermentation in a monoculture of a higher concentration of bifidobacteria in Yacon-containing PY Broth. Data represent means of triplicate determinations.

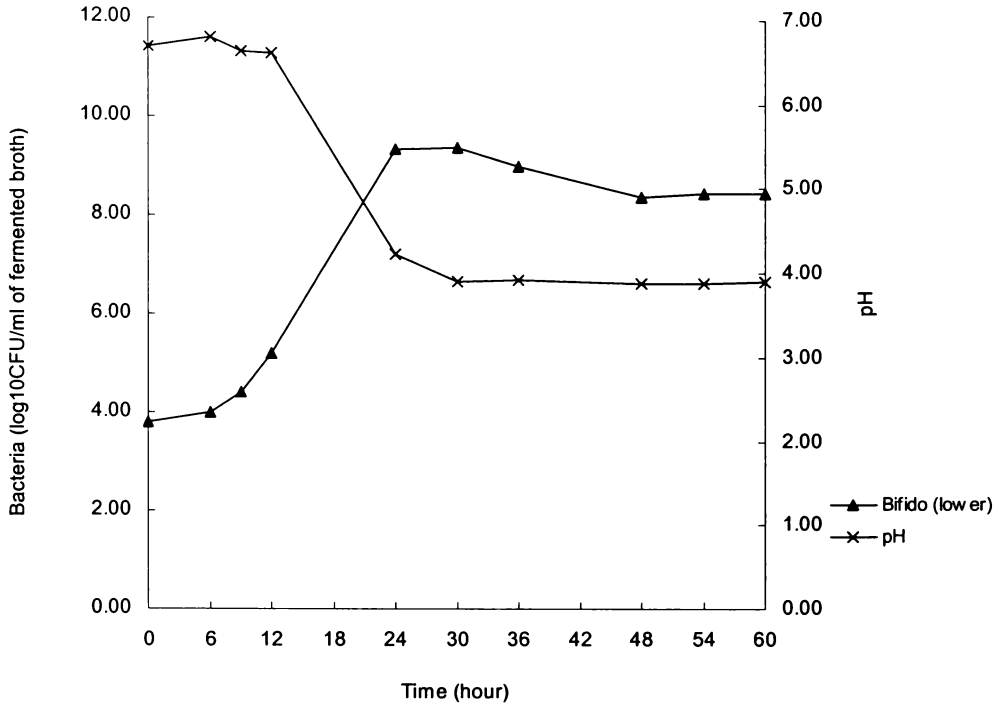


Figure 4.16 Growth curve of bifidobacteria (Bifido) and pH of fermented broth during 60-hour fermentation in a monoculture of a lower concentration of bifidobacteria in Yacon-containing PY Broth. Data represent means of triplicate determinations.

4.3.6 *Inhibition of in vitro growth of other potential intestinal pathogens by bifidobacterial strain P5-Po4-37*

Inhibition of *in vitro* growth on Raftilose and Yacon substrates, of the seven potential intestinal pathogens when co-cultured with bifidobacterial strain P5-Po4-37 was also investigated (**Table 4.4**). *E. coli* served as a reference control. The growth of all the test strains, with the exception of *Enterococcus faecalis* R65, was completely inhibited after 48-hour co-culture with bifidobacterial strain P5-Po4-37, reaching 100% inhibition rate. A slightly lower inhibitory effect, but still a high inhibition rate (99.86%), on *Enterococcus faecalis* R65 was observed after 48-hour incubation with bifidobacterial strain

P5-Po4-37 in Raftilose- containing PY broth. The pH in each tube was also measured after 48-hour growth and showed a decrease from the initial value of 7.0 ± 0.2 to approximately 4.0 or below in all the co-culture fermentation tubes. On the other hand with monocultures of the potential intestinal pathogens, the pH remained at a much higher level than that of co-cultures throughout the 48-hour fermentation.

Table 4.4 Inhibition of *in vitro* growth of other potential intestinal pathogens by bifidobacterial strain P5-Po4-37. Data represent means of triplicate determinations of % inhibition of test strains with individual standard error of means and final pH in the co-cultures and monocultures with standard errors of differences of means.

Strains of potential intestinal pathogens	**% inhibition of intestinal pathogens**		**Final pH in co-cultures**		**Final pH in monocultures**	
	Raftilose	**Yacon**	**Raftilose**	**Yacon**	**Raftilose**	**Yacon**
Clostridium perfringens R70	100	100	4.00	3.91	5.21	6.50
Enterobacter aerogenes R62	100	100	3.88	3.80	5.58	5.43
Enterococcus faecalis R65	99.86 ± 0.06	100	3.84	3.84	4.38	5.57
Escherichia coli NZRM 916	100	100	4.03	3.96	5.19	5.95
Klebsiella pneumoniae R54	100	100	3.89	3.77	4.23	5.26
Salmonella Dublin ATCC 15480	100	100	3.94	3.86	5.03	6.07
Salmonella Menston R71	100	100	3.96	3.88	5.03	5.90
Standard errors of differences of means			0.0094		0.1468	

4.4 Discussion

Three commercial fructooligosaccharide products, together with 18 bifidobacterial isolates from sheep faeces were investigated in a series of mixed culture studies to determine their capacity for and mechanisms of, inhibitory action against *E. coli*. *In vitro*, the inhibition on the growth of other potential intestinal pathogens by sheep faceal bifidobacteria was also investigated.

Before the mixed culture studies, two pure culture studies were carried out. In one, seven potential intestinal pathogens were investigated for their capacity for *in vitro* utilisation of Fibrulose, Raftilose, AG, and Fibruline. These bacteria were selected on the basis of their potential adverse influences on host health. Under normal conditions they may have little detrimental effect on host health; but nevertheless have a potential to become pathogenic for example when the host becomes stressed. The results indicated that all of the potential pathogens trialled grew well and utilised the test oligo- and polysaccharides with the exception of *Clostridium perfringens* R70 which did not utilise any of the test carbohydrates (**Table 4.2**). Similar results have been obtained in some pure batch culture studies conducted by others (Hidaka *et al.*, 1986; Gibson and Wang, 1994a). These authors determined the utilisation of FOS by intestinal bacteria *in vitro*. In their studies, bacteria other than bifidobacteria, such as *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Salmonella* spp., *Clostridium perfringens*, and *Escherichia coli*, also grew, to varying extents, in media containing FOS as the sole carbohydrate source. Oyarzabal and Conner (1995) also compared the utilisation of FOS-50 and a pure formulation of FOS by *Salmonella* spp., which showed that all test *Salmonella* serotypes utilised FOS-50 for growth, but did not utilise the pure formulation of FOS. The authors explained that this difference was due to the different compositions between these two forms of FOS: FOS-50 is composed of 50% solids, 57% of which are FOS, 33% are glucose and fructose,

and 10% are sucrose. The presence of glucose, fructose, and sucrose in the formulation most likely accounted for the growth observed with *Salmonella* serotypes in medium containing FOS-50; in contrast, no mono- and disaccharides, which could serve as growth substrates for *Salmonella* spp., were present in the pure formulation of FOS. This explanation demonstrated that FOS itself cannot be utilised by *Salmonella* as a growth substrate. All the test FOS used in the present studies contain small quantities of mono- and disaccharides. The presence of these lower MW and DP sugars, while not FOS itself, may be the reason for the *in vitro* growth observed with the test, potential pathogenic, intestinal bacteria. Roberfroid and Slavin (2000) also highlighted this, in their review article: "*even if in pure cultures miscellaneous bacterial species have the capacity to use FOS as fermentation substrate, in mixed cultures mimicking the large bowel as well as in vivo in human volunteers, they may selectively stimulate the growth of bifidobacteria and thus be "bifidogenic" and classified as "prebiotics"*. Similar speculation was presented by Oyarzabal and Conner (1995), who presumed that the mono- and disaccharides in FOS products can be absorbed in the upper part of the intestinal tract when FOS is fed to humans and animals, and that intact FOS molecules reach the lower part of the GIT, where they selectively stimulate the growth of bifidobacteria, and these in turn exert an inhibitory effect on the potential intestinal pathogens and putrefactive bacteria. A number of clinical studies have also been carried out and these results showed that bifidobacteria utilise NDOs more rapidly than species of pathogens and are the dominant bacteria in the human intestine (Hidaka *et al.*, 1990). Based on these explanations and hypotheses, it appears that *in vitro* pure culture experiments of intestinal bacteria in media containing FOS are just basic screens for the capacity for the test strains to utilise the FOS. However, it cannot be concluded that FOS is not a good food or feed ingredient for human or animal health merely because they can also be utilised by some potential intestinal pathogens. It is highly likely that mono- and disaccharides will be removed in the upper part of the GIT.

In the second series of pure culture studies, 117 bifidobacterial strains isolated from sheep faeces, and 4 reference strains of *Bifidobacterium* spp.: *B. bifidum* DSM 20 239, *B. pseudolongum* subsp. *globosum* DSM 20 092, *B. animalis* DSM 20 104, and *B. boum* DSM 20 432, were screened for their capacity for *in vitro* utilisation of Yacon, Raftilose, AG, or Fibruline on modified RB agar plates. Fibrulose was substituted by Yacon in this study and in subsequent mixed culture studies as Yacon has similar chemical characteristics to that of Fibrulose and is a local product. The results demonstrated that Raftilose and Yacon exhibit strong fermentability with most of the test strains and were consequently selected for use as carbon sources in the subsequent mixed culture studies. Fibruline was also tested in the mixed culture studies regardless of the absence of marked fermentation patterns. This was because of the significant "bifidogenic" effect on the growth of bifidobacteria obtained in the *in vivo* experiment (see Chapter 3). The test strains were stimulated by Raftilose and Yacon, however, as they were reluctant to utilise either Fibruline or AG, this seemed to indicate that utilisation of oligo- and polysaccharides by bifidobacteria is determined by the DP of the substrates. These results are in agreement with those obtained by Gibson and Wang (1994a), Tashiro *et al.* (1997), and Bielecka *et al.* (2002). In those studies, low DP FOS containing traces of mono- and disaccharides stimulated the *in vitro* growth of bifidobacteria, whereas high DP and highly purified inulin preparations did not. The results of the present study also indicated that the degrees of substrate utilisation differed between different test strains of bifidobacteria, which might belong to different species within the genus *Bifidobacterium*. If the ability of bifidobacteria to ferment FOS is a species-dependent feature (Bielecka *et al.*, 2002), this should be taken into consideration in future work with only the species possessing strong fermentation activity on FOS being selected as probiotics. At this stage of the study, eighteen strains with strong fermentation patterns and exhibiting typical growth and physiological characteristics of bifidobacteria were selected for use in mixed culture studies. It is also notable from the results of the present study that one reference culture of bifidobacteria, *B. bifidum* DSM 20 239,

did not grow in media containing any test oligo- and polysaccharides. This result is in agreement with the studies by Hidaka *et al.* (1986); Gibson and Wang (1994a); Hartemink and Rombouts (1997); and Bielecka *et al.* (2002). These studies showed fairly slow or negligible growth of *B. bifidum* strains in media containing FOS and most of the other investigated oligo- and polysaccharides such as inulin, transgalactosyloligosaccharides, galactosyllactose, isomalto-oligosaccharides, raffinose, lactulose, and lactitol.

On the basis of the results from two pure culture studies, a series of mixed culture studies were then carried out to investigate three FOS products and 18 bifidobacterial strains isolated from sheep faeces for their capacity to inhibit the growth of potential intestinal pathogens.

In the first preliminary experiment, five 4-strain mixtures of bifidobacteria comprising the 18 bifidobacterial isolates plus 2 reference cultures of bifidobacteria, *B. pseudolongum* subsp. *globosum* DSM 20 092 and *B. animalis* DSM 20 104, were evaluated. Their inhibitory activities against the *in vitro* growth of *E. coli* when growing in PY media containing Yacon, Raftilose, Fibruline or no carbohydrate were compared by measurement the inhibition of *E. coli* after a 48-hour anaerobic incubation at 37°C. *E. coli* was selected as a representative of the test potential intestinal pathogens because it is a normal inhabitant of the mammalian GIT and is commonly used as an indicator to assess the hygiene and cleanliness of many food processes. It is also easy to cultivate *in vitro* and exhibits rapid growth. In addition, the inhibition of *E. coli* by bifidobacteria has been reported by Wang and Gibson (1993), which further supports the use of *E. coli* an ideal reference strain for selecting sheep faecal bifidobacterial isolates for their inhibitory features. The results of this preliminary experiment showed that two of the five groups significantly inhibited the growth of *E. coli* ($P < 0.001$), compared with the other 3 groups (**Figure 4.1**). The 8 strains in the two groups with inhibitory activities against *E. coli* were

therefore selected for use in the second preliminary experiment. As the inhibition of *E. coli* on Raftilose and Yacon were significantly higher than that on Fibruline and control ($P < 0.001$), Fibruline was eliminated from subsequent experiments. Similarly, the 8 bifidobacterial isolates were evaluated and compared for their inhibitory activities against the *in vitro* growth of *E. coli* when growing in PY media containing Raftilose and Yacon by measurement of the inhibition of *E. coli* after a 48-hour anaerobic incubation at 37°C. Six of the eight strains showed 100% inhibitory activities against the growth of *E. coli*; consequently these six strains were promising candidates for use as probiotic products in the future research.

The pH of the fermented broth in each tube was also determined in these two preliminary experiments, both of which showed clear negative correlations between pH of fermented broth and the rank transformed or angular transformed inhibition rate of *E. coli* (**Figure 4.3** and **Figure 4.4**). Based on the data in these figures, the effective pH value required for complete inhibition of the growth of *E. coli* was approximately 4.0. Compared with the initial pH in the media which was 7.0 ± 0.2, the marked decrease in pH and the clear negative correlations obtained between pH of fermented broth and the rank transformed or angular transformed inhibition rate of *E. coli*, showed that the inhibitory effect against *E. coli* by the bifidobacterial strains isolated from sheep faeces was probably an acid-dependant process.

Further investigations in bacterial growth kinetics and production of SCFA in co-culture of bifidobacteria and *E. coli* in Raftilose-containing media were carried out to elucidate the acid-dependant mode of action of the antibacterial effect of the sheep faecal bifidobacteria. One of the six most inhibitory strains, P5-Po4-37, was selected for use in this experiment because its maximum biomass was reached and 100% inhibition rate of *E. coli* was obtained within the trial period. Two different concentrations of cells of this bifidobacterial strain were co-cultured

with *E. coli* anaerobically at 37°C for 60 hours. Each of the above bacteria was also mono-cultured under the same conditions as pure culture controls. The growth curve for each test bacterium in mono-cultures or co-cultures and the pH change were obtained during a 60-hour fermentation (**Figure 4.5 – 4.9**).

The interactions between *E. coli* and higher concentration of bifidobacteria and the pH change were monitored by determination of bacterial growth kinetics during a 60-hour co-culture of these two bacteria (**Figure 4.6**). The production of fermentation acids during this co-culture fermentation was also determined periodically (**Figure 4.10**). The exponential phase of bifidobacterial growth lasted from 6 to 12 hours; coinciding with this the pH decreased markedly, while *E. coli* grew well, reaching the stationary phase. During this phase a rapid increase in concentration of acetic acid from 1.26 to 18.69 mmol/L and a smaller increase in concentration of lactic acid from 5.16 to 14.03 mmol/L were observed (**Figure 4.10**). The concentrations of these two acids achieved at this point may not be high enough to exert a bactericidal effect on the growth of *E. coli*. After 12 hours bifidobacteria entered the stationary phase and reached maximum population at 24 hours. During this period, pH continued to decrease to its lowest value at 24 hours, and *E. coli* also began to decrease rapidly to a low level but was not completely inhibited. The determination of fermentation acids during this phase (**Figure 4.10**) showed marked increases of both acetic acid and lactic acid, reaching near their highest values at 24 hours, 74.83 and 57.05 mmol/L, respectively. Continued fermentation from 24 to 60 hours saw bifidobacteria beginning to decrease slightly, pH remained at a low level, and *E. coli* reached zero after another 6 hours. During this period, the concentrations of acetic and lactic acids increased further reaching 84.32 and 61.47, respectively (**Figure 4.10**).

Low pH and high concentrations of fermentation acids have a bactericidal potential, especially against Gram-negative bacteria. Because the undissociated

species of the fermentation acids increase as the pH declines, it has generally been assumed that the antimicrobial activity of fermentation acids at low pH was caused by their undissociated species (Salmond *et al.*, 1984). Acetic and lactic acids are produced by bifidobacteria, in the theoretical ratio of 3:2. Acetic acid has a stronger antimicrobial activity than lactic acid, which is not only due to the greater quantities of acetic acid produced by bifidobacteria, but perhaps more importantly, to its smaller dissociation constant (K_a). This means that at the same pH, an organic acid with a smaller K_a value presents a larger proportion in the undissociated form, and thus has a stronger antimicrobial activity (Savage, 1977; Rasic and Kurmann, 1983; Modler *et al.*, 1990; Tamime, 1999). Just as pH is the negative logarithm of the $[H^+]$ concentration, pK_a is the negative logarithm of the dissociation constant K_a. Acetic acid has a pK_a of 4.76 while lactic acid has a pK_a of 3.87. At a pH of 4.0, which was the lowest pH value achieved after 24 hours of co-culture of *E. coli* and the higher concentration of bifidobacteria in the present studies, 74.83 mmol/L of the acetic acid and 57.05 mmol/L of the lactic acid were produced, respectively. The concentrations of the dissociated and undissociated species in the acetic and lactic acids can be calculated using the Henderson-Hasselbalch equation ($pH = pK_a + \log [A^-]/[HA]$, where A^- and HA are the dissociated and undissociated species; Russell and Diez-Gonzalez, 1998) which relates dissociation to pH and pK_a. Using this equation, at pH 4.0, 63.74 mmol/L of the acetic acid was present in the undissociated form which is 5.75 times the concentration of its dissociated form, while 24.28 mmol/L of the lactic acid were present in the undissociated form, which is 0.74 times the concentration of its dissociated form. It is possible therefore that, the mechanism of the antagonistic activities of the sheep faecal bifidobacteria is due to the production of the organic acids by bifidobacteria, in particular, the high concentrations of undissociated acetic acid.

Regarding the toxic effect of the undissociated fermentation acids, the traditional explanation is that undissociated fermentation acids can pass across the cell

membrane and dissociate in the more alkaline interior, which causes an accumulation of the anionic species. This fermentation anion accumulation is dependant on the pH gradient (ΔpH) across the membrane and the change of intracellular potassium concentration. Fermentation acid-resistant bacteria, in general, Gram-positive lactic acid-producing bacteria, have low ΔpH and high intracellular potassium concentration, which counteract the fermentation acid anions; whereas the acid-sensitive bacteria such as *E. coli*, are able to decrease intracellular pH to only 6.1 before growth ceases. This limited decrease and a relatively small increase of the potassium levels can only partially counteract the toxic effect of the fermentation anion accumulation which will eventually inhibit the growth of *E. coli* (Russell and Diez-Gonzalez, 1998). Another 6 hours were still required for complete inhibition of the growth of *E. coli* after the pH reached the lowest values and near maximum concentrations of acetic and lactic acids were attained. This may result from the time-required for the bactericidal process to occur as undissociated acetic acid passes across the cell membrane, dissociates in the more alkaline interior, anionic species accumulate, and toxic effects on *E. coli* are expressed. Bifidobacteria began to slightly decrease from 24 to 60 hours because bifidobacteria are also adversely affected by the accumulation of their own metabolic by-products.

The difference between the two experiments shown in **Figure 4.6** and **Figure 4.7** was the initial inoculum size of bifidobacteria. This difference caused some differences in the fermentation results. As shown in **Figure 4.7**, when a lower concentration of bifidobacteria was co-cultured with *E. coli*, a similar inhibitory effect against *E. coli* by bifidobacteria was observed. However, compared with the growth curves observed in **Figure 4.6**, a longer lag phase and a longer exponential phase was achieved for bifidobacteria. The exponential phase could be divided into two parts. The first period was from 12 to 24 hours, in which the number of bifidobacteria increased markedly to 10^8 CFU/mL of fermented broth, similar to the initial counts of bifidobacteria shown in **Figure 4.6**, while pH

decreased slowly; the concentrations of acetic and lactic acids increased slowly (**Figure 4.11**) and *E. coli* remained at the stationary phase during this period. The second period was from 24 to 30 hours, in which the changes of all the determined parameters were quite similar to that which happened during the exponential phase of the higher bifidobacteria inoculum (**Figure 4.6**). This longer exponential phase observed in **Figure 4.7** indicated that *E. coli* competed for nutrition with Bifiodobacteria during this period, and bifidobacteria, which was inoculated with lower counts than *E. coli*, exhibited longer lag and exponential phases before reaching the required high number to inhibit the growth of *E. coli*. Interestingly, with the higher inoculum level, the populations of bifidobacteria increased by only approximately 1.30 $\log_{10}$ cycles (**Figure 4.6**); whereas with the lower inoculum level, the populations of bifidobacteria increased by approximately 5.62 $\log_{10}$ cycles (**Figure 4.7**), both of which, however, reach the same level of maximum viable counts at approximately 10^9 CFU/mL. One possible explanation for reaching the same maximum number of bifidobacteria with different inoculum sizes may be that the same amount of the substrates were used in these two experiments and became exhausted when sustaining 10^9 CFU/mL. Alternatively the metabolic by-products may have accumulated to become inhibitory to bifidobacteria when the bifidobacteria population reached 10^9 CFU/mL. Alternatively the bifidobacterial density may be limited to 10^9 CFU/mL by cell proximity. The explanation for the different stimulation sizes is as discussed by Roberfroid and Delzenne (1998). They suggest that the relationship between increase in bifidobacteria and the dose or nature of NDO is not correlative. It is the initial number of bifidobacteria in the faeces, before supplementing the diet with the NDO, which may influence the size of the stimulation. There is a negative correlation between the increase in bifidobacteria and their initial population, that is, the lower the initial count of bifidobacteria, the larger the stimulation. This phenomenon is also observed by Rycroft *et al.* (2001) in their *in vitro* batch fermentation experiment. The results in **Figure 4.6** and **Figure 4.7** both demonstrated that the effective populations of

bifidobacterial strain P5-Po4-37 for inhibiting the growth of *E. coli* were approximately 10^9 CFU/mL. To achieve this number, 1.0% of Raftilose was used as an effective dose *in vitro*.

The growth curve and the pH change of the higher bifidobacteria inoculum levels in monoculture (**Figure 4.8**) were the same as those in co-culture with *E. coli* (**Figure 4.6**), which indicated that the presence of *E. coli* did not inhibit the growth of bifidobacteria, and the pH change is due to the presence of bifidobacteria; whereas the growth curve and the pH change of the lower bifidobacteria inoculum in co-culture with *E. coli*, when compared with monoculture, were observed (**Figure 4.7**) to have a delay of 6 hours to start the exponential phase, a delay of 6 hours to start the stationary phase, and a delay of 6 hours to reaching the lowest pH level (**Figure 4.9**). This may be attributed to the presence of *E. coli* which competed for nutrition, mono- and disaccharide components, in the FOS fermentation broth.

Inhibition of *in vitro* growth of other potential enteric pathogens was also investigated in co-culture with bifidobacterial strain P5-Po4-37 in media containing Raftilose and Yacon under anaerobic condition at 37°C for 48 hours. These results (**Table 4.4**) showed bifidobacterial strain P5-Po4-37 also exerted strong antagonistic activities against *Clostridium perfringens* R70, *Enterobacter aerogenes* R62, *Enterococcus faecalis* R65, *Klebsiella pneumoniae* R54, *Salmonella* Dublin ATCC 15480, and *Salmonella* Menston R71. The final pH values associated with inhibition of growth of these bacteria were also approximately 4.0. This suggests that the mechanism for the inhibitory action of bifidobacterial strain P5-Po4-37 against these bacteria may be also due to the production of acetic and lactic acids.

In summary, Raftilose and Yacon both selectively stimulate the growth of sheep faecal bifidobacteria and so are "bifidogenic" in mixed culture studies *in vitro*.

They also potentiate the inhibitory action of bifidobacterial strain P5-Po4-37 against the test potential intestinal pathogens and therefore prove that they are "prebiotics", that is, "*nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon, that can improve host health*" (Gibson and Roberfroid, 1995). The principal mechanism for the inhibitory action of bifidobacterial strain P5-Po4-37 is the production of acetic and lactic acids. At this stage, such a demonstration has been made convincingly only *in vitro*. A combination of Raftilose or Yacon as a prebiotic carbohydrate (with a 1.0% final concentration as the effective dose), and the most inhibitory bifidobacterial strain P5-Po4-37 (with 10^9 CFU/mL as the effective dose) as an effective probiotic strain looks promising as a synbiotic to manipulate sheep hindgut microflora.

Chapter 5

SUMMARY DISCUSSION, CONCLUDING REMARKS AND SUGGESTIONS FOR FUTURE RESEARCH

The intestinal microflora of mammals influence host health and well-being. In a healthy state, these microorganisms establish an equilibrium between predominant beneficial flora such as bifidobacteria and lactobacilli, and low populations of potential pathogenic bacteria such as *E. coli* and *Clostridium* spp. However, the composition of the intestinal flora can be altered by many endogenous and exogenous factors such as aging, stress, diet, environment, and use of medicines (Holzapfel *et al.*, 1998). Such factors may shift the balance of the intestinal flora away from potentially beneficial bacteria, and towards a predominance of potentially harmful or pathogenic microorganisms. This shift may, in turn, result in a variety of clinical disorders and even cancer. The host is also more susceptible to infections by transient enteropathogens such as *Salmonella*, *Campylobacter*, *E. coli* and *Listeria* spp.

There is, therefore, increasing interest in manipulation of the composition of the gut microflora towards a more salutary community, that is, an increase in numbers and activities of bacterial groups such as bifidobacteria and lactobacilli, that may have health-promoting properties. Probiotics, prebiotics, or synbiotics are widely used to obtain this effect.

Probiotics, are *live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance* (Fuller, 1989). Many microorganisms have been used or considered for use as probiotics. The most

typical active components of probiotic products are strains of LAB, particularly *Lactobacillus* spp. and *Bifidobacterium* spp., which have been demonstrated, by a variety of *in vitro* and *in vivo* studies, to exert various nutritive and therapeutic benefits in humans and animals. These include: suppression of potential pathogens and putrefactive bacteria, maintenance and improvement of normal intestinal conditions, prevention and/or remediation of enteric infections, alleviation of constipation in the elderly, suppression of some cancers, stimulation of the immune system, enhancement of lactose digestion, reduction in serum cholesterol levels, and production of certain nutritional substances (Modler *et al.*,1990; Ballongue, 1993; Yaeshima, 1996; Duffy *et al.*, 1999). However, such beneficial changes may be transient, and the intestinal colonisation by exogenous bacteria may be limited and of short duration.

An alternative method is to stimulate selective growth of indigenous gut bacteria by feeding the host prebiotics. A prebiotic is *a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve host health* (Gibson and Roberfroid, 1995). A number of non-digestible oligosaccharides (NDOs) have now been developed for use as prebiotics. Among them, fructooligosaccharide (FOS) have been the most thoroughly investigated. FOS are good *in vitro* carbon sources utilised by some isolated intestinal bacteria; and *in vivo*, escape enzymic hydrolysis in the upper gastrointestinal tract, reaching the large intestine without change to their structure, thereby, becoming potential substrates for fermentation by the microflora, especially by the bifidobacteria in the lower gut. By stimulating bifidobacteria, FOS may stimulate a variety of health benefits in human and animals, as discussed above.

In addition to probiotics and prebiotics, another approach that future research will focus on is the combination of both probiotics and prebiotics as synbiotics. A

synbiotic may be defined as *a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare* (Gibson and Roberfroid, 1995). As stated in this definition, the use of synbiotics may offer the dual/synergistic benefits of both approaches; the use of a selective substrate (prebiotic) may help long term persistence of growth and/or activities of both the exogenous live microorganisms (probiotics) and endogenous bacteria (Roberfroid, 1998; Gibson, 2001).

To date, the application of these approaches and their potential beneficial effects have been largely studied in humans and some other monogastric animals. Very few studies, however, have been made to determine their effects on ruminant intestinal microflora.

The overall purpose of the present project was to test four commercial food-grade oligo- and polysaccharides (three FOS products and one polysaccharide control, Arabinogalactan) for their potential application in manipulating hindgut microflora of sheep through an abomasal supplementation model. These prebiotics were expected to modify hindgut microflora of sheep by selective stimulation of the health-promoting bacterial groups, lactobacilli and bifidobacteria, and selectively suppress the potential intestinal pathogens and putrefactive bacteria *E. coli* and sulphite-reducing clostridia. This effect, reduced pathogen carriage, is of value to New Zealand agriculture (meat industry) by potentially reducing the risk of carcass contamination with enteric bacteria, and is also important in improving animal health, well-being, and digestion.

In the first stage of the project, an enzyme-based identification protocol was developed for the identification and large-scale screening of presumptive

bifidobacteria isolates from the gut contents or faecal samples. In this protocol, the detection of the enzyme fructo-6-phosphate phosphokatolase (F6PPK) activity was optimised, for a more rapid and sensitive enzyme reaction, this was achieved by using cells in the exponential growth phase, using Triton X-100 for F6PPK release to enhance colour development and test sensitivity, using shaking -incubation for the enzyme reaction, and using an enzyme reaction time at 37°C of 45 minutes.

The F6PPK test played an integral part in the detection, isolation, differential enumeration, and identification of sheep faecal bifidobacteria, which were carried out in the subsequent animal experiment. The presence of other sheep hindgut bacteria: lactobacilli, total anaerobes, *E. coli*/*Enterobacteriaceae*, and sulphite reducing clostridia were concurrently investigated to determine the bifidogenic effect (promotion of bifidobacteria by prebiotics) and the associated antimicrobial effect (suppression of pontential pathogens due to the increase of the populations of bifidobacteria) of the four oligo- and polysaccharides on sheep hindgut microflora. Twelve fistulated sheep were managed in a balanced, two Latin square, cross-over design experiment, which was run in 5 consecutive periods, with each of 5 treatments (Arabinogalactan, Fibruline, Raftilose, Yacon, and "acidified saline" as a no carbohydrate-introduction control) administered to two sheep in each period. Each period consisted of a 1 week of stabilisation to the pelleted diet (no oligo- and polysaccharides), followed by 14 days of daily abomasal supplementation of oligo- and polysaccharide/acidified saline, followed by an approximately 12 day recovery period of normal pelleted diet. Microbiological and chemical analysis were carried out on day -4 of the "stabilisation" period; days 3, 9 of the "treatment supplementation" period, and days 15, 16, 19 & 26 after cessation of infusions in each period. Raftilose, Yacon, and Fibruline, all belonging to the FOS group, exerted significant bifidogenic effects on sheep faecal bifidobacteria after 9 days of daily dosing. Raftilose resulted in the highest promotion of bifidobacteria by 2.128 log units,

reaching the highest counts of bifidobacteria at approximately 10^7 CFU/g of faeces. These three FOS products were selected as promising bifidogenic factors for use in ruminants. In this study, the effective dose and the time required for exerting a bifidogenic effect was 7 g/d for at least 9 days. In contrast, no significant changes of the populations of bifidobacteria were observed in AG-treated sheep, which demonstrated that AG cannot be used as a bifidogenic factor in the sheep hingut microbial ecosystem. Raftilose and Yacon also significantly increased the number of lactobacilli, by 1.286 and 1.138 log units, respectively, reaching approximately 10^7 CFU/g of faeces, after 9 days of daily dosing; Fibruline increased lactobacilli after 9 days of administration, but did not reach a significant level; AG did not elevate the populations of lactobacilli. Lactobacilli are closely related to bifidobacteria and are another group of health-promoting bacteria, exerting various health effects on humans and animals, and thereby served as additional indicator bacteria in this study for determining the prebiotic effect of the test oligo- and polysaccharides. All four carbohydrate treatments increased the number of total anaerobes to approximately 10^7 to 10^8 CFU/g of faeces after 9 days of daily dosing in comparison with the no carbohydrate control. This was almost certainly due to the increase in the number of bifidobacteria and lactobacilli, both of which are relatively dominant genera in the gut of humans and animals. In summary, the effective stimulation on bifidobacteria, lactobacilli, and total anaerobes by FOS has been demonstrated; however, the other determined groups of gut microflora: sulphite reducing clostridia and *E. coli/Enterobacteriaceae* still remained at the pre-trial level after the treatment by the test oligo- and polysaccharides.

Further *in vitro* assays were carried out to test whether or not the bifidobacterial strains, which had been isolated from sheep, would inhibit the growth of potential intestinal pathogens in the fermentation broth containing the bifidogenic FOS selected from the animal experiments. Eighteen bifidobacterial isolates with strong fermentation patterns for the test oligo- and polysaccharides and exhibiting

typical growth and physiological characteristics of bifidobacteria were selected to be used in a series of *in vitro* antagonism studies. In the preliminary experiment, the 18 isolates plus 2 reference cultures of bifidobacteria were divided into 5 groups with 4 strains combined to make a mixture in each group. The *in vitro* antagonistic activities of the 5 mixtures of bifidobacteria against *E. coli* in Peptone Yeast Extract (PY) broth containing Yacon, Raftilose, or Fibruline as the primary carbon source were compared. After 48-hour anaerobic co-culture at 37°C, the inhibition of *E. coli* by each mixture of bifidobacteria was measured. The two most inhibitory mixtures of bifidobacteria were selected and the eight individual strains in the two mixtures were further assessed and compared for their *in vitro* antagonistic activities against *E. coli* in PY broth containing Yacon or Raftilose as primary carbon source. Fibruline was eliminated due to its low antagonistic activity by bifidobacteria. The six most inhibitory isolates, showed 100% inhibitory effects against *E. coli*, which made them particularly promising for selection as probiotic products. The pH of the fermented broths was also determined in these two preliminary experiments, both of which showed a clear negative correlation between pH of the fermented broth and the ranked transformed or angular transformed inhibition rate of *E. coli*. This demonstrated that the inhibitory effect against *E. coli* by the bifidobacterial strains isolated from sheep faeces is an acid-dependant process. One bifidobacterial strain, P5-Po4-37, selected from the six most inhibitory isolates, was subsequently investigated for its *in vitro* antagonistic activity against *E. coli* by determination of bacterial growth kinetics in co-culture with *E. coli* in PY broth containing Raftilose or Yacon under anaerobic conditions at 37°C for up to 60 hours. In this experiment, two different concentrations of bifidobacteria, 10^7 to 10^8 CFU/mL and 10^3 to 10^4 CFU/mL, were incubated with 10^4 to 10^5 CFU/mL of *E. coli*. After 30 and 48-60 hours of incubation, the growth of *E. coli* was completely inhibited by the higher and the lower concentration of bifidobacteria, respectively. In contrast, the presence of *E. coli* did not affect the growth of bifidobacteria. Fermentation products were assessed in Raftilose containing PY fermented broth which showed

that the inhibitory activity of strain P5-Po4-37 was primarily due to the production of acetic and lactic acids. Acetic acid in particular, exerted bactericidal activity against *E. coli* because of the presence of a higher proportion of the undissociated species of the acid at low pH produced by fermentation. With the higher inoculum level, the populations of bifidobacteria increased by only approximately 1.30 $\log_{10}$ cycles; whereas with the lower inoculum level, the populations of bifidobacteria increased by approximately 5.62 $\log_{10}$ cycles, both of which, however, reached the same maximum viable counts at approximately 10^9 CFU/mL. This result is in agreement with the hypothesis that the initial number of bifidobacteria may influence the size of the stimulation (Roberfroid and Delzenne, 1998). The same maximum viable counts of approximately 10^9 CFU/mL were achieved in the both co-cultures as the same amount of the substrates were used in these two experiments and became exhausted when sustaining maximum viable counts of 10^9 CFU/mL, as metabolic by-products accumulated to become inhibitory to bifidobacteria when bifidobacteria reached 10^9 CFU/mL, and/or as the bifidobacterial density may be limited to 10^9 CFU/mL by cell proximity. 10^9 CFU/mL were effective populations for bifidobacterial strain P5-Po4-37 to exert an antagonistic effect against *E. coli*. Inhibition of *in vitro* growth of other potential enteric pathogens was also investigated in co-culture with bifidobacterial strain P5-Po4-37 under anaerobic condition at 37°C for 48 hours; this showed that bifidobacterial strain P5-Po4-37 also exerted strong antagonistic activities against *Clostridium perfringens* R70, *Enterobacter aerogenes* R62, *Enterococcus faecalis* R65, *Klebsiella pneumoniae* R54, *Salmonella* Dublin ATCC 15480, and *Salmonella* Menston R71. These findings also indicated that Yacon and Raftilose potentiate an inhibitory action of bifidobacterial strain P5-Po4-37 against potential intestinal pathogens.

The *in vivo* studies and *in vitro* studies showed different antimicrobial influences on potential intestinal pathogens concomitant with the increase in bifidobacteria by the test fructooligosaccharides. The bifidogenic FOS appeared to have no

significant effects on *E. coli*/*Enterobacteriaceae* and sulphite reducing clostridia *in vivo*; whereas *in vitro*, they potentiate antagonistic effects of sheep faecal bifidobacteria against the test enteric pathogens. These different findings may be explained in several ways. The first is likely to be that the complex *in vivo* ecosystem is composed of a vast number and variety of microorganisms and growth substrates. These microorganisms and substrates participate in a multitude of interactions that may confound *in vivo* measurement. As determined by various molecular methods (Section 1.1.5, Chapter 1), a more detailed and complete understanding of the intestinal microflora has been obtained. However, only a limited number of culturable bacterial species (bifidobacteria and lactobacilli) of the intestinal microflora have been investigated for their ability to utilise the prebiotics. Lactobacilli, although numerically important in the proximal digestive tract, comprise less than 1% of the total gut microflora. Similarly, although bifidobacteria account for more than 70% of the faecal microflora of human infants within one month of age, they also represent less than 1% of the DAPI total counts or of the total 16S rRNA (Langendijk *et al.*, 1995; Sghir *et al.*, 2000). How these minor members of the microflora produce the previously mentioned health effects is still questioned. One possible explanation may be that some presently unknown or uncultivated bacterial species in addition to bifidobacteria are also able to utilise prebiotics. Apajalahti *et al.* (2002) revealed *in their studies* that inulin in the diet primarily affected previously unknown bacteria in the mouse cecum in addition to stimulation of bifidobacteria and suppression of clostridia by using culture-independent microbial community analysis. Further studies are required to increase the understanding of such unknown or uncultivated species to therefore select a vast array of new candidate probiotics.

In contrast, the *in vitro* experiments included only the target bacteria and the growth substrates, and were carried out under well-controlled conditions.

Therefore, *in vitro* experiments were conducted in this project to identify the possible mode of action *in vivo*.

Second, the composition and dynamics of intestinal microflora may be unique in each individual. It must be concluded that different bifidobacterial isolates from different sheep utilised the test oligo- and polysaccharides to differing degrees. This suggests that the test strains of bifidobacteria may belong to different species of the genus *Bifidobacterium*, and that the ability of bifidobacteria to ferment FOS is probably a species-dependent feature (Bielecka *et al.*, 2002). The six most inhibitory strains of bifidobacteria: P2-Po2-76, P2-Po2-80, P3-Pre1-68, P5-In2-42, P5-Po2-23, and P5-Po4-37, were isolated from the same sheep (**Appendix 1**). This further suggested that the inhibitory activity of bifidobacteria is a species-dependent feature and that individual sheep have different bifidobacteria, exerting various degrees of inhibitory effects. In future work the species of bifidobacteria possessing effective fermentation patterns on FOS and the greatest inhibitory effects will be selected as probiotics. Further *in vivo* work administering these selected probiotics to sheep is required to support the robust data obtained *in vitro*.

The third reason to explain the different results obtained *in vivo* and *in vitro* is that different populations of bifidobacteria were obtained. *In vivo*, Raftilose, Yacon, and Fibruline exerted significant bifidogenic effects on sheep faecal bifidobacteria after 9 days of daily dosing, with Raftilose producing the highest counts of approximately 10^7 CFU/g of faeces. *In vitro*, maximum viable counts of approximately 10^9 CFU of bifidobacteria per mL were obtained in fermentation broths containing either Raftilose or Yacon. This suggested that the effective populations of bifidobacteria for inhibiting the growth of potential intestinal pathogens is approximately 10^9 CFU/g or mL of product or gut content. Thus the maximum number of bifidobacteria achieved by FOS administration *in vivo* (approximately 10^7 CFU/g of faeces) may be insufficient for metabolic activities of bifidobacteria to interfere with other intestinal microorganisms. Other studies

have also shown that the effective dose of bifidobacteria to 10^9 CFU/mL. Tohyama *et al.* (1982) observed that when rats were inoculated with trans-galactosylated oligosaccharides and *B. breve*, activities of detrimental enzymes such as nitroreductase, urea amidohydrolase, and L-lysine decarboxylase were significantly reduced, production of noxious substrates such as indole and cadaverine in the intestine decreased, and there was also a significant drop in indican, indole-3-acetate, *p*-cresol, piperidine and cadaverine in the urine. The minimum effective dose of *B. breve* was 10^9 per day. Homma *et al.* (Homma *et al.*,1967; Homma, 1988) observed that the administration of fermented milks containing 10^9 bifidobacteria/mL resulted in a reduction in the total cholesterol level in the hypercholesterolemic human subjects. Tojo *et al.* (1987) studied the effects of *B. breve* on Campylobacter-induced enteritis. When 3×10^9 colony forming units of *B. breve* were given daily, together with antidiarrheal medication, *C. jejuni* was eradicated from the stools, and the normal intestinal flora in Campylobacter-induced enteritis patients was restored. The different level of maximum populations of bifidobacteria were achieved *in vivo* and *in vitro* was probably due to the different concentrations of FOS used. *In vitro*, 1.0% final concentration of FOS was used as carbon source in fermentation media, which stimulated bifidobacteria to reach approximately 10^9 CFU/mL, whereas *in vivo*, 7 gram of FOS administered in each sheep was diluted by normal feed and water intake to give a hindgut FOS concentration well below 1.0%, and the FOS might also be utilised by other sheep hindgut bacteria which competed for nutrients with bifidobacteria, these being the limiting factors for the growth of bifidobacteria. Therefore, only 10^7 CFU/g of faeces of bifidobacteria was attained *in vivo*, which was insufficient to exert an antimicrobial effect against intestinal pathogens. Further *in vivo* studies with higher doses of the bifidogenic FOS will be carried out to determine the relationship between the dosages of FOS supplements and the antimicrobial effect on sheep faecal pathogens.

In conclusion, the six most inhibitory strains of sheep faecal bifidobacteria and two most bifidogenic FOS (Yacon and Raftilose) were selected in this project which could be used as promising pro- and prebiotic products for the manipulation of ruminant hindgut microflora. Future research will focus on *in vitro* and *in vivo* evaluation of synbiotic effects, of combining the bifidobacteria strains with the most inhibitory activities at an effective dose of 10^9 CFU/mL, with the FOS with the most bifidogenic properties and possibly an increased dose, on sheep/ruminant hindgut microflora. It is proposed to test whether or not this synbiotic combination can suppress the growth of potential intestinal pathogens concurrrently with increasing bifidobacteria in the sheep/ruminant hindgut. To facilitate this experimental programme, molecular techniques for detection and enumeration of intestinal bacteria, in particular, bifidobacteria will be developed and applied to overcome difficulties with classical culture-based techniques and provide a better picture of the interactions between more members of the intestinal microflora. Knowledge of the interaction between a modified intestinal flora and the host is a prerequisite for future gut nutritional modulation studies i.e. the further research and development of pro, pre, and synbiotics. Since it would be necessary for gut microflora modulating formulations to survive passage through the rumen in normal usage, a rumen protective delivery system and the commercialisation of products will be developed subsequently.

Appendix 1

RESULTS OF FRUCTOSE-6-PHOSPHATE PHOSPHOKETOLASE AND IN VITRO FERMENTATION TESTS USING REFERENCE BIFIDOBACTERIA AND PRESUMPTIVE BIFIDOBACTERIAL STRAINS ISOLATED FROM SHEEP

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep.

No.	Isolate No.	Source	F6PPK test*	Which treatment used in *in vivo* test	*In vitro* fermentation test**					
					Yacon	Fibruline	Raftilose	AG	Raffinose	Basal Media
1	P1-Pre 1-41	Sheep 7	2+	Yacon	2+	-	-	-	1+	-
2	P1-Pre 1-57	Sheep 11	2+	Fibruline	1+	-	-	+/-	2+	-
3	**P1-Pre 1-60*** **	**Sheep 11**	**3+**	**Fibruline**	**+/-**	**-**	**1+**	**-**	**1+**	**-**
4	P1-Pre 1-61	Sheep 11	2+	Fibruline	1+	-	-	1+	-	-
5	P1-Pre 1-64	Sheep 12	2+	Yacon	2+	-	-	-	1+	-
6	P1-Pre 1-68	Sheep 12	2+	Yacon	2+	-	-	+/-	-	-
7	P1-Post 4-57	Sheep 6	2+	AG	2+	-	+/-	-	2+	-
8	P1-Post 4-58	Sheep 6	1+	AG	2+	-	+/-	+/-	1+	-
9	P1-Post 4-59	Sheep 6	1+	AG	2+	-	3+	-	-	-
10	P1-Post 4-61	Sheep 6	1+	AG	2+	-	3+	+/-	+/-	-
11	P1-Post 4-124	Sheep 11	2+	Fibruline	1+	-	+/-	1+	1+	-
12	P1-Post 4-137	Sheep 12	1+	Yacon	2+	-	-	+/-	-	-
13	P1-Post 4-138	Sheep 12	0+	Yacon	2+	-	-	+/-	-	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

14	**P2-Post 2-1**	**Sheep 1**	**3+**	**Raftilose**	**2+**	**-**	**1+**	**+/-**	**2+**	**-**
15	**P2-Post 2-10**	**Sheep 2**	**3+**	**Fibruline**	**2+**	**-**	**1+**	**+/-**	**2+**	**-**
16	P2-Post 2-11	Sheep 2	3+	Fibruline	2+	-	1+	+/-	2+	-
17	P2-Post 2-12	Sheep 2	3+	Fibruline	2+	-	-	+/-	2+	-
18	P2-Post 2-14	Sheep 2	3+	Fibruline	2+	-	1+	+/-	2+	-
19	**P2-Post 2-30a**	**Sheep 5**	**3+**	**Control**	**2+**	**-**	**1+**	**-**	**1+**	**-**
20	P2-Post 2-30b	Sheep 5	2+	Control	2+	-	-	-	-	-
21	P2-Post 2-38	Sheep 6	2+	Fibruline	2+	-	-	-	+/-	-
22	P2-Post 2-41	Sheep 6	1+	Fibruline	2+	-	-	-	-	-
23	P2-Post 2-42	Sheep 6	2+	Fibruline	2+	-	-	-	-	-
24	P2-Post 2-43	Sheep 6	2+	Fibruline	2+	-	-	-	+/-	-
25	**P2-Post 2-76**	**Sheep 10**	**4+**	**Raftilose**	**2+**	**1+**	**3+**	**-**	**2+**	**-**
26	**P2-Post 2-80**	**Sheep 10**	**4+**	**Raftilose**	**2+**	**1+**	**3+**	**-**	**2+**	**-**
27	P2-Post 2-82	Sheep 11	3+	AG	1+	-	1+	+/-	+/-	-
28	P2-Post 2-83	Sheep 11	3+	AG	1+	-	1+	-	1+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

29	P2-Post 2-84	Sheep 11	3+	AG	1+	-	-	+/-	+/-	-
30	P2-Post 2-92	Sheep 11	3+	AG	2+	-	+/-	+/-	1+	-
31	**P2-Post 2-96**	**Sheep 12**	**1+**	**Control**	**1+**	**-**	**+/-**	**+/-**	**+/-**	**-**
32	P2-Post 2-97	Sheep 12	1+	Control	1+	-	-	+/-	+/-	-
33	P2-Post 2-PS1	Sheep 6	2+	Fibruline	2+	-	-	-	+/-	-
34	P3-Pre 1-31	Sheep 6	0+	Yacon	2+	-	-	-	-	-
35	P3-Pre 1-32	Sheep 6	1+	Yacon	1+	-	+/-	+/-	+/-	-
36	P3-Pre 1-36	Sheep 6	1+	Yacon	2+	-	-	-	-	-
37	P3-Pre 1-45LARGE	Sheep 7	3+	Control	2+	-	1+	-	2+	-
38	**P3-Pre 1-45SMALL**	**Sheep 7**	**3+**	**Control**	**2+**	**-**	**1+**	**-**	**2+**	**-**
39	**P3-Pre 1-68**	**Sheep 10**	**3+**	**AG**	**3+**	**1+**	**3+**	**+/-**	**2+**	**-**
40	**P3-In 3-15**	**Sheep 6**	**2+**	**Yacon**	**2+**	**-**	**2+**	**-**	**2+**	**-**
41	P3-In 3-17	Sheep 6	1+	Yacon	2+	-	1+	-	2+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

42	P3-In 3-21	Sheep 6	1+	Yacon	1+	-	1+	-	2+	-
43	**P3-In 3-22**	**Sheep 6**	**1+**	**Yacon**	**1+**	**-**	**1+**	**-**	**2+**	**-**
44	P3-Post 1-19	Sheep 6	1+	Yacon	3+	-	1+	-	3+	-
45	P3-Post 1-20	Sheep 6	0+	Yacon	3+	-	1+	-	3+	-
46	P3-Post 1-22	Sheep 6	0+-1+	Yacon	3+	-	1+	-	2+	-
47	P3-Post 2-17	Sheep 6	0+	Yacon	1+	-	1+	-	2+	-
48	P3-Post 3-24	Sheep 5	1+	AG	3+	-	1+	-	3+	-
49	P3-Post 3-24'	Sheep 5	1+	AG	1+	-	+/-	-	1+	-
50	P3-Post 3-29	Sheep 6	1+	Yacon	1+	-	+/-	-	+/-	-
51	P3-Post 3-30	Sheep 6	1+	Yacon	1+	-	+/-	-	1+	-
52	**P3-Post 3-31**	**Sheep 6**	**1+**	**Yacon**	**1+**	**-**	**+/-**	**-**	**1+**	**-**
53	P3-Post 3-38	Sheep 6	2+	Yacon	1+	-	1+	-	1+	-
54	P3-Post 3-59	Sheep 10	3+	AG	2+	+/-	2+	-	1+	-
55	P3-Post 3-60	Sheep 10	3+	AG	3+	+/-	2+	-	+/-	-
56	**P4-Pre 1-17**	**Sheep 6**	**2+**	**Raftilose**	**1+**	**-**	**1+**	**-**	**1+**	**-**
57	P4-Pre 1-19	Sheep 6	1+	Raftilose	1+	-	2+	-	1+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

58	P4-Pre 1-36	Sheep 9	0+	Yacon	-	-	-	-	-	-
59	P4-Pre 1-38	Sheep 10	3+	Control	3+	+/-	3+	-	2+	-
60	P4-In 1-24	Sheep 6	1+	Raftilose	2+	-	1+	-	2+	-
61	P4-In 1-27L	Sheep 6	0+	Raftilose	3+	-	+/-	-	2+	-
62	P4-In 1-27S	Sheep 6	1+	Raftilose	3+	-	-	-	1+	-
63	P4-In 2-18	Sheep 6	1+	Raftilose	2+	-	+/-	-	1+	-
64	P4-In 2-19Flat	Sheep 6	0+	Raftilose	2+	-	-	-	-	-
65	P4-In 2-19Convex	Sheep 6	0+	Raftilose	1+	-	-	-	-	-
66	P4-In 2-40LARGE	Sheep 10	3+	Control	2+	2+	2+	-	1+	-
67	P4-In 2-40SMALL	Sheep 10	3+	Control	2+	+/-	2+	-	2+	-
68	P4-In 3-21	Sheep 5	1+	Raftilose	1+	-	+/-	-	1+	-
69	P4-In 3-21'	Sheep 5	1+	Raftilose	1+	-	1+	-	2+	-
70	P4-In 3-22	Sheep 6	0+	Raftilose	1+	-	1+	-	2+	-
71	P4-In 3-29	Sheep 6	0+	Raftilose	1+	-	+/-	-	1+	-
72	P4-In 3-33	Sheep 6	1+	Raftilose	1+	-	1+	-	2+	-
73	P4-In 3-40	Sheep 6	1+	Raftilose	1+	-	1+	-	2+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

74	P4-In 3-41	Sheep 6	1+	Raftilose	1+	-	2+	-	2+	-
75	**P4-In 3-46**	**Sheep 6**	**1+**	**Raftilose**	**1+**	**-**	**2+**	**-**	**1+**	**-**
76	P4-In 3-52	Sheep 6	0+	Raftilose	1+	-	2+	-	2+	-
77	P4-In 3-52'	Sheep 6	0+	Raftilose	1+	-	2+	-	2+	-
78	P4-In 3-82	Sheep 10	3+	Control	2+	1+	2+	+/-	2+	-
79	P4-Post 1-11	Sheep 6	0+	Raftilose	2+	-	1+	-	1+	-
80	P4-Post 1-15	Sheep 6	1+	Raftilose	1+	-	+/-	-	2+	-
81	P4-Post 1-17	Sheep 6	0+	Raftilose	1+	-	1+	-	+/-	-
82	P4-Post 1-23	Sheep 6	1+	Raftilose	1+	+/-	1+	-	1+	-
83	P4-Post 1-37	Sheep 10	3+	Control	2+	-	2+	+/-	2+	-
84	P4-Post 2-57	Sheep 10	3+	Control	2+	2+	2+	-	3+	-
85	P4-Post 3-32	Sheep 6	1+	Raftilose	1+	-	2+	-	2+	-
86	P4-Post 3-34	Sheep 6	0+	Raftilose	1+	-	2+	-	2+	-
87	P4-Post 3-35	Sheep 6	0+	Raftilose	1+	-	2+	-	2+	-
88	P5-Pre 1-19	Sheep 6	0+	Control	1+	-	-	-	-	-
89	P5-Pre 1-21	Sheep 6	0+	Control	2+	-	-	-	3+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

90	P5-Pre 1-25	Sheep 6	1+	Control	2+	-	1+	-	1+	-
91	P5-Pre 1-29	Sheep 6	1+	Control	1+	-	+/-	-	2+	-
92	P5-Pre 1-29'	Sheep 6	1+	Control	1+	-	2+	-	3+	-
93	P5-In 2-20	Sheep 6	1+	Control	1+	-	+/-	-	+/-	-
94	P5-In 2-23	Sheep 6	1+	Control	1+	-	+/-	-	1+	-
95	**P5-In 2-42**	**Sheep 10**	**3+**	**Yacon**	**2+**	**1+**	**2+**	**-**	**1+**	**-**
96	P5-In 3-19	Sheep 5	1+	Fibruline	1+	-	-	-	-	-
97	P5-In 3-22	Sheep 5	1+	Fibruline	1+	-	-	-	-	-
98	P5-In 3-23	Sheep 5	0+	Fibruline	1+	-	+/-	-	1+	-
99	P5-In 3-25	Sheep 6	0+	Control	1+	-	+/-	-	1+	-
100	P5-In 3-28	Sheep 6	2+	Control	2+	-	-	-	+/-	-
101	P5-In 3-38	Sheep 10	3+	Yacon	2+	+/-	2+	-	2+	-
102	P5-Post 1-16	Sheep 10	3+	Yacon	3+	1+	3+	-	3+	-
103	P5-Post 1-33	Sheep 6	0+	Control	2+	-	+/-	-	1+	-
104	P5-Post 2-9	Sheep 6	1+	Control	2+	-	1+	-	3+	-
105	P5-Post 2-13	Sheep 6	0+	Control	2+	-	1+	-	3+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

106	**P5-Post 2-23**	**Sheep 10**	**2+-3+**	**Yacon**	**3+**	**1+**	**2+**	**+/-**	**2+**	**-**
107	P5-Post 3-16	Sheep 6	1+	Control	2+	-	-	-	-	-
108	P5-Post 3-17	Sheep 6	1+	Control	2+	-	1+	-	+/-	-
109	**P5-Post 3-22**	**Sheep 6**	**2+**	**Control**	**2+**	**-**	**+/-**	**-**	**1+**	**-**
110	P5-Post 3-25	Sheep 6	0+	Control	1+	-	+/-	-	1+	-
111	P5-Post 3-26	Sheep 6	1+	Control	2+	-	1+	-	2+	-
112	P5-Post 3-27	Sheep 6	0+	Control	2+	-	+/-	-	2+	-
113	P5-Post 3-31	Sheep 6	0+	Control	2+	-	+/-	-	2+	-
114	P5-Post 3-32	Sheep 6	0+	Control	2+	-	+/-	-	2+	-
115	P5-Post 3-57	Sheep 10	3+	Yacon	3+	+/-	2+	-	1+	-
116	P5-Post 3-60	Sheep 10	3+	Yacon	3+	+/-	2+	-	1+	-

Appendix 1 Results of fructose-6-phosphate phosphoketolase and *in vitro* fermentation tests using reference bifidobacteria and presumptive bifidobacterial strains isolated from sheep (Continued).

117	**P5-Post 4-37**	**Sheep 10**	**2+-3+**	**Yacon**	**3+**	**1+**	**3+**	**-**	**3+**	**-**
	B. bifidum		2+		N/G	N/G	N/G	N/G	N/G	N/G
	B. pseudolongum subsp. *Globosum*		1+		1+	-	1+	-	-	-
	B. animalis		3+		2+	-	1+	+/-	2+	-
	B. boum		3+		2+	-	-	-	-	-

* In F6PPK tests, "0+" ~ "4+" represent various degrees of colour development: "0+" yellow/brown, "1+" light reddish brown, "2+" reddish brown, "3+" reddish purple, "4+" stronger reddish purple observed than "3+"

** In *in vitro* fermentation tests, "-" ~ "3+" represent various degrees of colour changes of carbohydrate fermentation agar: "-" no colour change on agar, "+/-" slight colour change, but no fermentation zone observed, "1+" a colour change from purple to yellow with a small fermentation zone, "2+" a colour change from purple to yellow with a medium fermentation zone; "3+" a colour change from purple to yellow on the whole agar area. "N/G" represents no growth of the test strain in media containing the test carbohydrate.

*** The selected strains are indicated in bold.

References

Akiyama, K., Hosono, S., Takahashi, E., Ishizeki, S., Takigawa, I., Imura, S., Yamauchi, K., Yaeshima, T., Hayasawa, H. and Shimamura, S. **(1994a).** Effects of oral administration of *Bifidobacterium breve* on development of intestinal microflora in extremely premature infants. *Acta Neonatol. Japon.* **30**: 130-137.

Akiyama, K., Shimada, M., Ishizeki, S., Takigawa, I., Imura, S., Yamauchi, K., Hatano, M., Abe, N., Yaeshima, T., Hayasawa, H. and Shimamura, S. **(1994b).** Effects of administration of *Bifidobacterium* in extremely premature infants. *Acta Neonatol. Japon.* **30**: 257-263.

Alles, M. S., Hautvast, J. G. A. J., Nagengast, F. M., Hartemink, R., Van Laere, K. M. J. and Jansen, J. B. M. J. **(1996).** Fate of fructo-oligosaccharides in the human intestine. *Br. J. Nutr.* **76**: 211-221.

Anderson, K. L., Nagaraja, T. G., Morril, J. L., Avery, T. B., Galitzer, S. J., and Boyer, J. E. **(1987).** Ruminal microbial development in conventionally or early-weaned calves. *J. Anim. Sci.* **64**: 1215.

Anderson, P. H., Matthews, J. G., Berrett, S., Brush, P. J. and Patterson, D. S. P. **(1981).** Changes in plasma enzyme activities and other blood components in response to acute liver damage in cattle. *Research in Veterinary Science.* **31**: 1-4.

Apajalahti, J. H. A., Kettunen, H., Kettunen, A., Holben, W. E., Nurminen, P. H., Rautonen, N. and Mutanen, M. **(2002).** Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Appl. Environ. Microbiol.* **68**: 4986-4995.

Armstrong, D. G. (1984). Antibiotics as feed additives for ruminant livestock. In: *Antimicrobials and Agriculture* (ed. Woodbine, W.). pp. 331-347. Butterworths, London.

Armstrong, D. G. (1986). Gut-active growth promoters. In: *Control and Manipulation of Animal Growth* (eds. Buttery, P. J., Lindsay, D., and Haynes, N. B.). pp. 21-37. Butterworths, London.

Avery, S. M. and Bell, R. G. (1991). Lactic acid bacteria. In: *Microbiological methods for the Meat Industry.* (2nd ed. Cook, R. L.) pp. 9.7-2. The Meat Industry Research Institute of New Zealand. Hamilton. New Zealand.

Avery, S. M. and Cook, R. L. (1991). Sulphite Reducing Anaerobes. In: *Microbiological methods for the Meat Industry.* (2nd ed. Cook, R. L.) pp. 7.11-3. The Meat Industry Research Institute of New Zealand. Hamilton. New Zealand.

Bach Knudsen, K. E. and Hessov, I. (1995). Recovery of inulin from Jerusalem artichoke (*Helianthus tuberosus L.*) in the small intestine of man. *Br. J. Nutr.* **74**: 101.

Bahaka, D., Neut, C., Khattabi, A., Monget, D. and Gavini, F. **(1993).** Phenotypic and genomic analysis of human strains belonging or related to

Bifidobacterium longum, *B. infantis* and *B. breve*. *Int. J. Syst. Bacteriol.* **43**: 565-573.

Bailey, J. S., Blankenship, L. C. and Cox, N. A. **(1991).** Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. *Poult. Sci.* **70**: 2433-2438.

Balken, J. A. M., van Dooren, Th. J. G. M., Van den Tweel, W. J. J., Kamphuis, J. and Meijer, E. M. **(1991).** Production of 1-kestose with intact mycelium of *Aspergillus phoenicis* containing sucrose-1^F-fructosyltansferase. *Appl. Microbiol. Biotechnol.* **35**: 216-221.

Ballongue, J. (1993). Bifidobacteria and probiotic action. In: *Lactic Acid Bacteria* (eds. Salminen, S. and von Wright, A.). pp. 357-428. Marcel Dekker Inc. New York.

Barnsley, E. A. (1975). The bacterial degradation of fluoranthrene and benzo[α]pyrene. *Can. J. Microbiol.* **21**: 1004-1008.

Barthomeuf, C. and Pourrat, H. (1995). Production of high-content fructooligosaccharides by an enzymatic system from *Penicillium rugulosum*. *Biotechnol. Lett.* **17**: 911-916.

Bartlett, J. G. (1992). Antibiotic-associated diarrhea. *Clin. Infect. Dis.* **15**: 573-581.

Beerens, H. (1990). An elective and selective isolation medium for *Bifidobacterium* spp. *Lett. Appl. Microbiol.* **11 (3)**: 155-157.

Benno, Y. and Mitsuoka, T. (1986). Development of intestinal microflora in humans and animals. *Bifidobacteria Microflora*. **5**: 13–25.

Benno, Y., Sawada, K., and Mitsuoka, T. **(1984).** The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. *Microbiol. Immunol.* **28**: 975–86.

Bernet, M. F., Brassart, D., Neeser, J. R. and Servin, A. L. **(1993).** Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl. Envir. Microbiol.* **59**: 4121-4128.

Bhat, P., Albert, M. J., Rajan, D., Ponniah, J., Mathan, H. and Baker, S. J. **(1989).** Bacterial flora of the jejunum: A comparison of luminal aspirate and mucosal biopsy. *J. Med. Microbiol.* **13**: 247-256.

Bibiloni, R., Pérez, P. F. and de Antoni, G. L. **(2000).** An enzymatic-colorimetric assay for the quantification of *Bifidobacterium*. *J. Food Prot.* **63 (3)**: 322-326.

Bielecka, M., Biedrzycka, E. and Majkowska, A. **(2002).** Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Res. Int.* **35**: 125-131.

Bohnhoff, M., Drake, B. L. and Miller, C. P. **(1954).** Effect of streptomycin on susceptibility of the intestinal tract to experimental salmonella infection. In: *Proceedings of the Society for Experimental Biology and Medicine* **86**: 132-137.

Bornet, F. R. J. (1994). Undigestible sugars in food products. *Am. J. Clin. Nutr.* **59**: 763S-769S.

Bouhnik, Y., Flourié, B., Riottot, M., Bisetti, N., Gailing, M. F., Guibert, A., Bornet, F. and Rambaud, J. C. **(1996).** Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutr. Cancer.* **26**: 21-29.

Braun O. H. (1981). Effect of consumption of human milk and other formulas on intestinal bacterial flora in infants. In: *Gastroenterology and Nutrition in Infancy* (ed. Lebenthal B.). pp. 247–51. Raven Press. New York.

Briet, F., Achour, L., Flourié, B., Beaugerie, L., Pellier, P., Franchisseur, C., Bornet, F. and Rambaud, J. C. **(1995).** Symptomatic response to varying levels of fructo-oligosaccharides consumed occasionally or regularly. *Eur. J. Clin. Nutr.* **49**: 501-507.

Brighenti, F., Casiraghi, M. C., Pellingrini, N., Riso, P., Simonetti, P. and Testolin, G. **(1995).** Comparison of lactulose and inulin as reference standard for the study of resistant starch fermentation using hydrogen breath test. *Ital. J. Gastroenterol.* **27**:122–128.

Bryant, M. P., Small, N., Bouma, C., and Robinson, I. **(1958).** Studies on the composition of the ruminal flora and fauna of young calves. *J. Dairy Sci.* **41**: 1747.

Buddington, R. K., Williams, C. H., Chen, S. C. and Witherly, S. A. **(1996).** Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. *Am. J. Clin. Nutr.* **63**: 709-716.

Bullen, C. L., Tearle, P. V. and Stewart, M. G. **(1977).** The effect of `humanised' milks and supplemented breastfeeding on the faecal flora of infants. *J. Med. Microbiol.* **10**: 403-413.

Burton, J. P. (2000). The development of molecular methods for the identification and enumeration of bifidobacteria. Ph.D. Thesis. *Universtiy of Otago.* Dunedin. New Zealand.

Campbell, M. K. (1999). *Biochemistry.* Saunders College Publishing. Philadelphia.

Chang, J. H., Kwon, I. K. and Kim, H. U. **(1983).** Studies on the bifidobacteria in breast-fed Korean infant gut. *Korean J. Dairy Sci.* **5**: 111.

Chatterjee, D., Roberts, A. D., Lowell, K., Brennan, P. J. and Orme, I. M. **(1992).** Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. *Infect. Immun.* **60**: 1249–1253.

Chen, W. and Liu, C. (1996). Production of β-fructofuranosidase by *Aspergillus japonicus*. *Enzyme Microb. Technol.* **18**: 153-160.

Clevenger, M. A., Turnbull, D., Inoue, H., Enomoto, M., Allen, J. A., Henderson, L. M., and Jones, E. **(1988).** Toxicological evaluation of neosugar: genotoxicity, carcinogenicity, and chronic toxicity. *J. Am. Coll. Toxicol.* **7**: 643.

Collins, F. M. and Carter, P. B. (1978). Growth of Salmonellae in orally infected germfree mice. *Infection & Immunity.* **21**: 41-47.

Collins, M. D. and Gibson, G. R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* **69(5)**: 1052S-1057S.

Colombel, J. F., Cortot, A., Neut, C. and Romond, C. **(1987).** Yoghurt with *Bifidobacterium longum* reduces erythromycin-induced gastrointestinal effects. *The Lancet.* **2**: 8549.

Coudray, C., Bellanger, J., Castiglia-Delavaud, C., Rémésy, C., Vermorel, M. and Rayssiguier, Y. **(1997).** Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance calcium, magnesium, iron and zinc in healthy young men. *Eur. J. Clin. Nutr.* **51**: 375-380.

Crittenden, R. G. and Playne, M. J. (1996). Production, properties and application of food-grade oligosaccharides. *Trends Food Sci. Technol.* **7**: 353-361.

Crociani, J., Grill, J. P., Huppert, M. and Ballongue, J. **(1995).** Adhesion of different bifidobacteria strains to human enterocyte-like Caco-2 cells and comparison with *in vivo* study. *Lett. Appl. Microbiol.* **21**: 146-148.

Cummings J. H. (1994). Non-starch polysaccharides (dietary fibre) including bulk laxatives in constipation. In: *Constipation* (eds. Kamm, M. A. and Lennard-Jones, J. E.). pp. 307-314. Wrightson Biomedical Publishing Ltd. Petersfield. United Kingdom.

Cummings J. H. and Englyst, H. N. (1991). Measurement of starch fermentation in the human large intestine. *Can. J. Physiol. Pharmacol.* **69**: 121-129.

Cummings J. H., Macfarlane, G. T. and Englyst, H. N. **(2001).** Prebiotic digestion and fermentation. *Am. J. Clin. Nutr.* **73 (suppl)**: 415S-420S.

Cummings, J. H. (1995). Short chain fatty acids. In: *Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology* (eds. Gibson, G. R. and MacFarlane, G. T.). pp. 101-130. CRC Press. Boca Raton, FL.

Cummings, J. H. and MacFarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bact.* **70**: 443-459.

Cunningham D. V. M. and James G. (1997). *Textbook of Veterinary Physiology.* W.B. Saunders Company. Philadelphia.

Cushnie, G. H., Richardson, A. J. and Sharman, G. A. M. **(1981).** Procedures and equipment for the production and rearing of gnotobiotic lambs. *Lab. Anim.* **15**: 199-204.

Davidson, M. H., Synecki, C., Maki, K. C. and Drenman, K. B. **(1998).** Effects of dietary inulin in serum lipids in men and women with hypercholesterolemia. *Nutr. Res.* **3**: 503-517.

de Vries, W. and Stouthamer, A. H. (1967). Pathway of glucose fermentation in relation to the taxonomy of bifidobacteria. *J. Bacteriol.* **93**: 574.

de Vries, W., Gerbrandy, S. J. and Stouthamer, A. H. **(1967).** Carbohydrate metabolism in *Bifidobacterium bifidum. Biochim. Biophys. Acta.* **136**: 415-425.

Deguchi, Y., Morishita, T. and Mutai, M. **(1985).** Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. *Agric. Biol. Chem.* **49 (1)**: 13-19.

Delzenne, N. M. and Roberfroid, M. B. (1994). Physiological effects of non digestible oligosaccharides. *Food Sci. Technol.* **27**: 1-6.

Delzenne, N., Aertssens, J., Verplaetse, H., Roccaro, M. and Roberfroid, M. **(1995).** Effect of fermentable fructo-oligosaccharides on mineral, nitrogen and energy digestive balance in the rats. *Life Sci.* **57**: 1579-1587.

Donohue, D. and Salminen, S. (1996). Safety of probiotic bacteria. *Asia Pac. J. Clin. Nutr.* **5**: 25-28.

Duffy, L. C., Leavens, A., Griffiths, E. and Dryja, D. **(1999).** Perspectives on bifidobacteria as biotherapeutic agents in gastrointestinal health. *Digestive Diseases and Sciences.* **44(8)**: 1499-1505.

Ellegärd, L., Andersson, H. and Bosaeus, I. **(1997).** Inulin and oligofructose do not influence the absorption of cholesterol, or the excretion of cholesterol, Ca, Mg, Zn, Fe, or bile acids but increases energy excretion in ileostomy subjects. *Eur. J. Clin. Nutr.* **51**: 1.

Eysssen, H. and van Eldere, J. (1984). Metabolism of bile acids. In: *The Germ-Free Animal in Biomedical Research* (eds. Coates, M. E. and Gustafsson, B. E.). pp. 291-316. Laboratory Animals. London.

Fan, X. R., Li, G. W. and Shen, P. **(1988).** *Microbiological Experiments* (2nd ed.). pp. 180. Higher Education Publishing. Beijing. China.

Faure, J. C., Schellenberg, D., Bexter, A. and Wurzner, H. P. **(1982).** Barrier effect of *Bifidobacterium longum* on *Escherichia coli* in the germ-free rat. *Int. J. Vit. Nutr. Res.* **52 (2)**: 225-230.

Findlay, A. L. R. (1998). Fermentation in the ruminant stomach. [On-line] http://www.physiol.cam.ac.uk/staff/findlay/fermdig.htm

Finegold, S. M., Sutter, V. L. and Mathisen, G. E. (1983). Normal indigenous intestinal flora, In: *Human Intestinal Microflora in Health and Disease.* (ed. Hentges, D. J.). pp. 3-31. Academic Press, New York, N.Y.

Fiordaliso, M. F., Kok, N., Desager, J. P., Goethals, F., Deboyser, D., Roberfroid, M. and Delzenne, N. **(1995).** Oligofrutose-supplemented diet lowers serum and VLDL concentrations of triglycerides, phospholipids and cholesterol in rats. *Lipids.* **30**: 163-167.

Fonty, G., Gouet, P., Jouany, J. P. and Senaud, J. **(1987).** Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J. Gen. Microbiol.* **133**: 1835-1843.

Fooks, L. J., Fuller, R., and Gibson, G. R. **(1999).** Prebiotics, probiotics and human gut microbiology. *International Dairy Journal.* **9(1)**: 53-61.

Franks, A. H., Harmsen, H. J. M., Raangs, G. C., Jansen, G. J., Schut, F. and Welling, G. W. **(1998).** Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **64(9)**: 3336-3345.

Freter, R. (1955). The fatal enteric cholera infection in the guinea pig achieved by inhibition of normal enteric flora. *Journal of Infectious Diseases*. **97**: 57-65.

Freter, R. (1956). Experimental enteric shigella and vibrio infection in mice and guinea pigs. *Journal of Experimental Medicine*. **104**: 411-418.

Freter, R. (1974). Interactions between mechanisms controlling the intestinal microflora. *Am. J. Clin. Nutr.* **27**: 1409-1416.

Fujita, K., Hara, K., Hashimoto, H. and Kitahata, S. **(1990).** Purification and some properties of β-fructofuranosidase I from *Arthrobacter* sp. K1. *Agric. Biol. Chem.* **54 (4)**: 913-919.

Fujita, K., Kuwahara, N., Tanimoto, T., Koizumi, K., Lizuka, M., Minamiura, N., Furuichi, K. and Kitahata, S. **(1994).** Chemical structures of heterooligosaccharides produced by *Arthrobacter* sp. K71 β-fructofuranosidase. *Biosci. Biotechnol. Biochem.* **58**: 239-243.

Fujiwara, S., Hashiba, H., Hirota, T. and Forstner, J. F. **(1997).** Proteinaceous factor(s) in culture supernatant fluids of bifidobacteria which prevents the binding of enterotoxigenic Escherichia coli to gangliotetraosylceramide. *Appl. Environ. Microbiol.* **63(2)**: 506-512.

Fuller R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* **66**: 365-378.

Gavini, F., Van Esbroeck, M., Touzel, J. P., Fourment, A. and Goossens, H. **(1996).** Detection of fructose-6-phosphate phosphoketolase (F6PPK), a key enzyme of the bifid-shunt, in *Gardnerella vaginalis*. *Anaerobe*. **2 (3)**: 191-193.

Gibson, G. R. (1999). Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *J. Nutr.* [On-line]. http://www.nutrition.org/ **129**: 1438S-1441S.

Gibson, G. R. (2001). Prebiotics for Gut Health. [On-line]. http://www.ifis.org/forum/March_2001/prebiotics4health_real.html

Gibson, G. R. and Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* **125**: 1401-1412.

Gibson, G. R. and Wang, X. (1994a). Bifidogenic properties of different types of fructooligosaccharides. *Food Microbiol.* **11**: 491-498.

Gibson, G. R. and Wang, X. (1994b). Enrichment of bifidobacteria from human gut contents by oligofructose using continuous culture. *FEMS Microbiol. Lett.* **118**: 121-128.

Gibson, G. R. and Wang, X. (1994c). Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J. Appl. Bacteriol.* **77**: 412–420.

Gibson, G. R., Beatty, E. B., Wang, X. and Cummings, J. H. **(1995).** Selective stimulation of bifidobacteria in the human colon by fructo-oligofructoses (GFn+Fm) and inulin. *Gastroenterology.* **108**: 975-982.

Gibson, G. R., Willis, C. L. and Loo, J. V. **(1994).** Non-digestible oligosaccharides and bifidobacteria – implications for health. *Int. Sugar Jnl.* **96 (1150)**: 381-387.

Gilliland S. E. and Kim H. S. (1984). Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. *J. Dairy Sci.* **67**:1-6.

Gilliland, S. E. and Speck, M. L. (1977). Deconjugation of bile acid by intestinal lactobacilli. *Appl. Environ. Microbiol.* **33**: 15.

Glick, M. C., Sall, T., Zilliken, F. and Mudd, S. **(1960).** Morphological changes of *L. bifidus* var. penn. produced by a cell-wall precursor. *Biochim. Biophys. Acta.* **37 (2)**: 363-365.

Goddard, P., Fernandez, F., West, B., Hill, J. M. and Barnes, P. **(1975).** The nuclear dehydrogenation of steroids by intestinal bacteria. *J. Med. Microbiol.* **8**: 429-435.

Gopal, P. K., Prasad, J., Smart, J. and Gill, H. S. **(2001).** *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *Int. J. Food Microbiol.* **67(3)**: 207-216.

Goto, K., Fukai, K., Hikida, J., Nanjo, F. and Hara, Y. **(1995).** Isolation and structural analysis of oligosaccharides from yacon (*Polymnia sonchifolia*). *Biosci. Biotechnol. Biochem.* **59(12)**: 2346-2347.

Grizard, D. and Barthomeuf, C. (1999). Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health. *Reprod. Nutr. Dev.* **39**: 563-588.

Grönlund, M. M., Arvilommi, H., Kero, P., Lehtonen, O. P. and Isolauri, E. **(2000).** Importance of intestinal colonisation in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0–6 months. *Archives of Diseases in Children* **83**: F186–F192.

Guarner, F. and Schaafsma G. J. (1998). Probiotics. *Int. J. Food Microbiol.* **39(3)**: 237-238.

Hammer, H. F., Fine, K. D., Santa Ana, C. A., Porter, J. L., Schiller, L. R. and Fordtran, J. S. **(1989).** Carbohydrate malabsorption. Its measurement and its contribution to diarrhea. *J. Clin. Invest.* **84**: 1056-1062.

Harmsen, H. J. M., Wildeboer-Veloo, A. C. M., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels., J. G. and Welling, G. W. **(2000).** Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification detection methods. *JPGN.* **30**: 61-67.

Hartemink, R. (1999). Prebiotic effects of non-digestible oligo-and polysaccharides. *Ph.D. Thesis*. Wageningen Agricultural University. The Netherlands.

Hartemink, R. and Rombouts, F. M. (1997). Gas formation from oligosaccharides by the intestinal microflora. In: *Proceedings of the International Symposium "Non-digestible oligosaccharides: healthy food for the colon?"*. pp. 57-66. 4-5 December. 1997. Wageningen. the Netherlands.

Hartemink, R., Kok, B. J., Weenk, G. H. and Rombouts, F. M. **(1996).** Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. *J. Microbiol. Methods.* **27(1)**: 33-43.

Havenaar, R. and Huis in't Veld, J. H. J. (1992). Probiotics: a general view. In: *The Lactic Acid Bacteria.* (Vol. 1, ed. Wood, B. J. B.) pp. 151-170. Chapman & Hall. London.

Havenaar, R., Ten Brink, B., and Huis in't Veld, J. H. J. **(1992).** In: *Probiotics,*

The Scientific Basis. (ed. Fuller, R.). pp. 209-224. Chapmann & Hall. London.

Hedges, A. J. and Linton, A. H. (1988). Olaguindox resistance in the coliform flora of pigs and their environment: an ecological study. *J. Appl. Bact.* **64**: 329.

Hidaka, H., Eida, T., Takizawa, T. Tokunaga, T. and Tashiro, Y. **(1986).** Effects of fructooligosaccharides on intestinal flora and human health. *Bifidobacteria Microflora.* **5**: 37-41.

Hidaka, H., Hirayama, M. and Sumi, N. **(1988).** A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* **52**: 1181-1187.

Hidaka, H., Hirayama, M., Tokunaga, T. and Eida, T. **(1990).** The effects of undigestible fructooligosaccharides on intestinal microflora and various physiological functions on human health. In: *New Developments in Dietary Fiber* (eds. Furda, I. and Brine, C. J.). pp. 105-117. Plenum Press. New York.

Hill, M. J. (1995). Carbohydrate metabolism. *In: Role of Gut Bacteria in Human Toxicology and Pharmacology* (ed. Hill, M.J.). pp. 95-104. Taylor and Francis. London.

Hill, M. J., Drasar, B. S., Aries, V., Crowther, J. S., Hawksworth, G. and Williams, R. E. O. **(1971).** Bacteria and aetiology of cancer of the large bowel. *The Lancet.* **1**: 95-100.

Hobson, P. N. (ed.) **(1988).** *The rumen Microbial Ecosystem.* Elsevier. Barking.

Holdeman, L. V., Cato, E. P., and Moore, W. E. C. **(1977).** Chromatographic procedures for analysis of acid and alcohol products. In: *Anaerobe Laboratory Manual* (4th ed.). pp 134–136. Anaerobe Laboratory. Virginia Polytechnic Institute and State University. Blacksburg. Virginia. U. S. A.

Holdeman, L. V., Good, I. J. and Moore, W. E. C. **(1976).** Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. *Appl. Environ. Microbiol.* **31:**359-375.

Holzapfel, W. H. and Schillinger, U. (2002). Introduction to pre- and probiotics. *Food Res. Int.* **35**: 109-116.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J. H. J. **(1998).** Overview of gut flora and probiotics. *Int. J. Food Microbiol.* **41(2)**: 85-101.

Homma, N. (1988). Bifidobacteria as a resistance factor in human beings. *Bifidobacteria Microflora.* **7 (1)**: 35-43.

Homma, N., Nishihara, K. and Isoda, K. **(1967).** Antifidus cocci, their biological properties and clinical significance. *Mschr. Kinderheilk.* **115 (4)**: 296.

Hoover, D. G. (1999). *Bifidobacterium.* In: *Encyclopedia of Food Microbiology* (eds. Robinson, R. K., Batt, C. A. and Patel, P. D.) pp. 210-217. Academic Press. London. UK.

Hungate, R. E. (1966a). *The rumen and its microbes.* Academic Press. New York.

Hungate, R. E. (1966b). The rumen protozoa. In: *The Rumen and its Microbes.* pp. 206-240. Academic Press. New York.

Husain, I., Poupard, J. A. and Norris, R. F. **(1972).** Influence of nutrition on the morphology of strain of *Bifidobacterium bifidum*. *J. Bacteriol.* **III**: 841-844.

Ikeda, T., Kurita, T., Hidaka, H., Michaleck, S. M. and Hirasawa, M. **(1990).** Low-cariogenicity of the tetrasaccharide nystose. *Gen. Pharmacol.* **21**: 175-179.

Jenny, N. V., Günther, E. and Jann, K. **(1977).** Mitogenic stimulation of murine spleen cells: Relation to susceptibility to Salmonella infection. *Infect. Immun.* **15**: 26–32.

Jiang, T., Mustapha, A. and Savaiano, D. A. **(1996).** Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J. Dairy Sci.* **79 (5)**: 750–757.

Jung, K. H., Li, J. Y., Yoo, S. J., Lee, J. H. and Yoo, M. Y. **(1987)**. Production of fructosyltransferase from *Aureobasidium pullulans*. *Biotechnol. Lett.* **9**: 703-708.

Karney, T. L., Johnson, M. C. and Ray, B. **(1986).** Changes in the lactobacilli and coliform populations in the intestinal tract of calves from birth to weaning. *J. Anim. Sci.* **63 (Suppl. 1)**: 446-447.

Keen, J. E., Urlich, G. A. and Elder, R. O. **(1999)**. Paper presented at the 80th Conference of Research Workers in Animal Diseases. Chicago. IL. 7-9 November.

Kim H. S. and Gilliland S. E. (1983). *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* **66**:

959-966.

Kirjavainen, P. V., Apostolou, E., Arvola, T., Salminen, S. J., Gibson G. R. and Isolauri, E. **(2001).** Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunol. Med. Microbiol.* **32:** 1–7.

Kleessen, B. and Blaut, M. (2000). Oligofructose and long-chain inulin affect microflora and short-chain fatty acids in rats associated with a human faecal flora. In: *Functional Food Challenges for the New Millennium. The 5th Karlsruhe Nutrition Congress.* pp. 22. 22-24 October. 2000.

Kleessen, B., Sykura, B., Zunft, H. J. and Blaut, M. **(1997).** Effect of inulin and lactose on faecal microflora, microbial activity, and bowel habit in elderly constipated persons. *Am. J. Clin. Nutr.* **65**: 1397-1402.

Kohwi, Y., Imai, K., Tamura, Z. and Hashimoto, Y. **(1978).** Antitumor effect of *Bifidobacterium infantis* in mice. *Gann.* **69 (5)**: 613-618.

Kojima, M., Suda, S., Hotta, S. and Hamada, K. **(1970).** Induction of pleomorphology and calcium ion deficiency in *Lactobacillus bifidus*. *J. Bact.* **102**: 217-220.

Kok, N., Roberfroid, M. and Delzenne, N. **(1996a).** Dietrary oligofructose modifies the impact of fructose on hepatic triacylglycerol metabolism. *Metabolism.* **45**: 1547-1550.

Kok, N., Roberfroid, M., Robert, A. and Delzenne, N. **(1996b).** Involvement of lipogenesis in the lower VLDL secretion induced by oligofructose in rats. *Br. J. Nutr.* **76**: 881-890.

Koo, M. and Rao, V. (1991). Long term effect of bifidobacteria and Neosugar on precursor lesions of colonic cancer in mice. *Nutr. Cancer.* **16**: 249-257.

Kurmann, J. A. (1983). The development and significance of new cultures with bifidobacteria as an example. *N. Eur. Dairy J.* **48**: 65.

Kurmann, J. A. and Rasic, J. L. (1991). The health potential of products containing bifidobacteria. In: *Therapeutic Properties of Fermented Milks* (ed. Robinson, R. K.). pp. 117-158. Elsevier Science Publishers Ltd. London.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. and Welling, G. W. **(1995).** Quantitative fluorescence *in situ* hybridization of *Bifidobacterium spp.* with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl. Envir. Microbiol.* **61**: 3069-3075.

Lee Y. K. and Salminen S. (1995). The coming age of probiotics. *Trends Food Sci. Technol.* **6**: 241-245.

Lilly, D. M. and Stillwell, R. H. (1965). Probiotics: Growth promoting factors produced by microorganisms. *Science.* **147**: 747-748.

Linton, A. H., Hedges, A. J. and Bennet, B. M. **(1988).** Monitoring of resistance during the use of olaquindox as a feed additive on commercial pig farms. *J. Appl. Bact.* **64**: 311.

Lutz, T. and Scharrer, E. (1991). Effect of short-chain fatty acids on calcium absorption by the rat colon. *Exp. Physiol.* **76**: 615-618.

MacFarlane, G. T. and Cummings, J. H. (1991). The colonic flora, fermentation and large bowel digestive function. In: *The Large Intestine: Physiology, Pathophysiology and Disease* (eds. Phillips, S. F., Pemberton, J. H. and Shorter, R. G.). pp. 51-92. Raven Press. New York. NY.

MacFarlane, S. and MacFarlane, G. T. (1995). Proteolysis and amino acid fermentation. In: *Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology* (eds. Gibson, G. R. and MacFarlane, G. T.). pp. 75-100. CRC Press. Boca Raton, FL.

McFarlene, G. T. and Gibson, G. R. (1994). Metabolic activities of the normal colonic microflora. In: *Human Health: Contribution of Microorganisms* (ed. Gibson ,S. A. W.). pp. 17-38. Springer, Frankfurt, Germany.

Mann, G. V. and Spoerry, A. (1974). Studies of a surfactant and cholesteremia in the Maasai. *Amer. J. Clin. Nutr.* **27**: 464.

Martini, M. C., Lerebours, E. C., Lin, W. J., Harlander, S. K., Berrada, N. M., Antoine, J. M. and Savaiano, D. A. **(1991).** Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on *in vivo* lactose digestion. *Am. J. Clin. Nutr.* **54**: 1041-1046.

Matteuzzi, D., Crociani, F. and Brigidi, P. **(1983).** Antimicrobial susceptibility of *Bifidobacterium*. *Ann. Microbiol.* **134 A**: 339-349.

Meghrous, J., Euloge, P., Junelles, A. M., Ballongue, J. and Petidemange, H. **(1990).** Screening of *Bifidobacterium* strains for bacteriocin production. *Biotechnol. Lett.* **12 (8)**: 575-580.

Metchnikoff E. (1907). *The Prolongation of Life.* Optimistic studies.

Butterworth-Heinemann. London.

Mevissen-Verhage, E. A. E., Marcelis, J. H., Harmsen-van Amerongen, W. C. M., De Vos, N. M., Berkel, J. and Verhoef, J. **(1985).** Effect of iron on development of the neonatal gut flora during the first week of life. *Eur. J. Clin. Microbiol.* **4**: 14-18.

Mitsuoka, T. (1982). Recent trends in research on intestinal flora. *Bifidobacteria Microflora.* **1(1)**: 3-24.

Mitsuoka, T. (1990). Bifidobacteria and their role in human health. *J. Ind. Microbiol.* **6**: 263-268.

Mitzutani, T. and Mitsuoka, T. (1979). Effect of intestinal bacteria on incidence of liver tumors in gnotobiotic C3H/HE male mice. *J. Natl. Cancer Inst.* **63**: 1365-1370.

Mizota, T. (1996). Functional and nutritional foods containing bifidogenic factors. *Bulletin of the international dairy federation.* N° **313**: 31-35.

Modler, H. W. (1994). Bifidogenic Factors — Sources, Metabolisms and Applications. *Int. Dairy Journal.* 383-407.

Modler, H. W., McKellar, R. C. and Yaguchi, M. **(1990).** Bifidobacteria and bifidogenic factors. *Can. Inst. Food Sci. Technol.* **23 (1)**: 29-41.

Modler H. W. and Villa-Garcia L. (1993). The growth of *Bifidobacterium longum* in a whey based medium and viability of this organism in frozen yoghurt with low and high levels of developed acidity. *Cult. Dairy Prod. J.* **28:** 4-8.

Molis, C., Flourié, B., Ouarne, F., Gailing, M. F., Lartigue, S., Guibert, A., Bornet, F. and Galmiche, J. P. **(1996).** Digestion, excretion and energy value of fructooligosaccharides in healthy humans. *Am. J. Clin. Nutr.* **64**: 324-328.

Moore, W. E. C. and Holdmann, L. V. (1974). Human faecal flora: The normal flora of 20 Japanese Hawaiians. *Appl. Microbiol.* **27**: 961-979.

Munoa, F. J. and Pares, R. (1988). Selective medium for isolation and enumeration of *Bifidobacterium* spp. *Appl. Environ. Microbiol.* **54 (7)**: 1715-1718.

Nathan, C. F. (1987). Secretory products of macrophages. *J. Clin. Invest.* **79**: 319–323.

Nelson, G. M. and George, S. E. (1995). Comparison of media for selection and enumeration of mouse fecal flora populations. *J. Microbiol. Methods.* **22:** 293-300.

Nielsen, O. H., Jorgensen, S., Pedersen, K. and Justesen, T. **(1994).** Microbial evaluation of jejunal aspirates and faecal samples after oral administration of bifidobacteria and lactic acid bacteria. *J. Appl. Bacteriol.* **76**: 469-474.

Nogare, D. (1991). Southwestern International Medicine Conference: Septic shock. *Am. J. Med. Sci.* **302**: 50–65.

Nurmi, I. E. and Rantala, M. (1973). New aspects of *Salmonella* infection in broiler production. *Nature.* **241**: 210-211.

Ochi, Y. and Miyairi, K. (1943). Studies on swine influenza. I. The observation of natural outbreak and isolation of viruses. *Jpn. J. Vet. Sci.* **5**: 243-275.

Ohta, A., Motohashi, Y., Ohtsuki, M., Hirayama, M., Adachi, T. and Sakuma, K. **(1998).** Dietary fructooligosaccharides change the concentration of calbindinD9K differently in the mucosa of the small and large intestine of rats. *J. Nutr.* **128**: 934-939.

Ohta, A., Ohtsuki, M., Baba, S. and Takizawa, T. **(1995a).** Effects of fructooligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. *J. Nutr. Sci. Vitaminol.* **41**: 281-291.

Ohta, A., Ohtsuki, M., Baba, S., Adachi, T., Sakata, T. and Sakaguchi, E. **(1995b).** Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *J. Nutr.* **125**: 2417-2424.

Ohta, A., Ohtuki, M., Takizawa, T., Inaba, H., Adachi, T. and Kimura, S. **(1994).** Effect of fructooligosaccharides on the absorption of magnesium and calcium by cecectomized rats. *Int. J. Vitam. Nutr.* **64**: 316-323.

Oklahoma State University. (2002). Digestion in the ruminant. [On-line] http://www.ansi.okstate.edu/course/3543/Chapter%2011.1%20Digestion%20in%20the%20Ruminant1_files/frame.htm

Oku, T., Tokunaga, T. and Hosoya, N. **(1984).** Nondisgestibility of a new sweetner fructooligosaccharide neosugar in rat. *J. Nutr.* **114**: 1574-1581.

O'Sullivan, D. J. (1999). Methods of analysis of the intestinal microflora. In: *Probiotics: a Critical Review* (ed. Tannock, G. W.). pp. 23-44. Horizon Scientific Press. Wymondham. UK.

O'Sullivan, D. J. and M. J. Kullen (1998). Tracking of probiotic bifidobacteria in the intestine. *International Dairy Journal.* **8**: 513-525.

O'Sullivan, M. G. (1996). Metabolism of bifidogenic factors by gut flora - an overview. *Bulletin of the international dairy federation.* N° **313**: 23-30.

Oyarzabal, O. A. and Conner, D. E. (1995). *In vitro* fructooligosaccharide utilization and inhibition of *Salmonella* spp. by selected bacteria. *Poultry Science.* **74**: 1418-1425.

Parker R. B. (1974). Probiotics, the other half of the antibiotic story. *Anim Nutr. Health.* **29**:4-8.

Parker, D. S. and Armstrong, D. G. (1987). Antibiotic feed additives and livestock production. *Proc. Nutr. Soc.* **46**: 415.

Patel, V., Saunders, G. and Bucke, C. **(1994).** Production of fructooligosaccharides by *Fusarium oxysporum. Biotechnol. Lett.* **16**: 1139-1144.

Petschow, B. W. and Talbott, R. D. (1990). Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. *J. Clin. Microbiol.* **28**: 287-292.

Pettoello, M. M., Guandalini, S., Ecuba, P., Corvino, C. and di Martino, L. **(1989).** Lactose malabsorption in children with symptomatic *Giardia lamblia* infection: feasibility of yoghurt supplementation. *J. Pediatr. Gastroenterol.* **9**: 295-230.

Pierre, F., Perrin, P., Champ, M., Bornet, F., Meflah, K. and Menanteau, J. **(1997).** Short-chain fructo-oligosaccharides reduce the occurrence of colon tumors and develop gut-associated lymphoid tissue in Min mice. *Cancer Res.* **57**: 225.

Playne M. J. and Crittenden, R. (1996). Commercially available oligosaccharides. *Bulletin of the International Dairy Federation.* N° **313**: 10-22.

Poch, M. and Bezkorovainy, A. (1988). Growth-enhancing supplements for various species of the genus *Bifidobacterium. J. Dairy Sci.* **71**: 3214-3221.

Pond, W. G., Church, D. C. and Pond, K. R. **(1995).** *Basic Animal Nutrition and Feeding* (4th ed.). John Wiley & Sons. New York.

Pot, B. *et al.* (1994). Taxonomy of lactic acid bacteria. In: *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications* (eds. De Vuyst, L. & Vandamme, E. J.). pp. 13-90. Chapman and Hall. London.

Poupard, J. A., Husain, I. and Norris, R. F. **(1973).** Biology of the bifidobacteria. *Bacteriol. Rev.* **37**: 136-165.

Prapulla, S. G., Subhaprada, V. and Karanth, N. G. **(2000).** Microbial production of oligosaccharides: a review. *Adv. Appl. Microbiol.* **47**: 299-343.

Rada, V. (1997). Detection of *Bifidobacterium* species by enzymatic methods and antimicrobial susceptibility testing. *Biotechnol. Tech.* **11 (12)**: 909-912.

Raibaud, P. (1992). Bacterial interactions in gut. In: *Probiotics: The Scientific Basis.* (ed. Fuller, R.). pp. 9-28. Ch. 2.Chapman & Hall. London.

Rao, V. A. (2001). The prebiotic properties of oligofructose at low intake levels. *Nutr. Res.* **21**: 843-848.

Rasic, J. L. and Kurmann, J. A. (1983). *Bifidobacteria and Their Role.* Birkhauser Verlag. Basel.

Rasic, J. L. J. (1983). The role of dairy foods containing bifido- and *acidophilus bacteria* in nutrition and health. *N. Eur. Dairy J.* **48**: 80-88.

Reddy, B. S. (1999). Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. *J. Nutr.* **129**: 1478S-1482S.

Reddy, B. S., Hamid, R. and Rao, C. V. (1997). Effect of dietary oligofructose and inulin on colonic preneoplastic aberrant crypt foci inhibition. *Carcinogenesis.* **18**: 1371-1374.

Reddy, B. S., Watanabe, K., Weisburger, J. H. and Wynder, E. **(1977).** Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. *Cancer Res.* **37**: 3238-3242.

Reddy, B. S. and Rivenson, A. (1993). Inhibitory effect of *Bifidobacterium longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen. *Cancer Res.* **53**: 3914-3918.

Rémésy, C., Levrat, M. A., Gamet, I. and Demigne, C. **(1993).** Cecal fermentations in rats fed oligosaccharides (inulin) are modulated by dietary calcium level. *Am. J. Physiol.* **264**: G855-G862.

Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M. A., Tanaka, R., Watanabe, K. and Tannock, G. W. **(2002).** Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. *Applied and Environmental Microbiology.* **68 (5)**: 2420-2427.

Rietschel, E. T. and Brade, H. (1992). Bacterial endotoxins. *Sci. Am.* **267**: 54–61.

Roberfroid, M. (1998). Prebiotics and synbiotics: concepts and nutritional properties. *Br. J. Nutr.* **80 (Suppl. 2)**: S197-S202.

Roberfroid, M. and Slavin, J. (2000). Nondigestible oligosaccharides. *Crit. Rev. Food Sci. Nutr.* **40(6)**: 461-480.

Roberfroid, M. B. and Delzenne, N (1998). Dietary fructans. *Ann. Rev. Nutr.* **18**: 117.

Roberfroid, M., Gibson, G. R. and Delzenne, N. (1993). The biochemistry of oligofructose, a nondigestible fiber: an approach to calculate its caloric value. *Nutr. Rev.* **51**: 137-146.

Rogosa, M. (1974). *Bifidobacterium*. In: *Bergey's Manual of Determinative Bacteriology.* (8th Ed. Buchanan, R. E. and Gibbons, N. E.). pp. 669. Williams and Wilkins Co. Baltimore, M. D.

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* **130(2S)**: 396S-402S.

Rotimi V. O. and Duerden B. I. (1981). The development of the bacterial flora in normal neonates. *J. Med. Microbiol.* **14**: 51–62.

Rowland, I. R. and Grasso, P. (1975). Degradation of *N*-nitrosamines by intestinal bacteria. *Appl. Microbiol.* **29**:7-12.

Rowland, I. R., Rummey, C. J., Coutts, J. T. and Lievense, L. **(1998).** Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen induced aberrant crypt foci in rats. *Carcinogenesis.* **2**: 281-285.

Roy, D. (2001). Media for the isolation and enumeration of bifidobacteria in dairy products. *Int. J. Food Microbiol.* **69(3)**: 167-182.

Rumessen, J. J., Bode, S., Hamberg, O. and Gudmand-Hoyer, E. **(1990).** Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on bloodd glucose, insulin, and C-peptide responses in healthy subjects. *Am. J. Clin. Nutr.* **52**: 675-681.

Russell, J. B. and Diez-Gonzalez, F. (1998). The effects of fermentation acids on bacterial growth. *Adv. Microbial Physiol.* **39**: 205-234.

Rycroft, C. E., Jones, M. R., Gibson, G. R. and Rastall, R. A. **(2001).** A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J. Appl. Microbiol.* **91**: 878-887.

Saarela, M., Lähteenmäki, L., Crittenden, R., Salminen, S., and Mattila-Sandlholm, T. **(2002).** Gut bacteria and health foods - the European perspective. *Int. J. Food Microbiol.* **78**: 99-117.

Saavedra, J., Bauman, N. A., Oung, I., Perman, J. A. and Yolken, R. H. **(1994).** Feeding *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hopital for prevention of diarrhoea and shedding of rotavirus. *The Lancet.* **344**: 1046-1049.

Salmond, C. V., Kroll, R. G. and Booth, I. R. **(1984).** The effect of food preservatives on pH homeostasis in *Escherichia coli. J. Gen. Microbiol.* **130**: 2845-2850.

Samona, A. and Robinson, R. K. (1991). Enumeration of bifidobacteria in dairy products. *J. Soc. Dairy Technol.* **44**: 64-66

Savage, D. C. (1977). Microbial ecology of the gastrointestinal tract. *Ann. Rev. Microbiol.* **31**: 107-133.

Savage, D. C. (1986). Gastrointestinal microflora in mammalian nutrition. *Ann. Rev. Nutr.* **6**: 155.

Scardovi, V. (1986). Genus *Bifidobacterium*. In: *Bergey's Manual of Systematic Bacteriology* (eds. Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt, J. G.). pp. 1418-1434. Williams & Wilkins. Baltimore.

Scardovi, V. and Trovatelli, L. D. (1965). The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus *Bifidobacterium*. *Ann. Microbiol.* **15**: 19-29.

Scharrer, E. and Lutz, T. (1990). Effects of short chain fatty acids and K on absorption of Mg and other cations by the colon and caecum. *Z. Ernahrungswiss.* **29**: 162-168.

Schwab, J. H. (1977). Modulation of the immune response by bacteria. In: *Microbiology* (ed. Schlessinger, D.). pp. 366–373. American Society for Microbiology. Washington D. C.

Scrimshaw, N. S. and Murray, F. B. (1988). Lactose tolerance and milk consumption. *Am. J. Clin. Nutr.* **48**: 1083-1159.

Sebald, M., Gasser, F. and Werner, H. **(1965).** DNA base composition and classification, application to group of bifidobacteria and to related genera, *Ann. Inst. Pasteur.* **109**: 251-269.

Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P. and Dore, J. **(2000).** Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl. Envir. Microbiol.* **66**: 2263-2266.

Shah, N. P. (2001). Functional Foods from probiotics and prebiotics. *Food Technol.* **55 (11)**: 46-53.

Shortt, G. (1997). Innovative ingredients for optimum gut health. *The European Food & Drink Review*. Winter 1997. 31-35.

Sigma-Aldrich. (2002). Biological Detergent Theory and Application. [On-line]. http://www.sigmaaldrich.com/Brands/Sigma/Biological_Detergents/Detergent_Theory.html

Skinner, S. J. (2001). The effect of abomasal oligosaccharide supplementation on the intestinal microflora of sheep: analysis of oligosaccharides. *M.Sc. Thesis*. The University of Waikato. New Zealand.

Smith, C. J. and Bryant, M. P. (1979). Introduction to metabolic activities of intestinal bacteria. *Am. J. Clin. Nutr.* **32**: 149-157.

Smith, H. W. (1965). The development of the flora of the alimentary tract in young animals. *J. Pathol. Bacteriol.* **90**: 495-513.

Smith, H. W. and Tucker, J. F. (1975). The effect of feeding diets containing permitted antibiotics on the faecal excretion of *Salmonella typhimurium* by experimentally infected chicks. *Journal of Hygiene. Cambridge.* **75**: 293-301.

Sonoik, K., Mada, M. and Mutai, M. **(1986).** Selective agar medium for counting viable cells of bifidobacteria in fermented milk. *J. Food Hyg. Soc. Jap.* **27**: 238-244.

Sperti, G. S. (1971). *Probiotics.* Avi Publishing Co.West Point. CT.

Stanton, C., Gardiner, G., Meehan, H., Collins, K., Fitzgerald, G., Lynch, P. B. and Ross, R. P. **(2001).** Market potential for probiotics. *Am. J. Clin. Nutr.* **73(2S)**: 476S-483S.

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.

Stone-Dorhow, T. and Levitt, M. D. (1987). Gaseous response to ingestion of a poorly absorbed fructo-oligosaccharide sweetener. *Am. J. Clin. Nutr.* **46**: 61-65.

Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G. R., Collins, M. D. and Doré, J. **(1999).** Direct analysis of genes encoding 16S rRNA from complex

communities reveals many novel molecular species within the human gut. *Appl. Envir. Microbiol.* **65**: 4799-4807.

Symons, H. (1997). Bifidobacteria. Danone World Newsletter N°16. [Online]. http://www.danonevitapole.com/extranet/vitapole/portail.nsf/ACCUEIL?OpenForm&Seq=1

Szylit, O. and Andrieux, C. (1993). Physiological and pathophysiological effects of carbohydrate fermentation. *World Rev. Nutr. Diet.* **74**: 88-122.

Tamime, A. V. (1999). Biology of bifidobacteria. In: *Encyclopedia of Food Microbiology* (eds. Robinson, R. K., Batt, C. A. and Patel, P. D.) pp. 1355-1360. Academic Press. London. UK.

Tannock, G. W. (1997). Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D. *Trends Biotechnology.* **15(7)**: 270-274.

Tannock, G. W. (2000). The intestinal microflora: potentially fertile ground for microbial physiologists. *Adv. Microbial Physiol.* **42**: 25-46.

Taper, H. S., Delzenne, N. and Roberfroid, M. B. **(1997).** Growth inhibition of transplantable mouse tumors by nondigestible carbohydrates. *Int. J. Cancer.* **71**: 1109.

Tashiro, Y., Oike, H., Aramaki, M., Hirayama, M. and Adachi, T. **(1997).** *In vitro* fermentation of fructooligosaccharides in comparison with other oligo- and polysaccharides. In: *Proceedings of the International Symposium "Non-digestible oligosaccharides: healthy food for the colon?"* pp. 128. 4-5 December. 1997. Wageningen. The Netherlands.

Teraguchi, S., Uehara, M., Ogasa, K. and Mitsuoka, T. **(1978).** Enumeration of bifidobacteria in dairy products. *Jap. J. Bact.* **33 (6)**: 753-761.

Tohyama, K., Tanaka, R., Kobayashi, Y. and Mutai, M. **(1982).** Relationship between the metabolic regulation of intestinal microflora by feeding *Bifidobacterium* and host hepatic function. *Bifidobaceria Microflora.* **1**: 45.

Tojo, M., Oikawa, T., Morikawa, Y. Yamashita, N., Iwata, S., Satoh, Y., Hanada, J. and Tanaka, R. **(1987).** The effects of *Bifidobacterium breve* administration on Campylobacter enteritis. *Acta Paediatr. Jpn.* **29**: 160-167.

Tomomatsu, H. (1994). Health effects of oligosaccharides. *Food Technol.* **48 (10)**: 61-65.

Tzannis, S. T. (1991). Tutorial on cell disruption. [On-line]. http://www.esb.ucp.pt/~bungah/working/disrupt.htm

University of Alberta. (1998). The ruminant digestive system. [On-line]. http://www.afns.ualberta.ca/dairy/dp475-5a.htm

University of Kentucky. (2002a). Digestive tract of the monogastric mammal. [On-line]. http://www.ca.uky.edu/agripedia/classes/asc106/gimono.htm

University of Kentucky. (2002b). Digestive tract of the ruminant. [On-line]. http://www.ca.uky.edu/agripedia/classes/asc106/girumin.htm

Usami, S., Ishii, T., Kirimura, K., Vehara, K. and Chen, J. **(1991).** Production of β-fructofuranosidase showing fructose-transferring activity by *Penicillium firequentans* (*P. glabrum*). *J. Ferment. Bioeng.* **72**: 303-305.

Ushijima, T., Takahashi, M. and Ozaki, Y. **(1985).** Fourteen selective media facilitate evaluation of populations of coexisting fixed bacterial strains of enteric pathogens and normal human faecal flora. *J. Microbiol. Meth.* **4**: 189-195.

van Eldere, J. and Eysssen, H. (1984). Metabolism of cholesterol. In: *The Germ-Free Animal in Biomedical Research* (eds. Coates, M. E. and Gustafsson, B. E.). pp. 317-332. Laboratory Animals. London.

Vandenbergh, P. A. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol. Rev.* 12: 221-238.

vanLoon, D. (1976). *The Family cow.* Pownal: Garden Way Publishing.

Wang X. (1993). Comparative aspects of carbohydrate fermentation by colonic bacteria. *Ph.D. thesis.* University of Cambridge. UK.

Wang, X. and Gibson, G. R. (1993). Effect of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J. Appl. Bacteriol.* **75**: 373-380.

Ward, D. M., Bateson, M. M., Weller, R. and Ruff-Roberts, A. L. **(1992).** Ribosomal RNA analysis of microorganisms as they occur in nature. *Adv. Microb. Ecol.* **12:**219-286.

Wilson, K. H. and Blitchington, R. B. (1996). Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* **62:**2273-2278.

Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* **51**: 221-271.

Yaeshima, T. (1996). Benefits of bifidobacteria to human health. *Bulletin of the International Dairy Federation*. N° **313**: 36-42.

Yamashita, K., Kawai, K. and Itakura, M. **(1984).** Effect of fructo-oligosaccharides on blood glucose and serum lipids in diabetic subjects. *Nutr. Res.* **4**: 961-966.

Yamazaki, S., Kamimura, H., Momose, H., Kawashima, T. and Ueda, K. **(1982).** Protective effect of *Bifidobacterium*-monoassociation against lethal activity of *Escherichia coli*. *Bifidobacteria Microflora*. **1**: 55-59.

Yamazaki, S., Machii, K., Tsuyuki, S., Momose, H., Kawashima, T. and Ueda, K. **(1985).** Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial invasion. *Immunology*. **56**: 43-50.

Yamazaki, S., Tsuyuki, S., Akashiba, H., Kamimura, H., Kimura, M., Kawashima, T. and Ueda, K. **(1991).** Immune response to *Bifidobacterium*-monoassociated mice. *Bifidobacteria Microflora*. **10**: 19-31.

Yoshita, M., Fujita, K., Sakata, H., Muronon, K., Iseki, K. **(1991).** Development of the normal intestinal flora and its clinical significance in infants and children. *Bifidobacteria Microflora*. **10**: 11–27.

Younes, H., Demigné, C., Behr, S. R., Garleb, K. A. and Rémésy, C. **(1996).** A blend of dietary fibers increases urea disposal in the large intestine and lowers urinary nitrogen excretion in rats fed a low protein diet. *J. Nutr. Biochem.* **7**: 474-480.

Younes, H., Garleb, K., Behr, S., Rémésy, C. and Demigné, C. **(1995).** Fermentable fibers or oligosaccharides reduce urinary nitrogen excretion by increasing urea disposal in the rat cecum. *J. Nutr.* **125**: 1010-1616.

Yun, J. W. and Song, S. K. (1993). The production of high content fructooligosaccharides from sucrose by the mixed-enzyme system of fructosyltransferase and glucose oxidase. *Biotechnol. Lett.* **15**: 573-576.

Yun, J. W., Lee, M. G. and Song, S. K. **(1994).** Batch production of high-content fructooligosaccharides from sucrose by the mixed-enzyme system of β-fructofuranosidase and glucose oxidase. *J. Ferment. Bioeng.* **77**: 159-163.

Ziolecki, A. and Briggs, C. A. E. (1961). The microflora of the rumen of the young calf. II. Source, nature and development. *J. Appl. Bacteriol.* **24**: 148-63.

Zoetendal, E. G., Akkermans, A. D. L. 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. Envir. Microbiol.* **64**: 3854-3859.