CHAPTER 1

GENERAL INTRODUCTION

1. Prebiotic Oligosaccharides

The prebiotics currently in use are non-digestible, but fermentable carbohydrates, especially oligosaccharides, by selective strains of human microflora. Preliminary findings of several recent human clinical trials indicated that prebiotics may indeed prove to be a clinically beneficial dietary supplement (Gibson, 2004). The combination of selective effect on the microflora and fermentation should lead to several benefits for health such as protective effects against colorectal cancer (Cummings *et al*., 2001). The dietary intake of oligosaccharides is difficult to estimate, but may reach 3-13 g d^{-1} per person (for fructo-oligosaccharides), depending on the human populations (Delzenne *et al*., 2003).

The term prebiotic was first 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, that can improve the host health". The prebiotic concept considers that many potentially health-promoting microorganisms, such as bifidobacteria and lactobacilli, are already resident in the human colon. Prebiotics must be stable in the acid of the stomach, reach the colon where they are then selectively fermented by positive bacteria and they must not be absorbed in the small intestine (Roberfroid, 2001).

The traditional means of manipulating the gut flora was by the use of probiotics. The term probiotic was first defined by Parker (1974) as 'organisms and substances which contribute to intestinal microbial balance'. Later, Fuller (1989) re-defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. A synbiotic is defined as 'the mixtures of pro- and prebiotic, which beneficially affect the host, by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract' (Gibson and Roberfroid, 1995). Examples of health promoting effects and mechanism of probiotics, prebiotics and synbiotics are summarized in Table 1.1.

Table 1.1 Health promoting effects and mechanisms of probiotic bacteria and prebiotic substances

Beneficial Effect	**Mechanism**	**References**
Reduce risk of pathogen infection	Decreased pH and produced bacteriocins- inhibited growth of undesirable bacteria	Lawhon *et al*. (2002) Tzortzis *et al*. (2004)
Improve mineral absorption	Decreased pH- increased mineral (esp. calcium) solubility	Scholz-Ahrens *et al*. (2001) Gudiel-Urbano and Goni (2002)
Decrease lipids and cholesterol	Increased short-chain fatty acid (SCFA)-modulated lipogenesis Lowering of pH & biles precipitation Suppression of hepatic triglyceride and very low density lipoprotein (VLDL) synthesis	Taylor and Williams (1998) Delzenne and Kok (2001)
Decrease risk of cancer	Increased butyrate- fuel for colonocytes and cell differentiation Decreased bile acid formation Decreased genotoxic metabolites & enzymes and carcinogens	Reddy (1998), Brady *et al*. (2000) Sanderson *et al*. (2004)
Immune system	Direct contact of lactic acid bacteria or bacterial product with immune cells in the intestine Production of SCFA	Salminen *et al*. (1998) Schley and Field (2002)
Reduce constipation	Faecal bulking & fibre-like effects	Hara *et al*. (1994), Mizota (1996)
Reduce diarrhoea	Reduction in pathogens causing infection	Gibson and Wang (1994a)
Aid in irritable bowel syndrome (IBS)	Lactobacilli inhibited *Candida albicans*, thought to be the IBS causative organism	Madden and Hunter (2002)

There are three main types of carbohydrates that are non-digestible in the human small intestine: non-starch polysaccharides (NSP), resistant starch (RS) and non-digestible oligosaccharides (NDO, Voragen, 1998). Any dietary material that resists digestion in the upper gut and enters the large intestine is a candidate prebiotic (Crittenden *et al*., 2001), however at the present time the only molecules known to act as prebiotics are carbohydrates, particularly oligosaccharides (Gibson and Roberfroid, 1995; Roberfroid *et al*., 1998). Many oligosaccharides are not metabolised in the human small intestine (i.e. they are NDO), which is the first requirements of a prebiotic, and pass to the colon quantitatively. When the prebiotic arrives in the colon, certain members of the indigenous microflora must ferment it selectively which is the most important property of a prebiotic (Gibson and Roberfroid, 1995).

One of the biggest blocks to the development of prebiotics is our lack of knowledge of the structure-function relationships in these molecules. A useful prebiotic would; easily incorporate into appropriate food vehicles, possess multiple functions, have activity at low dosages, be non-carcinogenic, have a low calorific value and target the distal colon. It is possible to identify certain properties that the oligosaccharides should possess in order to achieve these attributes (Table 1.2).

Typically, oligosaccharides are sugars consisting of between 2 and 10 saccharide units. Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides or through enzymatic transfer reactions from lower molecular weight sugars. Various aspects of the production and properties of food grade oligosaccharides and their prebiotic effects have been tested using *in vitro* methods (i.e. batch and three-stage continuous culture) and *in vivo* methods (i.e. animal models and human clinical trials). Prebiotic oligosaccharides currently commercialized as food ingredients include fructo-oligosaccharides, galacto-oligosaccharides, lactosucrose, isomalto-oligosaccharides, gentio-oligosaccharides and xylo-oligosaccharides (Rastall, 2000).

Table 1.2 Design parameters for enhanced activity prebiotics

Desirable attribute in prebiotic	**Properties of oligosaccharides**
Active at low dosage	Highly selectively and efficiently metabolised by *Bifidobacterium* and/or *Lactobacillus* sp.
Lack of side effects	Highly selectively metabolised by beneficial bacteria but not by gas producers, putrefactive organisms, etc.
Persistence through the colon	High molecular weight, correct choice of glycosyl residue, chemical modification
Varying viscosity	Available in different molecular weights and linkages
Good storage and processing stability	Possess 1-6 linkages and pyranosyl sugar rings
Fine control of microflora modulation	Selectively metabolised by restricted species
Varying sweetness	Different monosaccharide composition
Inhibits adhesion of pathogens	Possess receptor sequence

Source: Rastall and Gibson (2002)

The current commercial available prebiotic oligosaccharides are as follow (Crittenden and Playne, 1996; Rastall, 2000; Rastall and Gibson, 2002):

1.1 Lactulose

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a synthetic disaccharide in the form of Gal β1-4 Fru, produced by catalytic isomerization from lactose and the producers are now in Germany, Denmark, Austria, Italy, Japan, UK and Netherlands (Schumann, 2000). Since the 1950s, lactulose has been used for the treatment of specific medical conditions in humans (Petuely, 1957 cited by Schumann, 2000). Lactulose is generally classified as a drug and it is a prebiotic experimentally but is not used commercially as a food ingredient.

It has been used as a laxative as it is not hydrolyzed or absorbed in the small intestine (Tamura, 1983; Modler, 1994). The bifidogenic nature of lactulose at doses of 10 g d^{-1} has been confirmed in a double-blind placebo-controlled study (Tuohy *et al*., 2000). Lactulose fermented by anaerobic bacteria, especially *Bacteroides* spp. in the colon yields lactic, acetic and butyric acid and gas, especially molecular hydrogen (H_2, Bongaerts *et al*., 2005).

1.2 Inulin and Fructo-oligosaccharides (FOS)

Inulin and fructo-oligosaccharides are β(2-1) fructans, which contain a mixture of oligo- and polysaccharides. They are almost all linear chains of fructose having the structure GF_n (with G=glucosyl unit, F=fructosyl unit and n=number of fructosyl units linked to one another, De Leenheer and Hoebregs, 1994). In native chicory inulin, the number of fructose units linked together ranges from 2 to more than 60, with average degree of polymerization (DP) in the order of 10-12. Inulin having an average DP of about 25 is also available commercially from Orafti (Tienen, Belgium) as Raftiline HP, for "High Performance". Oligofructose obtained from inulin contains GF_n and F_n chains with number of fructosyl units ranging from 2 to 9, whereas oligofructose as produced from sucrose only has GF_n forms with number of fructosyl unit between 2 and 4 (Bornet, 1994; De Leenheer and Hoebregs, 1994).

Fructans are, after starch, the most abundant non-structural natural polysaccharides. They are present in a wide variety of plants and some bacteria and fungi. Plants containing inulin and oligofructose primarily belong to either Liliales, e.g. leek, onion, garlic and asparagus, or the Compositae, such as Jerusalem artichoke, dahlia, yacon and chicory.

Oligofructose is produced using two different manufacturing techniques. Orafti produces oligofructose by partial enzymatic hydrolysis of chicory inulin (using a specific endo-inulinase), followed by spray-drying whereas BMI (France) synthesizes it from sucrose using fructosyltransferase (Bornet, 1994). Fructo-oligosaccharides (FOS) are short-chain oligofructose, represented as Glu α1-2[β Fru 1-2]$_n$ where n = 2-9. They are considered important prebiotic substrates mainly because of their economy of manufacture and reproducibility of their prebiotic effect (Crittenden and Playne, 1996). At least 88% of inulin and oligofructose reach the caecum (Cummings and

Macfarlane, 2002) and are hardly digested during *in vivo* tests with either human saliva or rat pancreatic homogenate (Ellegard *et al*., 1997).

FOS are not degraded or absorbed in the upper human gastrointestinal tract and they enter the colon intact where they are metabolisable by the resident microbiota. The effects of FOS have been studied both *in vitro* (i.e. batch and continuous culture) and *in vivo* (i.e. animals and human trials) as shown in Table 1.3. The results have consistently shown the prebiotic nature of this substrate (Wang and Gibson, 1993; Djouzi and Andrieux, 1997; Bouhnik *et al*., 1999).

1.3 Galacto-oligosaccharides (GOS)

Galacto-oligosaccharides, also called transgalacto-oligosaccharides (TOS), are non-digestible, galactose-containing oligosaccharides of the form Glu β 1-4 [β Gal 1-6]$_n$ where n= 2-5. They are produced from lactose by means of an enzymatic conversion (Crittenden and Playne, 1996). Two types of enzyme can theoretically be used to produce GOS from lactose: glycosyltransferases, which need a sugar nucleotide as a donor and glycosidases, which utilise galactose as a donor. The first type of enzyme is not available on an industrial scale and commercially only the glycosidases, namely β-galactosidases derived from bacteria (*Bacillus circulans*), moulds (*Aspergillus niger* or *oryzae*) and yeasts (*Kluyveromyces lactis* or *fragilis*) are used. The enzyme catalyses both hydrolysis and polymerization with the equilibrium being dependent on the biological source of enzyme and the reaction conditions.

For research purposes, enzymes derived from many other microorganisms such as bifidobacteria have also been used giving GOS yields of 24.7-47.6% within 7 h reaction (Laere *et al*., 2000; Rabiu *et al*., 2001). GOS has also been synthesized from lactose by β-galactosidase in AOT/isooctane reverse micelles at pH 7.0 and 45°C giving a maximum yield of 51.2%, w/w (Chen *et al*., 2001).

As galacto-oligosaccharides are not hydrolyzed and subsequently not absorbed in the small intestine, they can be considered as low-calorific carbohydrates at 1.73 kcal g^{-1} (Sako *et al*., 1999). In most human studies GOS have shown a bifidogenic effect after consumption of 2.5 to 15 g GOS a day (Table 1.4).

Table 1.3 Studies on effects of inulin and fructo-oligosaccharides

Subjects	Substrate	Dose	Duration	Results	Reference
12 adults	Neosugar	4 g d^{-1} (in controlled diet)	25 d	Sig.↑ aerobes, enterobacteria and bifidobacteria. Only the later decreased after feeding stopped.	Buddington *et al*. (1996)
10 senile adults	Inulin	20 g d^{-1}, then 40 g d^{-1}	8 d, then 11 d	Sig.↑ bifidobacteria, For 40 g d^{-1}, sig.↓ enterococci, bacteroides and enterobacteria	Kleessen *et al*. (1997)
54 adults	Inulin	10 g d^{-1} (supple-mented into diet)	8 weeks	Sig.↓ insulin, plasma triglyceride	Jackson *et al*. (1999)
40 adults (8 in each group)	Neosugar	0, 2.5, 5, 10 or 20 g d^{-1}	7 d	Sig. ↑Bifidobacteria with 5, 10 and 20 g d^{-1} Sig. correlation with dose & ↑ bifs. Gas occurred at 20 g d^{-1}	Bouhnik *et al*. (1999)
8 adults	Raftilose L60	8 g d^{-1} (in controlled diet), then 8 g d^{-1} (in normal diet)	14 d, then 21 d	Sig.↑ bifidobacteria and lactobacilli, ↓ bacteroides, coliforms and *Clostridium perfringens*	Menne *et al*. (2000)
Cats	Raftilose or Raftiline GR	0, 3, 6, 9 % (w/w)	12 d	For 3%, lowering pH, SCFA higher in oligofructose supplemented diet	Hesta *et al*. (2001)

Table 1.4 Studies on effects of galacto-oligosaccharides

Subjects	Substrate	Dose	Duration	Results	Reference
12 men	Oligomate[a]	0, 2.5, 5 or 10 g d^{-1}	1 week for each dose	Bifidobacteria ↑ as dose increased. For 10 g d^{-1}, sig.↑ bifidobacteria and lactobacilli	Ito *et al*. (1990)
12 men	TD[b]	15 g d^{-1}	6 d	Sig.↑ bifidobacteria and lactobacilli, sig. ↓ bacteroides. sig.↓ propionate and isobutyrate	Ito *et al*. (1993)
HFA[c] rats	TOS[d]	5% (w/w)	7 weeks	Sig.↑ bifidobacteria, SCFA and acetate, Sig.↓ staphylococci and streptococci	Morishita and Konishi (1994)
8 adults	TOS	10 g d^{-1}	21 d	Sig.↑ bifidobacteria, Sig.↓ breath H_2	Bouhnik *et al*. (1997)
6 adults	TOS	15 g d^{-1}	14 d	Sig.↑ total counts on MRS[e], no change in bifidobacteria	Teuri *et al*. (1998)
Human-colonic-batch culture	Oligomate 55 (50% GOS)	10% (w/v)	24 h	Sig. ↑ bifidobacteria, no sig.↓clostridia and lactobacilli by FISH[f]	Rycroft *et al*. (2001b)

[a] Trade name of GOS

[b] Transgalactosylated disaccharides

[c] Human faecal flora associated rats

[d] Transgalactosylated oligosaccharides

[e] Agar for enumeration of lactic acid bacteria and bifidobacteria

[f] Fluorescent *in situ* hybridization

Bifidobacteria and lactobacilli use GOS as a substrate and the major end products of bacterial fermentation are short-chain fatty acid (lactate, acetate, propionate) and GOS is completely fermented in the human colon (Salminen *et al*., 1998).

1.4 Soybean oligosaccharides (SOS)

Soybean oligosaccharides are non-reducing α-galactosyl sucrose derivatives (Crittenden and Playne, 1996). They were developed by Calpis Food Industry Co., Japan. The predominant oligosaccharides in soybeans are raffinose (trisaccharide) and stachyose (tetrasaccharide). SOS are able to reach the colon and are thought to stimulate bifidobacteria *in vivo* (Oku, 1994).

In vitro experiments using human faecal bacteria have shown that SOS are utilized primarily by bifidobacteria (with the exception of *B. bifidum*) and lactobacilli, but also to a limited degree by bacteroides, clostridia and *E. coli* (Yazawa and Tamura, 1982). Benno *et al*. (1987) administered 15 g d^{-1} raffinose to seven healthy adults and observed a significant increase in bifidobacteria while total bacterial counts remained stable. The addition of low concentration of SOS to a two-stage continuous culture of faecal bacteria resulted in a threefold increase in the proportion of bifidobacteria in the total bacterial count (Saito *et al*., 1992). Studies on these effects of soybean-oligosaccharides are summarized in Table 1.5.

1.5 Lactosucrose (LS)

Lactosucrose is a non-reducing oligosaccharide, in a mixture of lactose and sucrose which produced by the transfer of a fructosyl residue from sucrose to the glucose moiety of lactose by action of β-fructofuranosidase (Tamura, 1983). Lactosucrose was developed by Hayashibara Biochemical Laboratory Co., Japan.

A pure culture study compared lactosucrose with lactulose, FOS, SOS, raffinose and glucose for its utilization by various intestinal bacteria. Six bifidobacteria and three lactobacilli strains grew to the same extent on lactosucrose and glucose, whereas all the other organisms tested preferred glucose (Hara *et al*., 1994).

Lactosucrose has been evaluated in three human volunteer trials involving different types of subject. In normal healthy adults, LS fed at 3 g d^{-1} resulted in a significant, 0.7 log, increase in bifidobacteria, together with a significant, 0.6 log,

decrease in bacteroides (Ohkusa *et al*., 1995). This study showed no change in short-chain fatty acids.

A feeding trial involving constipated adults who were fed 0.32 g kg^{-1} of their body weight per day resulted in a significant, 0.7 log, increase in bifidobacteria and a large and significant, 4.5 log, decrease in clostridia. Acetate and butyrate increased, but propionate, isobutyrate, valerate and isovalerate remained unchanged (Kumemura *et al*., 1992). Studies on effects of lactosucrose are summarized in Table 1.6.

Table 1.5 Studies on effects of soybean oligosaccharides

Subjects	**Substrate**	**Dose**	**Duration**	**Results**	**Reference**
7 adults	Raffinose	15 g d^{-1}	4 weeks	Sig.↑ bifidobacteria, Sig.↓ bacteroides and clostridia	Benno *et al*. (1987)
6 adults	SOS[a]	10 g d^{-1}	3 weeks	Sig.↑ bifidobacteria and lactobacilli, large ↓ clostridia and peptostreptococci	Hayakawa *et al*. (1990)
7 adults	SOS (supplemen-ted in drink)	10.6 g d^{-1}	3 weeks	Sig.↑ bifidobacteria, slight ↓ bacteroides, Sig. ↓ NH_3 and β-glucuronidase, large ↓ indole, sketole, phenol and p-cresol	Wada *et al*. (1992)
Human colonic-batch culture	SOS (35%)	10% (w/v)	24 h	Sig.↑ bifidobacteria, No sig.↓ clostridia and lactobacilli by FISH	Rycroft *et al*. (2001b)

[a] Soybean oligosaccharides extract containing 23% stachyose and 7% raffinose

Sig. = significance

Table 1.6 Studies on effects of lactosucrose

Subjects	Substrate	Dose	Duration	Results	Reference
13 senile adults	55% lactosucrose	10.3 g d^{-1}	3 weeks	Sig.↑ bifidobacteria, ↓ bacteroides, eubacteria and peptococci. Sig. ↓ NH_3, sketole, indole and p-cresol. Sig. ↑ acetate and Sig. ↓ butyrate	Kumemura *et al*. (1992)
8 cats	95% lactosucrose	175 mg d^{-1}	2 weeks	Sig.↑ bifidobacteria, lactobacilli, Sig.↓ clostridia, enterobacteria, fusobacteria, staphylococci and *Spirochaetae*.	Terada *et al*. (1993)
8 adults	95% lactosucrose	3 g d^{-1}, then 6 g d^{-1}	1 week, then 1 week	Sig.↑ bifidobacteria, Sig. ↓ bacteroides and clostridia. Sig.↓ β-glucuronidase, ↓ azoreductase and nitrate reductase. Sig.↑ SCFA, lactate and acetate	Hara *et al*. (1994)
50 rats	94.6% lactosucrose	10% (w/w) supplem-ented in diet	4 weeks	Large Sig.↑ bifidobacteria, Sig↑ coliforms, Sig.↑ NH_3 Lowering pH Sig.↓ glucuronidase	Bielecka *et al*. (2002)

1.6 Isomalto-oligosaccharides (IMO)

Isomalto-oligosaccharides (IMO) are composed of glucose monomers linked by α(1-6) glucosidic linkages. IMO are derived from starch by a two-step enzymatic process and are mixtures of α(1,6) glucosides such as isomaltose, isomaltotriose, panose and isomaltotetraose. Firstly, starch is hydrolyzed to α(1-4) malto-oligosaccharides by α-glucosidase. Secondly, glucosyl residues are transferred to produce α(1-6) linked IMO using the same enzyme. IMO were developed by Hayashibara Biochemical Laboratory Co, Japan in 1982 and marketed by Hayashibara as well as Showa Sangyo and Nikken Chemicals. The effective daily dose of IMO is 13 g. A commercial mixture known as Isomalto-900 has been produced by incubating α-amylase, pullulanase and α-glucosidase with corn starch. The major oligosaccharides in this mixture are isomaltose (Glu α1-6 Glu), isomaltotriose (Glu α 1-6 Glu α1-6 Glu) and panose (Gluα1-6 Glu α1-4 Glu).

A mixture containing 58% IMO was produced from liquefied corn syrup by the hydrolyzing and transglycosylation activities of *Bacillus stearothermophilus* maltogenic amylase (BSMA) alone or 68% IMO obtained when BSMA and α-glucanotransferase (α-GTase) were reacted simultaneously (Lee *et al*., 2002a).

The attractiveness of IMO as prebiotics ought to lie in their cost effectiveness, as starch type dextrans are readily available and can be converted to IMO. A part of IMO was digested by isomaltase (70-80% compared to those of maltose) in the human jejunum and the residual IMO was fermented by intestinal flora, in particular bifidobacteria, but not by *E. coli* and most of the *Clostridium* species in the colon (Kohmoto *et al*., 1988; Olano-Martin *et al*., 2000). Bacterial growth in pure culture on isomaltose, isomaltotriose, panose and Isomalto-900 has been tested (Kohmoto *et al*., 1988). Several of bifidobacteria metabolised the tested sugars except for *Bifidobacterium bifidum* and all bacteroides utilized all the tested sugars but fewer clostridia grew on the Isomalto-900 than on raffinose. Isomalto-900 resulted in greater increases in bifidobacteria than did raffinose. *In vivo* experiments showed an increase of faecal bifidobacteria counts in elderly people on intake of an IMO product (Yatake, 1993). Studies on effects of IMO are given in Table 1.7.

Table 1.7 Studies on effects of isomalto-oligosaccharides

Subjects	Substrate	Dose	Duration	Results	Reference
6 men	Isomalto-900 (64% IMO)	20 g d^{-1}	10 d	Sig.↑ bifidobacteria, Sig.↑ (but smaller) bacteroides	Kohmoto *et al*. (1988)
18 senile adults	Isomalto-900	20 g d^{-1}	14 d	Sig.↑ bifidobacteria, Sig.↑ (but smaller) bacteroides	Kohmoto *et al*. (1988)
12 men	Isomalto-900	6.7, 10.1, 13 or 15.3 g d^{-1}	10 d for each dose	↑ bifidobacteria for all doses but Sig. ↑ only for 15.3 g d^{-1}	Kohmoto *et al*. (1991)
9 men	Isomalto-900	15.6 g d^{-1}	14 d	Sig.↑ bifidobacteria, all others bacteria trended to increase	Kohmoto *et al*. (1991)
2 groups of 7 men	IMO2[a] or IMO3[b]	10, 20 then 5 g d^{-1}	12 d for each dose	IMO2-Sig.↑ bifidobacteria at 20 g d^{-1}, no effect at 5 or 10 g d^{-1} IMO3-Sig.↑ bifidobacteria with all three doses	Kaneko *et al*. (1994)
Human colonic-batch culture	IMO (91%)	10% (w/v)	24 h	Sig.↑ bifidobacteria, not Sig.↑ bacteroides, slightly ↓ lactobacilli and clostridia	Rycroft *et al*. (2001b)

[a] Fraction of Isomalto-900, containing 63.8% isomaltose and 22.6% other disaccharides (nigerose and kojibiose).

[b] Fraction of Isomalto-900, containing 27.7% panose, 12.1% isomaltotriose and 50.1% tetra-, penta- and hexasaccharide components.

Sig. = significance

1.7 Gluco-oligosaccharides

Gluco-oligosaccharides with differing structures can be made using glycosyltransferases or β-glucosidases (Crittenden and Playne, 1996). Gentio-oligosaccharides (GEOS) consisted of β(1-6)-D-glucosidic linkages and are made by Nihon Shokuhin Kako Co. (Playne and Crittenden, 1996) and sold under the trade name Gentose.

Gluco-oligosaccharides are produced by transferring glucose molecules from a sucrose donor to a maltose acceptor using the glucosyl-transferase from *Leuconostoc mesenteroides* to form a mixture of different sized gluco-oligosaccharides. In addition, beta-linked gluco-oligosaccharides may be extracted from oat β-glucans and FDA recently accepted this product as a functional food, although there is no evidence that they act as prebiotics (Valette *et al*., 1993). Studies on effects of gluco-oligosaccharides are summarized in Table 1.8.

1.8 Xylo-oligosaccharides (XOS)

Xylo-oligosaccharides are low molecular weight reducing oligosaccharides, chains of xylose residues linked by β(1-4) bonds and mainly consist of xylobiose, xylotriose and xylotetraose (Hopkins *et al*., 1998). Xylo-oligosaccharides can be manufactured by enzymatic hydrolysis of xylan from corncobs (Crittenden and Playne, 1996), oats (Jaskari *et al*., 1998) and wheat arabinoxylan (Yamada *et al*., 1993). Commercially available XOS are manufactured and marketed by Suntory Ltd., Japan.

XOS were metabolised by the majority of bifidobacteria and lactobacilli while in a human trial showed significant increases in faecal bifidobacteria (10-31%) and deceases in bacteroides after the consumption of 1-2 g d^{-1} XOS and level dropped after administration ceased (Okazaki *et al*., 1990). Howard *et al*. (1995) studied the effect of 4.2 g d^{-1} XOS on the colonic microbiota of mice, but did not observe any increase in bifidobacterial numbers. The same group reported a significant increase in bifidobacteria levels upon administration of 5 g d^{-1} XOS in human volunteers. XOS stimulated caecal and faecal bifidobacteria at a higher level than did fructo-

oligosaccharides and at 6% of the diet, XOS produced higher SCFA levels (Campbell *et al*., 1997).

Table 1.8 Studies on effects of gluco-oligosaccharides

Subjects	Substrate	Dose	Duration	Results	Reference
Rats	Gluco-oligosacch-arides	2% (w/w)		Slight↑ bifidobacteria, no change in bacteroides and clostridia, Sig.↑ SCFA	Djouzi *et al*. (1995)
Rats	Gluco-oligosacch-arides	4% (w/w)	4 weeks	No Sig. change in all bacteria tested, Sig. ↑ SCFA, ↓H_2, ↑CH_4, slighly ↓NH_3, Sig. ↓β-glucuronidase	Djouzi and Andrieux (1997)
Human colonic-batch culture	Oligodex-tran[a]	10% (w/v)	48 h	Sig.↑ bifidobacteria, butyrate produced ranges from 5-14.85 mmol l^{-1}	Olano-Martin *et al*. (2000)
Human colonic-gut model	Oligodex-tran[b]	10% (w/v)	21 d	In vessel 1, Sig.↑ bifidobacteria and lactobacilli Vessel 3, maximum butyrate for 21.70 mmol l^{-1}	Olano-Martin *et al*. (2000)

[a] Oligodextran (MW≤74kDa) derived from controlled hydrolysis of native dextran

[b] Contains (g kg^{-1}) DP2, 154.6; DP3, 264.6; DP ≥ 11,358.5

1.9 Miscellaneous

Sugar alcohols such as lactitol, sorbitol and maltitol may also be candidate prebiotics. In particular, lactitol (4-O-β-D galactopyranosyl-D-glucitol) has been

tested in humans (Ballongue *et al*., 1997). Dietary fibres (e.g. from wheat, maize, rice, soybean, guar gum, pectin) have a beneficial effect on gut function and colonic bacteria but non-specific stimulatory effect and are therefore not true prebiotics (Kolida, 2003). Both pectin and pectic oligosaccharides have been reported to induce apoptosis of human colonic adenocarcinoma cells (Hotchkiss *et al*., 2003) as well as inhibiting the E. coli O157:H7 shiga toxin (Olano-Martin *et al*., 2003). Sources of plant polysaccharide such as arabinogalactan which is a major component of sugar beet, cellulose which is a polysaccharide found in many higher plants and xylan which is found in wheats and oats as well as many woods are being investigated as sources of novel prebiotics (Rastall and Maitin, 2002; Rastall and Hotchkiss, 2003). Resistant starch is not digestible in the upper gut and is metabolised by bifidobacteria and lactobacilli; however the fermentation may be too generalized to warrant classification as a prebiotic (Wang *et al*., 2002) but may be a good source for butyrate production (Cummings *et al*., 2004). Sialyloligosaccharide, manufactured by Taiyo Kagaku Co., Ltd. (Japan) and a food ingredient derived from hen egg yolk, is a component of glycoconjugates (mainly glycoproteins and glycolipids) on the cell surface. It has been involved in a variety of physiological effects such as cell adhesion, inhibition of rotavirus infection, *Helicobactor pyroli*, neutralization of cytotoxin and improving of performance learning. This additive is used in infant formula, baby food and dietary supplements (product sheet of Taiyo Kagaku Co., Ltd). Chitosan oligosaccharides (COS) with DP of 2-8 had prebiotic effect on the *Bifidobacterium bifidum* and *Lactobacillus* sp. COS composed of dimer (33.6%), trimer (16.9%), tetramer (15.8%), pentamer (12.4%), hexamer (8.3%), heptamer (7.1%) and octamer (5.9%; Lee *et al*., 2002b).

To compare oligosaccharides of all the different groups, the minimum effective doses needed to elicit a prebiotic effect in human may be compared (Table 1.9). The data suggested that there are large differences in the minimum dose needed for different oligosaccharides. More studies are needed for lactulose, lactosucrose and XOS as these were effective at lowest doses of all the substrates. As the studies all used different subjects, diets and methods, substrate effect is difficult to compare.

Table 1.9 Minimum effective doses for a prebiotic effect in humans for various oligosaccharides

Oligosaccharides	**Minimum effective dose (g d^{-1})**
Raftilose L60	8
Raftilose P95	15
Neosugar	10
Inulin	20 for significant increase in bifidobacteria
	40 for significant increase in bifidobacteria and decrease in bacteroides, coliforms and enterococci
Lactulose	3
95% Lactosucrose	3
Oligomate	10
Transgalactosylated oligosaccharides	10
Transgalactosylated disaccharides	15
Soybean oligosaccharide extract	10
Raffinose	15
Isomalto-900P	13
Xylo-oligosaccharides	1

Source: Rycroft (2001)

2. Manufacturing of Prebiotic Oligosaccharides

Oligosaccharides are increasingly being recognized as useful tools for the modulation of the colonic microflora, especially for the level of bifidobacteria and lactobacilli. The structures of commercial available prebiotic oligosaccharides are summarized in Table 1.10.

Table 1.10 Structure of commercial available prebiotic oligosaccharides

Oligosaccharides	**Structure**
Lactulose	Galβ1-4Fru
Fructo-oligosaccharides (FOS)	Fruβ2-1Fru$_n$, n=1-3
	Gluα1-2[βFru1-2]$_n$, n = 2-9, average 4-5
Inulin, Raftiline LS	Fruβ2-1Fru$_n$ n=1->60
	Gluα1-2[βFru1-2]$_n$, n > 10, average 10-12
Galacto-oligosaccharides (GOS)	Gluα1-4[βGal1-6]$_n$, n= 1-4, average 2
Soybean oligosaccharides (SOS)	Raffinose (Galα1-6Glu1-2βFru)
	Stachyose (Galα1-6Galα1-6Gluα1-2βFru)
Lactosucrose	Galβ1-4Gluα1-4Gluα-2βFru
Isomalto-oligosaccharides (IMO)	Glcα1-6[Gluα1-6]$_n$, n ≥ 1, average 1-2
Gentio-oligosaccharides (GEOS)	Gluβ1-6Glu
Xylo-oligosaccharides (XOS)	Xylβ1-4[Xyl]$_n$, n= 2-7

Source: Rastall and Gibson (2002)

2.1 Extraction

The simplest approach to the manufacture of prebiotics is to extract them from a biological material. This is currently commercially performed with inulin extracted from chicory (De Leenheer, 1994) and with raffinose and starchyose extracted from soybeans are already commercially performed (Koga *et al*., 1993), but the extraction yields are quite low.

Inulin is a β2↔1 linked fructan found in a variety of plants. The commercial source in Europe is chicory, with the largest manufacturer being Orafti in Tienen, Belgium. The chicory extraction process is similar to the sugar beet process (De Leenheer, 1994). Chicory chips are extracted with hot water and the extracted chip sold as animal feed. Protein, peptides, colloids and phosphates are then removed by liming and carbonatation at alkaline pH. The inulin is then demineralised by anion and cation exchange chromatography and decolourised by chromatography on activated carbon. The final products are then sterilized, concentrated and spray-dried.

Soybean oligosaccharides are also extracted from their biological source with no further modification. They are isolated from soybean whey and then concentrated to a 75% (w/v) syrup containing 35% oligosaccharides (Crittenden and Playne, 1996). SOS are extracted commercially from soybean whey by using reverse osmosis (RO) and nanofiltration (NF) membrane. Concentrations of the total oligosaccharides at 10% (w/v) and 22% (w/v) were obtained in a batch operation conditions (Matsubara *et al*., 1996).

Kim *et al*. (2003) reported the optimal conditions for extraction and purification of SOS from defatted soybean meal (DSM). For extraction, the optimal ratio of water to DSM and optimal temperature were 5:1 and 50°C, respectively, use of stirring process (without any further grinding) and a 10% ethanol-water solution. To purify the SOS, ultrafiltration was used. More than 90% of the protein was removed from the extracts at a volume concentration ratio (VCR) of 3-5. The percentages of fructose, sucrose, raffinose and stachyose in permeate for a VCR of 5 were 38.6, 51.4, 54.2 and 52.6%, respectively. A VCR of 5 was the most effective for protein removal and SOS recovery. In Japan, soybean oligosaccharides are manufactured by the Calpis Food Industry Co.

2.2 Chemical Approach

Oligosaccharides can be synthesized chemically (Garegg, 1990) but the processes are very complicated due to the many protection and deprotection steps that are necessary for regioselective synthesis, processes are also labour intensive as well as producing unwanted colour and flavour compounds that have to be removed in additional refining steps. The number of steps increases with the size of the oligosaccharides, so that, while synthesis of a disaccharide may require 5-7 steps, a trisaccharide may require more than ten steps. Total yields are often low and large-scale production is not applicable. In addition, steriospecific reactions giving the correct anomer (α or β) are often difficult (Bucke, 1996; Wong *et al*., 1995).

Lactulose is the only prebiotic manufactured using chemical rather than enzymatic approaches (Timmermans, 1994). The reaction is a base-catalyzed

isomerization of the glucosyl moiety of the lactose to form fructose by the Lobry de Bruyn-Alberda van Ekenstein reaction in below.

$$\text{Gal}\beta1\rightarrow4\text{Glc} \xrightarrow[HO^-]{} \text{Gal}\beta1\rightarrow4\text{Fru}$$

Sodium hydroxide can be used as the base in the reaction. Borate can also be used which preferentially forms a strong complex with the furanose forms of the lactulose (Battermann, 1997). Morinaga Industry is the largest producer in Japan, while Solvay is the major European producer of lactulose.

2.3 Enzymatic Approach

The manufacturer of oligosaccharides depends rather heavily on the use of enzymes to bring about either hydrolysis of polysaccharides or the synthesis of oligosaccharides.

2.3.1 Polysaccharide hydrolysis

Polysaccharide degradation has much potential to manufacture prebiotic oligosaccharides from various biological sources. The aim of this approach is a controlled partial hydrolysis to give oligosaccharides of specific molecular weights, resulting in useful rheological properties and technological applications. Currently, only two prebiotics are commercially manufactured by this method, inulin-derived fructo-oligosaccharides (De Leenheer, 1994) and xylan derived xylo-oligosaccharides (Playne and Crittenden, 1996).

Fructo-oligosaccharides can be derived from chicory inulin by partial enzymatic hydrolysis by fungal inulinase (De Leenheer, 1994). The composition of inulin-derived FOS is consisting of degree of polymerization (DP) 1, 2, 3, 4 and ≥5 ratio of 7, 9, 45, 14 and 25 % (dry weight basis), respectively. The largest producer of inulin-derived FOS in Europe is Orafti.

Xylo-oligosaccharides are manufactured by enzymatic hydrolysis of xylan from corncobs (Koga *et al*., 1993) and other sources could be used, such as oat spelt xylan (Jaskari *et al*., 1998) or wheat arabinoxylan. The xylan is hydrolyzed extensively to the disaccharide xylobiose and smaller quantities of higher oligosaccharides. The xylobiose can be further purified by membrane processes to remove xylose and high molecular weight components.

Non-prebiotic malto-oligosaccharides could also be produced from amylose and soluble starch by maltohexaose-forming amylase produced from *Bacillus clausii* BT-21 at pH 9.5, 55°C (Duedahl-Olesen *et al*., 2000).

Partial hydrolysis of dextran (Mountzouris, 1999) and pectin (Olano-Martin *et al*., 2001) was successfully performed by controlled hydrolysis using endo-glycanases in enzyme membrane reactors (EMR). It was possible to convert dextran into different oligodextran preparations with average molecular weights varying from trisaccharide up to 12,000 Daltons. These have proven to be useful prebiotics in gut model systems (Olano-Martin *et al*., 2000) with selective fermentation extending through to the third vessel (modelling the distal colon). This system could also be applied for bacterial extracellular polysaccharides (EPS).

2.3.2 Enzymatic synthesis

Future developments in the large-scale synthesis of oligosaccharides will most likely be in the area of enzymatic synthesis. The use of enzymes in synthesis of complex carbohydrates offers several advantages over chemical methods. A wide variety of regiospecific reactions can be catalyzed very efficiently without protection of the hydroxyl groups. These take place under mild conditions, often at room temperature and close to neutral pH, and organic solvents and hazardous chemicals or catalysts can be avoided (Rastall and Bucke, 1992). Two main types of enzymes are used to catalyze oligosaccharide synthesis: hydrolases (glycosidases E.C. 3.2) and transferases (glycosyltransferases E.C. 2.4). Most of these enzymes are of plant or microbial origin.

2.3.2.1 Glycosyltransferases

Although glycosyltransferases are often difficult to isolate, these enzymes have been successful purified and are being used in the synthesis of oligosaccharides *in vitro*. Glycosyltransferases can be divided into those which utilize sugar nucleotides as donors and those which use other sugars such as sucrose as a glycosyl donor. General glycosyltransferases such as galactosyltransferases, sialyltransferases, fucosyltransferases and mannosyltransferases have been employed for oligosaccharides (Rastall and Bucke, 1992). Glycosyltransferases provide a high selectivity for the acceptor substrate and also provide a high yield (Wong *et al*., 1995). Due to their unique features, glycosyltransferases are suitable for synthesis of high degree of polymerization oligosaccharides. However, the problems of using these enzymes are its availability, moreover, the sugar nucleotide donors are expensive (Ichikawa *et al*., 1992).

Some prebiotics such as IMO, TOS, FOS and lactosucrose are manufactured by enzymatic transfer reaction (Nakakuki, 1993). The reactions utilized cheap sugars as a donors and acceptors. The simplest reactions are the manufacture of FOS and lactosucrose. Fructo-oligosaccharides are made commercially by Meiji Seika in Japan and in Europe by collaboration between Meiji Seika and Eridania Beghin Say (Beghin-Meiji Industries). Fructosyltransferase from *Aureobasidium pullulans* or *Aspergillus niger* is used to build up higher fructo-oligosaccharides from a 60% (w/v) sucrose solution at 50-60°C in an immobilized cell-based reactor (Kono, 1993; Yun, 1996). All of the sucrose-derived FOS terminates in a non-reducing glucose residue. Higher product purities can be achieved by removal of the glucose and sucrose by ion-exchange chromatography (Kono, 1993).

TOS are manufactured from galactose by the action of β-galactosidase (Crittenden, 1999). The galactosyl moiety is transferred from lactose to another molecule of lactose acting as an acceptor to build up higher oligosaccharides. A concentrated lactose solution is used and product oligosaccharide DP values range between 2 and 6 depending on the enzyme used. Three products are available with slightly differing composition; TOS (Tanaka *et al*., 1983), Oligomate 55 (Ito *et al*., 1990) and transgalactosylated disaccharides (TD, Ito *et al*., 1993). The products are

complex mixtures principally containing β1→3 and β1→6 linkages. The largest manufacturers of TOS are Yakult Honsha and Snow Brand in Japan and Borculo Domo Ingredients in Europe.

Lactosucrose is made by a similar approach to the transgalacto-oligosaccharides. The frucosyl residue is transferred from the sucrose to the 1-position of the glucose moiety in the lactose. Lactosucrose is produced commercially by Hayashibara Shoji Inc. and Eusaiko Sugar refining Co. in Japan.

Malto-oligosaccharides could produce in similar approach by action of cyclomaltodextrin glycosyltransferases (CGTases) from *Thermoanaerobacter* sp. and uses soluble starch as donor. The conversion of starch into acceptor products was in the range of 63-79% within 48 h. The degree of polymerization of malto-oligosaccharides formed could be modulated by the ratio of starch: D-glucose provided; at a ratio of 1:2 (w/w), the reaction was quite selective for the formation of G2-G3 (Olano-Martin *et al*., 2001).

2.3.2.2 Glycosidases

The normal function of glycosidases is to hydrolyze and therefore degrade oligosaccharides, but certain glycosidases have long been known to be capable of glycosyltransferase activity. They can synthesize glycosidic bonds using underivatized sugars (Bucke *et al*., 1999). Glycosidases are classified as exoglycosidases, which act on glycosidic linkages at the non-reducing end of saccharide chains, or endoglycosidases acting on glycosidic linkages within saccharides (Ichikawa *et al*., 1992). Glycosidases are generally not specific for acceptor sugars, allowing novel hetero-oligosaccharides to be synthesized by co-condensation of two monosaccharides or a monosaccharide and oligosaccharide (Prapula *et al.,* 2000). Production of hetero-oligosaccharides can be promoted over the production of homo-oligosaccharides by increasing the percentage of acceptor sugar in the mixture, but total yield of oligosaccharide product is decreased (Rastall and Bucke, 1992). In addition, different linkages may be obtained by using different sources of the enzyme (Suwasono and Rastall, 1998). Generally glycosidases are used for the synthesis of shorter

oligosaccharides while glycosyltransferases are suitable for synthesis of higher oligosaccharides (Rastall and Bucke, 1992).

Many studies have been carried out to utilize galactosidases (Ajisaka *et al*., 1988; Ajisaka and Fujimoto, 1989; Ajisaka *et al*., 1994; Yoon and Ajisaka, 1996), glucosidases (Anindyawati *et al*.., 1995), *N*-acetylhexosaminidases (Singh *et al*., 1995a; Singh *et al*., 1995b), and mannosidases (Johansson *et al*., 1989; Rastall and Bucke, 1992).

The high yield of linear malto-oligosaccharides produced by the maltogenic α-amylases from *Penicillium expansum* and *Aspergillus oryzea* on hydrolysis of starch has been reported. Both enzymes catalyze transglycosylation reactions during the concentration-dependent degradation of malto-oligosaccharides produced on hydrolysis of starch. α-Amylase from *P. expansum* acted to give maltotriose whereas α-amylase from *A. oryzae* only switches from unimolecular to multimolecular events at high concentration of the trisaccharide (Doyle *et al*., 1999).

3. Dextrans

Dextrans are a class of polysaccharides synthesized from sucrose by bacterial enzymes such as dextransucrases, glucansucrases or glucosyltransferases. Dextrans are D-glucans connected by α-1→6 glucosidic linkages in the main chains and a variable amount of α1→2; 1→3; or 1→4 branch linkages (Robyt, 1986). Dextrans are synthesized extracellularly by a large numbers of bacteria grown on media containing sucrose. These bacteria are in the genera *Leuconostoc*, *Streptococcus* and *Lactobacillus* (Sidebotham, 1974). In addition, the enzyme dextrin-dextranase (DDase) isolated from *Gluconobacter oxydans* could synthesize dextran from short chain glucans (see detail in section 4.2), and chemical synthesis have also been reported (Reese and Parrish, 1966).

Dextrans were classified into three classes based on their structural features (Robyt, 1986): Class-1 dextrans that contain a main chain of contiguous α-1→6 linked glucosyl residues, with branching at positions 2, 3 or 4; Class-2 dextrans that contain non-consecutive α-1→3 and α-1→6 linkages and α-1→3 branch linkages (known as alternan); and Class-3 dextrans that contain consecutive α-1→3 linkages (known as

mutans if they are water insoluble) and α-1→6 branch linkages. The most commonly encountered polysaccharides known as dextrans belong to Class-1 (Robyt, 1986).

Dextrans became industrially significant after the Second World War when they were used as a blood plasma substitute (Foster, 1968b) and they have had widespread use by the pharmaceutical industry thereafter. Dextrans are used for restoring blood volume (dextrans with an average molecular weight of 70 kDa), assisting blood flow (dextrans with an average molecular weight of 40 kDa) and as anticoagulants (dextrans with an average molecular weight of 7.3 kDa, Foster, 1968a; Alsop, 1983; Robyt, 1986; Lapasin and Pricl, 1995). Dextran derivatives such as cross-linked dextran, dextran sulfate, mercaptodextran and iron-dextran were applied as molecular sieves in chromatography, anticoagulants, antiviral agents, environmental clean up agents for heavy metal contamination and for treating blood deficiencies respectively.

3.1 *Leuconostoc mesenteroides* B-512F dextrans

The Gram positive coccus, *Leuconostoc mesenteroides* B-512F is the dextran producing bacterium most extensively studied and is commonly used for the commercial production of dextran in the US and Europe. This strain produces a dextran containing 95% α-1→6 and 5% α-1→3 glucosidic linkages (Alsop, 1983). This dextran is the most acceptable for clinical use (Foster, 1968b). Native dextran is a dextran polymer obtained from a medium containing sucrose as the only carbohydrate and it has a molecular weight of several million Dalton (Foster, 1968a).

Dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase, E.C. 2.4.1.5), is an extracellular enzyme that catalyzes the conversion of sucrose to dextran (Robyt and Walseth, 1979). This enzyme does not require cofactors or high energy phosphorelated intermediates (Monsan and Lopez, 1981) for synthesizing dextran. The exact mechanism of dextran synthesis by the enzyme has been described by Robyt (1992).

Commercial production of dextran by *Leuconostoc mesenteroides* B-512F is carried out under unstirred and non-aerated conditions. The optimum pH for cell growth, enzyme production and enzyme action are 7.0, 6.0-6.9 and 5.2, respectively (Alsop, 1983; Landon and Webb, 1990). The culture medium becomes highly viscous with a reduction in viscosity if left to complete synthesis. This is due to partial

degradation of native dextran to a smaller molecular size caused by 'autolytic' activity of the strain (Jeanes *et al*., 1957). Optimal sucrose concentration used in unstirred, non-aerated batch culture fermentation of *L. mesenteroides* B-512F was 170-180 g l^{-1} (Alsop, 1983).

Studies on the effect of pH and aeration on dextran production found that the maximum dextran yield was obtained when the oxygen transfer rate in the reactor was equal to the oxygen uptake of the organism at culture pH of 5.5 (Lazi *et al*., 1993). Production of dextran by cell-free enzyme was reported by Alsop (1983). Some parameters such as poor inoculum, medium sterilization, use of K_2PO_4 and mixing affected to dextransucrase resulted in lower productivity of dextran (Landon and Webb, 1990; Pennell and Barker, 1992). Aeration the medium with carbon dioxide gas resulted in higher enzyme production and type of yeast extract also influenced on enzyme yield.

The first industrial scale of dextran production using traditional method gave 95.5% yield (Ajongwen and Barker, 1993) and the native dextran produced has very high molecular weight of up to 50,000 kDa. However, native dextran is not suitable for applications, especially for pharmaceutical applications which require lower molecular weight dextran. Hence, native dextran is degraded to give lower molecular weight dextran using several methods by acid hydrolysis (Landon and Webb, 1990), fractionation and purification using solvent precipitation (acetone, ethanol), chromatography and ultrafiltration (Also, 1983; Barker *et al*., 1984). A dextran with a MW of 20-100 kDa was produced by the action of *L. mesenteroides* and a dextranase producing *Lipomyces starkeyi* (Kim *et al*., 1996). Fermentation on sucrose gave 20-30% (w/v) dextran concentration and the MW of dextran produced was monitored by gel permeation chromatography until the desired MW was reached. Cells were removed from the viscous culture by centrifugation and dextran precipitated by iso-propanol (Kim and Day, 1994).

Control of dextran MW could be achieved using cell-free dextransucrase and sucrose in the presence of other carbohydrates, known as acceptors. The acceptor could be either a low molecular weight oligosaccharide or low molecular weight dextran. The yield and MW distribution depend mainly on the concentration of

sucrose in the reaction mixture and the types and concentration of acceptor (Robyt, 1992; Monsan and Paul, 1995).

3.2 Enzymatic hydrolysis of dextran

Dextranases (1,6-α-D-glucan-6-α-glucanohydrolases; E.C. 3.2.1.11) are enzymes that specifically hydrolyze the α1→6 linkages in dextrans. Depending on their action pattern, dextranases have been characterized as exo-dextranases and endo-dextranases. Exo-dextranases cleave monomer units from the non-reducing terminal end of the polymer whereas the endo-dextranases break bonds randomly in the interior of the polymer (Wheatley and Moo-Young, 1977). Kinetic studies of dextranases on dextran hydrolysis found that the exo-dextranase alone had very little effect on the MW distributions of the hydrolysates compared to a significant shift towards lower MW obtained with the endo-dextranase (Wheatley and Moo-Young, 1977). The action of glucoamylase (E.C. 3.2.1.3) on dextrans has been considered similar to an exo-dextranase (Kobayashi and Matsuda, 1978).

Endo-dextranase produced from *Penicillium* species is suitable for use in food applications (Cheetham, 1987; Chaplin and Bucke, 1992), especially the extracellular endo-dextranases from *P. funiculosum* and *P. lilacinum* which have been extensively studied and used commercially (Fulcher and Inkerman, 1976; Das and Dutta, 1996; Rogalski *et al*., 1998). Most of these enzymes have optimal pH and temperature of 5.0-5.5 and 45-60°C, respectively. The action of endo-dextranses are limited by the presence of α-1→3 branch points (Sidebotham, 1974), and the presence of α-1→4 branch points which are inaccessible to hydrolytic action (Robyt, 1992).

4. *Gluconobacter oxydans*

Gluconobacter oxydans belongs to the *Acetobacteraceae* which contains two well-defined genera, the genus *Acetobacter* and *Gluconobacter* (De Ley *et al*., 1984). These two genera are able to oxidize D-lactate and ethanol to acetate, using the pentose phosphate cycle, with the formation of 2-ketogluconate and they are acid resistant (De Ley, 1961). The key difference is that *Gluconobacter* does not overoxidize ethanol to CO_2 and H_2O via acetate and does not oxidize lactate to CO_2 and H_2O, whereas *Acetobacter* does (De Ley *et al*., 1984).

The genus *Gluconobacter* contains only one species, *G. oxydans*, which has been subdivided into five subspecies: *oxydans*, *industrius*, *suboxydans*, *melanogenes* and *sphaericus*. However, *Acetobacter suboxydans*, *Acetobacter oxydans* and *Acetobacter capsulatus* are as the same biotype as *G. oxydans* (De Ley and Kersters, 1964; De Ley, 1970).

4.1 Physiological properties

Gluconobacter is Gram-negative, obligatory and has a strictly respiratory type of metabolism using oxygen as the terminal electron acceptor. Colonies are pale and optimum temperature for growth is 25-30°C. Cells can grow at pH 3.4-6.3 and the optimum pH is 5.5-6.0. Cells are ellipsoidal to rod shaped, single, in pairs or chains could be found. They can be motile or non motile, the motile cells having 3-8 polar multitrichous flagella (De Ley *et al*., 1984). They are strongly catalase positive and oxidation of ethanol to acetic acid occurs although it is sometimes weak and slow. They do not oxidize acetate and lactate to CO_2 and are spoilage organisms in beer and wine (De Ley, 1970).

Glucose and several other substrates (Fig. 1.1) can be oxidized by two pathways. The first pathway involves an initial phosphorylation and then oxidation via the pentose phosphate cycle enzymes, whereas the other pathway involves a direct non-phosphorylative mechanism (Olijve and Kok, 1979). *G. oxydans* is entirely dependent on the pentose phosphate pathway for the assimilation of sugars or sugar alcohols. There is no evidence of an active Krebs (tricarboxylic acid) cycle and several important components of the normal Embden-Meyerhof pathway are also missing (Greenfield and Claus, 1972).

G. oxydans is able to carry out limited oxidation of a broad range of organic compounds (such as sugars, alcohols and acids) via direct non phosphorylative oxidation pathway. As a result, strong ketogenesis and acid formation from polyols are among other interesting properties of *Gluconobacter* that generates industrial important products. These oxidation are mainly due to membrane bound dehydrogeneses, while the oxidation products are almost quantitatively excreted into the medium (Kersters *et al*., 1965).

Among the interesting oxidation products (Figure 1.1) formed are dihydroxyacetone from glycerol (Hauge *et al*., 1955) and L-sorbose from D-sorbitol (Stouthamer, 1959). These two ketones are industrially important as sorbose is a precursor for vitamin C synthesis and dihydroxyacetone is a tanning agent used for cosmetic purposes (Ohrem and Voss, 1996).

Glucose can be oxidized directly to gluconic acid (gluconate) and this may be further oxidized to 2-ketogluconate or 5-ketogluconate (Kersters *et al*., 1965).

4.2 *Gluconobacter oxydans* NCIMB 4943

G. oxydans NCIMB 4943 is the strain that will be used for this study. This strain has been given different names depending on the culture collection e.g. *Acetobacter capsulatus* ATCC 11894, *Gluconobacter oxydans* NCTC 4943 or formerly *Gluconobacter oxydans* NCIB 4943.

Hehre and Hamilton (1951) confirmed that the carbohydrate slime produced by *Acetobacter capsulatum* (re-named as *Gluconobacter oxydans*) from maltodextrins was a dextran which was serologically related to a dextran produced by *L. mesenteroides*. However, the structure of the dextrans were revealed to differ from each other by periodate oxidation and dextranase digestion (Hehre and Hamilton, 1947; 1951). This was the first evidence for biological convertibility of starch class carbohydrates (dextrins, $\alpha 1 \rightarrow 4$-D-glucosidic) to dextran class carbohydrates ($\alpha 1 \rightarrow 6$-D-glucosidic).

Dextran produced by *G. oxydans* from dextrins was achieved by the action of enzyme named as dextran-dextrinase (DDase). It was considered as trans-glucosylation type enzyme, which acted on only partially hydrolysed starch or glycogen (dextrins) and maltotetraose was the smallest substrate that allowed dextran synthesis. They proposed that the mode of action of DDase was transfer of non-reducing terminal glucosyl residues from the donor dextrins to the non-reducing terminal of an acceptor in a growing dextran chain (Hehre and Hamilton, 1951). This enzyme can potentially produce oligodextrans from maltotriose (0.5%), malto-oligosaccharides (DP 4-12, 0.25%) and soluble starch (1%), respectively.

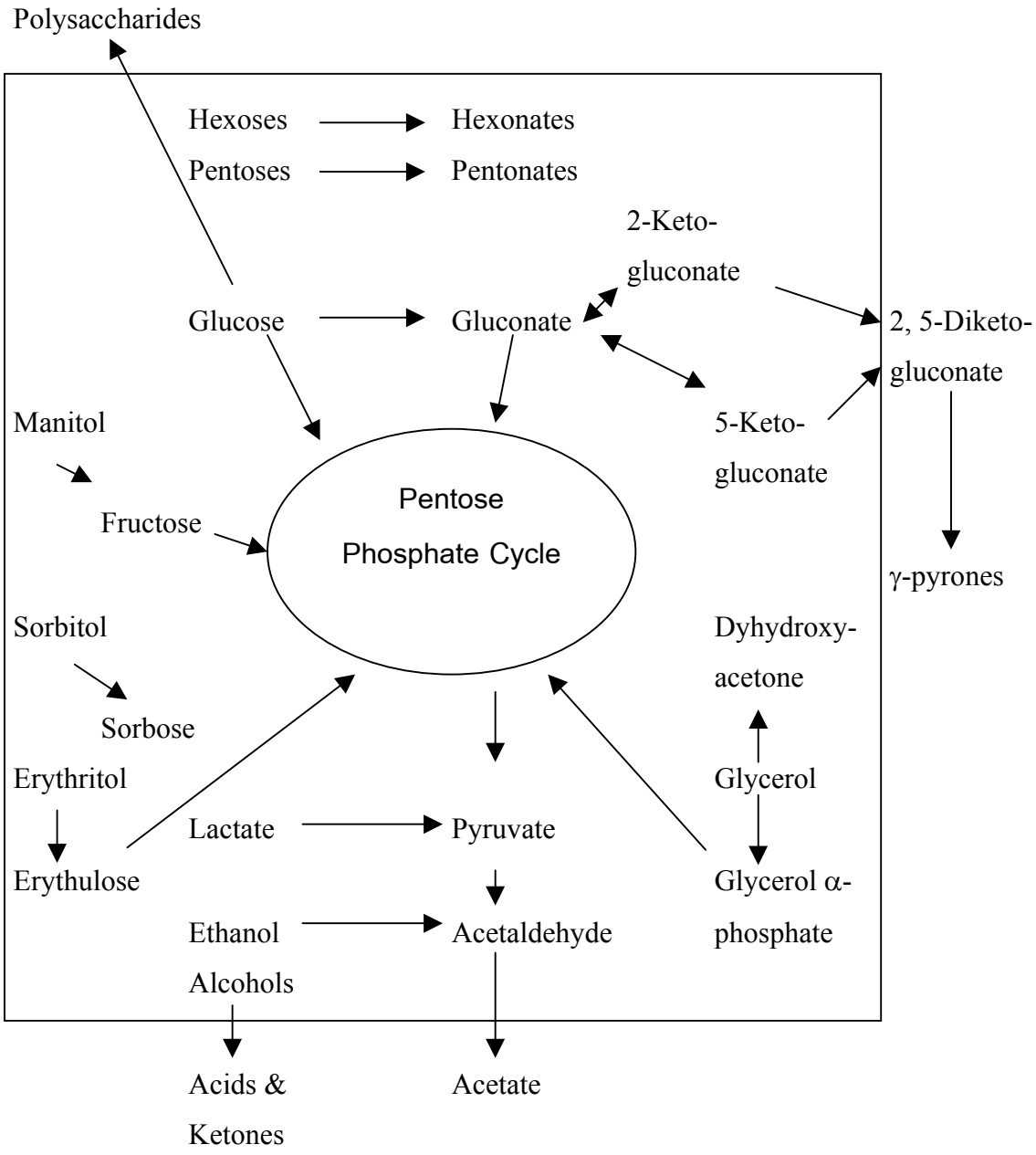

Figure 1.1 Simplified map for the carbohydrate metabolism of *Gluconobacter oxydans*
Source: Adapted from De Ley (1961) and Stouthamer (1959)

Kooi and Grange (1954) patented production of dextran by *A. capsulatum* from starch. They described the process that involved starch hydrolysis to give starch hydrolysate (dextrose equivalent, DE 15-30), followed by addition of 5% yeast extract and adjustment of the medium pH to 5.5-6.8. Sterile medium was then inoculated with *A. capsulatum* or *A. viscosum* and cultured for 72 h and agitation at 25-30°C. The

product was recovered by ethanol precipitation (37-43%, v/v). The maximum dextran yield (35%) was obtained when culture pH reached 4.4 and starch hydrolysate (DE 17) was used.

Recovery and purification of DDase from *A. capsulatum* was patented by Gilkison and Kooi (1957). Firstly, the bacteria was grown on medium containing carbohydrate material (starch hydrolysate, glucose, sorbitol) and a proteinaceous material (yeast extract), then enzyme was separated from the gluconic acid, proteinaceous material, and residual carbohydrate by adding ammonium sulfate, or 50-80% (v/v) water-miscible organic liquid (acetone, alcohol). To induce DDase production a suitable carbohydrate source was starch hydrolysate (DE=17) with the addition of 0.4% (w/v) dextran into medium.

A few decades later, a Japanese group re-opened the subject of production of dextran via the enzyme DDase from *A. capsulatus* ATCC 11894, presently called *G. oxydans* NCIB 4943 (Yamamoto *et al*., 1992). They re-named the enzyme dextran-dextrinase (suggested by Hehre and Hamilton, 1951) to dextrin-dextranase, despite the former name describes better the mechanism of action and the former name will be used in this thesis.

Yamamoto and co-workers (1992) purified DDase using three purification steps. Firstly, enzyme was extracted from the bacterial cells by n-butanol saturated in water with DDase partitioning into the water phase. Secondly, DDase was further purified by hydrophobic chromatography followed by gel permeation chromatography using 40% ethylene glycol in acetate buffer as eluent. DDase was intracellular, rather hydrophobic, having a molecular weight of 300,000 Daltons by SDS-PAGE, and the optimal temperature and pH were 37-45°C and 4.0-4.5, respectively.

Three transglycosylation modes (Figure 1.2) were seen with DDase: (1) from α-1,4 linkage to α-1,6 linkage which was the main mode of action of the enzyme; (2) from α-1,4 linkage to α-1,4 linkage; and (3) from α-1,6 linkage to α-1,6 linkage (Yamamoto *et al*., 1993a).

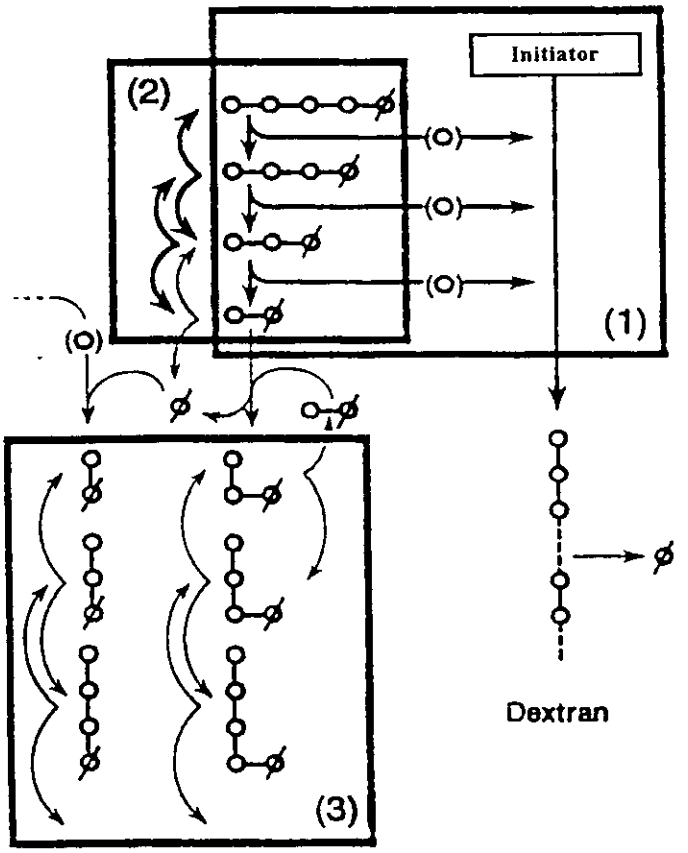

Figure 1.2 Summary of DDase modes of action. *Action (1):* from α-1→4 linkage to α-1→6 linkage. Non reducing terminal glucosyl residues are transferred to acceptors to become dextran. *Action (2):* from α-1→4 linkage to α-1→4 linkage. *Action (3):* from α-1→6 linkage to α-1→6 linkage
Symbols: O glucosyl residue; Ø glucose or reducing terminal glucosyl residue; (O) transferring glucosyl residue; — α-1→4 glucosidic linkage; | α-1→6 glucosidic linkage. Arrows indicate (⟹) fast and (⟶) slow reaction

Source: Yamamoto *et al.* (1992)

Dextran produced from maltotetraose was shown to be different from the commercial *Leuconostoc mesenteroides* dextran upon dextranase digestion (Yamamoto *et al.*, 1992). As a result, the structure of the native dextran synthesized by DDase action on maltotetraose, was further investigated by enzymatic and chemical analyses (Yamamoto *et al.*, 1993d). Methylation analysis showed that the native

dextran consisted of 6.23% α-1→4 branching points and 6.53% α-1→4 linked glucosyl residues in α-1→6 glucosyl linear chains.

Studies on substrate specificity of DDase demonstrated that the enzyme could act on maltose and isomaltose in addition to starch and dextrin. However, the enzyme did not act on other gluco-disaccharides. When salicin was used as glucosyl acceptor, glucosyl residues were transferred to salicin with maltose, isomaltose, starch and dextrans as glucosyl donors. On the other hand, when starch as a glucosyl donor was allowed to react with various saccharides, glucosyl residues from starch were transferred to D-glucose, D-xylose and oligosaccharides that had glucosyl or xylosyl residues at the non-reducing terminal (Yamamoto *et al*., 1994).

Dextran could be produced from starch or low-degree hydrolyzed starch by DDase when a de-branching enzyme such as pullulanase or isoamylase coexisted in the reaction mixture. Yields of dextran were in the range of 55-60% of the amount of starch initially added, within 7 hours reaction time (Yamamoto *et al*., 1993c). It was found that the yields of dextran from reduced malto-oligosaccharides were higher than from the respective malto-oligosaccharides (Yamamoto *et al*., 1993b).

5. The Human Large Intestine and The Gut Microflora

Initially sterile, the gastrointestinal tract of man is quickly colonized within hours of birth. This well-controlled colonization involves many different organisms which find their own particular niches along the length of the gastrointestinal tract (GI), such that different groups of microorganisms colonized different levels of the intestinal tract. The highest colonization of the gastrointestinal tract occurs in the colon. Here, the bacterial flora is dominated by strict anaerobes such as bacteroides, eubacterium, bifidobacterium, peptostreptococcus and peptococcus (Gibson and Roberfroid, 1995).

Epidemiological studies and laboratory research have indicated an association between the metabolic activity of the intestinal flora and cancer of the large bowel (Gibson and Roberfroid, 1995). It had been suggested that procarcinogens could be mediated by gut bacterial enzymes. Colonic cancer has been attributed to the toxic effect of high levels of ammonia produced by microbial metabolism of protein.

Diet materials are firstly degraded by bacterial endo-glycosidases, proteases and amino-peptidases to smaller oligomers and their sugar and amino acid components. Any dietary material that enters the large intestine can be considered as candidate prebiotic material, including resistant starch, dietary fibre, proteins and lipids. In practice prebiotics are confined to 'short chain carbohydrates, with a degree of polymerization (DP) of two or more, soluble in 80% ethanol and not susceptible to digestion by pancreatic and brush-border enzymes' (Quigley *et al*., 1999).

These intermediates are then fermented to short chain fatty acids (SCFA), organic acids gases and other metabolic end products by colonic bacteria (Gibson and Roberfroid, 1995). Malabsorbed fermentable material is converted to hydrogen by multiple species of colonic bacteria (Macfarlanes and Cummings, 1991). This hydrogen can be absorbed and excreted in the breath, passed through the rectum, or utilized by other bacterial species for the reduction of carbon dioxide to methane, or sulphate to sulphite (Gibson *et al*., 1991) affecting the environment or the pathological condition of the colon (Levitt *et al*., 1995).

5.1 Colon cancer

Colorectal cancer is the second commonest cause of death from cancer in developed countries. Incidence of cancer in large intestine was found to be 100 times higher than in small intestine (Morotomi *et al*., 1990; Sanderson *et al*., 2004). The prevalence of cancer appears to rise after the age of 40 and then becomes exponential thereafter (EUCAN, 1999). Rates of colorectal cancer in developing countries are much lower than in developed countries. The incidence for males in 1998 in more developed countries is 37.3 per 100,000 people, compared to 9.91 per 100,000 in less developed countries (Globocan, 2001). Several hypotheses exist to explain this dramatic difference, including dietary intake and infectious challenges in childhood (Pitari *et al*., 2003).

Presently, there are a large number of biomarkers available for assessing colon cancer risk in dietary intervention studies, which are validated to varying degrees. These include colonic mucosal markers, faecal water markers and immunological markers. Overwhelming evidence from epidemiological, *in vivo*, *in vitro* and clinical

trial data indicates that a plant-based diet can reduce the risk of chronic disease, particular cancer (Rafter, 2002).

Ingestion of viable probiotics or prebiotics is associated with anticarcinogenic effects, one mechanism of which is the detoxification of genotoxins in the gut. This mechanism was shown experimentally in animals with use of the rat colon carcinogen 1,2-dimethylhydrazine and by determining endpoints that range from tumorgenesis to induction of DNA damage (Wollowski *et al*., 2001). There is some experimental evidence that secondary bile salts are involved in colonic carcinogenesis, and that non digestible oligosaccharides can decrease the faecal concentration, probably through colonic pH reduction (Marteau and Boutron-Ruault, 2002).

Apart from their selective effect on microbial growth, prebiotics affect microbial activity in other ways. When studied in a 3-stage, compound continuous culture model of the colon, FOS caused profound and rapid change in microbial enzyme activities in which the azoreductase [NAD(P)H dehydrogenase (quinone); E.C. 1.6.99.2] and nitroreductase expression increased and a small increase in arylsulfatase was observed (McBain and Macfarlane, 1997). These changes have been described as dysbiotic because increased activities of these enzymes are thought to favor carcinogen formation in the colon. Buddington *et al*. (1996) studied of 12 human subjects on a controlled diet for 42 days showed 80% reduction in β-glucuronidase and a 90% reduction in nitroreductase with the addition of 4 g d^{-1} of short-chain FOS (2-4 fructosyl residues).

5.2 Composition of the colonic and faecal flora

The human large intestinal microflora is acquired at birth. The inoculum may be derived either from the mother's vaginal or faecal flora or from the environment. Initially, facultative bacteria species, such as *Escherichia coli* or streptococci, are transferred. Subsequently, their activities create a highly reduced environment, which allows the development of the strictly anaerobic bacteria that will later dominate the colon. Interestingly, these bacteria are protective with regard to large intestinal infections, some species are, however harmful (Figure 1.3) (Edwards and Parrett, 2002).

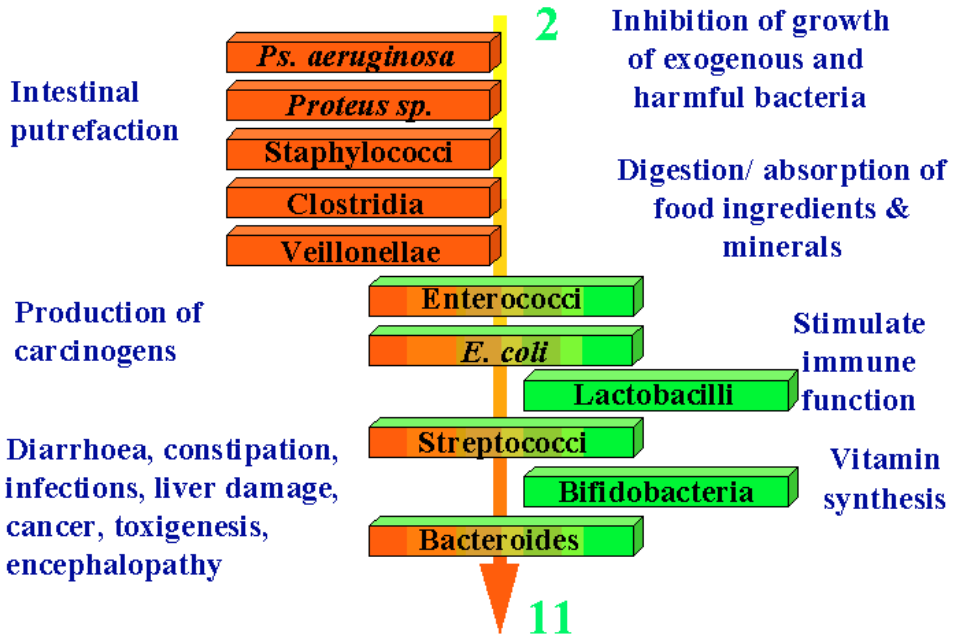

Figure 1.3 Composition of the human colonic microflora and the activities of beneficial, benign and harmful bacterial components. Beneficial bacteria are represented in green colour and harmful bacteria are in red colour

Source: the work of Prof. Dr. R.A. Rastall

Approximately 10^{14} microorganisms populate the human gastrointestinal tract. This is over ten times the total number of human cells in the body (Finegold *et al.*, 1983). It has been estimated that over 500 different species of microorganism reside in the human gut (Gibson and Roberfroid, 1999). The upper intestine (stomach, duodenum and jejunum) is generally less than 10^5 organisms ml^{-1} of content. The ileum contains approximately 10^7 bacterial cells ml^{-1} where most of intestinal lactobacilli reside here. The total concentration of bacteria in the human large intestine is thought to compromise about 95% of total cells in the body, representing 10^{12} cells (g dry weight of contents)$^{-1}$ (Gibson and Roberfroid, 1999). Members of the microbiota that have been commonly found in faecal samples are shown in Table 1.11. *Bacteroides* and the *Clostridium coccoides-Eubacterium rectale* group are dominant bacteria accounting for 50% and 70% of total bacteria in the faeces enumerated by

FISH (Franks *et al*., 1998) and oligonucleotide probes (Sghir *et al*., 2000), respectively.

Table 1.11 The adult colonic microflora composition as determined by bacterial culture and molecular methods

Genus		**Percentage of total anaerobes**			
		Culture methods		**Molecular methods**	
Bacteroides	G- rods	30	56	28	37
Bifidobacterium	G+ rods	11	4	4.8	1
Eubacterium	G+ rods	26	14	10.8	6
Fusobacterium	G- rods	8	0.1	N/A	
Ruminococcus	G+ rods	4	9	10	
Clostridium	G+ rods	2	2	22.7	
Lactobacillus	G+ rods	2	1	0.01	1
Streptococcus	G+ cocci	2	6	N/A	N/A
Enterobacteriaceae	G- rods	N/A	N/A	0.2	1
Peptostreptococcus	G+ cocci	9	4	N/A	N/A
Others	N/A	3	1	9.4	N/A
		Moore& Holdeman (1974)	Finegold *et al*. (1983)	Harmsen *et al*. (2002)	Sghir *et al*. (2000)

G+: Gram positive, G-: Gram negative, N/A: not applicable, species were not enumerated

Source: Moore&Holdeman (1974); Finegold *et al*. (1983); Harmsen *et al*. (2002); Sghir *et al*. (2000)

The microbiota of the colon is mostly anaerobic (A ratio of 1000:1 anaerobes:aerobes or facultative anaerobes). The ascending (proximal) colon features active saccharolytic fermentation; high substrate availability with high bacterial growth rates; the concentration of total short-chain fatty acid content is about 127 mmol l^{-1}; the pH is low in ranges 5.4-5.9 and rapid transit. In the transverse colon, total short-chain fatty acid content is about 117 mmol l^{-1} and the pH is approximately 6.2. In the descending (distal) colon there is little carbohydrate fermentation; predominant bacterial metabolism is proteolytic and the end products are phenols, indoles and ammonia; total short-chain fatty acid concentration is about 90 mmol l^{-1}; the neutral pH ranges from 6.6-6.9 and slow transit (Smith and Morton, 2001).

5.2.1 Bifidobacteria

Bifidobacteria has seen a revival of interest since the mid-1980s due to their expanded use as additives in products that are now marketed as probiotic cultures. Tissier (1900) isolated a Y-shaped irregular non-spore forming Gram-positive rod from the faeces of a child and named it *Bacillus bifidus* and later Holland (1915 cited by Ballongue, 1993) renamed it *Lactobacillus bifidus*. Bifidobacteria, however, do not conform to the definition of lactic acid bacteria as they produce more than 90% lactic acid as metabolic end products. Bifidobacteria are saccharolytic bacteria, produced lactic acid and acetic acid as products in the ratio of 3:1 (Mogensen, 2000). A key determinative assay to distinguish bifidobacteria from lactobacilli is the fructose 6-phosphate phosphoketolase (F6PPK) assay. This enzyme splits the hexose phosphate to erythrose 4-phosphate and acetyl phosphate; bifidobacteria have this enzyme, lactobacilli do not (Hoover, 2000).

Over the years, the taxonomy of the bifidobacteria has changed many times. In Bergey's Manual of Determinative Bacteriology 8th edition (1975), the bifidobacteria was classified as belonging to the *Actinomycetaceae* family and comprised of 11 species. Later developments in analytical tools, especially genetic tools, led to new classifications. In the 9th edition of Bergey's Manual of Determinative Bacteriology (1994), the genus *Bifidobacterium* comprises of 24 different species and then 6 more

species have been added and today the group comprises of 30 species (Mogensen, 2000).

Bifidobacteria, unlike other gut bacteria, have a specialized pathway that allows them a selective advantage over other bacteria in the gut, i.e. the fructose-6-phosphate shunt or bifidus pathway (Roberfroid *et al*., 1993) (Figure 1.4).

It is thought that possession of this pathway allows non-digestible oligosaccharides to be selectively fermented by bifidobacteria although this can only be part of the mechanism for the prebiotic effect.3). F6PPK= Fructose-6-phosphate phosphoketolase.

Bifidobacteria can easily be confused with lactobacilli and often referred to as a member of the lactic acid bacteria; however, bifidobacteria are not closely related to any of the traditional lactic acid bacteria (LAB) used in the production of fermented foods. For example, compared to lactobacilli, bifidobacteria are not as acid-tolerant, nor can their growth be termed 'facultative anaerobic'.

Bifidobacterium spp. lack the enzymes aldolase and glucose-6-phosphate $NADP^+$ oxidoreductase (De Vries *et al*., 1967) and are therefore unable to carry out the usual glycolysis pathway or the hexose monophosphate shunt pathway. The bifidus pathway depends on the presence of F6PPK (Roberfroid *et al*., 1993) and allows the production of 2.5 ATP from 1 mole of hexose such as glucose. Bifidobacteria therefore can compete with many other bacterial groups found in the gut that are potentially harmful. The theoretical amounts of SCFA produced are 1 M acetate and 3 M lactate from 1 M of glucose, however due to the conversion of pyruvate into formate, ethanol and acetate in expense of lactate these ratios are rarely seen.

The growth of nine species of *Bifidobacterium* on media containing glucose, xylose, XOS, xylan or FOS as the sole carbon source were compared in pure culture (Palframan *et al*., 2003). The bifidobacteria differed in fermentation profiles when tested on the different carbohydrates. All species grew to their highest final optical density (OD) on a glucose containing medium, with the exception of *B. catenulatum* which demonstrated a preference for xylose over glucose, XOS over FOS and XOS over xylose. In general, high lactate production correlated with low formate production and low lactate concentrations were obtained at higher levels of formate. Bifidobacteria may alter their metabolic pathways based upon the carbohydrates that

are available for their use (Palframan *et al*., 2003). More details of bifidobacteria can be found in the review by Mogensen (2000).

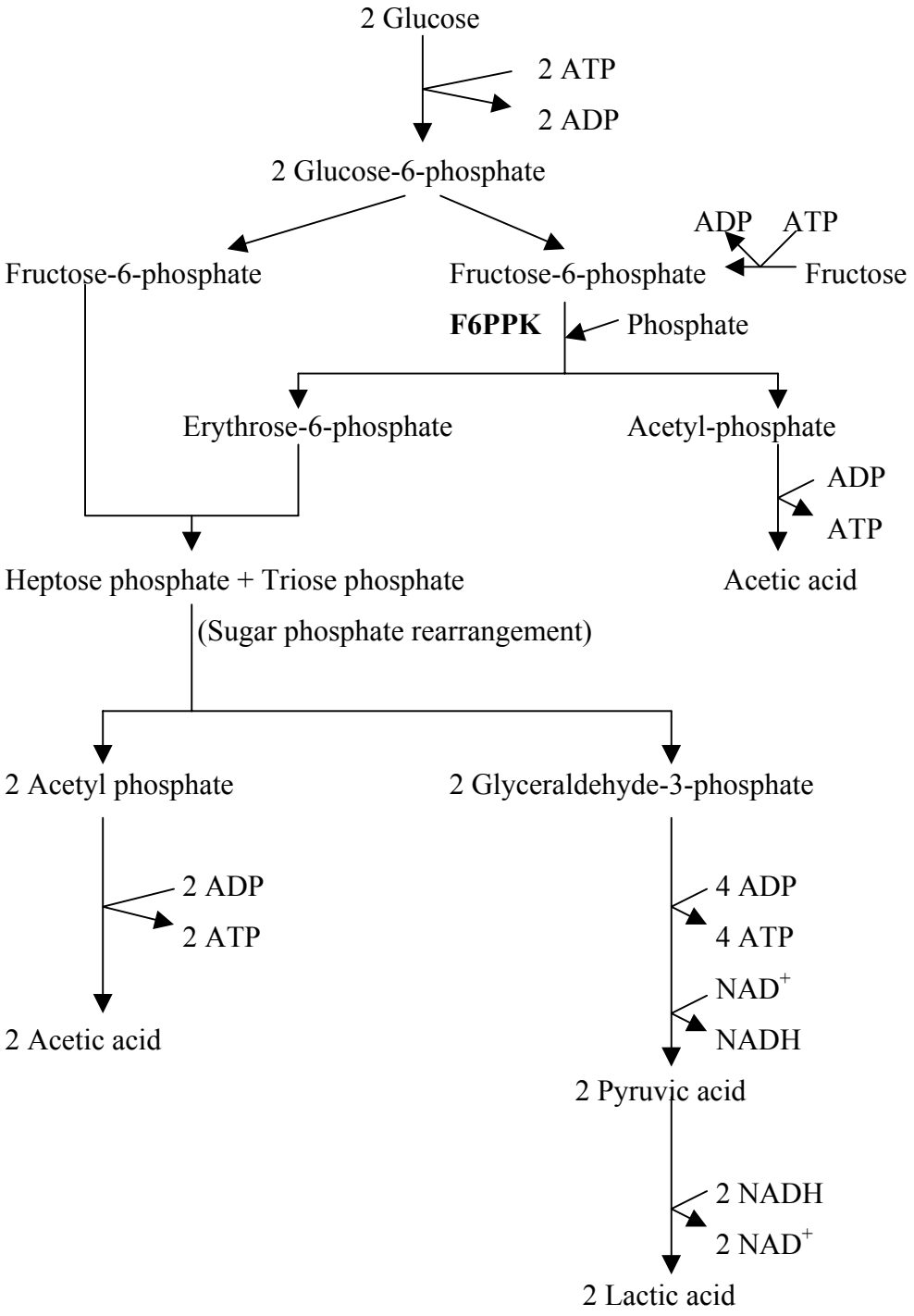

Figure 1.4 The 'Bifidus' pathway for hexose fermentation in bifidobacteria

Source: Roberfroid *et al*. (1993)

5.2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporing, fermentative cocci or rod, which produce lactic acid as their major end product during the fermentation of carbohydrates.

Lactobacillus spp. are usually found in low levels of the commensal intestinal flora of healthy individuals and are the most commonly studied probiotic bacteria. *Lactobacillus acidophilus*, *Lactobacillus johnsonii* and *Lactobacillus caseii* are frequently used to produce fermented milk products. These organisms usually resist gastric acidity and bile salts well. Generally, their survival rate in the gastrointestinal tract is estimated to be between 2-5% and they achieve sufficient concentrations (10^6-10^8 cfu ml^{-1}) in the colon. Depending on the strain, they have varying gut adhesion capacities, and favourable effects on lactose digestibility to prevent diarrhoea. Some strains are now known to stimulate the body's immune defence (Richardson, 1996). Benefits of *L. acidophilus* are secretion of lactic acid which lowers the pH of the intestinal content and helps to inhibit the development of invasive pathogens such as *Salmonella* spp. or strains of *Escherichia coli* and metabolism of any residual lactose in the gut content, so helping to avoid discomfort for consumers with low levels of indigenous lactase (Itsaranuwat *et al*., 2003).

Tzortzis *et al*. (2004) reported that *L. mucosae*, *L. acidophilus* and *L. reuteri*, when grown on sugar mixtures consisting of α-glucosidase (DP 1-4) could produce antimicrobial compounds active against all three of the above pathogens *in vitro*. This effect could not be attributed to a single ingredient of those sugar mixtures and was synergistic. This inhibition displayed a dose-response and was more active at acidic pH.

The use of lactobacilli, especially *Lactobacillus reuteri* as probiotics was reviewed by Casas and Dobrogosz (2000) and Mattila-Sandholm (2000).

5.2.3 Bacteroides

Bacteroides ferment polysaccharides or other dietary carbohydrates to form acetate, succinate, propionate and to a lesser degree lactate. The predominant species

is *Bacteroides fragilis*. Under glucose-limited conditions acetate and propionate are mainly produced (Caspari and Macy, 1983). However, in carbon-excess conditions high levels of acetate and succinate are produced (Macfarlane and Gibson, 1997).

5.2.4 Clostridia

Fermentation by saccharolytic clostridia produces mainly acetate, lactate and butyrate. Lactate is produced directly from pyruvate, while acetate and butyrate production is mediated by the formation of acetyl-CoA from pyruvate, which acts as a very important precursor for their production (Roger, 1986). In the presence of excess substrate, two acetyl-CoA are converted to butyrate yielding one ATP, while under substrate-limited conditions thermodynamic efficiency favours acetate production since one acetyl-CoA is converted yielding one ATP (Macfarlane and Gibson, 1997). In lactate producing clostridia, such as *Cl. perfringens*, high substrate concentrations increase production of lactate and reduce formation of acetate and H_2.

5.2.5 Eubacteria

The phylogenic tree of the genus *Eubacterium* is in a state of re-organization based on 16S rRNA information. One group of the *Eubacterium* genus is branched between cluster IV and III (Collins *et al*., 1994) where cellulolytic clostridia species are located. Eubacteria are metabolically very important in the large intestine, second only to *Bacteroides*, with *Eubacterium rectale* being most prevalent. Eubacteria, along with clostridia and *Fusobacterium* spp., are the main butyrate producers, it must borne in mind that lactobacilli and bifidobacteria do not produce butyrate, in the large intestine (Gibson and Roberfroid, 1999; Manning and Gibson, 2004).

5.3 Metabolic activities of the human gut microflora

One role of microorganisms in the colon is to salvage energy by breaking down carbohydrates and protein not digested in the upper gut, providing substrates for metabolism by human tissue. The metabolic capacity of the colon is comparable to that of the liver, since many microbial enzymes are active in the colon, and have a

broad range of substrates. Two major metabolic activities are encountered: fermentative metabolism of diverse substrates and anaerobic respiration (Cummings and Macfarlane, 1997). The majority of intestinal bacteria use the glycolytic pathway or Embden Meyerhof Parnas (EMP) pathway to ferment carbohydrates. The carbohydrate is converted to pyruvate in a series of reactions summarized as:

$$\text{Glucose} + 2\ NAD^+ + 2\ ADP + 2\ Pi \rightarrow 2\ \text{Pyruvate} + 2\ NADH + 2\ ATP$$

The pyruvate produced in the above pathway is the central intermediate of colonic fermentation. The metabolic routes of pyruvate conversion to acetate, butyrate and propionate are presented.

Alternative routes to metabolize hexoses employ the Entner Duodoroff (ED) pathway, which yields two NADPH and one ATP per two pyruvates and although not advantageous for anaerobic bacteria can be important for some substrates (Gottschalk, 1988), and the pentose phosphate pathway which uses NADPH and pentoses for biosynthetic purposes. Pentose fermentation can proceed by the transketolase and transaldolase reaction of the pentose cycle or by a phosphorolytic cleavage (phosphoketolase). All these alternative routes have in common the glucose-6-phosphate dehydrogenase. In all fermentation strategies pyruvate is the central intermediate, and depending on the species and the substrate availability it can be converted to various end products (Figure 1.5).

The following synoptic stoichiometric reaction has been suggested to describe carbohydrate fermentation in the human colon:

$$59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ \text{Acetate} + 22\ \text{Propionate} + 18\ \text{Butyrate} + 96\ C0_2 + 188\ H_2$$

In a study by Barcenilla *et al*. (2000), 74 isolates of butyrate-producing bacteria were obtained from the human microflora and these produced more than 2 mM butyrate *in vitro*. Eighty percent of the butyrate-producing isolates fell within the XIVa cluster Gram-positive bacteria as defined by Collins *et al*. (1994) and Willems *et al*. (1996), with the most abundant group (42%) clustering with *Eubacterium rectale*, *Eubacterium ramulus* and *Roseburia cecicola.*

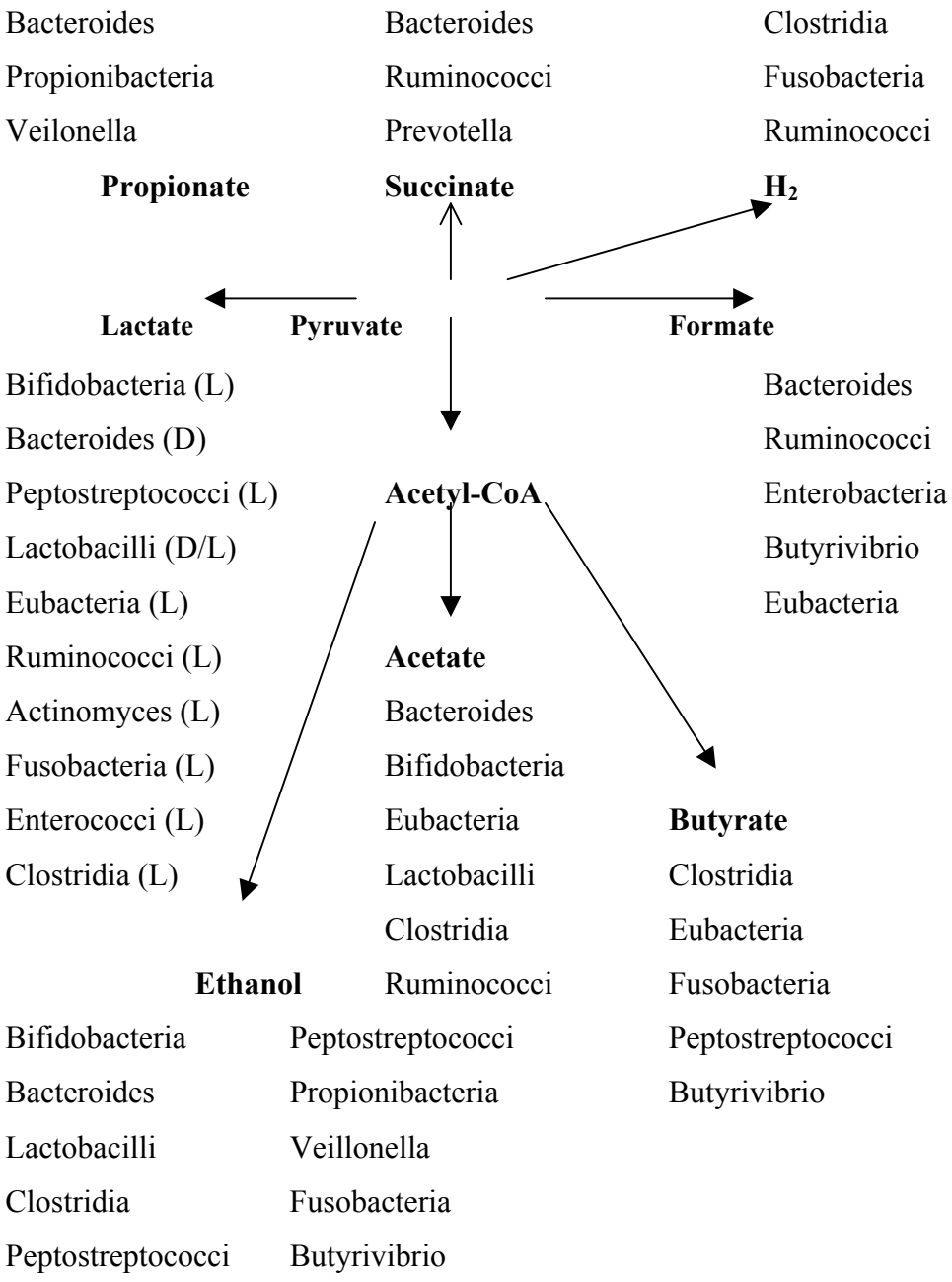

Figure 1.5 Simplified overview of pyruvate metabolism in relation to SCFA formation and electron sinks, by the large intestine microflora

Source: Gibson *et al*. (1998)

The clusters defined by 16S rRNA analyses, lower butyrate-producer fell within the clusters I, IV, XV, XIVa, XV and XVI (Pryde *et al*., 2002). Fifty percent of the butyrate-producing isolates were net acetate consumers during growth, suggesting that

they employ the butyryl coenzyme A-acetyl coenzyme A transferase pathway for butyrate production. Butyrate is formed from two molecules of acetyle CoA yielding acetoacetyl CoA, which is then converted, via the intermediates L(+)-β-hydroxybutyryl CoA and crotonyl CoA, to butyryl CoA (Gottschalk, 1979). Therefore, butyryl CoA may yield butyrate via butyrate kinase or via butyryl CoA:acetyl CoA transferase (Pryde *et al*., 2002) (Figure 1.6).

For oligosaccharides to be utilized by the bifidus pathway they must first be hydrolyzed into monomers, this cleavage can occur in a number of ways. Firstly bifidobacteria could produce extra-cellular glycosidases which are released from the cell and cleave the oligosaccharides externally to the cell. The resulting monomers can then be transported into the cell, utilizing monosaccharide transport mechanisms. Alternatively oligosaccharides can be cleaved by cell surface associated glycosidases with the resulting monomers again transported into the cell as above. The latter may be the most efficient as all resulting monosaccharides will be fermented in the cell and none are lost into the environment, where other non-probiotic organisms will be able to ferment them (Perrin *et al.,* 2001). The transport of oligosaccharides by bifidobacteria is of key importance in the design of novel prebiotics. A oligosaccharide that is transported into the bifidobacteria cell intact will elicit a greater prebiotic effect than one where resulting monosaccharides are transported into the cell, as other non-target organisms will not be able to ferment resulting monosaccharides (Perrin *et al*., 2001).

5.4 Carbohydrates: main substrates for bacterial fermentation

Carbohydrates are categorized as digestible or non-digestible. Digestible carbohydrates are the various sugar-containing molecules that can be digested by amylase or the saccharidases of the small intestine to monosaccharides that can be absorbed from the intestine. The predominant digestible carbohydrates in foods are starch, sucrose, lactose (in some people) and maltose. α-Amylase, which hydrolyses the α-1,4 linkages in starch, is secreted in the mouth from saliva glands and from the pancreas into the small intestine. Human saliva contains less than 1% total solids and probably 50 different proteins, the major ones being the enzyme α-amylase, which active at pH of 6.2-8.0. Human saliva is able to hydrolyze up to 75% of digestible

starch before inactivated by acid in stomach, which consists of HCl giving pH below up to 2.0 (1.5×10^{-1} M H^+, Smith and Morton, 2001).

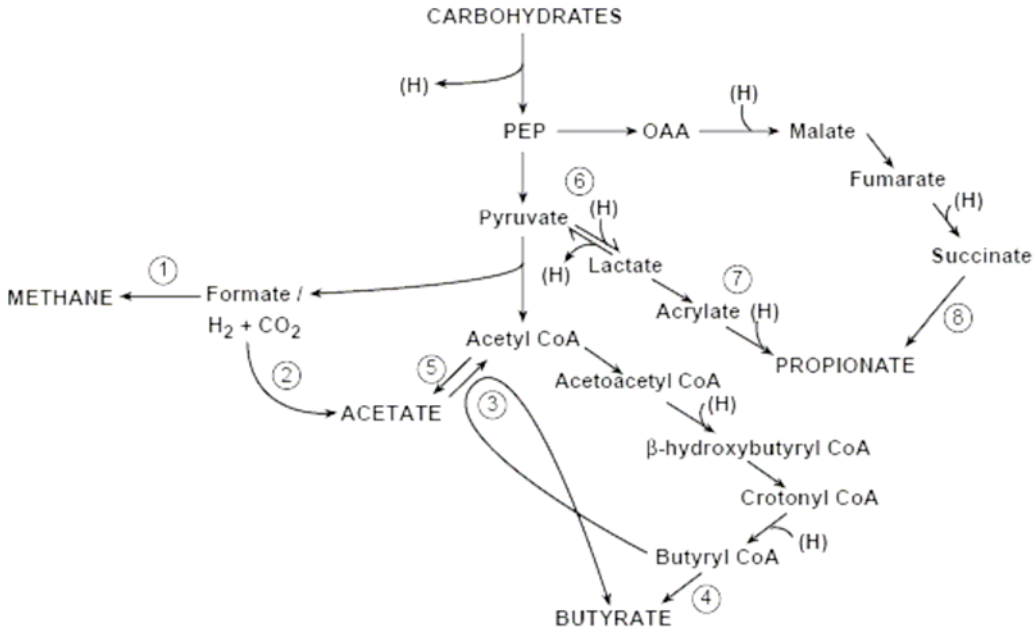

Figure 1.6 Schematic representation of pathways for carbohydrate fermentation in the large intestine. 1 = Methanogenesis, 2 = reductive acetogenesis, 3 = butyryl CoA : acetate CoA transferase, 4 = phosphotransbutyrylase/butyrate kinase, 5 = phosphotransacetylase/acetate kinase, 6 = lactate dehydrogenese, 7 = acrylate pathway, 8 = succinate decarboxylation

Source: Pryde *et al*. (2002)

The action of amylase produces smaller carbohydrate segments i.e. maltose, maltotriose, maltotetraose that can be further hydrolyzed to sugars by enzymes at the brush border of the intestinal cells to glucose. Brush border enzymes consist of mainly intestinal sucrase-isomaltase (α-glucosidase) and pancreatic α-amylase and minority of glucoamylase, maltase, isomaltase, sucrase and lactase (Smith and Morton, 2001). Non-digestible carbohydrates cannot be digested by the enzymes in the small intestine and are the primary components of dietary fibres such as non-starch polysaccharides, resistant starch, non-digestible oligosaccharides (prebiotics).

These substances are fermented in colon by the colonic microflora to produce mainly SCFA and gasses. Survey data from various populations showed that faecal SCFA were produced in the order of acetate > propionate > butyrate ranging from 2.75-83.6, 1.20-32.3 and 0-19.0 mg g^{-1} wet faeces, respectively (Schneeman, 2002; Topping and Clifton, 2001).

Dietary carbohydrates, which are absorbed as hexose sugars, (glucose, fructose) have a calorific value of 3.9 kcal g^{-1} (16.3 kJ g^{-1}) and their cellular metabolism produces ~38 ATP mol^{-1}. The colorific value of nondigested but fermented carbohydrates varies between 0 and 2.5 kcal g^{-1} and they may produce up to 17 mol ATP mol^{-1} of fermented sugar moiety. On the basis of biochemical balance charts for carbon atoms, metabolic pathways and energy yields to the host, the calorific value of a fructosyl residue in chicory inulin and oligofructose has been calculated to be ~25-35% that of a fully digested and absorbed fructose molecule. It is recommended that chicory inulin and oligofructose, like all of other carbohydrates that more or less completely fermented in the human colon, should be given a calorific value of 1.5 kcal g^{-1} (6.3 kJ g^{-1}, Roberfroid, 1999).

5.5 Products of fermentation

5.5.1 Short-chain fatty acid (SCFA)

In vitro, yields vary from 40-60% (g of SCFA per 100 g of substrate) with molar ratios of acetate between 60-80, propionate 14-22, butyrate 8-23 (Cummings and Englyst, 1995). Lactate, mainly formed by bifidobacteria and lactobacilli, seldom accumulates in the colon where it is oxidized by other bacteria such as sulfur-producing bacteria and propionibacteria. Concentrations of SCFA are highest in the caecum and proximal colon, and decrease towards the distal colon. The amount of SCFA produced in the human colon is very difficult to determine despite some studies which indicate that 300-500 mmol are produced each day.

SCFA are rapidly absorbed by epithelial cells and favour the absorption of sodium and water. SCFA absorption is aided by protons generated from intraluminal hydration of CO_2 and secreted into the lumen by Na/H exchange. They are subsequently either metabolised by colonocytes (butyrate) or they enter the portal circulation. Acetate passes through the liver to peripheral tissues where it is

metabolised by muscle, kidney, heart and brain. Utilization of acetate represents the principal means for the body to obtain energy from the carbohydrates not digested nor absorbed in the small intestine. Propionate is largely cleared by the liver and affected to changes in cholesterol levels in human feeding studies (Todesco *et al*., 1991). SCFA can regulate the processes of cellular proliferation and programmed cell death (apoptosis) *in vitro* and in colorectal crypts *in vivo* (Smith *et al.,* 1998; Johnson, 2002).

Butyrate is almost entirely metabolised by epithelial cells, and is the principal source of energy for the colonocytes (Bugaut and Bentejac, 1993). Oxidation of n-butyrate is involved in absorption of ions (Roediger and Moore, 1981), mucus synthesis (Finnie *et al*., 1995), lipid synthesis for membranes (Roediger *et al*., 1992), and detoxification processes in colonocytes (Ramakrishna *et al*., 1991). Butyrate is also of great importance when considering its effect on cell growth and differentiation (Mortensen and Clausen, 1996; Macfarlane and Gibson, 1997; Cook and Sellin, 1998).

Approximately 55-67% (by weight) of galactosucrose or xylosylfructoside was transformed to organic acids, mainly acetic, propionic, and *n*-butyric acids in batch cultures using pig caecal bacteria whereas feeding of lactosucrose or IMO to fish resulted in higher production of SCFA (Kihara *et al*., 1995).

Kajiwara *et al*. (2002) reported large amounts (mM) of lactic acid produced by *B. infantis* ATCC 15697 using honey (35.1), FOS (39.0), GOS (46.6) and inulin (36.6) whereas large amount (mM) of acetic acid produced by *B. adolescentis* ATCC 15700 using honey (85.8) and FOS (97.3) or produced by *B. infantis* ATCC 15697 using GOS (103.9) and inulin (100.6).

Shiga *et al.* (2002) reported that nondigestible carbohydrate (water-soluble soybean fibre) enhanced calcium absorption in rats correlating positively with the concentration of total SCFA in caecal contents and negatively with caecal pH. Acetic, propionic and butyric acid contents were 28.5-32.9, 11.6-15.7 and 4.92-6.66 μM g^{-1}, respectively.

Perrin *et al*. (2001) reported that resistant starch or fructo-oligosaccharides generated a high stable concentration of butyrate and suppressed the appearance of aberrant crypt foci (ACF) in a rat model. Rising concentration of acetate in the distal ileum provides a signal for invasion gene expression by the production of acetyl-

phosphate in the cytoplasm of *Salmonella typhimurium* resulted in inhibition of this pathogenic bacteria (Lawhon *et al*., 2002).

5.5.2 Gas production

During fermentation, gas is eliminated through the lungs and partially expelled in breath as well as flatus. When gas production is low (<200 ml d^{-1}) the majority of gas (65%) is excreted in breath; this proportion falls to 20% when the production rate is > 400 ml d^{-1} (Cummings and Macfarlane, 1997). Composition of flatus varies with diet and individuals, reflecting the fact that the bacterial flora differs markedly individuals. Hydrogen is the main gas formed during fermentation, and it is used by many bacteria as a source of reducing power. It results mainly from reduction of pyruvate, formate or reduced pyridine nucleotides. Hydrogen is also further metabolised by colonic bacteria. It is used as an electron donor by methanogenic, acetogenic and sulfur-reducing bacteria (Wolin and Miller, 1983; Gibson *et al*., 1998).

Kihara and Sakata (2002) studied short-chain fatty acid and gas production from various oligosaccharides and they found that constant rates of gas production (ml min^{-1}) were 1.1, 1.6, 1.5, 1.5, 1.2, 1.3, 1.1, 2.1, 1.1, 1.6, and 1.7 for blank, gentio-oligosaccharide, 4′-galactosyllactose, 6′-galactosyllactose, lactulose, isomalto-oligosaccharide, kestose, lactosucrose, xylo-oligosaccharide, soybean-oligosaccharide and raffinose, respectively.

6. Methods for Evaluating Prebiotics

6.1 *In vitro* methods

In vitro models of the gut are often used to screen effects that different prebiotics and dietary fibres, probiotics and synbiotics can exert on the colonic microflora. The systems enable the changes in colonic microflora, in terms of numbers and metabolism, to be assessed in a rapid screening process. Models do not always provide an accurate model of what occurs *in vivo* as they lack mucosa and colonocytes.

6.1.1 Batch cultures

Different prebiotics and dietary fibres can be screened using batch cultures to ascertain how prebiotics affect the colonic microflora. Glass vessels containing a

medium capable of supporting the growth of the colonic microflora are used. The substance to be tested is added just before the addition of a faecal slurry (1% w/w total volume), which is used as a representation of the colonic microflora. The vessel is maintained under anaerobic conditions at 37°C and sampled periodically. Static batch cultures are generally used with small volumes (40 ml total), these are non-pH controlled, so they are best suited to the initial screening process. Stirred pH-controlled batch cultures can then be used to obtain more detailed information at a pH representative of distal regions of the colon.

Arrigoni *et al*. (2002) studied the fermentability of commercial wheat germ (Biogerm®), inulin (Raftiline®) and lactulose and the growth of bifidobacteria using fresh human faeces under anaerobic conditions in batch cultures. It was found that total SCFA produced with lactulose, inulin and wheat germ were 12.1, 11.1 and 10.4 mM g^{-1}, respectively. Proportion (%) of acetate, propionate and butyrate produced compared to total SCFA were 57, 20 and 17, for lactulose; 60, 15 and 24 for inulin and 47, 4 and 8 for wheat germ. Culture pH on all substrates decreased dramatically from 6.6 to 5.8, 6.0 and 6.5 at 24 h incubation for lactulose, inulin and wheat germ, respectively. Total bacteria and bifidobacteria counts of lactulose, inulin and wheat germ were 1.28×10^9 and 1.01×10^9, 1.12×10^9 and 1.30×10^8, 1.09×10^9 and 1.37×10^8 cell g^{-1} at 24 h incubation, respectively. The proportion of bifidobacteria among total bacteria was highest on inulin (34%) at 8 h incubation however lactulose resulted in the highest proportion (33%) at 24 h incubation. They concluded that the fermentability of wheat germ was less than inulin and lactulose respectively. Moreover, they found that the proportion of bifidobacteria in the total microflora of fresh faecal samples enumerated with FISH technique were calculated to be approximately 3.5-9.0%.

6.1.2 Three-stage continuous culture (gut model)

A three-stage continuous culture is a system with varying pH's through the vessels, as would be found in different colonic regions (Macfarlane *et al*., 1998). This model is fed from the first vessel; hence substrate availability differs between regions, as would occur naturally in the colon. This model has been validated with sudden death victims and hence provides a good method for testing substances of interest

(Macfarlane *et al*., 1998). The three-stage continuous culture system has a relatively slow turn over, so is generally used following preliminary screening in batch cultures. The colonic epithelial cells are, however, covered in a mucous layer; which provides a niche for many different bacteria but that cannot be incorporated into the *in vitro* model. This needs to be considered when interpreting data of *in vitro* models.

Sghir and co-workers (1998) studied prebiotic effects in continuous culture (semi-defined, anaerobic medium containing 5 g l^{-1} FOS, dilution rate of 0.1 h^{-1}, pH 5.5) followed over a 21 day period after inoculation with blended human faeces from four healthy adults. Results showed that SCFA concentrations decreased abruptly one day after inoculation while lactate concentrations increased. Classical methods of enumeration using selective media showed that the proportion of total culturable counts represented by bifidobacteria and lactobacilli increased from 11.9% on day one to 98.1% on day 21. However, molecular methods using genus-specific 16S rRNA oligonucleotide probes indicated that the bifidobacterial population maintained a level between 10-20% of total 16S rRNA during the first 6 day and disappeared rapidly when the maximum concentration of lactate was reached. Lactobacilli increased until day nine and remained at a high level (20-42% of total 16S rRNA) until day 21. They suggested that (1) both bifidobacteria and lactobacilli can utilize FOS, (2) lactobacilli can out-compete bifidobacteria in continuous culture at pH 5.2-5.4 when FOS is the primary carbon and energy source and (3) bifidobacteria can grow faster on FOS than lactobacilli under controlled conditions.

6.2 *In vivo* methods

Animal models, in particular rats, have been used extensively to study effects of dietary substances on the colonic microflora and on biomarkers of colon cancer. Rats associated with a human faecal flora have provided a model of the mature human colonic microflora (Rumney and Rowland, 1992). The human-flora associated rat colonic microflora does respond to fibre supplementation in the diet and the similar result was confirmed in human trials (Mallett *et al*., 1987). However, a rat does not always respond to the same way that a human would. For example, in rats cancers occur much quicker, i.e. after exposure to one carcinogen, and whist this facilitates studies, it also means that effects cannot be directly extrapolated to humans.

A human study is the ultimate way to investigate effects of prebiotics on the colonic microflora. However, it is necessary to carry out initial screening *in vitro* before dietary intervention is acceptable.

Bielecka *et al*. (2002) studied the selection of probiotics (*B. longum*, *B. pseudolongum*, *B. bifidum*, *B. animalis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. globosum*, *B. adolescentis*, *B. angulatum* and *B. sp*) and prebiotics (FOS, oligofructose (Raftilose P95) and inulin (Fructafit IQ)) for synbiotic formation. Fructo-oligosaccharide, oligofructose and inulins stimulated highest growth of *B. longum* KN29.1 (1.2×10^9), *B. animalis* J38 (1.2×10^9), *B. longum* KN29.1 (7.1×10^8). Highest acidifying activity (final pH) appeared in media containing FOS, oligofructose and inulin with *B. animalis* KD12 were 4.58, 4.49 and 5.06, respectively. The highest numbers of bifidobacteria were found in the group of rats receiving oligofructose (10.48 log cfu g^{-1}) and total count of mesophilic aerobic bacteria from 7.79 to 8.81 log cfu g^{-1} was not different in tested and control group. Bifidobacteria as well as oligofructose or synbiotics administered to animals did not significantly influence the number of anaerobic and aerobic bacteria spores.

Bielecka *et al.* (2002) studied the effect of feeding 10% cellulose (control), fructo-oligosaccharide (FOS), lactulose (LAC), corn dextrins (DEX) or resistant corn starch (RS) on the human flora associated rats for 4 weeks. Counts (log cfu g^{-1} faeces) of bifidobacteria on cellulose, FOS, LAC, DEX and RS were 9.21±0.65, 9.50±0.33, 10.58±0.39, 9.16±0.79 and 10.42±0.60, respectively. They were dominating among the groups of microflora determined, followed by aerobic and facultative anaerobic mesophilic bacteria at the level of ~10^8 cfu g^{-1}, and coliforms ~10^7 cfu g^{-1}. The mean number of spores of either proteolytic or saccharolytic bacteria was not higher than 10^2 cfu g^{-1}. Caecal weight, caecal walls weight and caecal content of rats supplemented with FOS, LAC and DEX had significantly higher values compared to the control diet. The faecal pH was lowest in rats fed with DEX (6.00). The activities (U g^{-1} faecal content) of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and β-glucuronidase in the rats caeca were 0.34 (FOS), 13.94 (LAC), 8.84 (DEX), 2.67 (DEX) and 3.81 (RS), respectively. Administration of bifidobacteria to nineteen elderly patients together with oligofructose improved the bifidogenic effect by 1.4 log cfu g^{-1} of faeces (Bielecka *et al*., 2002).

6.3 Molecular microbiology methods

The evaluation of the prebiotic potential of oligosaccharides has been performed using conventional microbiological techniques. Gut microbiology is usually carried out by plating faecal microorganisms onto selective agars designed to recover the numerically predominant groups of bacteria. However, the agars used are only semi-selective and do not recover non-culturable bacteria. In fact, it is estimated that up to 60-80% of the observable bacteria in the colon cannot be cultured (Suau *et al*., 1999).

Recent advances in molecular techniques based around the 16S ribosomal RNA (16s rRNA) molecule have greatly changed this situation (Woese *et al*., 1990). The polymerase chain reaction coupled with denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis (PCR-DGGE/TGGE) provides a method whereby the bacterial communities in large numbers of samples can be compared efficiently and effectively (Tannock, 2002; Blaut, 2004). Bacterial changes in response to prebiotics can be determined through molecular gene probing procedures. Techniques used to detect the probes involve radioactive hybridization or fluorescent procedures. The latter method is known as fluorescent *in situ* hybridization (FISH). Molecular sequencing and probing techniques currently under development will allow a very detailed understanding of the ecological interactions between microorganisms and the effect on such ecosystems of prebiotics.

6.3.1 Fluorescent *in situ* hybridization (FISH)

The 16S rRNA molecule has become an important tool in molecular phylogeny studies due to the fact that it contains regions with different degrees of sequence variation, some of which give diagnostic information. Hence, 16S rRNA is an excellent molecule for looking at evolutionary relationships, and also an excellent molecule for distinguishing between different unknown bacterial species. Utilizing the unique nature of 16S rRNA between different bacterial groups means that probe libraries can be created (Olsen *et al*., 1986; Amann *et al*., 1995). FISH probes are highly specific probes that bind selectively to their target sequence. Successful fluorescence staining of a cell is possible and if there is a high abundance of the target species or groups in a mixed culture then they can be identified. Probes were created

with varying specificity, some can be used to identify at species level, whereas others may be more genus specific (Franks *et al*., 1998; Harmsen *et al*., 2002).

The number of available and validated probes is sufficient to cover the microbial community of the gut at the group level to approximately 90%. In future, the proportion of probes targeting faecal bacteria at a species level will increase and hence the resolution and the potential relevance of the results will be improved (Blaut, 2004). Around 80% of intestinal microbial population has been described (Franks *et al*., 1998), allowing detection of bacteria on different phylogenetic levels (Amann *et al*., 1995; Raskin *et al*., 1994). However, it is difficult to study the complex microflora of the gut with probes on a species level due to the great diversity of this ecosystem and the detection limits of this technique (10^6 ml^{-1}, McCartney, 2002). Another limitation of FISH is poor fluorescence due to an insufficient permeability of the cell envelope (Bidnenko *et al*., 1998) or due to a low content of ribosomes, which indicates that the cells are in a metabolically inactivate state (Fegatella *et al*., 1998).

Comparative studies between traditional microbial culturing methods and the FISH technique, have shown that modern methods enable a clearer overall picture to be seen of the colonic microflora. A study conducted by Harmsen *et al*. (2000) found that plate counts gave approximately 10-fold lower counts than did FISH. In addition, the inability to culture microorganisms present in samples introduces bias into the enumeration process of the microflora. Franks *et al*. (1998) showed that the total bacterial plate counts of *Bifidobacteria* spp. were very similar to that of the FISH counts. This may mean that *Bifidobacteria* spp. is largely culturable. However, as other microbial groups are not so easily culturable, the total bacterial numbers were thought to be lower than that given by staining with DAPI (4', 6-diamidino-2-phenylindole) and direct microscopic counting, thus leading to an over-estimation of bifidobacteria within the total colonic microflora population. FISH can be used to enumerate bacteria that are unculturable, hence giving a more detailed analysis of the colonic bacterial composition. In this study, bacterial enumeration was conducted using the probes listed in Table 1.12.

Table 1.12 FISH probes used in the bacterial enumeration in this study

Probe	**Sequence**	**Target genus**
DAPI	Nucleic acid stain 4', 6-diamidino-2-phenylindole	Total counts
Bif 164	5'-CAT CCG GCA TTA CCA CCC-3'	*Bifidobacterium* spp.
Bac 303	5'-CCA ATG TGG GGG ACC TT-3'	*Bacteroides* spp.
Lab 158	5'-GGT ATT AGC A(T/C)G TGT TTC CA-3'	*Lactobacillus/ Enterococcus*
Erec 482	5'-GCT TCT TAG TCA GGT ACC G-3'	*Clostridium coccoides-Eubacterium rectale* group
His 150	5'-TTA TGC GGT ATT AAT CT(C/T) CCTTT-3'	*Clostridium perfringens*/ *histolyticum* subgroup

Source: Rinne *et al*. (2005)

The 16S rRNA of bacteria in the bifidobacterium group has a common region, allowing generation of genus specific probes. The probe Bif 164 when tested for its ability to hybridize to different species of *Bifidobacterium* matched with all the bacteria in the database that are known to exist in the human intestine (Langendijk *et al*., 1995). Other bacterial species on the database with similar to rRNA sequences, i.e. up to three mismatches, are not known to be present within the human intestinal tract. Hence, the Bif 164 probe should be very specific for all known *Bifidobacterium* species found in a human faecal sample.

The probe Lab 158, as used to detect species belonging to the *Lactobacillus* genera, also detects nearly all species of the genera *Enterococcus*, *Pediococcus*, *Weissella*, *Vagococcus*, *Leuconostoc*, and *Oenococcus*. Of these genera only *Enterococcus* and *Lactobacillus* are found in the human gastrointestinal tract, hence for faecal and colonic enumeration the Lab 158 probe can therefore be used as relevant

to both. However, the permeability of this probe to the microbial cell seemed to be low thus hybridization is easily got a problem.

The probe Erec 482 is used for detection of *Eubacterium rectale* and the *Clostridium coccoides* subgroup, which usually accounts for about one third of the colonic microflora population in healthy adults (Franks *et al*., 1998). This group represents *Clostridium* cluster XVI a and b, which have been identified as a source of butyrate producing bacteria, as 80% of the butyrate producing species isolated were of the clostridia XVI a cluster (Barcenilla *et al*., 2000). Some of these bacterial groups are pathogenic or toxigenic bacteria particular clostridia.

The probe Bac 303 designed by Manz *et al*. (1996) targets the genus *Bacteroides*, with the exception of the human faecal microorganism, *Bacteroides putredinis*. The *Clostridium histolyticum* subgroup that can be enumerated using His 150 consists of *Cl. perfringens*, *Cl. histolyticum*, *Cl. putrificum*, *Cl. butyricum*, *Cl. carnic*, *Eubacterium multiform* and other related species (Harmsen *et al*., 1999; Franks *et al*., 1998). *Cl. difficile*, the principle etiological agent of pseudomembranous colitis is not represented in this group.

In the last few years, an automated microscopic detection of single cells by image analysis has been developed (Jansen *et al*., 1999). In principle, this analysis consists of image acquisition, image storage, object recognition and object enumeration. Automated image acquisition requires a high precision motorized microscope stage whose movements in three dimensions can be programmed. Images are captured at predetermined positions using a charge coupled device (CCD) camera and stored as electronic files. Jansen and colleagues (1999) were the first ones who introduced an automated microscopy based image analysis system in gut microbiology for the analysis of intestinal bacteria in faecal samples (Jansen *et al*., 1999).

With this approach the proportion of out-of-focus images was approximately 1%. In view of the improvements in computer technology since that time it is certain that the speed and accuracy of this analysis has greatly improved. Once the captured images have been stored in digital format, the fluorescing cells in the image have to be identified based on their fluorescence, size and shape (Blaut, 2004). Flow cytometry (FCM) has found widespread application in medical diagnostics. FCM has been used for counting of bacterial cells. A combination of FCM and FISH was successfully

optimized for use in conjugation with rRNA targeted oligonucleotide probes. Taken together, FISH-FCM is a very powerful method for the enumeration of faecal bacteria, the main advantage being the spread of analysis. This feature makes FISH-FCM really suitable for high throughput analysis of feacal samples (Blaut, 2004).

7. Aims and Objectives

This research aims to investigate the optimal medium and environmental factors for oligodextrans production, influence of reaction parameters on the molecular weight distribution and chemical structure of the oligodextrans, susceptibility of the oligodextrans to acidic conditions and amylases digestion, and potential of the oligodextrans as a butyrogenic prebiotic.

7.1 Optimization for the production of oligodextran using the culture medium method and the cell suspension method, effect of cell concentration, buffer pH and temperature, effect of substrate type and its concentration.

7.2 Studies on the influence of reaction parameters on the molecular weight distribution and chemical structure of the oligodextran obtained.

7.3 Studies on the susceptibility of oligodextran to enzymatic digestion.

7.4 Studies on the persistency of oligodextran to acid hydrolysis.

7.5 Scaled up to provide sufficient oligodextran to evaluate prebiotic properties.

7.6 To evaluate on fermentability of oligodextran by human microflora in stirred, pH-controlled batch culture.

7.7 To evaluate on fermentability of oligodextran by human microflora in three-stage continuous system.