In vitro fermentation of mixtures of indigestible carbohydrates by the human faecal bacteria

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AUTHOR'S DECLARATION

I declared that the work contained in this thesis is original, and is the work of one author, Mohammad Khalid Khan. The information reported from the other authors has been quoted with their name and source of publication.

All the laboratory analysis and data processing was carried out by the author, under the supervision of Dr Christine A Edwards.

(M K Khan)

I certify that the work reported in this thesis has been performed by M K Khan and that during the period of study he has fulfilled the conditions of the ordinances and regulation governing the Degree of Doctor of Philosphy.

(Dr Christine A Edwards)

DEDICATION

I dedicate this thesis to my family whose support and patience was a constant source of fortitude for me during this research work. The work is also dedicated to my late father and late brother who would have been proud of me at this moment. The work is also dedicated to my mother and parents-in-law, without whose support and prayers I would have not been able to complete this work.

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CHAPTER 2: Methods

CHAPTER 6: Fermentation of mixtures of indigestible carbohydrates

CHAPTER 7: General Discussion

LIST OF TABLES AND FIGURES

TABLES

FIGURES

Summary

Indigestible carbohydrates are fermented in the human large intestine, producing short chain fatty acids (SCFA) and gases. SCFA, in addition to the provision of energy, have significant effects on the physiology of the human body. n-Butyrate in particular may help prevent colonic disease. The rate of SCFA production and the resultant profile of SCFA is characteristic of each carbohydrate. The physical properties of such carbohydrates are also very important in regulating the small intestinal nutrient absorption and transit time. Indigestible carbohydrates differ considerably in their physico-chemical properties, which will have a significant impact on their rate of fermentation, the amount and profile of SCFA. A carbohydrate with intermediate fermentability may have the best action because it should produce a high concentration of SCFA and contribute to stool output. It would be more beneficial to colonic health by facilitating the production and absorption of higher amounts of SCFA at a distal colonic site.

Several indigestible carbohydrates have interesting therapeutic properties but large amounts may have undesirable side effects such as bloating, osmotic diarrhoea and low organoleptic properties. Considering such idiosyncratic actions, it is unlikely that any single carbohydrate would provide ideal therapeutic actions without having some undesirable effects. In this study, the approach of fermenting different combinations of carbohydrates was adopted. Since, it is not always possible to feed patients meals containing such supplements, the characteristics of the constituent carbohydrates, such as their solubility and viscosity were considered in relation to developing a supplement mixture for tube feeding.

Aim of this thesis was to evaluate mixtures of indigestible carbohydrates *in vitro* to predict their effects on gut function. In this study, I investigated the effect of combining carbohydrates with different fermentative properties and their interactive influences, reflected in the end products from *in vitro* fermentation. The study focused on the rate of fermentation and fermentability of such mixtures and the SCFA produced to gain an index of the likely site of fermentation in the colon.

In view of the difficulties and limitations of *in vivo* methods, an *in vitro* model was used, with human faeces incubated in a basic salts medium. Despite the simplicity of this method, it provided useful information about the rate and extent of carbohydrate fermentation and production of SCFA. It is obvious that the physiological conditions of such *in vitro* models may change, resulting in altered bacterial metabolic activity. Therefore, experiments were designed to explore the limitations of the *in vitro* model, in this way, allowing adaptation and corrections where necessary.

For validation of the *in vitro* model, incremental portions 25, 50, 75, 100mg of rapidly fermenting lactulose and slowly fermenting ispaghula were used. The production of SCFA progressively declined with increasing amounts of lactulose, showing a significant difference between actual SCFA concentration from fermentation of 100mg and that calculated from portions of 25mg lactulose. There was no significant effect with ispaghula. This study suggested 50mg substrate per 10ml incubation volume as the best amount of a rapidly fermenting carbohydrate to be used in *in vitro* fermentation models.

This study also showed an inhibition of the fermentation of lactulose by ispaghula (44% inhibition of SCFA production in presence of 75mg of ispaghula). This study also suggested that the best substrate ratio of two carbohydrates in a mixture was 50mg of each carbohydrate. Such a mixture produced significantly more of the three principal SCFA than other mixtures of lactulose and ispaghula. This combination was confirmed with mixtures of raftilose and guar gum, which in 50:50 mixtures produced similar SCFA as cultures of 100mg raftilose. Such mixtures also gave no further change in pH after 8 hours, suggesting prevention of further deterioration of physiological conditions in cultures. Combining two carbohydrates with contrasting fermentation properties, in such proportions will be more effective, as both carbohydrates in a mixture would have the optimum chance for their role in the mutual interaction.

In this study, different carbohydrates were tested to determine their potential use in mixtures. Raftilose, guar gum, gum arabica, ispaghula and pectin were selected for such mixtures. Raftilose and pectin were rapidly fermenting, and guar gum showed an intermediate rate of fermentation. Gum arabica was fermented slowly and ispaghula was fermented very slowly. Ispaghula was selected for mixtures also because of its specific effects on stool output and transit time. Raftilose was a good producer of n-butyrate, whereas, good producers of propionate included raftilose; guar gum and gum arabica. These carbohydrates are soluble in water and could be easily incorporated into the liquid food supplements for tube feeding if low viscosity products are used.

The main aim of this thesis was to produce a mixture of carbohydrates which would delay but preserve butyrate production from rapidly fermenting carbohydrates such as raftilose.

This was achieved in several mixtures but mostly those containing raftilose and ispaghula. In general, mixtures of carbohydrates were fermented more slowly than raftilose alone. Overall, ispaghula was the most effective in slowing the rate of fermentation compared with pectin or gums. Mixing raftilose with ispaghula or guar gum gave the best preservation of n-butyrate and propionate production. The rate of n-butyrate production was less rapid in mixed cultures of three carbohydrates (raftilose, ispaghula and pectin) than cultures of 100mg raftilose but production of n-butyrate was preserved.

In summary, ispaghula and raftilose in two-carbohydrate mixtures and ispaghula, pectin and raftilose in three-carbohydrate mixtures delayed the release of butyrate with no loss in butyrate production despite using less raftilose. Such an effect *in vivo* may be helpful in prolonging n-butyrate production and may move butyrate further round the colon, at the same time reducing the potential adverse effects of raftilose. Moreover, the addition of pectin (or guar gum) may add the therapeutic effect of delaying nutrient absorption in the small intestine as well. These studies have identified at least two mixtures (raftilose & ispaghula; raftilose, ispaghula & pectin) worthy of study in more detail in man.

CHAPTER 1

Literature review and background information

1.1 Introduction

A wide range of carbohydrates is found in nature. Mostly, dietary carbohydrates are products of photosynthesis, almost exclusively derived from plant sources. Animal sources contribute insignificantly in dietetic terms, the only major exception to this, is lactose from milk. Carbohydrates have served as a fundamental source of energy since the first day of life on the earth and they are still a main source of energy in the human diet, providing 40 to 85 percent of energy in the human diet, depending on the affluence of the particular society (Englyst & Kingman, 1993). Over the past few decades in affluent societies of the West, a change has occurred from diets rich in carbohydrates to diets poor in carbohydrate and rich in fat. This change is related to an increase in chronic diseases like cardiovascular disease and cancer. Awareness of the importance of dietary fibre and other indigestible carbohydrates in human health emphasises the need to return to more traditional diets, but dietary change back to original diet patterns, is occurring with unsatisfactory slow momentum. Current recommendations for Western society are for a 50 to 100 percent increase over the present intake of non starch polysaccharides or dietary fibre and dietary starch (Webb 1995).

Carbohydrates offer a wide range of physicochemical properties which are based on their underlying chemical structure, interactions with other carbohydrates and with other molecules through hydrogen bonding, ionic effects and the formation of complexes with lipids and proteins (Chinachoti, 1995). The actions of

carbohydrates in the human gut are related to these properties. There are three important strands of nutrition related research regarding carbohydrates;

1) familiarity with the chemistry of carbohydrates.
2) an understanding of physiological role of carbohydrates.
3) validating the relationship between the dietary carbohydrates and chronic diseases.

In this review, I will consider the information available from published literature related to these areas of carbohydrate research.

1.2 Classification of carbohydrates

Carbohydrates are polyhydroxyaldehydes and ketones, with an empirical formula $C_n(H_2O)_n$, and their derivatives. Carbohydrates differ in their physicochemical properties, degree of polymerisation, molecular structure, spatial configuration of the molecule, water solubility and viscosity. They can be classified in a number of ways depending, for example, on the number of their monomers in a molecule, on their source, on their physicochemical characteristics, or on a nutritional basis. McCance and Lawrence (1929) proposed that carbohydrates should be classified in two groups on the basis of their digestibility and absorption as carbohydrate in the human small intestine. A carbohydrate should be termed as available carbohydrate if it is digested and absorbed in the small intestine. Whereas, carbohydrates that are indigestible or unabsorbed in the small intestine, should be termed as unavailable carbohydrates. According to these authors the implication of "availability or unavailability" pointed to availability or unavailability of the

carbohydrate for absorption, not for energy. The principal dietary carbohydrates are listed in the table 1.1.

1.2.1 On basis of number of monomers

Carbohydrates are classified according to the number of monomers in a single molecule (Englyst & Kingman, 1993) as follows;

Monosaccharides contain a single monomer in a molecule and examples include glucose, fructose, and galactose. These three monosaccharides have the same number and type of atoms but in different spatial arrangements, resulting in different degree of sweetness. Free glucose is not very abundant in nature but it is present in small amounts in fruits and vegetables. Fructose is found in fruits, vegetables and honey.

Disaccharides contain two monomers in a molecule. The two monomers may be the same, for example, in case of maltose which is the product of starch hydrolysis. Each molecule of maltose is constituted by two monomers of glucose. The two constituting monomers may be different as in the case of sucrose (glucose: fructose), lactose (glucose: galactose), lactulose (galactose: fructose).

Oligosaccharides contain 3-10 monomers in a single molecule. Oligosaccharides are mostly produced synthetically or by the processing of higher molecular weight carbohydrates, for example, maltodextrins are produced from partial hydrolysis of the starch. Common examples of oligosaccharides are raffinose, stachyose and verbascose which are found in nature as free sugars, especially in

leguminous plant seeds. The fructo- and galacto-oligosaccharides (present in breast milk) and some inulins are also examples of oligosaccharides (Cummings & Englyst, 1995). Fructans are composed of 3-50 fructose residues, attached to a single glucose molecule. Fructans with 3-10 monomeric residues, the oligofructans, predominate in cereal grains (Englyst & Kingman, 1993).

Polysaccharides contain more than 10 monomers in a molecule e.g. amylose, cellulose, amylopectin and fructan with more than 10 residual monomers. A polysaccharide can be defined on the basis of three important factors including the type of constituent monomers, nature of the inter-residual glycosidic bonding and sometimes, sequence of residues. They may be homo-polysaccharides containing identical residues or they may be hetero-polysaccharides containing different monomeric residues (Bohinsky, 1983). Starch and cellulose both are homo-polysaccharides, being polymers of glucose residues. Starch is a mixture of amylose and amylopectin. Amylose is a linear chain molecule containing α 1,4 bond as inter-residual linkage while amylopectin, on the other hand, is a branched chain molecule containing, in addition to inter-residual linear α 1,4 bonds, a small number of inter-residual α 1,6 bonds at the branching sites. The glucose residues in cellulose are linked by β 1,4 bonds. Human α amylase can break α 1,4 bonds found in starch but, cannot break β1,4 bonds found in cellulose. Thus, due to the different nature of glycosidic linkages of glucose residues, starch, in general, is digestible and cellulose is indigestible in the human small intestine.

1.2.2 On basis of physical functionality in plants

Polysaccharides in plants may be classified on the basis of their physical functionality (Asp et al., 1992) into following groups;

i) Structural carbohydrates are found mostly as components of the cell wall, for example, cellulose and hemicellulose, pectic substances.

ii) Storage polysaccharides are formed and stored in the plant to avoid osmotic pressure after synthesis of high amounts of the sugars in plant tissues. They include mainly starch and fructans found in seeds, root tubers and other plant tissues, and in some plants they are present in the form of non-starch polysaccharides, for example, galactomannans (e.g. guar gum) in the cluster bean. In seeds, storage polysaccharides act as the stored fuel for metabolic activities of the new sapling.

iii) Protective polysaccharides are found in the form of naturally occurring gums and mucilages. These are usually viscous substances found in the cell sap. These polysaccharides offer protection from desiccation to the plants. Gum arabica is an example of such carbohydrates.

1.2.3 On basis of complexity of molecule

Monosaccharides and disaccharides may be termed **simple sugars** because of the simplicity of their molecular structure, while higher molecular weight carbohydrates may be classified as **complex carbohydrates** (Chinachoti, 1995). Whitney et al., (1998) described the monosaccharides and disaccharides as simple carbohydrates (or sugars) and polysaccharides as complex carbohydrates. Complex carbohydrates include oligosaccharides, starch, non starch polysaccharides and the various types of gums and mucilage. The British

Nutrition Foundation Task Force on Carbohydrates (1990) included starch and non-starch polysaccharides, but not oligosaccharides, in the category of complex carbohydrates. The complexity of the carbohydrate molecule is not used frequently as the basis for the carbohydrate classification because the

Table 1.1 Main carbohydrates in the human diet (adopted and modified from Asp, 1995)

Monosaccharides	**Disaccharides**	**Oligosaccharides**	**Polysaccharides**
		α Galactosides	**Starch**
Glucose	Sucrose		
Fructose	Lactose	Raffinose	Amylose
Mannose	Lactulose	Stachyose	Amylopectin
Galactose	Maltose	Verbascose	Modified starches in foods e.g dextrins, etc.
Ribose			
Deoxyribose		**Fructans** (Oligomers)	
			Non Starch Polysaccharides
		Fructooligosaccharide	Cellulose
			Hemicellulose
			Pectin
			β Glucans
			Fructans (Polymers)
			Gums e.g. Guar gum, gum arabic etc.
			Mucilages e.g. Ispaghula

impracticability of its use for nutritional and physiological information. Any such use will lead to big confusion about their digestibility. For example, lactulose is disaccharide and a simple carbohydrate by the definition of the complexity but it is not a digestible carbohydrate in the small intestine. In contrast, a digestible part of starch is included in the complex carbohydrates with the other indigestible carbohydrates. On basis of such confusion, the complexity

of the carbohydrate molecule is not used so much for the classification purpose now.

1.2.4 On nutritional (or physiological) basis

Carbohydrates may be classified on a nutritional (or physiological) basis as digestible or indigestible depending on their possible digestion by the digestive enzymes in the small intestine. The extent and site of break down of the carbohydrate in the human intestine has very important implications in their physiological role in the body.

1.2.4.1 Digestible carbohydrates

Digestible carbohydrates are easily broken down into their constituent monomers by the digestive enzymes in human small intestine. Most disaccharides, such as sucrose and higher molecular weight digestible starch and glycogen are examples of digestible carbohydrates. Monosaccharides such as glucose, fructose, etc., do not need digestion and they are absorbed directly by the epithelial cells. The epithelial cells of the intestinal wall and cells in the liver convert fructose and galactose into glucose, thus ultimately yielding the primary fuel, glucose, to the body tissues (Whitney et al.,1998). Fructose, sometimes, may not be so easily absorbed in the small intestine and may cause diarrhoea after entering the large intestine (Caspary, 1986).

Starch is the main polysaccharide ingredient of the human diet consisting of two main macromolecules; amylose and amylopectin (Cummings & Englyst, 1995). Normally a starch granule is composed of 14-27 percent amylose and 73-86 percent of amylopectin (Chinachoti, 1995). Amylose is conformed in a helical

structure containing six anhydroglucose residues in each turn with interchain hydrogen bonding. The helical structure of the amylose renders it more compact and less accessible to the enzymatic action compared with more open, branched amylopectin. Dietary starch is classified in three major groups, based on the rate of their digestibility *in vitro* (Englyst et al., 1992);

Rapidly Digestible Starch (RDS)
Slowly Digestible Starch (RDS)
Resistant Starch (RS)

RDS is rapidly digested in the human small intestine and it is found mostly in freshly cooked starchy foods like breads, potatoes (Englyst & Kingman, 1993). SDS is also completely digested in the human small intestine but at much slower rate compared with RDS. SDS is not easily accessible to the amylolytic enzymes and such starch is present in partially milled seeds or in foods with more compact and dense texture such as pasta. Resistant starch is not digestible in the small intestine (this category is discussed in later sections in more detail). All these three types may be present in the same food but the amount of each type varies depending on the source of starch and processing of the food (Englyst et al., 1992).

1.2.4.2 Indigestible carbohydrates

Indigestible carbohydrates resist the action of the pancreatic enzymes in the human small intestine. Consequently, they reach the large intestine where they are fermented by the gut bacteria into short chain fatty acids (SCFA), gases and some other organic compounds. The extent of the bacterial fermentation of these carbohydrates in the colon differ widely from each other. Some of these

carbohydrates may not be fermented by bacteria at all, some may be fermented partially resulting in the loss of their physical properties, whereas, still others may be fermented completely. Indigestible carbohydrates include most of the higher molecular weight complex carbohydrate and some lower molecular weight simple carbohydrates. Some of these indigestible carbohydrates are also termed as pre-biotics; a concept introduced recently (Gibson & Roberfroid, 1995). According to these authors "*a prebiotic is nondigestible 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, and thus improves host health.*"

1.2.4.2.1 Simple indigestible carbohydrates

Due to gastrointestinal disorders, or because of lack of genetic expression in some subjects, disaccharides like lactose and other simple sugars may escape digestion in the small intestine and become available for bacterial fermentation in the colon. Lactose requires the brush border enzyme, lactase, for break down into its component monosaccharides glucose and galactose, before absorption. In lactose intolerant subjects, either lactase production is insufficient or they lack the genetic expression of lactase altogether due to some genetic disorder. Unabsorbed large amounts of lactose may exceed the fermentation capacity of gut bacteria and result in an increase of osmolarity in the gut (Wiggins, 1984). Higher osmolarity of lactose may cause an accumulation of large volumes of fluid in the gut lumen, consequently leading to osmotic diarrhoea. However, most individuals with lactase deficiency can tolerate lactose without causing diarrhoea because of an adequate fermentation capacity.

Lactulose, a semi synthetic disaccharide that is formed in milk by a heating process, is also indigestible in the small bowel but it is metabolised by the gut bacteria. It is a water soluble compound of low molecular weight and, therefore, its solution has high osmotic pressure (Wiggins, 1984), and is used therapeutically to treat constipation (Lactulose is described in detail in later chapters).

1.2.4.2.2 Oligosaccharides

Some oligosaccharides including raffinose, stachyose, fructo- and galacto-oligosaccharides and some inulins, are indigestible. These are readily soluble in water. Oligosaccharides range from 2 to 8 g/d in different Western diets and mainly originate from legumes, onions, artichokes and root vegetables (Englyst & Kingman, 1993). Oligofructose and inulin are not degraded in the small intestine and therefore, reach the colon intact (Bach Knudsen & Hessov, 1995). *In vitro* studies showed that these carbohydrates are fermented by the colonic bacteria (Wang &Gibson 1993). The importance of oligosaccharides emerged in late 1980s as a result of belief that they may affect microbial function in human large intestine in a beneficial direction (Hidaka et al., 1986). Oligosaccharides with such beneficial effects have been classified as pre-biotics, (Gibson & Roberfroid, 1995). Among such oligosaccharides, fructo-oligosaccharides meet all the criteria of being ideal candidates for classification as prebiotics. The importance of the fructo-oligosaccharides is due to their properties of stimulating the growth of "human friendly" bifidobacteria in the colon (Hidaka et al., 1986; Mitsuoka et al., 1987). These findings were confirmed by a study showing that bacteria

beneficial to human health, such as bifidobacteria, grow well on the fructo-oligosaccharides in comparison with the other substrates (Wang &Gibson 1993). The fructo-oligosaccharides including oligofructose commercially known as raftilose [ORAFTI s.a. - Aandorenstraat 1-B-3300 TIENEN, BELGIUM] (Gibson & Roberfroid, 1995) is good example of prebiotic.

1.2.4.2.3 Polysaccharides

The polysaccharides resistant to digestion in the human small intestine, comprise two sub groups;

Resistant Starch
Non-starch Polysaccharides.

1.2.4.2.3.1 Dietary fibre

Before this clear classification of indigestible polysaccharides into two categories, there was a large volume of scientific information in different fields related to carbohydrates which led to the hypothesis of dietary fibre. It was Hipsley (1953) who used the term "Dietary Fiber" for the components of cell wall that resisted the digestion in human small intestine. In 1970s Burkitt, Trowell and Southgate independently contributed to the concept of dietary fibre, resuscitating and supporting the term coined by Hipsley (1953). Trowell's definition of dietary fibre went through an evolution in its botanical aspect from "skeletal remains of plant cells" (Trowell, 1972) to "remnants of the plant cell wall" (Trowell, 1975), then to "structural polymers of plant cell wall" (Trowell, 1976) and later to a redefinition by Trowell et al., (1976) which included all indigestible polysaccharides and lignin present in the cell rather that present in the cell wall only. By this definition, all other indigestible compounds, including

proteins, tannins and cutin in plant cell wall, were excluded from the concept of dietary fibre whereas all indigestible polysaccharides were included, regardless of their botanical origin within the cell. This definition allowed inclusion of storage polysaccharides such as guar gum, etc. The physiological aspect of this definition remained the same since Trowell et al., (1976) described dietary fibre as resistant to the digestive process in human small intestine.

The latest definition of dietary fibre, proposed by Trowell et al., (1976) was widely accepted but another dispute came forth about this definition. Trowell et al., (1978) did not exclude non cell wall polysaccharide such as resistant starch from his definition of dietary fibre. By virtue of this definition, resistant starch has to be included within the dietary fibre category. On the other hand, digestible starch, which does not agree with the physiological concept of dietary fibre, has to be excluded from the definition of dietary fibre. This could create a confusion about the categorical position of digestible and resistant starch. Overcoming this discrepancy, Englyst et al., (1987a) proposed the substitution of 'non starch polysaccharides' as an equivalent for the term of 'dietary fibre'. This is a more realistic definition of dietary fibre because it is not in conflict with the original physiological criterion of dietary fibre concept, i.e. being resistant to the digestive enzymes in small intestine. At the same time it excludes resistant starch, protein and other non carbohydrate polymers from the original definition of the dietary fibre, which was not very clear about the position of these polymers. This new definition is more emphatic about the role of plant cell wall polysaccharides, thus not deviating much from the original definition of dietary fibre. However, there

is still much discussion, especially in USA, of whether resistant starch and fructo-oligosaccharides should be included in the dietary fibre (personal communication -Prof D Gordon, Food Science, South Dakota, USA).

1.2.4.2.3.2 Resistant starch

In the plant cell, starch is contained within granules of different shapes and sizes depending on the source species. For example, in rice and oats these starch granules are tiny in size while those in potatoes and banana, are large in size. The starch molecules are arranged in partially crystalline structures within granules which, in the case of amylopectin, have clusters of interchain branching points and regions with short chains. Although the constituent polymers of the starch are hydrophilic, the starch granules are not water soluble due to their semi-crystalline form (Eliasson & Gudmundsson, 1996).

This partially crystalline structure has distinct patterns on x-ray diffraction. Three main crystalline forms have been described: Types A, B, and C.
Type A is thermodynamically the most stable form and found in cereal starches, except in high amylose varieties. Type B is characteristic of starches in banana, roots and other tubers like potatoes. This is a crystal type also found in high amylose varieties of the cereals and retrograded starch. Type C is found in legumes such as bean and peas. The size and crystalline nature of starch granules influence their susceptibility to pancreatic enzymes. In general, starch granules with B and C type of crystallinity, tend to be more resistant to the pancreatic amylase.

Until the findings in early 1980s, starch was considered completely digestible in the small intestine. However, starch is now known not to be completely digested in the small gut by α amylase and pullulanase (an enzyme that can hydrolyze α 1-6 glycosidic bonds). Normally starch is digestible in the small intestine but a portion of the dietary starch is resistant to the digestive enzymes for a number of reasons and is readily available for the fermentation in the colon. This finding led to the development of the concept of the resistant starch (Englyst & Cummings, 1987a). Resistant starch has been defined by EURESTA (European Food Linked Agro-Industrial Research - Concerted Action on Resistant starch) as: *"The sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals"* (Asp, 1992). The incomplete digestion of the starch in the small gut has been demonstrated by different methodologies in several studies including the measurement of breath hydrogen (Anderson et al., 1981; Levitt et al., 1987; Christl et al., 1992), intubation of the ileum (Stephen et al. 1983), studies with ileostomists (Sandberg et al., 1981; Englyst & Cummings, 1987b). Three main types of the resistant starch are so far described (Englyst et al., 1992); **RS1** refers to starch granules that are physically trapped in the food matrix. In this case, intact cell structures such as cell wall, prevent the complete swelling of starch granules and as result, may hinder the access of amylase to the starch (Würsch et al., 1986). Mostly this category of resistant starch is found in whole or partially milled grains, seeds or legumes and also in some very compact processed foods like pasta. Starch in this case is inaccessible for the digestive enzymes in the gut. This type of the resistant starch can be affected largely by food processing and also by lack of chewing (Muir & O'Dea, 1992).

RS2 refers to raw starch granules with B-type crystalline structure which affects the digestibility of the starch by resisting the swelling and gelatinization of the granules during cooking (Würsch et al., 1986), which renders it indigestible. RS2 is mostly found in raw potatoes, banana and raw high amylose maize. These are very large starch granules with low SA/volume ratio, being resistant to digestion in small intestine because of the intact structures in food and specific crystalline pattern (B-type) of the starch granules.

RS3 refers to amylose and amylopectin forms of dietary starch which may retrograde (re-crystallised) when cooled down after cooking. When the starch is boiled in excess of water, it goes through a process of gelatinisation which consists of swelling and disruption of the starch granules. On cooling, both soluble and partially soluble components of starch in the gelatinised granules re-crystallise and this process of re-crystallisation is termed as retrogradation of the starch. The retrograded starch is highly resistant to the digestion in the small intestine. For example, in ileostomy samples from human subjects, 3% of undigested starch contents were reported after eating freshly cooked potatoes as compared with 12% undigested starch after eating cooked and cooled potatoes (Englyst & Cummings, 1987b). In the latter case, retrogradation of the starch could be a possible reason for higher amounts of resistant starch. Retrograded starch characteristically forms the B-type crystalline pattern.

The RS 1 & 2, mainly depend on the food industry which by the controlling the level of its processing, has a major role in determining the amount of the dietary starch available for the fermentation. Fermentation of resistant starch is much

slower compared with most dietary fibre (Achour et al ., 1996). A semi-purified retrograded amylose (RS3) was less fermentable with the lowest production of SCFA compared with raw potato starch (Edwards et al., 1996). Thus, resistant starch may resist, not only amylolytic action in the small intestine, but may resist even the degradation by bacteria in the large intestine as well.

1.2.4.2.3.3 Non starch polysaccharides

Non starch polysaccharides include main structural components of the plant cell wall i.e. Cellulose, hemicellulose and pectin, different plant gums, various mucilages. Gums such as guar gum, gum arabica are examples of indigestible carbohydrates that are chemically related to non starch polysaccharides (NSP) and are included in the definition of the dietary fibre. Intake of non starch polysaccharides, ranges from 12.4 /day in the diet of British people (Englyst & Kingman, 1993). Non starch polysaccharides can be grouped into two major categories on basis of their solubility in water;

First, water soluble non starch polysaccharides that may form gels or viscous solutions, include different gums, pectin. Second, water insoluble non starch polysaccharides including cellulose.

Proportions of both types vary in different foods, the proportion of the soluble non starch polysaccharides is higher in fruits and vegetables compared to that in wheat fibre and brown rice. The water soluble NSP constitute the major portion of non starch polysaccharides in fruits, oats, rye, barley and legumes while water insoluble NSP are predominant in vegetables, bran of wheat and other cereal grains (Englyst et al., 1988; Englyst et al., 1989). Insoluble NSP are more

resistant to the bacterial fermentation in the human colon than soluble fibres (Spiller et al., 1980; Edwards, 1990). Classification of indigestible carbohydrates on the basis of their water solubility, may, in turn, depend on intermolecular bonds, spatial arrangement and packing of the monomers in a molecule of higher molecular weight complex carbohydrates. The regularity of a linear chemical structure increases the strength of the non-covalent bonds, thus, stabilising the ordered conformation. If the backbone has irregularity or the branching occurs, such bonds are weak, thus making the dissociation of ordered structure and promoting the solubility (Thibault et al., 1992). Spatial arrangement and packing of the monomers in a molecule, is a very important property in determining the physiological role of a particular complex carbohydrate. The stability of ordered and regular shapes of the polysaccharide chains after exposure to the excess water depends on the interaction of a number of factors, the most important of which is charge. Many polysaccharides have charged groups, such as COO^-, which repel one another, thus favouring expansion and destabilisation of coils in solution rather than a compact and ordered molecular structure (Morris, 1992). For example, uronic acid (a major constituent of pectin) will tend to repel polysaccharides from each other and thus will favour solubilisation of the complex molecule (Thibault et al., 1992).

1.3 The human colon and its environment

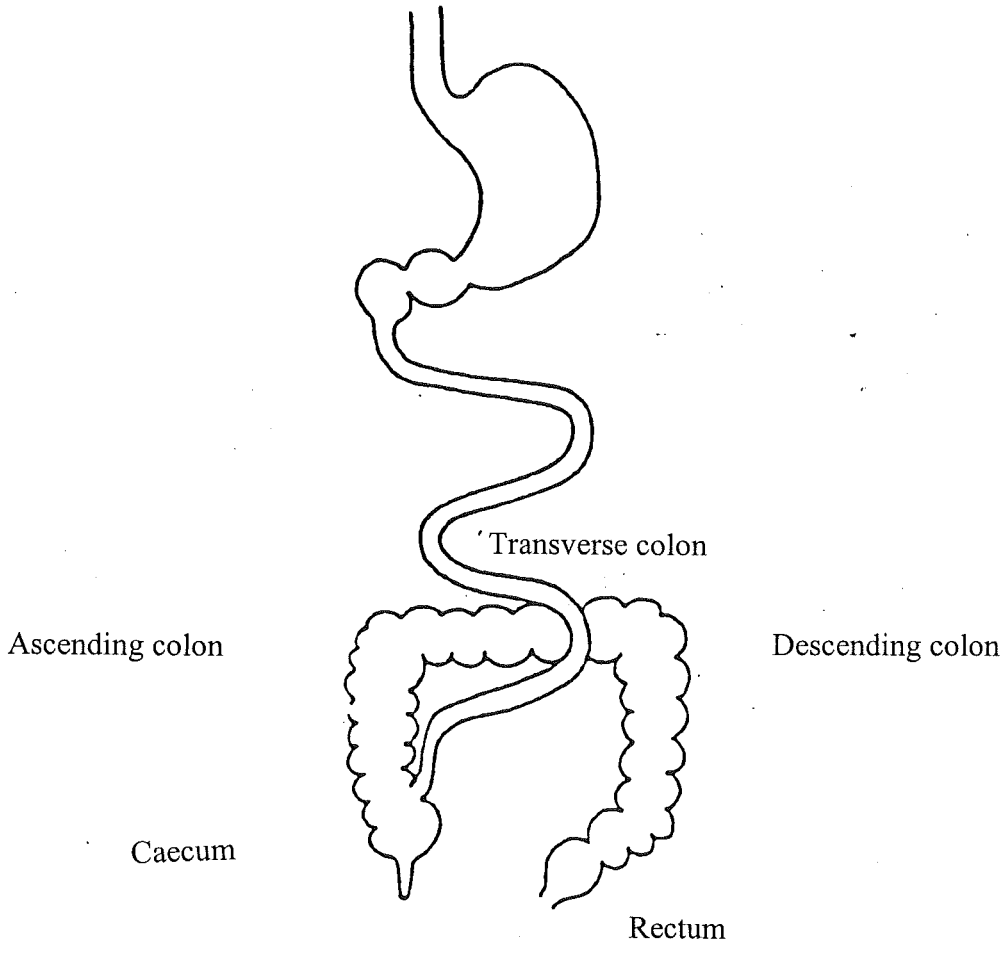
Carbohydrates resistant to the intestinal digestive enzymes reach the large intestine where they are fermented by the gut bacteria to organic metabolites, most of which are absorbed through the epithelium of the large intestine. In this

way, carbohydrates provide energy to the body tissues in the form of either monosaccharides resulting from digestion in the upper gut or through formation of different metabolites such as short chain fatty acids by the fermentation in the lower gut. The colon is about 100 to 150 cm in length (Christensen, 1989) and 6 cm in diameter. The human colon is haustrated throughout most of its length and can be, regionally, divided into proximal ascending colon, traverse colon, distal descending colon and sigmoid colon (Lacy, 1991). The ascending colon is 15cm, traverse colon 50 cm, descending colon 25 cm and the S-shaped sigmoid colon is 40 cm in length. The rectum, the final part of the digestive tube, starts from recto-sigmoid junction and ends in the anal canal (Fig. 1.1). The human large intestine can be divided arbitrarily in three functional regions;

1) Caecum and proximal colon as the site for carbohydrates fermentation
2) Traverse colon as site for absorption and the motility check point
3) Distal colon, and rectum which acts mainly as the reservoir of the residual waste material ready for excretion (Edwards, 1997).

There are differences in the anatomy, absorptive characteristics, neural and blood supply of different regions of the human colon (Edwards, 1993). The mucosa covers the luminal surface of the colon. Unlike the small intestine, the colon is not convoluted and no villi are present. As a result, the absorptive epithelial surface of the colon, despite a greater diameter of colon, is only about $1/30^{th}$ that of the small intestine. However, the longer transit time in the colon gives an opportunity for substantial absorption to take place. The total amount of the material entering the colon is about 1.5 kg per day while average stool weight,

Fig. 1.1 The human gastrointestinal tract



- Caecum & ascending colon – carbohydrate fermentation.
- Transverse colon – absorption & motility check post.
- Dscending colon & rectume – water & SCFA absorption; protein fermentation; reservoir of waste material ready for excretion.

in the UK, is 120 g/d (Cummings & Macfarlane, 1991). The average wet weight of colonic contents is about 220g in the western populations with a range of 58 to 904 g (Banwell et al., 1981), consisting mainly of bacteria. The microbial community in the human gut, is diverse and complex comprising of approximately 400 to 500 species (Finegold et al., 1983). The majority of this microbial population consists of anaerobic bacteria which outnumber the aerobic bacteria by 1000 to 1 (Gustafsson, 1982). Substrate, required daily for the maintenance of such a huge microbial population, is provided from intrinsic as well as extrinsic sources, including dietary carbohydrates, proteins and peptides.

1.4 Colon and fermentation

When undigested carbohydrates reach the colon, they may be broken down anaerobically by the colonic bacteria. This process of anaerobic bacterial degradation of carbohydrates is termed fermentation (MacFarlane, 1991). Bacterial fermentation in the human colon facilitates the salvage of the unabsorbed energy. The colon has two main functions;

First, it is the site of fermentation of different substrates reaching the colon in partially or fully intact form. Secondly, it is the major site for the absorption of water, electrolytes and other end products from bacterial fermentation. The cells lining of the human large intestine are unable to transport actively either glucose or amino acids.

The active transport of sodium across the gut mucosa (promoted by SCFA absorption) is accompanied by the re-absorption of the water from gut lumen to blood. Caecal content is approximately 86% water, falling to 77% water in the

sigmoid rectum (Cummings & Macfarlane, 1991). This process of water absorption renders the colonic contents more concentrated with the passage through the gut. Absorption of water and other metabolites in the colon, prevents the osmotic diarrhoea which can cause a severe depletion of total body potassium. The nutrient depleted and concentrated colonic contents, reach the rectum and are stored there for excretion. In this way, the colon may be regarded as a semi continuous fermenter because of its fermentation pattern, receiving a semi-continuous supply of substrate from the upper gut which is fermented by the colonic microorganisms. The body tissues clear most of the end products of this fermentation process for further metabolism while part of the end products and residual material are excreted with the faeces. The bacterial population is also replenished continuously by the carbohydrate fermentation, if abundant nitrogen is present.

The right colon is the area of an extensive carbohydrate fermentation producing large amounts of the short chain fatty acids (Cummings et al., 1987), creating an environment with low pH due to higher concentration of organic acids in colon. The subsequent absorption of SCFA from colon and simultaneous secretion of HCO_3, causes an increase in colonic pH, lowest (i.e. pH 5.6) in the caecum and gradually rises to about pH 6.6 in the descending colon, towards the distal end (Cummings et al.,1987). As the gut contents move towards left side of the colon, they become gradually nutrient-depleted. Nitrogen contents are 6% of dry matter in all regions of the gut content whilst total carbohydrates fall from 20% of content in the caecum to 11% in the sigmoid rectum (Cummings & Macfarlane,

1991). This gradual depletion of the carbohydrate substrate in gut, affects the fermentation reactions in gut lumen resulting in lower production of short chain fatty acids at the distal colonic site. Although concentrations of SCFA vary throughout the gut, their molar ratios seem to show little change (Cummings et al., 1987).

1.5 Products of fermentation

Intestinal microflora adopt the Embden-Meyerhoff pathway for degradation of carbohydrates into their end products. Major end products of carbohydrate fermentation are short chain fatty acids and some other organic compounds, different colonic gases and replenished bacterial mass.

1.5.1 Short chain fatty acids

Acetate, propionate and n-butyrate are the most important short chain fatty acids (SCFA), resulting from the fermentation of carbohydrates and these account for approximately 85-95% of total SCFA in all regions of human colon (Cummings et al., 1987). The amount of net total SCFA produced per gram substrate fermented varies from 34 to 59 percent in stoichiometric terms (Englyst et al., 1987b).

There is evidence, mainly from *in vitro* studies, that the SCFA profile is carbohydrate specific, i.e. some carbohydrates are more butyrate predominant. For example, starch fermentation produced more butyrate (Englyst et al., 1987b; Wang & Gibson, 1993), whereas, others are more propionate predominant, such as guar gum (Adiotomre et al., 1990). Concentration of these SCFA is highest in the human caecum and proximal colon, declining progressively towards the distal

site of the large intestine (Cummings et al., 1987). This shows carbohydrate fermentation is higher in the proximal colon compared with the distal colon. Some branched SCFA, including isobutyrate, isovalerate and nvalerate are produced as a result of protein fermentation in the gut (Macfarlane & Alison, 1986), chiefly they arise from de-amination of the branched chain amino acids. Protein fermentation is greatest in the left colon as it is clear from the higher ratio of the branched chain fatty acids in the left colon (Macfarlane,1991).

1.5.1.1 SCFA and their metabolic implications

Low concentration of short chain fatty acids in the peripheral blood circulation (Cummings et al., 1987) indicated that these acids were metabolised either in the liver or in the colonic tissues. Although each SCFA is metabolised differently and at several sites in the body, and the metabolism of short chain fatty acids differs for acetate, propionate and n butyrate, the liver is a major site of the expected impact of SCFA. In general, over 50% of short chain fatty acids are metabolised in this organ (Demigné & Rémésy, 1991). In physiologic conditions, propionate and n-butyrate in portal vein are almost quantitatively cleared by hepatic tissues. Acetate is cleared only partially by the liver, depending on its portal concentration and on the physiological state (i.e. fed, fasting, starvation, diabetic state) of the individual (Demigné & Rémésy, 1991). During their absorption, SCFA are partly metabolised by the colonic mucosa and partly transported to the liver for further metabolism. The oxidative breakdown of the SCFA after their absorption in the body provides the fuel for different physiological reactions in the body tissues.

Interest in SCFA production has been spurred by the emerging role of n-butyrate in colonic mucosal cell growth and metabolism (Kruh, 1982; Sakata, 1983; Smith, 1986), but also by the potential role of propionate in inhibiting hepatic cholesterol biosynthesis (Chen et al 1984). Short chain fatty acids provide about 60-70 % of estimated energy requirements of the colonocytes and colonic epithelium (Roediger, 1980; Roediger, 1982). The SCFA, once absorbed, have a relatively low calorie value per gram (acetate 3.49, propionate 4.96 and n-butyrate 6.16). Acetate is the major SCFA absorbed and reaching the peripheral tissues which limits the energy contribution by the SCFA even with large amounts of fibre (Rémésy et al., 1992). It is estimated that bacterial fermentation in the colon may contribute about 10% of daily energy requirement in the Western society (McNeil, 1984). Although SCFA may not be a major source of energy in the human, compared with other metabolites such as glucose, other fatty acids and amino acids, they may play an important role in the integrity and growth of intestinal mucosa and hepatic tissues in the human (Demigné & Rémésy, 1991). Short chain fatty acids have a number of effects which may be relevant to metabolic activities in human body as well as to colonic health and, thus influencing the intermediary metabolic reactions in the colonic epithelial, hepatic and peripheral tissues.

1.5.1.1.1 Acetate

Acetate is about 60% of the total SCFA produced from bacterial fermentation in human colon (Scheppach et al., 1991) and it has been used as the indicator for the

fermentation in the human colon in many studies (Pomare et al., 1985; Muir et al., 1995; Zavoshy, 1998). A number of metabolic roles have been ascribed to acetate in hind gut fermenting species, such as lipogenesis, however, in practice probably acetate acts simply as a source of energy for the tissues (Cummings, 1991). It is rapidly oxidised and acts as an important source of energy in the human peripheral tissues (Cummings & MacFarlane, 1991). Acetate has two particular features, which make it distinct from the other short chain fatty acids;

i) Acetate not a major energy source, but is nevertheless an important source of energy in the liver and peripheral tissues of body. It is the only SCFA reaching the peripheral blood circulation and passing to peripheral tissues for further metabolism in muscles. Significantly higher amounts of acetate were reported in portal blood compared with that in the peripheral venous blood system (Cummings et al., 1987), which is an indication of an uptake and utilisation of acetate by hepatic tissues. Despite, its considerable uptake by the hepatic tissues and rapid oxidation, free acetate is usually present in the peripheral blood system (Pomare et al., 1985).

ii) Acetate stimulates cell turnover in the entire gut and helps in healing the damaged intestinal mucosa in rats (Sakata 1987).

Apart from production by bacterial fermentation in colon, an endogenous synthesis of acetate takes place in body tissues. Pomare et al., (1985) noted significantly higher acetate (125.6±13.5 μmol/l) in arterial blood system compared with that in venous blood system (61.1± 6.9 μmol/l) in the fasting state in human subjects. Acetate is produced by liver in the fasting state (Scheppach et

al., 1991). In the fed state increasing amounts of plasma acetate are mostly derived from the bacterial fermentation in the colon (Pomare et al., 1985; Scheppach et al., 1991; Muir et al., 1995). Scheppach et al., (1991) also reported a significantly lowered fasting plasma acetate in ileostomy subjects (21.3±0.8 μmol/l) compared with normal control subjects (48.0±4.2 μmol/l). Considering these observations, it is evident that humans mainly derive their plasma acetate from the colonic fermentation but in the fasting state a marked endogenous synthesis probably compensates for the colonic fermentation. In reality endogenous synthesis is a result of fat degradation in the liver.

All these studies pointed to important facts related to the synthesis and metabolism of the acetate in the human body, i.e.

a) Colonic fermentation contributes significant amounts to plasma acetate whereas endogenous system of acetate synthesis becomes more important with prolonging starvation.
b) Metabolism of acetate occurs at both hepatic tissue and peripheral tissue level.

Acetate can be utilised by most tissues, except probably the brain and other nervous tissues and it is a good source of acetyl CoA in adipose tissue, where glycolysis is limited. In muscles, acetate may spare fatty acids and glucose, and has some vasodilatory effects (Demigné, 1991). Since fatty acids decrease the use of glucose in muscle, through an effect on hexokinase, glycolysis and pyruvate dehydrogenase, acetate may have a regulatory role in this regard. Acetate reduces serum free fatty acids (FFA) levels, which, in turn, may reduce

blood glucose levels because FFA compete with glucose for uptake by insulin sensitive tissues (Ferrannini et al., 1983).

Although the liver has a high capacity of acetate activation, it seems that that long chain fatty acids are sources of energy in the liver and acetate utilisation is channelled towards lipogenesis (Rémésy et al., 1992). Therefore, acetate is a primary substrate for lipid synthesis and when acetyl-CoA provision from glucose is limiting, acetate may become an effective precursor for lipogenesis. Acetate is activated to generate acetyl CoA in the hepatic tissues, thus acetate acts as a precursor for lipid synthesis in the fasting state i.e. when acetate concentrations are low in portal blood. Acetyl CoA, being used for citrate synthesis in hepatic cell mitochondria, enters the Krebs cycle causing an increase in the intracellular citrate (Berggeren, 1996). This increase in the concentration of citrate may inhibit pyruvate, in turn, acetyl CoA, formation from glucose, thus reducing the flux of glucose via the glycolytic route. In this way, acetyl CoA from acetate may spare the glucose for the central nervous systems and some other specialised cells such as red blood cells of the body because of their obligatory needs, as these tissues must be supplied with glucose as a source of energy.

Propionate and butyrate can completely inhibit the oxidation of acetate in hepatic tissues. In this way, the liver removes the propionate for glucose synthesis and n-butyrate for ketone body formation, whereas, acetate is not metabolised and it is spared for the utilisation in the extrahepatic tissues (Ballard, 1972). Therefore, it seems that acetate and propionate, after reaching the liver, influence the

metabolism of carbohydrates and lipid, probably in an opposing way. The ratio of acetate to propionate may be very important in the overall physiological role of the carbohydrate fermentation in the colon (Wolever, 1991).

1.5.1.1.2 Propionate

Propionate is an excellent precursor of gluconeogenesis and can be used to produce glucose in the body. It stimulates cell turnover in the gut and may help in healing the damaged gut epithelium (Sakata, 1989). Propionate has been studied extensively in animals (Thacker & Bowland, 1981; Boilla et al., 1981; Anderson & Bridges, 1982; Chen et al., 1984; Illman et al., 1988; Imaizumi et al., 1992; Berggren et al., 1996) but less information is available on its effect in humans (Venter et al., 1990; Todesco et al., 1991; Wolever et al., 1991). Some of these studies showed an inhibitory effect of propionate on hepatic cholesterol synthesis and such studies may be helpful in explaining the lipid lowering effect of the soluble dietary fibre (Chen et al., 1984; Illman et al., 1988). This cholesterol lowering effect is either due to inhibition of the hepatic cholesterol synthesis or because of the redistribution of cholesterol from plasma to liver (Illman et al., 1988). Berggren et al., (1996) noted significantly lower total hepatic cholesterol pools in obese hyper-insulinaemic rats by dietary ingestion or rectally infused propionate compared with control group. Chen et al., (1984) has shown an inhibition of cholesterol synthesis in isolated rat hepatocytes, hence this may result in plasma cholesterol lowering effect. However, this may not occur at physiological concentrations.

The liver is a main metabolic site for propionate, which is cleared by the hepatic tissues after absorption through the colonic mucosa, especially when large amounts are absorbed from gut. Hepatic clearance from the portal system is over 50%, resulting in very little appearance of propionate in the peripheral blood system (Cummings et al., 1987). Uptake of propionate may influence glucose metabolism and cholesterol synthesis in hepatic tissues and these effects are believed to be beneficial, reducing the fasting serum glucose and increasing the insulin sensitivity i.e. reducing the maximum insulin increments during the glucose tolerance (Venter et al., 1990; Todesco et al., 1991). Propionate is readily taken up and activated in hepatic tissues. This activation of propionate, unlike conversion of lactate into oxaloacetate via formation of the pyruvate (Wolver, 1995), does not depend on the rate controlling step of pyruvate carboxylase (Rémésy et al., 1992). Its activation generates propionyl CoA in mitochondria of hepatic cells. Propionyl CoA is transformed into methylmalonyl CoA with a subsequent conversion to the succinyl CoA which may enter the Krebs cycle and is then converted to the oxaloacetate via the formation of succinate. The resulting oxaloacetate is metabolised further to yield glucose.

Propionate is metabolised in an opposing way to the metabolism of acetate after reaching the liver and, therefore, influences the metabolism of carbohydrates and lipid. Propionate can completely inhibit the oxidation of acetate in hepatic tissues by blocking the oxaloacetate availability for the formation of citrate, by using it in gluconeogenesis in tissue cells. Propionate generated during colonic fermentation is unlikely to raise the plasma glucose but probably contributes

small amounts of glucose to the system. The rate of delivery of propionate is much faster in the experimental amounts (180mmol) used for rectal infusion in humans compared with that in the actual colonic fermentation (50-200mmol/24 hours) (Wolever, 1991).

Since most of the studies relating to its metabolic influences on serum lipid and glucose levels are carried using dietary supplementation of propionate, there may be different effects than if propionate was produced in the colon;

i) Propionate may act as an enzyme inhibitor of amylolytic activity, hence reducing the starch hydrolysis in the small intestine.

ii) It may cause a delay in gastric emptying (Blum et al., 1976).

iii) There may be a re-distribution of the cholesterol from plasma to the liver, responsible for the hypo-cholesterolaemic effects of dietary propionate.

iv) An increase in glycolysis and a simultaneous decrease in glucose generation in isolated rat hepatocytes (Anderson & Bridges, 1982), may be responsible for the plasma glucose lowering effect.

The results of Anderson & Bridges, (1982), were supported by Venter et al., (1990), who noted a reduced fasting serum glucose after dietary supplementation with propionate in healthy subjects. In this study total cholesterol levels did not show any change in man, although HDL cholesterol were increased by feeding 7.5g sodium propionate per day to a group of female volunteers.

1.5.1.1.3 n-Butyrate

Roediger (1980) identified n-butyrate as a preferred source of energy for the colonocytes *in vitro*. In his *in vitro* study, clear intra-colonic regional differences

were noted for n-butyrate utilisation, suggesting that n-butyrate is the major source of energy for the colonic mucosa, particularly of the distal colon. As lower molar ratios of butyrate were reported in portal veins compared with the colon of sudden death victims (Cummings et al., 1987), it seemed that surplus n-butyrate, not metabolised by colonocytes, enters the portal blood system and is effectively metabolised by the hepatic tissues.

The n-butyrate is considered particularly important because of its various effects on colonic mucosal health and it is implicated in reducing the risk of malignant changes in the colonic epithelium, in addition to providing the energy to the colonocytes in human. n-Butyrate has been studied extensively for its effect on the colonic epithelium at the cellular level (Kruh, 1982; Sakata, 1983; Smith, 1986). It is emerging as a promising nutritional agent in the treatment of mild to moderate distal ulcerative colitis. Beneficial effects of butyrate enemas on the colonic mucosa, were reported in patients with distal ulcerative colitis (Scheppach et al., 1992). In this study, irrigation with 100mmol/l sodium butyrate, decreased stool frequency significantly and stopped blood discharge in 9 out of 10 patients. Similarly the degree of histological inflammation decreased significantly. These results indicated a possible role of butyrate deficiency in pathogenesis of the distal ulcerative colitis.

In another study a lower ratio of butyrate was reported in enema samples from subjects with polyp-colon cancer, compared with that from normal subjects (Weaver et al., 1988). Since n-butyrate has been implied in direct gene expression, as explained later, its production at the site of tumour formation may

be a significant protective mechanism of certain dietary fibres. It seems that n-butyrate is of greater significance than the other SCFA in distal colon. Therefore, a dietary fibre, with n-butyrate predominant fermentation in the distal colon, may be very important and beneficial for colonic health. Reduced tumour mass was noted by feeding n-butyrate predominant fibres in a rat model (McIntyre et al., 1993). These authors reported a significant negative correlation between the concentration of faecal butyrate and tumour mass in distal colon. Clausen et al., (1991) supported these results by showing a reduced ratio of butyrate when different fibres were incubated *in vitro* with the faeces from patients with colonic adenomas and cancer as compared with healthy individuals. In this study, faecal bacteria from patients with colonic adenomas and cancers showed a significantly reduced capacity for producing butyrate which suggested that lack of butyrate may be important in the genesis of colonic neoplasia. On basis of these studies, it may be hypothesised that subjects characterised by a colonic flora with a relatively low capacity of butyrate synthesis, may have an increased risk of developing colonic adenomas and cancer. It is possible that this risk could be overcome by a diet rich in fermentable carbohydrates, producing high butyrate especially at a distal colonic site.

n-Butyrate possesses an anti-neoplastic property (Cummings, 1984) and because of this property, it is thought to interact with genetic events of the tumour cells, playing an important role in regulation of the rate of differentiation of colonic cancer cells (Kruh, 1982). n-Butyrate has properties of affecting the cellular activities including the arrest of cell growth, alteration of gene expression,

suppression of several cancer-specific properties of transformed cells, and modification of cell morphology and ultra-structure. The effects of n-butyrate are reversible, whereas, other SCFA are, in general, much less effective or not effective at all in inducing cellular changes (Kruh, 1982). A significantly diminished proliferation was noted in the mucosal cell by feeding healthy volunteers a diet supplemented with amylomaize, a source of higher n-butyrate ratios (van Munster et al., 1994). In this study, a significant increase in faecal SCFA was noted. However, n-butyrate was reported, in earlier studies, to reduce the DNA synthesis and suppress the proliferation in a variety of cell types (Leder & Leder 1975; Hagopian et al 1977; Borenfreund et al. 1980). In another study, butyrate showed a positive effect on cell proliferation (Sakata, 1989), stimulating cell turn over in gut which may lead to enhanced chances of transcriptional errors and that of production of more tumour cells. However, n-butyrate has also been shown *in vitro* to stimulate the apoptosis or programmed cell death in colonic tumour cells (Hague et al., 1993; Hague & Paraskeva, 1995). Similarly n-butyrate helps the healing of damaged mucosa in the gut and it is believed to play an important role in prevention of colonic cancer through this effect.

Interestingly when colon cancer cells were treated with n-butyrate, they started differentiating *in vitro* (Augeron & Laboisse, 1984; Whitehead et al., 1986). n-Butyrate has an important role in the modulation of the nucleic acid metabolism especially affecting the gene expression and cell growth. n-Butyrate induced differentiation in a range of mammalian cells including colorectal cancer cell lines (Whitehead et al. 1986). It is also effective in inducing phenotypic

maturation in cells derived from human colon carcinoma (Whitehead et al., 1986).

Butyrate has shown the potential to modulate gene expression directly. n-Butyrate showed an *in vitro* inhibition of histone deacetylase which, in turn, resulted in hyperacetylation of histone. The hyperacetylation of histone stabilises DNA structure by opening it and facilitating its accessibility to DNA repair systems. In this way n-butyrate facilitates the repair of DNA by affecting histone deacetylase and methylation of DNA (Candido, 1978; Smith, 1986). Another mechanism for the health implications of n-butyrate in colon cancer was that it suppressed the *in vitro* growth rate of isolated cancer cells from colon by prolonging the doubling time (Kruh, 1982; Whitehead et al., 1986). These findings were confirmed by subsequent studies reporting a reversible alteration in properties of cancer cell line by prolonging the doubling time and retarding the growth rate (Sakata, 1987; Gamet et al., 1992).

When we look at the results of different studies in relation to the effects of butyrate on cellular activity of colonic mucosa, interesting contrast in its effects are seen. On one hand, butyrate stimulates the growth of colonic mucosa, whereas, on the other hand, it inhibits the growth and induction of differentiation in colonic cancer lines (Hague et al., 1997). Higher production of n-butyrate, therefore, is of great importance and is identified with an significant role in human colonic health because of its capacity to affect the cell growth in healthy and diseased colonic epithelium. At the same time, not only a higher ratio of n-butyrate, but also the site of its generation, may be important in relation to

colonic mucosal health. Thus, it may be possible to prevent the incidence of colitis and cancer in human colon, by facilitating the production of more butyrate at more distal site. Incidence of tumours is more in the distal colon of human patients whereas SCFA production from carbohydrates fermentation declines as digesta passes from proximal colon towards more distal site. Therefore, it is important to facilitate higher production of n-butyrate at more distal site. In this thesis, the possibility of producing higher amount of butyrate at more distal colonic site will be explored. In this study, I will evaluate different mixtures of indigestible carbohydrates, to identify a mixture which could produce more butyrate at more distal site, by delaying the bacterial fermentation to an optimum extent and at the same time to avoid the undesirable side effects of large doses of such indigestible carbohydrates including bloating, distension, flatulence, abdominal pain, diarrhoea, etc.

1.5.2 Other organic acids

Sometimes other organic acids, as intermediate or end products, including electron sink products, e.g. lactate and succinate, may be produced to a lesser degree during bacterial fermentation in the human colon (Cummings et al., 1987). A very acidic environment in gut favours the production of lactate by fermentation activity of some bacterial species and production of lactate is affected by the speed of fermentation. When large quantities of rapidly fermenting carbohydrates are readily available, lactate production is favoured.

Lactate may be produced from pyruvate by the fermentation activity of some bacterial species. Although lactate may be produced to a lesser degree during

bacterial fermentation in the human colon (Cummings et al., 1987), the increased fermentation of a rapidly fermenting carbohydrate may reduce luminal pH which, in turn, inhibits the metabolic activity of the lactate utilising bacteria. This may increase the amounts of lactate in lumenal contents in the case of rapid fermentation. Lactate act as electron sink products in the metabolic chain reaction by accepting the electron from NADH in the form of H^+, making the NAD available for further metabolic reactions. Lactate is not a key intermediate in the fermentation process and, therefore, no significant amounts are found, especially after prolonged fermentation. Lactate is not as well absorbed as the other SCFA and its accumulation may increase the lumenal osmolarity, resulting in acceleration of transit, consequently loss of energy in faeces.

1.5.3 Fermentation of nitrogenous compounds

Protein may be fermented by gut microflora into other end products including phenols, indoles, amines, ammonia. Some of the metabolites resulting from protein fermentation in the colon, unlike the end products of bacterial fermentation of carbohydrates, are of potential hazard to the host (Cummings & Macfarlane, 1991). For example, NH_3 is produced as one of the main end product by de-amination of amino acids in the gut. Ammonia can affect DNA synthesis, reducing the life span of epithelial cells by a faster turnover. An enhanced cell proliferation may increase the chances of duplicating errors in DNA copying, unmasking latent DNA changes resulting from earlier mutagenesis (Visek, 1978). A replicating cell may be more vulnerable to carcinogenesis and the extent of this vulnerability may vary at different stages of replication. This concept is supported by the evidence of increased sensitivity of the newborn and

regenerating adult mammalian liver to the carcinogenic action of certain chemicals or radiation (Warwick, 1971). In this way, a high NH_3 concentration in the colon may select for neoplastic growth. Ammonia is rapidly absorbed from gut and detoxified by urea formation in human liver. Alternatively, NH_3 concentration may be reduced by increased bacterial fermentation of carbohydrate in the gut which may result in stimulation of bacterial growth (Cummings & MacFarlane, 1991).

Bacterial fermentation of tyrosine, produces phenols and p-cresol as the major phenolic compounds which act as co-carcinogens. Increased carbohydrate fermentation may decrease the production of these compounds by using higher amounts of amino acids for further growth of bacterial mass. This has been shown by a decreased phenol excretion in urine after an increased bacterial fermentation of carbohydrates in the human colon (Cummings & Macfarlane, 1991).

1.5.4 pH changes

Higher production of organic acids such as acetic, propionic, lactic acids, may result in the depression of pH in the gut, the lowest pH (i.e. pH 5.6) in gut, was noted in the caecum (Bown et al. 1974; Cummings et al.,1987). Such decreased pH has a number of effects on the activity of gut bacteria and on metabolic reactions in the human gut. The reduced pH may decrease the solubility and so the absorption of bile acids and fatty acids, resulting in the enhanced excretion of these metabolites with faeces. In this way, low pH mobilises the reserves of body

cholesterol for further synthesis of bile acids and fatty acids, and as a result the plasma cholesterol level is lowered.

A low pH may have a protective action against colonic cancer and higher faecal pH has been considered as a indicator for the risk of cancer incidence (Walker et al., 1986; Malhotra, 1982). Lower pH may help in reducing the potential carcinogenic agents in different ways, such as changing the ionic level, affecting the enzymic activity. An acidic pH may facilitate the conversion of potentially toxic ammonia (NH_3) into ammonium (NH_4^+) ion (Visek, 1978) which is not well absorbed from colon resulting in a reduced blood urea.

The intracellular and extracellular biochemical reactions in the bacterial cells, are catalysed by various enzymes, and lowered pH may affect the fermentation reactions by influencing the intracellular and extracellular enzyme activity of such bacterial cells. Changes in pH may affect the metabolism due to the different optimal pH requirements of enzymes involved in various metabolic reaction.s.For example, a decreased pH in the colon is considered helpful in reducing the synthesis of carcinogens such as conversion of primary bile acids into secondary bile acids through 7 α de-hydroxylation by the catalytic activity of a bacterial enzyme, 7α dehydroxlase (Thornton, 1981). This enzyme requires an optimum pH 7-8 and enzymatic conversion of primary bile acids into secondary bile acids by this enzyme, is inhibited at acidic pH (Nagengast et al., 1988).

Changes in pH can affect the physiological activities of the gut bacteria without affecting the numeric values of species. This may lead to different metabolic

pathways, for example, a lower *in vitro* pH (6.0) favoured the production of propionate rather than acetate (Edwards et al., 1985). A lowered pH inhibits the pathogenic bacteria such as *E. coli*, *Cl. perfringens*. and promotes the growth of bifidobacteria and lactobacilli which are beneficial bacteria in the human gut (Wang & Gibson, 1993). Similarly changes in pH may make conditions unfavourable for the optimal growth of certain bacterial species because the specific growth rates of different bacteria vary with growth conditions such as pH. For example, *lactobacilli* show optimal growth at an acidic pH (5.4 - 6.4) whereas optimal growth of Clostridium species is in pH (6.5 - 7.5). A number of Bacteroides species were well maintained at all pH from 5 to 7 in continuous anaerobic cultures (Edwards et al., 1985). In the same experiment, *lactobacilli* and *bifidobacteria* survived in cultures at pH 5 but disappeared with increasing pH from 5 to 6 and then to pH 7. *Cl. perfringens* demonstrated a reverse ability by disappearing with the decreasing pH from 7 to 6 and then to 5. In another *in vitro* experiment, *B. infantis* withstood the effects of acidic conditions at pH 5.0 and 4.5, but the same pH inhibited the growth of *E. coli* and *Cl. perfringens* completely (Wang & Gibson, 1993).

Changes in pH caused by fermentation may affect the absorption of various substrates or minerals. This may be due to the increased solubility of minerals. A higher solubility of calcium is reported in caecal contents of rats in presence of a lower pH, achieved by an increased fermentation of resistant starch RS2 (Younes et al., 1996). These authors reported 5 to 6 fold increase in absorption of calcium and also an increased caecal absorption of magnesium by feeding resistant starch.

This showed that the large intestine may act as a major absorptive site for calcium and magnesium, due to its acidic environment resulting from its fermentation capacity.

1.5.5 Production of gases

The gases resulting from bacterial fermentation in the colon include H_2, CO_2, CH_4 and H_2S. Total gas production may depend on the dietary intake of the individual, ranging from 0.5 to 4 l/d. H_2 is produced as a result of oxidation of pyruvate, formate or reduced pyridine nucleotides. Saccharolytic Clostridia produce H_2 from pyruvate via ferredoxin in butyrate fermentation while Enterobacteria produce H_2 through cleavage of pyruvate by pyruvate-formate lyase, the resulting formate is converted to CO_2 and H_2 (Cummings & MacFarlane, 1991). Many studies have extensively investigated the production of gases by colonic fermentation of carbohydrates (Anderson et al., 1981; Olesen et al., 1992; Rumessen, 1992).

Breath H_2 measurements have been adopted as an indirect measurement of colonic fermentation and in different studies, changes in breath H_2 and serum acetate levels were used as qualitative markers of colonic fermentation (Muir et al., 1995; Zavoshy, 1998). A rise in breath H_2 has been considered an indication of the fermentation activity in human large intestine although there has been a controversy about its quantitative interpretation. Measurement of breath hydrogen is not reproducible (Florent et al., 1985) and does not always relate directly to amount of carbohydrate being fermented (McBurney & Thompson, 1989; McBurney et al., 1990).

Colonic gases may be either excreted rectally or absorbed from colon, re-metabolised and then excreted via the lungs in breath. Gibson et al., (1990) described several ways of H_2 disposal including methanogensis, sulphate reduction, acetogenesis. H_2 may either be absorbed by the gut tissues and excreted in breath or re-metabolised by the gut bacteria in methanogenesis forming methane(Gibson et al., 1990) or utilised in sulphate reduction forming H_2S (Gibson et al., 1988; Gibson et al., 1990) or it may combine with CO_2 during acetogenesis producing acetate (Gibson et al., 1990; Lajoie et al., 1988). Carbon dioxide may be reduced to methane, and as a result of higher consumption of CO_2 for methanogenesis, may consequently lead to change of acetate to propionate ratios. All these studies showed that there are several possible routes for H_2 disposal *in vivo* depending on different factors such as composition of microbial population, sulphate availability, etc.

1.6 Actions of carbohydrates and important determinants

There are a number of factors, such as chemical composition, water solubility, viscosity, extent and rate of fermentation, end products from their degradation, gut motility which affect the ultimate physiological impact of each ingested carbohydrate. Some factors are inter-dependent and others are independent. The physiological role of each indigestible carbohydrate depends on the site, rate and extent of their fermentation. The most important factors are discussed below;

1.6.1 Water solubility and viscosity of carbohydrates

Water solubility is considered a very important factor for the fermentation characteristics of a carbohydrate. The soluble fibre content may determine the

rate and extent of fermentation (Cherbut et al., 1991). The water solubility of indigestible carbohydrates may result in higher fermentability. Soluble carbohydrates such as raftilose, pectin, lactulose, are more readily fermented than insoluble carbohydrates such as resistant starch, cellulose. Eighty five to 100 percent of pectin and gums are fermented, in comparison to 10 to 50 percent fermentation of cellulose and 30 to 40 percent fermentability of wheat bran which is a rich source of cellulose (Hill, 1995a). Therefore, the water solubility of the indigestible carbohydrates has important implications on metabolic activities in the body.

Many soluble carbohydrates for example pectin, guar gum are viscous. Guar gum is more viscous than pectin. Viscosity is an important determinant in the regulation of gastric emptying and small gut transit time. Fibres, such as guar gum, showed a very strong positive correlation between viscosity and mouth to caecum transit time (Jenkins et al., 1978; Edwards et al., 1987). Another metabolically important aspect of high viscosity is that it may reduce the interaction of nutrients with mucosal receptors which control motility reflexes and the secretion of enzymes and hormones. This anti-motility action may lead to the inhibition of the effects of intestinal contraction. This may then result in malabsorption or slower absorption of carbohydrates and other nutrients because of the reduced mixing of enzymes and substrates, and reduced movement of the nutrients to the epithelial surface (Read & Eastwood, 1992). Different concentrations of guar gum significantly reduced glucose absorption and insulin secretion in growing pigs (Ellis et al., 1995). In this study, an inverse relationship

between the rate of glucose absorption and the viscosity of jejunal digesta was found.

Viscous indigestible carbohydrates, such as guar gum, are considered the most effective in reducing blood glucose after a carbohydrate meal. They act by decreasing the rate of absorption in the small intestine (Jenkins et al., 1977; Blackburn et al., 1984; Leclere et al., 1994; Ellis et al., 1995). Viscous indigestible carbohydrates may also reduce serum lipid and cholesterol levels (Blake et al., 1997), which may be due to the trapping of bile acids and fat molecules in the human small intestine. These effects however, may be fibre specific. For example, guar gum reduced both blood glucose and the serum cholesterol levels (Ellis et al., 1995; Blake et al., 1997). Similarly guar gum had the greater effect on the flow of glucose and bile acid in an *in vitro* model. In contrast, pectin was predicted to be a good agent for lowering serum cholesterol but did not show any effect on glucose movement (Adiotomre et al., 1990).

1.6.2 Extent of fermentation

The fermentibility of a carbohydrate is a prime factor in its physiological action. The extent of *in vitro* fermentation of a carbohydrate can be determined partly by its solubility and its acidic polysaccharide content (Cherbut et al., 1991). Water insoluble carbohydrates, such as cellulose, are mostly resistant to the fermentation, whereas, water soluble pectin and gums are highly fermentable in the gut (Hill, 1995a). A readily fermentable carbohydrate will produce more SCFA than a slowly fermentable carbohydrate and such highly fermentable

carbohydrates have their main effects in the gut via the action of the SCFA produced during colonic fermentation.

Insoluble carbohydrates are generally less fermentable and thus affect gut function by physical influences such as increasing the volume of the colonic contents and stimulating propulsion. Ispaghula was fermented throughout the colon of the rat and it increased the volume of colonic contents, consequently resulting in increased stool weight (Edwards et al., 1992a). In another study ispaghula and wheat bran, both increased the stool output but in different ways; ispaghula increased the volume of the colonic contents, whereas, wheat bran did not show any effect on the colonic contents (Edwards & Eastwood, 1992). Possibly, the increase in colonic contents may be due to large water holding capacity of ispaghula (about 7g water per 1g of ispaghula; Dr. Edwards- personal communication), whereas, slowly fermentable wheat bran affected the stool output by increasing the propulsion of the colonic contents. One fact is very well established as these studies showed that the faecal bulking effect is more prominent when a non starch polysaccharide is resistant to the bacterial fermentation in large gut.

In fact, the stool bulking properties of fibre are complex and the mechanism varies with different individual fibres. In earlier studies, the water holding capacity of a particular carbohydrate was regarded as the most important determinant in the stool bulking. However it is the water holding capacity after fermentation of a carbohydrate that is most related to stool bulking (McBurney et al., 1985). Most of the carbohydrates with the largest water holding capacities

lost most of the water held after fermentation. Even the water holding capacity after fermentation is not considered as the ultimate determinant in the stool bulking. Some other factors may contribute to stool bulking. A diet with low residue is usually associated with slow transit time and small faecal output (Spiller et al., 1980) but some studies reported the stool bulking effect of high amounts of resistant starch (Scheppach et al., 1988a; Phillips et al., 1995). Resistant starch has very little water holding capacity and is reported to be mainly fermented in the colon. One explanation for this is an increase in the bacterial biomass. Increased fermentation in presence of large amount of protein, may result in an increased bacterial biomass in the gut. Since water is the main component of the bacterial cell, an increase in the bacterial mass may result in an increased stool output (Table 1.2).

In summary, the action of indigestible carbohydrates on gut function may be due to increased volume of gut contents, which in turn increases stool output. These functions are influenced by a number of factors such as the water holding capacity of the fibre remaining after fermentation, loss of organic matter, particle size, and stimulation of microbial growth increasing microbial mass.

A carbohydrate with intermediate fermentability may have the best action because it produces a higher concentration of SCFA as well as contributing to stool output (Table 1.2). Generally, such an ideal therapeutic effect is not possible with a single carbohydrate. To develop an ideal carbohydrate supplement, it will be logical to combine various indigestible carbohydrates with

different degrees of solubility, fermentability and residual water capacities, which is the main aim of this study.

Table 1.2 The fermentation of carbohydrates in relation to stool output.

Solubility of carbohydrates	**Extent of fermentation**	**Effect on stool output**
Insoluble carbohydrates	Poor fermentation	i) Most action is due to residual water holding capacity. ii) Stimulation of propulsion, e.g. wheat bran
Soluble carbohydrates	complete fermentation	No effect on stool output, e.g. lactulose
	complete fermentation	Increased biomass contributes to the increased stool output, e.g. cabbage fibre*
	slow or poor fermentation	Some residual water holding capacity contributes to increased stool output, e.g. ispaghula

* Stephen & Cummings, 1980

1.6.3 Rate & site of fermentation

The rate of fermentation of a carbohydrate is very important in determining the fate of resulting SCFA. The rate of fermentation of a carbohydrate is partly determined by its solubility and its content of acidic polysaccharides (Cherbut et al., 1991). A rapid fermentation of carbohydrate may result in the early production and absorption of end products such as SCFA, in the caecum and proximal colon. For example, lactulose is a water soluble and rapidly fermenting

carbohydrate; its fermentation starts in the caecum, producing higher amounts of SCFA, which are readily absorbed in proximal colon. Although lactulose is less effective in stool bulking due to its rapid fermentation and highly fermentable characteristics, large amounts of lactulose, which exceed the fermentation capacity of the gut, may result in the osmotic diarrhoea. In contrast, slowly fermenting carbohydrates, such as ispaghula, may be important resulting in increased volume of gut contents, stool bulking. As ispaghula undergoes fermentation throughout the colon (Edwards et al., 1992a), it may take the effects of fermentation to more distal colonic sites.

The site of fermentation, in conjunction with the rate of fermentation, is another important determinant of the metabolic role of a particular carbohydrate in human health. Fermentation of carbohydrates occurs more in the proximal colon and it declines gradually as colonic contents pass through towards the distal end of the colon. The distal colon is more prone to disease than the proximal colon. Therefore, a lower availability of carbohydrates for fermentation and the production of SCFA in the distal colon may be more important in relation to colonic disease. In this context, a slow rate of fermentation may result in the production and absorption of the SCFA at more distal colonic site. Such production and absorption of SCFA may provide energy over a prolonged period as well as affecting the physiology of colonocytes at more distal sites. On the other hand, an extremely slow fermentation may result in loss of un-degraded fibre and un-salvaged energy in faeces. Therefore, it is important to achieve a balance between the rate of fermentation and the transit time, so the carbohydrate

is fermented and SCFA are absorbed at a distal colonic site. This objective can be achieved by developing a mixture of carbohydrates with higher fermentability and an intermediate rate of fermentation.

1.6.4 Gut transit time

Colonic transit time plays an important role in different human pathologies. The rate of transit through the gut affects a number of gut functions directly including the extent of energy salvage and the absorption of different metabolites from gut contents. For example, a longer transit time gives more chance of fermentation and efficient absorption of SCFA than a short transit time which may cause a loss of unfermented food components and unabsorbed SCFA in faeces. The rate of transit also determines the duration of exposure of the colonic epithelium to different beneficial and harmful metabolites, which may be important in the aetiology of gut diseases.

Ideally, rate of fermentation must not exceed the transit time. There should be an optimised balance between the extent of fermentation, rate of fermentation and the transit time of a particular carbohydrate. For example, lactulose is rapidly fermented, producing higher amounts of SCFA. These SCFA are absorbed rapidly in the proximal colon and very little SCFA may reach the distal colonic sites. Although lactulose has different therapeutic benefits (discussed later), a high dose of lactulose, which exceeds the fermentation capacity of the gut, may result in the osmotic diarrhoea. In this case, there would be a loss of unfermented carbohydrate and unabsorbed SCFA (Wiggins, 1984). Either case is not desirable. It will be beneficial for colonic health to facilitate the production and

absorption of higher amounts of SCFA from fermentation of a rapidly fermenting carbohydrate such as lactulose, at a distal colonic site. Such an ideal situation may be achieved by combining rapidly fermenting lactulose with a slowly fermenting carbohydrate, such as ispaghula. A combination of carbohydrates with such contrasting fermentation properties may regulate the short transit time of the lactulose (Washington et al., 1998) by diluting its osmotic effect in gut and, at the same time, it may facilitate production of higher amounts of SCFA at a more distal colonic site.

Little is known about the mechanisms involved in the regulation of gut transit time. There may be a number of extrinsic and intrinsic factors. Physicochemical characteristics of fibres, such as particle size, solubility and water binding capacity, may be important in this regard but the exact mechanism is not identified yet. Intestinal transit time mainly depends on the type of diet ingested as a study reported an association of low residual diet with slow gut transit (Spiller et al., 1980). However, another study noted the importance of higher amount of water held after fermentation of a fibre, in relation to transit acceleration (McBurney et al., 1985). Different studies noted rapid gut transit and higher faecal output with wheat bran or ispaghula or a mixed high fibre diet (Cummings et al., 1976; Eastwood et al., 1978; Spiller et al., 1980). Other studies reported an inverse relationship between transit time and the faecal weight (Stephen et al., 1987; Ourfir et al., 1996) but it is not clear which of the two, is an independent factor with a primary role. This relationship is not always strict, as another study reported the possibility of an independent influence of feeding

viscous polysaccharides on these indices (Tomlin & Read, 1988). These authors noted a significant correlation between the stool frequency and transit time, but not faecal output.

Although fibres may exert mechanical influence in regulating transit through the gut, mechanical action cannot be the only mode of action, rather it seemed that there may be some other factors involved in this event. Possibly, colonic fermentation may be involved in regulating the intestinal transit via the production of SCFA and different colonic gases (Cherbut et al., 1991; Ourfir et al., 1996). Gases produced during colonic fermentation, may cause propulsion of gut contents by distension of the bowel. Different studies, although contradictory, implicated the SCFA in this regard. For example, an *in vitro* study reported that propionate and n-butyrate stimulated the contraction of muscle strips from rat colon (Yajima, 1985), whereas, another *in vitro* study reported an inhibition of colonic contraction by SCFA in rats (Squires et al., 1992). In the first of these studies, strips of colonic wall were used, which have lost the enteric neural connections and therefore, their response to the stimuli may be affected by this disruption of normal neural processes. There is much published information in this regard but it is beyond the scope of this thesis.

Still, another possible explanation is the action of bile acids or fatty acids which are adsorbed onto the fibres. When fibres are fermented in large intestine, these adsorbed acids are released and transformed into metabolites, such as deoxycholic acid, hydroxy fatty acid. Such metabolites have laxative properties and these may be involved in enhancing the transit through gut, as stimulation of

colonic propulsion by bile acids was noted in a study (Kirwan et al., 1975). Similarly large rectal contractions were reported in humans, after infusion of low concentration of deoxycholic acid into rectum of healthy individuals (Edwards et al., 1989). In this regard, a recent study is very important, summarising all the speculated factors, and establishing a relation between different fermentation events and transit time in gut (Ourfir et al., 1996). These authors showed an inverse relationship of whole gut transit time to production of SCFA, sulphate reducing bacteria, H_2 excretion and pH of the colonic contents.

1.7 Main factors affecting amount and profile of SCFA

The ultimate physiological role of indigestible carbohydrates depends mainly on the amount and type of the SCFA produced during the gut fermentation. The profile of SCFA depends on the type of substrate, bacterial species and the gut environment.

1.7.1 Gut microbial composition

There is growing evidence that intestinal bacteria play a vital role in both health and disease in human, affecting the bowel under normal circumstances as well as these link to specific digestive disorders. The microbial composition is one of the most important factors in determining the amounts and types of SCFA during fermentation in gut. Therefore, it is important to understand the association of gut bacteria with their host from the perspective of their beneficial effects and their role in maintaining the homeostasis, principally through fermentation. Bacteria use the unabsorbed food residues for their own metabolism and in the process new compounds are formed, such as SCFA, sulphides, secondary bile

acids, etc. Weaver et al., (1992) reported considerable variations of fermentation rate and production of gases between faecal cultures from different subjects emphasising the importance of the flora as the determinant of the end product of the fermentation of a substrate.

The human colon contains a diverse and complex microbial population of about 10^{11} to 10^{12} bacteria per gram of dry faeces. The majority of this microbial population consists of anaerobic bacteria which outnumber the aerobic bacteria by 1000 to 1 (Gustafsson, 1982) and this microbial community is comprised of approximately 400 to 500 species (Finegold et al., 1983). The bacterial profile of the gut depends on the internal environment of the gut which, in turn, depends on the intrinsic and the extrinsic sources of the substrate. The complexity and diversity of the gut microbial community in the human is due to the diversity in carbon and energy sources, available to the gut bacteria. The huge microbial population requires daily about 70 g of a substrate for their maintenance (Cummings, 1981). About 4.1g (3.2 - 5.7g) resistant starch (Asp et al., 1996) and 10 to 20g/day non-starch polysaccharides are provided from the food everyday (OPCS, 1990). Intrinsic sources like mucus, dead mucosal and bacterial cells also contribute to the substrate for microbial metabolism.

The majority of the microbial population in the human gut, is saccharolytic using carbohydrates as substrate for their metabolic activity and the principal bacteria, that degrade the polysaccharides, are probably gram negative anaerobes from the genus bacteroides (Salyers & Leedle, 1983). These are able to grow on a variety of polysaccharides because of their ability to synthesise a wide range of the cell-

associated polysaccharide degrading enzymes such as glycosidases, polysaccharides depolymerases (Macfarlane et al., 1990). Since high molecular weight carbohydrates cannot be absorbed and metabolised intracellular within the bacterial cell, extracellular enzymes degrade such large polymers to their constituent hexoses and pentoses including mainly glucose, galactose, arabinose, xylose and uronic acids. These small molecular weight products are then metabolised intracellularly by the gut bacteria (Tomlin et al., 1986; Englyst et al., 1987b). The extracellular and intracellular activity of these cell-associated enzymes means that the degradation of high molecular weight polymers is the part of a synergistic activity with enzymes from many different species. Only a small number (29 out of 286 strains) of anaerobic colonic bacteria were reported to ferment guar gum in isolation (Salyers et al., 1977 a, b). It is probable that only these strains can produce extracellular enzymes. Production of such enzymes is often induced by the presence of their substrate (Salyers & Leedle, 1983; Daly et al., 1993). In another *in vitro* study, 28 % of the Bacteroides strains were able to degrade guar gum (Tomlin et al., 1988). Pre-digestion by extracellular enzymes can make guar gum available to 33 percent more strains for fermentation (Tomlin et al., 1988).

Simple sugars, generated by extracellular enzymatic activity, which after entering the bacterial cell are converted to glucose or glucose-1-phosphate which enter glycolysis yielding pyruvate. Pyruvate is the main intermediate through which all carbohydrates pass during their fermentation to synthesise SCFA, colonic gases and other organic metabolites. Pyruvate is further metabolised through various

routes depending on the bacterial species present in the gut. It may be converted to acetate via formation of acetyl CoA or may produce succinate or lactate. Succinate is converted to methylmalonyl-CoA, an intermediate precursor of the propionate while lactate is reduced to propionyl-CoA, a precursor of the propionate, prior to the formation of propionate. The most common pathway for the formation of butyrate is reversal of β-oxidation.

Some bacteria degrade proteins, peptides and amino acids, producing branched SCFA such as iso-butyrate, iso-valerate and n-valerate (Macfarlane & Alison, 1986) from the de-amination of branched chain amino acids. Protein degradation is greatest in the left colon as evident from the higher ratio of the branched chain fatty acids in distal colon (Cummings et al., 1987). Methanogens and some other bacteria grow on the intermediate products of fermentation, such as hydrogen, lactate, succinate and ethanol and there is a competition between methanogens and sulphate reducing bacteria for H_2 (Gibson et al., 1990). The faecal flora in humans faeces is thought to be of four types dominated by one of these genera, such as bacteroides, or bifidobacterium, or eubacteria or a mixed flora from all genera (Gustafsson, 1982). Most of the colonic bacterial species belong to important genera, such as bacteroides, fusobacterium, bifidobacterium, eubacterium and gram-positive cocci. Numerically less predominant species are non-spore forming anaerobes from the genera bacteroides, eubacterium and bifidobacterium. Other quantitatively less important groups include lactobacilli, clostridium, enterobacteria and gram-positive cocci (Finegold et al., 1983).

Considering the metabolic role of bacteria in human colon, these microorganisms may be grouped in two broad categories of beneficial or pathogenic bacteria. For example, bifidobacterium and lactobacilli are considered beneficial to the human health, whereas, many enterobacteria and clostridia can be undesirable bacteria in respect to human health. Beneficial bacteria are considered to create conditions unfavourable for the growth of potentially pathogenic species (Edwards et al., 1985). Their health benefits may be due to their stimulatory effect on immune function, vitamin B synthesis, restoration of a normal flora after antibiotic therapy, and prevention of growth of pathogenic species in the human gut (Cummings & Englyst, 1995). Bifidobacteria are the major flora in breast fed babies, representing up to 95 % of total gut bacteria. They make up only 25 percent in the adult. This could be one mechanism whereby breast fed infants are protected against gut infections (Cummings & Englyst, 1995).

Recently the concept of the 'probiotic', was used for beneficial bacteria such as bifidobacteria, lactobacilli (Gibson & Fuller, 1998), and the term 'prebiotic', was used for the indigestible carbohydrates that stimulated the growth and/or activity of one or a limited number of beneficial bacteria in the human colon, thus improving the human health (Fuller, 1989; Gibson & Roberfroid, 1995). Due to beneficial effects in the human gut, addition of these probiotics to yoghurt is a common practice in many countries.

Different SCFA profile are characteristic of different microbial species, often used for species identification. For example, nbutyrate is produced by clostridia, eubacteria, fusobacteria and peptococci while propionate is produced by

bacteroides, clostridia, propionibacteria and veillonella (Macfarlane & Gibson, 1995). The bacterial species that are known to produce butyrate are not the most numerous species in the colon (Rao, 1995). Microorganisms may affect the SCFA profile in different ways. For example, there is competition for H_2 between methanogens and sulphate reducing bacteria. H_2 may be absorbed by the gut tissues and excreted in breath, or re-metabolised by the gut bacteria in methanogenesis or sulphate reduction forming H_2S, combined with CO_2 during acetogenesis producing acetate (Gibson et al., 1990; Lajoie et al., 1988). Higher consumption of CO_2 by methanogenesis may, in turn, change the acetate to propionate ratio. Similarly, the profile of different SCFA may be related to different metabolic pathways. Propionate formation involves the production of succinate as an intermediate whereas both acetate and n-butyrate directly result from pyruvate via acetyl CoA. Therefore such preferential use of metabolic routes, influences the profiles of end products. It is evident that the SCFA profile in the human gut depends on the gut microbial composition while this composition, in turn, depends on type of the substrate reaching the large intestine.

1.7.2 Substrate

The substrate is one of the prime determinants of the amount and profile of SCFA production from bacterial fermentation in the gut. The most important aspect of substrate availability for fermentation in colon is its unavailability to the digestive enzymes in the small intestine. Substrates which escape digestion in upper gut, reach the large intestine where they are degraded by the gut bacteria. Dietary factors, particularly fibre, affect the total number of bacteria in the colon and thus have an important effect on the bowel habit. The amount of material escaping

digestion in the small intestine varies and there is a direct relation between the amount of this substrate and the bacterial activity in the large intestine. Some indigestible carbohydrates are ingested as part of daily food and may include oligosaccharides, non starch polysaccharide and resistant starch. Others such as lactulose and ispaghula are ingested as part of special diet for gut therapy.

Of the many possible factors, physicochemical structure and amount of substrate are the most important in relation to production of different SCFA and other metabolites. Such factors are the easiest to manipulate to be of benefit in human health. For example, manipulation of the diet can reduce the amount of gas produced by bacterial fermentation, relieving a distressing symptom in IBS. The chemical nature and physical arrangement of sugars in the fibre matrix may control the rate and extent of fermentation, together with the SCFA produced by fermentation of these sugars (Salvador et al., 1993). However, other factors may also affect the end products of a fermentation reaction (sections 1.7.1 & 1.7.3). A number of *in vitro* and *in vivo* studies showed that the intermediate products and end products of fermentation have some relationship with the chemical and physicochemical characteristics of the fibres (Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993). For example, starch fermentation produced 29% n-butyrate compared with the 2 % n-butyrate by the fermentation of a more oxidised substrate such as pectin. Similarly, fermentation of pectin produced more acetate compared with the fermentation of starch (Englyst et al., 1987b; Scheppach et al., 1988b). Starch is a polymer of glucose, whereas, the main component of pectin is D-galacturonic acid, with L-rhamnose, L-arabinose and

D-galactose as the part of the structure. Some of the galacturonic acid units are esterified. The acidic polysaccharide content of the fibre was also important. When equal quantities of soluble fibre were compared, the richer the fibres were in acidic polysaccharides the more they were fermented (Cherbut et al., 1991). Fibres richest in pectic substances and uronic acids produced a higher ratio of acetate in most *in vitro* studies (McBurney & Thompson, 1987; Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993).

The ratios of SCFA produced by fermentation, are very important in the metabolic impact of a particular carbohydrate. Different indigestible carbohydrates have different SCFA profiles. Acetate is common to all the carbohydrates but a carbohydrate may be either propionate or n-butyrate predominant (Edwards & Rowland, 1992). Fermentation of ispaghula produced more propionate than n-butyrate, whereas, fermentation of resistant starch yielded more n-butyrate than propionate. Similarly, fermentation of resistant starch produced a higher ratio of n-butyrate than fermentation of pectin which produced a higher ratio of acetate (Englyst et al., 1987b). Other *in vitro* studies reported a higher proportion of propionate from the fermentation of guar gum and pectin (Adiotomre et al., 1990; Englyst et al., 1987b), whereas oat bran and wheat bran produced more butyrate proportionally (Adiotomre et al., 1990; McBurney & Thompson, 1987). In a vivo study, the propionate content of faeces was much higher in rats fed ispaghula compared to those fed a low fibre or wheat bran diet (Edwards & Eastwood, 1992).

As discussed previously, colonic bacteria act in synergy. The break down of higher molecular weight polysaccharides by some bacteria is necessary for the initiation of a fermentation reaction by other bacteria. Thus, the relative rate of de-polymerisation of complex carbohydrates, may influence the profile of fermentation end products. Enzymes are often induced and synthesised in accordance with the saccharides available, enabling the bacteria to switch from one substrate to another as the diet changes (Salyers & Leedle, 1983; Daly et al., 1993). When an indigestible carbohydrates is not a usual part of the normal diet, the extracellular enzyme necessary for its degradation may not be present in the gut and may need to be induced. Studies in man have shown that feeding a new fibre source for 1 week can demonstrate induction of such enzymes (Daly et al., 1993).

Oligosaccharides which are prebiotics (section 1.2.4.2 for definition), are different in their physical and other characteristics from resistant starch and non starch polysaccharides. Prebiotics produce a favourable environment for the friendly bacteria in the human colon. These indigestible carbohydrates also have therapeutic importance due to their specific SCFA profiles. Therefore, addition of prebiotics to the diet may cause proliferation of particular types of beneficial bacteria and may suppress the harmful bacteria which may become an important preventive or therapeutic measure.

1.7.3 Host related factors

Host related factors may also be important in the metabolic role of the carbohydrates and these factors can affect the end product profile of a particular

carbohydrate fermentation. For example, the age and gender of a host can affect the bacterial activity in the gut. Similarly, the host's dietary habits may also be important in determining the metabolic effect of a carbohydrate. Different dietary habits can influence the environment of gut producing different types of bacterial population and, in turn, different end product profiles. Different diets may influence the metabolic activity through a number of factors. For example, enzymes are often induced and synthesised in accordance with the saccharides available, thus enabling the bacteria to switch from one substrate to another as the diet changes (Salyers & Leedle, 1983). Higher amounts of resistant starch reduced the faecal pH, and produced higher faecal n-butyrate in human subjects (Phillips et al., 1995). As starch is a normal part of the diet especially in the Asian countries, it may influence fermentation events in human colon depending on the dietary habits of the individual. Similarly, pectin and cellulose are part of normal human diet and their amounts may vary, depending on the individual dietary habits.

The anatomy and physiology of the gut may influence the ultimate products of the fermentation by influencing the bacterial activity in the gut. Vegetarians, in general, have longer colons than omnivore human beings (Hill, 1995b). Similarly different hosts may have different transit time due to genetic reasons or individual dietary habits. A diet with low residue is associated with slow transit and low stool output (Spiller et al., 1980). A longer transit time will give more chance to bacteria for the degradation of a fibre. Whole gut transit time ranges from 20-140 hours with a mean of 60 hours in normal individuals in the UK (Cummings &

Macfarlane, 1991). An inverse relationship has been noted between whole gut transit time and sulphate reducing bacteria and H_2 excretion (Ourfir et al., 1996). Similarly, methane producing subjects had slower gut transit than non methane producing subjects (Edwards, 1997). Whereas, the production of methane and H_2S may depend on the amount of sulphate in colonic contents (Gibson et al., 1988). Therefore, intake of drugs or diet with high sulphate content or sulphur containing protein, can increase the growth of sulphate reducing bacteria with a simultaneous decrease in methane production, which will affect metabolic profiles, as discussed previously. All these studies, showed that transit of the digesta through the large gut strongly influenced the bacterial activity in the gut, and thus the end products of their metabolism. As stated above, drugs may influence the activity or growth of the gut bacteria. Antibiotics can suppress the activity of all or selective bacteria in the gut (Rao, 1995) and, therefore, administration of certain drugs can influence the SCFA profile by increasing the growth of certain bacteria and at the same time inhibiting the growth of the others. The gut bacterial populations and their metabolites may also depend on the immune system and antibiotic resistance of the particular host.

Other host related genetic factors have physiological implications in this regard. Some individuals are incapable of digesting simple carbohydrates. Such digestible carbohydrates reach the large gut where they may be fermented resulting in a different SCFA profile. Lactose intolerance is a good example in this regard (Caspary, 1986). In the case of increased amount in the large

intestine, lactose may lead to the osmotic diarrhoea, which may affect the SCFA formation.

1.8 Absorption of SCFA

The end products of fermentation reactions in gut are very important for the man because after their absorption, they affect different metabolic processes. Colonic epithelial cells are exposed to very high concentrations of total SCFA and other organic anions, up to 175 mmol/L in human. SCFA are rapidly absorbed and transferred from the gut lumen to blood at a very high rate through gut epithelial cells, faciliating the absorption of water and sodium (Ruppin et al., 1980), thus preventing an accumulation of SCFA. Acetate and propionate, after their absorption from gut, enter the portal blood system and are transported to the liver for further metabolism. n-Butyrate is a preferred fuel for human colonocytes (Roediger, 1980; Roediger, 1982), and is used mainly by the colonic mucosa.

In normal conditions, 90 to 95 percent of the SCFA from colonic fermentation, are absorbed during transit through the human gut (Cummings, 1981; Hill, 1995b) and as little as 5 to 10 percent of SCFA are excreted in faeces. When the production rate of SCFA exceeds the absorption rate of SCFA, total solute levels in faecal water, are increased. Rapid SCFA absorption is accompanied by the stimulation of sodium and water absorption from lumen, and in exchange there is an excessive accumulation of bicarbonate ions (Ruppin et al., 1980; Cummings, 1981; Engelhardt, 1995; Omaida et al., 1996). Transport of SCFA results in increased pH, creating a more alkaline environment in gut lumen. The absorption of SCFA facilitates absorption of excess water from the gut by acting as a

powerful driving force for water movement and such absorption may prevent the occurrence of osmotic diarrhoea.

Although precise mechanisms of SCFA absorption are not known in man with certainty, it is concentration dependent. It seems that absorption of the SCFA is most likely by a passive diffusion in the large intestine with considerable segmental differences in the permeability of the intestinal mucosa. Passive diffusion is a major route for the absorption of SCFA in the protonated form across the colonocyte membrane and most of the total SCFA absorption is reported through such passive diffusion (Ruppin et al., 1980; Engelhardt, 1995). These authors reported consistent results suggesting the existence of two mechanisms for SCFA absorption from the gut lumen. 1) non-ionic diffusion of protonated SCFA involving consumption of the CO_2; and, 2) cellular uptake by ionic diffusion of the Na or K salt of the SCFA.

Most studies are carried out with faecal parameters in order to depict the events in colonic fermentation because of the physical inaccessibility of the intact human gut. In view of the high percentage of SCFA absorption from the colonic lumen, interpretation of events in the large intestine on the basis of faecal SCFA is not an ideal approach. Adding normal amounts of indigestible carbohydrates to the diet may not produce any significant changes in the faecal SCFA, therefore, this approach will not present a true picture of intra-lumenal events. However, it is the easiest and least expensive mean of understanding events in the intact gut, especially in human.

1.9 Effects of indigestible carbohydrates in the human

The effects of different indigestible carbohydrates in the human body made it possible to some extent, to group the action of isolated indigestible carbohydrates or concentrated sources of these carbohydrates arbitrarily into two overlapping categories; First, their actions on gut function before bacterial degradation; and secondly, the physiological implications after the absorption of metabolites resulting from their bacterial fermentation.

1.9.1 Pre-absorption effects

The pre-absorption effects mainly include the physical influences of the indigestible carbohydrates in the gut either by their physical action on the gut function or by creating a specific physicochemical environment. However, the gut environment and the physical influences are interdependent (Table 1.4). These effects include changes in viscosity and bulking of gut contents, and pH changes in the gut (Table 1.3).

Absorption in the upper gut may also be affected mainly by the physical presence of soluble and insoluble non starch polysaccharides. Some water soluble carbohydrates delay gastric emptying and slow down the transit of digesta by increasing the viscosity of gut contents. This may affect the rate of absorption of glucose or other metabolites in upper gut (Blackburn et al., 1984). Therefore, the action of fibre on absorption in the small intestine must also be considered. Changes in viscosity or the volume of gut contents may affect intra-lumenal mixing which may be critical for enzyme-substrate interaction and the movement

of nutrients from the bulk phase to the absorptive gut epithelium (Edwards et al., 1988). A large volume of colonic contents may dilute carcinogenic and other

Table 1.3 Actions of non starch polysaccharides *in vivo*

Soluble Polysaccharides	Insoluble Polysaccharides
stomach & small intestine	
Delay in gastric emptying[1]	Accelerated intestinal transit[8]
Increased Satiety	
Lowering of plasma glucose[3]	
Reduced plasma lipids and cholesterol[4, 5, 6]	
Probable osmotic action with higher amounts[7]	
Large intestine	
Higher fermentability[2, 3]	Less fermentability[2, 3]
Enhanced bacterial growth[12]	Higher stool frequency[13]
Higher concentration of SCFA[12]	Higher stool output[13]
and volume of colonic gases[12]	Softer and wetter stool texture[13]
	Ease of stool passage and reduced risk
	of constipation[13]

1. Edwards et al., 1987; 2. Hill, 1995a; 3. Cherbut et al., 1991; 4. Topping, 1991; 5. Eastwood et al., 1986; 6. Anderson et al., 1991; 7. Wiggins, 1984; 8. Jenkins et al., 1978; 9. Jenkins et al., 1977; 10. Blackburn et al., 1984; 11. Leclere et al., 1994; 12. Edwards & Parrette, 1996; 13. Edwards et al., 1992.

harmful compounds such as bile acids and NH_3. Increased intake of insoluble dietary fibre may increase stool output and also increase the transit of digesta through gut. Ispaghula is an exception to this, as despite being a soluble carbohydrate, it increases the volume of gut contents and consequently, increases the gut motility and accelerates the passage of digesta through the gut. Insoluble and unfermented non starch polysaccharides increase stool bulk and make faeces softer and easier to pass.

Higher production of short chain fatty acids by carbohydrate fermentation, lowers the pH of lumenal environment. Different studies have reported that such low pH plays an important role in regulating the bacterial activity in the gut as well affecting the physical action of the gut. Low pH may also change the gut bacterial population, and the ionisation and absorption of metabolites (Wang & Gibson, 1993). Although certain dietary fibres hinder mineral absorption in the small intestine, increased fermentation of indigestible carbohydrates in gut, may increase mineral absorption in the large intestine (section 1.5.4 for detailed effects of pH). Higher amounts of short chain fatty acids produced during fermentation, may also increase the mucosal surface area of the proximal colon, increasing the absorption capacity at this site (Sakata, 1987).

1.9.2 Post-absorption effects

The post-absorption effects mainly include the physiological actions of the end products of bacterial reactions which are absorbed in the colon (Table 1.4). The major end products of carbohydrates break down, absorbed from the gut are

monosaccharides, especially glucose, from the small intestine and, then short chain fatty acids from the large intestine.

SCFA absorbed from the gut lumen are metabolised by the tissues of the human body. The post absorption physiological role of these SCFA is still not well understood. They have been implicated in different health related aspects such as irritable bowel syndrome (Heaton, 1992), colorectal carcinogenesis (Kruh, 1982), glucose tolerance (Anderson & Bridges, 1982; Venter et al., 1990), cholesterol metabolism (Chen et al., 1984; Venter et al., 1990; Wolver, 1995; Berggren et al., 1996), and energy metabolism (Rémésy et al., 1992; Molis et al., 1996), and in treatment of ulcerative colitis (Breuer et al., 1991). Propionate may affect carbohydrate and cholesterol metabolism, whereas, n-butyrate provides energy for colonocytes and may affect different cellular activities in the colonic epithelium, these effects will be discussed in detail in the next section (section 1.10).

Table 1.4 Possible actions of glucose and SCFA in the human body.

Glucose	SCFA
Energy source for body tissues Increases insulin production Glycogenesis Exclusive source of energy for brain, nervous system and some other tissues	Energy source for colonocytes and other tissues. Promote water and mineral absorption in the colon. Stimulation of bacterial growth in the colon. Protection against colonic cancer.

1.10 Two important physiological effects related to indigestible carbohydrates

The indigestible carbohydrates are believed to affect two major metabolic activities; lipids and carbohydrate metabolism, either via their actions in the gut or at the physiological level through metabolism of their end products. Knowledge about effects of dietary fibre on lipid and carbohydrate metabolism was mostly derived from the studies with single polysaccharides (Truswell & Beynen, 1992) and these isolated polysaccharides were used in larger amounts than that present in normal diets.

1.10.1 Lipid Metabolism

Soluble indigestible carbohydrates may lower blood cholesterol by different mechanisms such as altered bile acids metabolism and reduced rate of nutrient absorption by increased viscosity of gut contents and by inhibition of lipogenesis by SCFA. It is sometimes suggested that high fibre diets tend to be high in starch and low in fat, and that the reduction of LDL and triglycerides is due to the low fat content of a high fibre diet (Swain et al., 1990). However, there is a definite role of certain dietary fibres in reducing serum lipids, as not all non-starch polysaccharides have an effect. Different studies reported a reduction of 10-20 % in serum cholesterol after ingestion of different soluble fibres such as oat bran, beans, guar gum and pectin (Eastwood et al., 1986; Anderson et al., 1991). This would be sufficient to reduce the associated risk of cardiovascular disease. Oat bran significantly reduced serum total and LDL cholesterol (Kestin et al., 1990; Anderson et al., 1991; Keenan et al., 1991; Cara et al., 1992).

Although the exact mechanism of lipid lowering effect by soluble fibres is unknown, several possible mechanisms are suggested by different studies (Anderson, 1995). Fibres from different food sources have different modes of action. For example, oat bran increased total faecal sterol but reduced chenodeoxycholic acid in faeces, while beans showed completely the opposite effect on faecal bile acid contents. At the same time both sources of dietary fibre had a similar reducing effect on serum cholesterol (Anderson et al., 1984). The most widely believed explanation for this, is the binding or chelating of dietary cholesterol and bile acids by the fibres. This chelation of ions hinders the absorption or re-absorption of the dietary cholesterol and related substances in small intestine. Pectin, being a soluble fibre, binds bile acids to the charged sites in their structure resulting in increased faecal loss of these acids. This loss in the enterohepatic pool of bile acids has to be replenished by increased the de novo synthesis of bile acids from the cholesterol, thus decreasing cholesterol levels in the body. However, the alteration in bile metabolism and influence on the rate of absorption, may depend on the level of mixing of dietary fibre with the meal ingested. Wolver et al., (1994) showed a significant reduction in the blood cholesterol after feeding ispaghula as part of a meal but no effect from the same ispaghula eaten between meals. Similarly not all fibres show the same properties, however, soluble fibres such as oat bran, guar gum and pectin are more potent and effective in lowering of the plasma cholesterol in the human (Topping, 1991).

Soluble fibres are usually viscous and most are completely fermented by gut bacteria. Such fibres may be more effective in lowering serum cholesterol levels

by slowing absorption of lipids and other nutrients due to increased the viscosity of contents in the upper gut (Edwards et al., 1988). The products of their fermentation may also have an effect on the cholesterol level (section 1.10.1.1). However, Ahrens et al., (1986) reported a 50% reduction in serum cholesterol of mini pigs after feeding pectin orally but no effect when the same amount of the pectin was instilled directly into the caecum, suggesting no role of fermentation in the lipid lowering effect.

However, Fernandez showed that guar gum significantly altered endogenous hepatic cholesterol metabolism and cholesterol homeostasis by reducing the plasma LDL concentrations but cholesterol absorption was not affected or decreased (Fernandez et al., 1995). This suggests that multiple mechanisms are involved in down regulating hepatic and serum cholesterol. One mechanism may be by increasing the binding of bile acids as reported by other investigators (see above). These findings emphasise the importance of defining the physiological changes that are responsible for the onset of specific metabolic responses to different soluble fibres.

1.10.1.1 Propionate production

Propionate production from fermentation of carbohydrates in the colon may be another mechanism which could be involved in reducing serum and hepatic lipid level. Rectal infusions of propionate interacted with acetate partly blocking the rise in serum cholesterol noted when acetate was infused alone rectally (Wolever et al., 1991). In this way, the ratio of acetate to propionate may be very important in the overall physiological effect of carbohydrate fermentation in the colon

(Wolver, 1991). This author suggested that acetate and propionate, after reaching the liver, influence the metabolism of carbohydrates and lipid, probably in an opposing way. Acetate is a precursor of acetyl CoA resulting in lipogenesis, whereas, propionate hinders further metabolism of acetate. Thus propionate may inhibit lipogenesis, resulting in reduced hepatic cholesterol and other related metabolites.

Moreover, soluble fibers, such as ispaghula, guar gum, which reduce cholesterol synthesis are normally propionate predominant when fermented. Chen et al (1984) demonstrated a hypocholesterolaemic effect of dietary propionate. This suggested that SCFA, especially propionate, may exert the inhibitory effect on hepatic cholesterol synthesis seen with soluble fibres. However, the effects of propionate given orally may not be the same as the effects of propionate from colonic fermentation (refer to section 1.5.1.1.2). Propionate increased the synthesis and secretion of bile acid in isolated rat hepatocytes (Imaizumi et al., 1992) *in vitro* suggesting that altered bile acid metabolism by the action of propionate may be responsible for cholesterol lowering effect. However, studies with ileostomy models suggested different modes of action of dietary fibre such as reduced cholesterol absorption and an increased excretion of bile acids and cholesterol (Anderson, 1992; Zhang et al., 1992). Similar findings were reported in another study (de Deckere et al., 1995) which found higher total bile acid in caecal contents and in faeces of rats fed diets containing resistant starch (14%) than diets low in resistant starch (1%). As such loss of bile acids must be replenished by synthesis of new bile acids from the body's cholesterol pool, this

may be helpful in reducing the serum cholesterol. Yamashita et al., (1984) reported that fructo-oligosaccharides may also affect lipid metabolism, decreasing triglycerides and cholesterol in diabetic humans.

1.10.2 Carbohydrate Metabolism

Changes in the physical properties of gut contents such as viscosity and volume induced by the physical action of indigestible carbohydrates may be of importance in regulating carbohydrate metabolism and post-prandial responses. There are a number of ways in which indigestible carbohydrates may affect carbohydrate metabolism in the gut. These include affecting amylase activity and starch digestibility in the small intestine, decreasing small intestinal transit time, increasing the viscosity of intestinal contents and hindering enzyme accessibility to substrate. Most of the physiological effects of dietary fibre are attributed to their viscous nature (Edwards et al., 1988) which may play an important role in the gastrointestinal tract. Viscosity may hinder gastric emptying, reducing the glycaemic responses after food (Blackburn et al., 1984). Also increased viscosity in the small intestine may hinder the mobility of enzymes reducing their hydrolytic actions on starch and other food ingredients. Although increased viscosity may explain the effects in small intestine to some extent, too much emphasis on viscosity could be misleading because the physiological effects should be lost if viscosity is reduced due to processing of the food or fermentation in the large intestine. It has also been suggested that the viscosity of fibres, such as β glucan in cereals, may be less important than their structural properties for glycaemic responses. The structural properties may restrict the

availability of starch for the hydrolytic action of enzymes (Granfeldt et al., 1994; Liljeberg et al.,1994). For example, finely ground flour of the same cereal, will produce a greater glycaemic response than coarsely ground flour because starch and other simple carbohydrates in the coarse flour will be less accessible to hydrolytic enzymes.

There may be more than one mechanism for the influence of dietary fibre on carbohydrate metabolism and post-prandial responses. Some fibres may trap the water and nutrients, including water soluble sugars or may dilute the action of hydrolytic enzymes. Due to the high fibre matrix, mobility of absorbable nutrients may be restricted resulting in slower absorption of glucose from the gut and consequently a slower post-prandial response to a meal (Flourié, 1992). This may be very important in diabetes and other pathological conditions in humans.

Another important aspect is the effect of resistant starch which is not absorbed in the human small intestine. Therefore, starchy foods with higher amounts of resistant starch are expected to show a lower glycaemic response compared with starchy foods containing same amount of rapidly digestible starch. Achour et al., (1996) reported a higher post-prandial glucose and insulin response with digestible starch compared with retrograded starch from the same maize source in human subjects.

In summary, the lowering effect of fibre on plasma glucose is related to: a) stimulation of overall glucose utilisation; b) increased hepatic uptake of ingested

glucose, thus reducing the entry of glucose into the systemic circulation; or c) delayed or decreased glucose absorption from the gut (Flourié, 1992).

1.11 Carbohydrate fermentation and colonic diseases

Indigestible carbohydrates are suggested for the treatment of different diseases such as bowl cancer, diverticular diseases, ulcerative colitis, Crohn's disease, diabetes, obesity, CHD and gallstones. A number of studies have shown their importance in different colonic disorders. Recent research shows that interactions between bacteria, growing on indigestible carbohydrates, and the body's immune system, or interactions between chemicals produced by bacteria and the colonic lining, play an important role in long continued inflammations such as ulcerative colitis and Crohn's disease.

1.11.1 Colitis and Colonic Cancer

The large bowl is the second most common site for cancer in the British men and women. Epidemiological studies have shown that the diet plays a major role in the incidence of colorectal cancer. A survey showed a strong negative correlation between colorectal cancer incidence and the starch intake in 12 populations in different parts of the world (Cassidy et al., 1994). Similarly, higher incidence of the colon cancer was reported in populations consuming higher amounts of dietary fat and protein, whereas, the reverse was observed in populations consuming high amounts of dietary fibre and vegetables (Lipkin, 1988; Bingham, 1990). In controlled experimental studies, when animals were exposed to chemical carcinogens, bran was consistently reported to reduce the number of tumours (Klurfeld, 1990), showing a potential protective action of bran against

these chemically initiated cancers. A recent study supported the association of high consumption of refined cereals with an increased risk of cancers of the large bowel, the stomach, and other selected digestive and non-digestive sites (Chatenoud et al., 1999). This may be due to the lack of fibre in diet or may be due simply to association of such refined cereals with poor intake of fruits and vegetables. These contain anti-oxidant which are considered to play an important role in the prevention of colonic cancer (World Cancer Research Fund, 1997).

Although a low incidence of large bowel cancer is a general trend for populations with high fibre intakes, this negative relationship is very sharply reduced when the relationship is corrected for fat and meat intake (Webb, 1995). Vegetarian groups, such as 7th day Adventists in the USA, have lower incidence of bowel cancer compared to the other Americans. Vegetarians, however, consume a diet high in antioxidants but with no animal fat and meat. The incidence of bowl cancer is most strongly and positively correlated with meat and fat intake. For example, in Japanese populations incidence of bowl cancer had increased two fold with new dietary trends of consuming more meat, animal fat, dairy products instead of their traditional diet with high consumption of rice, over past few decades. Although there is no obvious change in fibre intake, there has been a substantial change in the calories derived from starch (Webb, 1995).

The hypothesis discussed above sounds plausible but the role of dietary fibre is not exclusively due to the displacement of fat contents. A more specific role cannot be ruled out, because there are other studies, correlating metabolites from fermentation of dietary fibres with human colonic health. In this regard, n-

butyrate has been studied extensively for its effect on the colonic epithelium at the cellular level (Kruh, 1982; Sakata, 1983; Smith, 1986; Scheppach et al., 1992; refer to section 1.5.1.1.3 for details). It has been noted that less availability or utilisation of SCFA, especially n-butyrate, may lead to colitis, ultimately resulting in colonic carcinogenesis. The majority of colon tumours are found in distal colon, and n-butyrate enemas were found positively effective in treatment of distal ulcerative colitis (Scheppach et al., 1992). Therefore, dietary fibre is likely to play a role in the prevention of large bowl cancer. Fibre might protect against the bowl cancer through a number of possible mechanisms;

1) Insoluble fibres may be protective through bulking of intestinal contents, increasing stool output and, in acute cases, causing a laxative effect (Burkitt, 1971; Stephen & Cummings, 1980). Such a bulking effect may result in the dilution of potential carcinogens and tumour promoters. Fibre may speed up intestinal transit and the elimination of carcinogens from the gut, reducing the exposure time of colonic mucosa to such carcinogens. Therefore, insoluble carbohydrate sources may be of help in reducing the risk of colonic cancer. Cummings et al., (1992) supported this hypothesis by reporting a significant inverse relation between stool weight and incidence of colon cancer in two cities of UK.
2) Fermentable fibres may lower colonic pH. A low pH has been implicated in a lower risk of colorectal cancer (Walker et al., 1986; Newmark & Lupton, 1990), which may act through control of different metabolic pathways. This was supported by the findings that a higher faecal pH was noted in patients with colorectal cancer than healthy human subjects (Vernia et al., 1989).

Increased fermentation of dietary fibre results in a lowered pH by producing different organic acids. This fall in colonic pH may be helpful in the prevention or reduced synthesis of carcinogens such as NH_3, secondary bile acids (Thornton, 1981; Bruce, 1987; Nagengast et al., 1988). These secondary bile acids, deoxycholic acid and lithocholic acid, were found to be tumour promoters in rats (Narisawa et al., 1974; Summerton et al., 1985). Secondary bile acids are derived from primary bile acids, cholic acid and chenodeoxycholic acid, by 7α dehydroxylation. The enzyme, 7α dehydroxlase, required for this conversion, is synthesised by obligate anaerobic bacteria and has an optimum pH 7-8 (MacDonald et al., 1978). Therefore, low pH resulting from increased colonic fermentation, may inhibit dehydroxylation of primary into secondary bile acids (van Munster et al., 1994; van Munster et al., 1995) which are potential carcinogens (section 1.5.4 for detail). Another hypothesis suggests that faecal pH becomes alkaline in presence of diets that are high in fat and protein, low in fibre, resistant starch and perhaps certain minerals. In this situation acidic lipids become more soluble and toxic to the colonic epithelium. This leads to increased epithelial proliferation and increased sensitivity to carcinogens (Bruce, 1987).

3) **Butyrate production:** A number of studies implicated the lower production of SCFA, especially n-butyrate, in the increased incidence of colorectal cancer in animals and human (Candido et al., 1978; Kruh, 1982; Sakata, 1983; Smith, 1986; Weaver et al., 1988; Clausen et al., 1991; Scheppach et al., 1992; McIntyre et al., 1993; Hague et al., 1993; Hague & Paraskeva, 1995). Therefore, it is thought that higher fermentation of indigestible carbohydrates,

and higher production of SCFA in colon may be beneficial in human colonic health. Since, the majority of human colonic tumours occur in the distal colon in humans (Eastwood, 1987), the incidence of colorectal cancer could be controlled by increased production of SCFA, especially n-butyrate, in more distal colonic sites. Increased production of SCFA could be achieved by increasing the bacterial fermentation of carbohydrates in more distal sites of the colon.

The aim of the study in this thesis is to evaluate *in vitro* fermentation of individual carbohydrates and their mixtures, and to predict their effects on gut function. The study will focus on their rate of fermentation to determine their expected site of *in vivo* fermentation. With the help of this *in vitro* study, I will be able to relate the fermentation characteristics of mixtures to their expected end products and the site of their maximum fermentation activity in the gut. This should help predict their action in human colonic health.

1.12 Why use mixtures of carbohydrates at all?

Considering the properties and specific actions of indigestible carbohydrates, it is important to study the fermentation characteristics of some of these carbohydrates in relation to their specific therapeutic effects. Interest in the physiological action of indigestible carbohydrates was initiated in early 70s (Trowell, 1972; 1976; Burkit, 1971). An immediate consideration with realisation of the metabolic importance of indigestible carbohydrates, was to provide these carbohydrates without disturbing the balance of other dietary contents. Many researchers considered supplementation of the isolated carbohydrates as a solution to the

problem. Such isolated polysaccharides like pectin, guar gum and locust bean gum were tested and approved for use in food preparations (Jenkins et al., 1976; Jenkins et al., 1977; Cummings et al., 1978)

The interesting metabolic effects of these indigestible carbohydrates attracted much attention. It is difficult to distinguish between the physical effects of fibre on gut functions and the metabolic effects of SCFA from fermentation *in vivo*. However, it is possible to predict some expected effects of these carbohydrates from *in vitro* studies. Previously different individual indigestible carbohydrates were studied extensively for their *in vitro* fermentation and *in vivo* metabolic implications (Englyst et al., 1987b; Adiotomre et al., 1990; Wang & Gibson, 1993; Younes et al., 1996). Most of the work is done *in vitro*, using these carbohydrates in isolated form, whereas, data from *in vivo* studies is usually lacking information about processing, cooking methods and other related aspects. The apparent clinical usefulness of such carbohydrates in large amounts, as required to be metabolically effective, will be of little value in view of their low organoleptic value, in addition to their adverse gastrointestinal effects. These carbohydrates have interesting therapeutic properties but, as stated above, large amounts of these carbohydrates have undesirable side effects such as bloating, osmotic diarrhoea. Considering the idiosyncratic actions of individual carbohydrates, it is unlikely that any single carbohydrate will provide ideal therapeutic actions without having some undesirable effects. A logical approach would be to combine different indigestible carbohydrates in mixtures of one or more carbohydrates in order to harvest their potential therapeutic benefits and

simultaneously avoid their undesirable side effects. Such mixed carbohydrates supplementation, could be considered ideal for use in major colonic problems. At present very little is known about the interaction of fibres with each other *in vitro* or *in vivo* (Washington, 1998). Therefore, a study is needed to investigate the fermentation and interaction of these carbohydrates in mixtures.

The questions about supplementation with these therapeutic carbohydrates in mixtures are; First, whether these carbohydrates affect the properties of each other when ingested in the mixtures and second, whether such effects are interactive or simply additive.

For example a slowly fermenting carbohydrate may alter the fermentation of a rapidly fermenting carbohydrate and vice versa. It may be beneficial to delay the fermentation of rapidly fermenting carbohydrates, which are generally higher SCFA producers. Similarly carbohydrates of different SCFA profile may affect the ability of each other in their production of a particular SCFA. In this way, higher production of a particular SCFA, especially n-butyrate, at more distal colonic site, may be of great benefit in the colonic diseases discussed above. Therefore, mixtures of indigestible carbohydrates should be between carbohydrates of different fermentability, rate of fermentation and other related characteristics. Since, it is not always possible to feed patients meals containing such supplement, the characteristics of the constituent carbohydrates, such as their solubility, and viscosity should be considered in relation to developing a supplement mixture for tube feeding.

In this study, I will investigate the effect of combining carbohydrates with different fermentative properties and look for their interactive influences, reflected in the end products of their fermentation *in vitro*. The ideal carbohydrate mixture should contain a slowly fermenting carbohydrate, a rapidly fermenting carbohydrate and will be a good source of n-butyrate. In addition to this, such mixture should be suitable for tube feeding.

CHAPTER 2

Methods

General methods, used throughout this thesis, are described in this chapter. Specific methods for each experiment are described in their respective chapter.

2.1 Measurement of fermentation

In vivo fermentation methods allow determination of the extent of fermentation of a dietary fibre, but it is almost impossible to predict the amount of SCFA produced during *in vivo* colonic fermentation in humans because of extensive absorption and metabolism in gut. Direct *in vivo* measurement of the end products from colonic fermentation is possible only when sampling is carried out directly from the colon over a number of short time periods. Such frequent collection of samples of colonic contents from the intact human gut, is not possible due to the inaccessibility of the proximal colon, the major site of the carbohydrate fermentation. The only published data, in this regard, have been obtained from victims of sudden death (Cummings et al., 1987). However, such data provide information only about the colonic environment and the metabolism at the time of death, and very little information about the dynamic metabolic environment of the colon.

2.1.1 *In vivo* methods

2.1.1.1 Human subjects

In humans, the intact gut is not easily accessible and procedures, such as intubation, which are very invasive and very expensive, are necessary. Such studies need strict medical supervision and are not practicable for routine assessment of the fermentability of carbohydrates in a normal research unit. In addition, the use of a tube may interfere with the normal functions of the human gut, and may alter transit time (Read et al., 1983). Speeding up the

transit may interfere with absorption in small intestine. The bowel preparation and washing prior to intubation may also interfere with the normal physiology of the gut (Edwards & Rowland, 1992).

Alternative *in vivo* methods using indirect measurements have been adopted including breath H_2 measurement, blood sampling for measuring acetate and other SCFA (Pomare et al., 1985; Muir et al., 1995; Zavoshy, 1998), and measurement of end products of fermentation in the human faeces (Scheppach et al., 1988b; Phillips et al., 1995). Most of these methods are limited in their interpretation. However, measurement of breath H_2 is the most commonly used method for measuring the colonic fermentation. An increase in breath H_2 is an indication of fermentation activity in the human large intestine but it does not yield any quantitative information about the important metabolites of colonic fermentation. There is controversy about the quantitative interpretation of data from breath H_2 (Florent et al., 1985; McBurney & Thompson, 1989; McBurney et al., 1990). A reduced breath H_2 was noted in human subjects after feeding lactulose for eight weeks, despite the presence of increased colonic fermentation (Florent et al., 1985). There are several alternate routes for the disposal of H_2, including acetogenesis and methanogenesis, and the use of H_2 by a terminal oxidiser such as sulphate, forming H_2S (Gibson et al., 1988). These make it more difficult to measure all the H_2 produced during the colonic fermentation and the use of such data as an index of fermentation.

Acetate in peripheral blood is another method for the assessment of the colonic fermentation. As the principal SCFA from colonic fermentation, only acetate reaches the peripheral blood system. Propionate is metabolised by the

liver while butyrate is cleared by the colonic epithelium. (Roediger, 1980; Roediger, 1982; Demigné & Rémésy, 1991). Therefore, measurement of only acetate is possible in the human because of the easy accessibility of peripheral circulatory system. Measurement of SCFA in portal blood systems is only possible at the time of postmortem (Cummings et al.,1987) or during surgery. Since only acetate reaches the peripheral blood system, measurements of peripheral blood do not provide a quantitative measure of all short chain fatty acids produced during colonic fermentation, especially butyrate and propionate. Quantitative measurement of all SCFA production was necessary in our study. In addition to this, the use of frequent blood samples was impracticable in a screening study like ours. Another limitation of this method of measuring acetate in peripheral system, is the endogenous synthesis of acetate by the human tissues which is indistinguishable from bacterial synthesis in the human gut (Pethick et al, 1981; Ballard, 1972).

In view of these difficulties, some studies have used measurement of metabolites in faeces as an index of *in vivo* fermentation products with different carbohydrates (Scheppach et al., 1988b). Measurement of faecal SCFA, produced by carbohydrate fermentation, is not very helpful in the interpretation of events taking place in the colon, especially in the proximal colon because most of the short chain fatty acids are absorbed by the intestinal epithelium during the transit of digesta. Only a minor proportion, up to about 10 percent of the short chain fatty acids, are excreted in faeces (Hill, 1995b). In rapidly fermenting carbohydrates these SCFA are readily absorbed in the proximal colon and only negligible amounts are excreted in the faeces. A rise in faecal SCFA may indicate the prolonged or slow fermentation of the

carbohydrate. A recent *in vitro* study reported that caecal and faecal inocula from the same rats produced different profiles of SCFA from the same substrates (Monsma & Marlett, 1995). Thus, the faecal SCFA profile cannot be considered with certainty as representative of events taking place in the large intestine and such data cannot be considered quantitatively representative of the SCFA produced in the colonic fermentation. Above all, control of the diet in human subjects is a difficult task, especially when the study involves a long term feeding of the test food. This difficulty can be overcome by the use of the animal models for study.

2.1.1.2 Animal models

In view of the difficulties faced during the direct studies in humans, animal models are frequently used for *in vivo* study. Rats and pigs are the animals which are used as models in most of such studies (Bach Knudsen et al., 1991; Mathers & Smith, 1993; Ellis et al., 1995; Monsoma & Mallete, 1995; Berggren, 1996; Djouzi & Claude, 1997). There are number of advantages in using animal models. Digestive and circulatory systems of animals are easily accessible, and animals can be fed a control diet under strict experimental conditions. The major limitation to this model is the difficulty of inter-laboratory comparison of such studies in the literature due to the variation of the basal diets from laboratory to laboratory (Edwards & Rowland, 1992). The basal diets used in animal feeding varied from zero (Edwards et al., 1992c) to 16g of fibre per day. The length of study period varied from less than 1 week to 18 months (Edwards et al., 1992c) and the level of fibre inclusion varied from 1.5g (Edwards et al., 1992a) to 10g per day (Nyman & Asp, 1988). Such variable conditions of different studies may make inter-

laboratory comparison very difficult because variable fibre contents of the basal diet may interfere with the effects of the test material. The adaptation period of the colonic bacteria in the rat to a new diet may be over 4 weeks (Walter et al., 1988). Extrapolating data obtained from animals to humans, however is also questionable, due to a large number of genetic and anatomical differences.

2.1.1.2.1 Rat model

The colon is a major site for fermentation in humans whereas, rats have a bigger caecum and are considered caecal fermenters. Although rats are easily housed and large numbers of animals can be used for replication to avoid individual variations, with a limited amount of test material. However, it will be very impracticable to screen large numbers of carbohydrates. For example, in this study for a single substrate I needed over 25 cultures (five incubation time periods, 5 replications) for one screening experiment. In this way, I would have to use hundreds of cultures. Therefore, it would be difficult to carry out such a study in rats because for quantitative measurement thousands of animals would be required to make measurements at short intervals, due to the rapid absorption and metabolism in vivo systems.

Moreover, a number of species differences exist between rat models and humans. Rats are not ideal models for the human colon, because rats have shorter transit time and, the pattern of digesta flow and SCFA production in their hind gut differ markedly from the human condition (Hume, 1995). Coprophagy is another problem which is difficult to prevent completely in rats. Rats may be better adapted to digest cereals, other food materials, etc., than humans, especially because the rat is more likely to eat uncooked foods.

In addition, rats have a smaller diameter of the pyloric sphincter compared with humans. This may affect the particle size of the food ingredients that pass from the stomach to the small intestine which may be important in the digestibility and subsequent fermentability in rat gut (Roe et al., 1996). Since metabolism in mammals has been shown to be strongly correlated to body mass and the length of the alimentary canal (Speakman, 1997), extrapolation of the data, achieved from experimentation in the rat, to humans may be misleading. Despite a good correlation between rats and humans for effects of dietary fibre on stool output (Edwards et al., 1992b; Nyman et al., 1986), the effects on SCFA production may be less comparable.

2.1.1.2.2 Pig model

Pigs are considered 'colon fermenters' and their body mass is also comparable to that of man but interpretation of fermentation data may be affected by ileal fermentation in the pigs. Besides, pig hind gut capacity is significantly larger (48% of total gut capacity) compared with that (17 % of total gut capacity) in the man (Hume, 1995). Animal models, especially pigs, need suitable facilities which may be impractical in some cases and are also expensive for screening purpose of large number of substrates as in our study.

2.1.2 *In vitro* methods

The human colon is not a closed system, and the absorption of the metabolites, in addition to faecal excretion, will have a different impact on the bacterial population than that measured in an *in vitro* system. The difficulty of sampling from intact colon and quantitative *in vivo* measurement of the short chain fatty acids in contents from different regions of the large intestine, results in the adoption of *in vitro* models for the study of carbohydrate

fermentation because *in vivo* methods are not suitable for initial prediction of the carbohydrates fermentation and its end product profiles. For this purpose simple, rapid and cost effective methods are needed.

Post mortem studies have shown that the composition of lumenal flora was relatively constant from proximal colon to rectum (Moore et al., 1978). The microbial composition in human faeces can be a good representative sample of the gut microbial population, although it is not as complex a population. Therefore, *in vitro* fermentation models, using human faeces in presence of a basic salts medium, is the simplest and cheapest method to screen carbohydrates. In this way, assessing the fermentation of a carbohydrate is more cost effective and practicable, and such methods offer an interpretable picture of fermentation events in the human gut such as SCFA profile, lactate and other bacterial products. Despite the simplicity of this method, it provides useful information about the extent of carbohydrate fermentation and relative proportions of the short chain fatty acids in cultures. This method is also helpful in getting an index of rate of fermentation by stopping the cultures after different time periods of incubation.

Such *in vitro* methods are rapid and very suitable for comparing large number of indigestible carbohydrates. These methods could be particularly useful when the aim of the study is the quantitative measurement of the fermentation products (Edwards et al., 1996) because, unlike the intact gut, there is no epithelial absorptive surface *in vitro* methods. On the other hand, this situation poses a limitation because of accumulation of the intermediary and end products and low pH, which may affect the metabolic activity in the cultures. Problems of changed physiological conditions in *in vitro* cultures will be addressed in this thesis.

The most important factors regarding *in vitro* fermentation methods, include consideration of source and size of inoculum, type of fermentation media, maintenance of anaerobic conditions, choice of buffer, mixing rate and sampling time. In the recent years research studies have started to consider these factors, whereas in the past little attention was paid to report the rationale of *in vitro* system. A number of *in vitro* methods used human faeces as the source of gut bacteria (Table 2.1). Some of these studies did not consider the necessity of including a source of nitrogen and basic essential minerals as a requirement for further growth of bacterial mass. These essential macro- and micro nutrients may affect the growth and survival of bacteria, as well their lag phase. Whereas, other methods ignored the need of buffering pH changes in cultures which could have adverse effects on the activity of the bacteria in such cultures. Still other methods did not adopt the shaking of cultures which could be helpful in dispersion and accessibility of substrate for the bacteria, and to mimic motility in the gut.

2.1.2.1 Limitations of *in vitro* methods

In vitro methods have two important limitations. In most of the studies, human faeces instead of inocula from the actual site of the fermentation i.e. colon, are used as source of the microorganisms. It can be argued that the faecal flora are not considered representative of the intestinal flora. Emerging data suggested that fermentation in the caecum and proximal colon may differ from that obtained with faecal inoculum. Monsma and Martlett (1995) reported different profiles and higher amount of short chain fatty acids produced by the same substrates, using the caecal inocula compared to faecal inocula from rats. In addition to this, a problem with using the faeces as

Table 2.1 A summary of the main characteristics of different *in vitro* fermentation methods. (adapted and modified from Barry et al., 1995; Edwards et al., 1996).

Reference	Inoculum (g/L)	Substrate (g/L)	*Buffer/salt solution	N_2-Source	Micronutrients	Shaking	Duration (Hours)
Adiotomre et al., 1990	40	10	P	tripticase	Yes	Yes	24
Barry et al.,1995	200	5, 10, 20	C/P	urea	Yes	Yes	0 - 24
Daly et al., 1993	25	1.25	$NaHCO_3$ solution	Tryptone	NaCl, KCl	None	24
Edwards et al., 1996	160	10	P	none	None	Yes	24
McBurney et al., 1985	___	5	C	-	Yes	___	24
McBurney et al., 1988	60	10	C/P	-	Yes		24
McBurney & Thompson 1987; 1989	13.3	10	C/P	trypticase peptone	Yes	swirled at intervals	0 - 24
Monsma & Martlett 1995	10 (rat faeces)	10.2	$NaHCO_3$, NaCl, K_2HPO_4	-	None	agitated at intervals	0 - 96
Mortensen & Nordgaard-Anderson 1993	166	10	$NaHCO_3$ solution	None	None	None	6, 24
Mortensen et al., 1988	200	~ 10 - 50	Na/K solution	None	None	None	0-72
Mortensen et al., 1991	166	2.5 - 30	Bicarbonate solution	None	None	None	6, 24
Rasmussen et al., 1987	167	up to ~50	None	None	none	None	0 - 72
Salvador et al., 1993	250	20	C/P	Urea	Yes	None	0 - 24
Stevenson et al., 1997b	16	2.5 - 10	None	None	Yes	Partly	0, 24
Tomlin et al., 1986; Tomlin & Read, 1988	50	5	chloride, bicarbonate solution.	Tryptone	None	None	21
Vince et al., 1990	250	10	Saline (0.9 %)	None	None	None	48
Weaver et al., 1989	50 & 100	10	$NaHCO_3$; KH_2PO_4	None	Yes	Yes	0 - 24

*C- for carbonate buffer; P- for phosphate buffer.

source of inoculum, is that, after defaecation, the faecal composition starts changing and this change continues through out incubation process.

Another objection to the use of faeces as source of fermenting bacteria, was the presence of any unfermented fibres or other fermentable components in faeces which might lead to erroneous results of fermentation of the test carbohydrate. A control without the addition of test material for each faecal sample, was helpful in solving this problem and providing the base level data in each incubation run (Mortensen et al., 1988). Despite these objections, in most cases, using faeces is the only practical and easy way of obtaining a source of human gut flora, and methods to use a representative inoculum from the exact site, may present almost the same practical difficulties which can be encountered in *in vivo* studies. McBurney and Thompson (1990) pointed out that some fibre preparations, such as wheat bran, may contain digestible carbohydrates and protein. Such preparations are suitable for *in vivo* use because digestible substrates are absorbed in the small intestine but *in vitro* this is not the case and it will be necessary to pre digest such preparations before their use in *in vitro* fermentation.

The *in vitro* systems lack the ideal *in vivo* conditions, for example, they lack an absorptive epithelial surface. This single limitation leads to the accumulation of end product such as SCFA, gases, consequently resulting in the creation of an imbalance of the *in vitro* conditions by changing pH and partial pressure of H_2 in a closed system (Edwards & Rowland, 1992). This may lead to changes in the physiological conditions of cultures, probably

affecting bacterial composition or at least the bacterial metabolic activity in culture. The internal physiological conditions of an *in vitro* system may start conditions of an *in vitro* system may start changing early and continue to do so until the end of incubation and it is unlikely that the environmental conditions remain optimal for bacterial metabolic activity because of this. As a result, the metabolic activities of the microbes may under go transformation due to the changed experimental conditions in cultures, which cannot be maintained critically. For example, a number of Bacteroides species were well maintained at all the pH from 5 to 7 in continuous anaerobic cultures, whereas, lactobacilli and bifidobacteria survived in cultures at pH 5. However, these species disappeared with the increasing pH from 5 to pH 7. Similarly *Cl. perfringens* disappeared with the decreasing pH from 7 to 5 (Edwards et al., 1985). In another *in vitro* experiment, *B. infantis* demonstrated the ability of withstanding an acidic pH 5.0 and 4.5, whereas, the same pH inhibited the growth of *E. coli* and *Cl. perfringens* completely (Wang & Gibson, 1993). In this way, the bacterio-static conditions may be initiated in *in vitro* batch cultures. The impact of changed physiological conditions can be judged by three methods;

1) stopping cultures at different time points.
2) using the increasing amounts of individual carbohydrates in cultures.
3) chromatographic determination of the residual carbohydrate in cultures.

I used all these three approaches in this study to investigate changes in the production of SCFA in cultures. In this way it will be possible to ascertain the extent and rate of fermentation by comparing the accumulation of end products at different

time points, as the time of incubation has a consistent effect on the total SCFA production (Monsma & Marlett, 1995).

2.1.2.2 Why to use *in vitro* system at all?

Despite these limitations, *in vitro* systems provide useful information on the extent and product of fermentation (Table 2.2). Using a well planned *in vitro* fermentation system provides the opportunity to infer information about the site and rate of SCFA production in the large bowel. An *in vitro* study can assess the rate of fermentation, by sampling the cultures at regular intervals, and thus getting a basic idea of fermentation rate. Thus *in vitro* models offer the ease to determine the rates of SCFA production, the extent of production at each time point and the maximum production of SCFA by conducting *in vitro* fermentation for several periods of time (Monsma & Marlett, 1995). The *in vitro* systems also offer the possibility of measuring the production of gases. Another advantage of *in vitro* fermentation is that it is a good screening tool, facilitating the quantitative measurement of individual short chain fatty acids, which is necessary for the screening purpose, and also this is one of the requirements in my study.

The simplest and cheapest *in vitro* method is *in vitro* fermentation using human faeces as an inoculum in presence of basic salts fermentation medium, for assessing the fermentability of a carbohydrate and determining SCFA profile, lactate and other bacterial products (Adiotomre et al., 1990). This method of fermentation was adopted in the study by Edwards et al., (1996). In this study, a simpler method was developed for study of *in vitro* fermentation and it was tested in eight laboratories with faecal inocula from a total of 40 subjects. Trends of the fermentation were same and comparable

between most of the laboratories. The variation in the mean results between the laboratories was of the similar or less magnitude than the intra-laboratory data. The method used by Edwards et al., (1996) has taken good account of the properties of human colon, thus mimicking the *in vivo* environment of the human colon. This method consisted of three important characteristics including size of the inoculum, shaking of cultures and addition of buffer to the culture. Shaking was helpful in mixing cultures and stopping deposits of the sedimentation of substrates in bottom of culture bottles. Mixing also stops building up the preferential

Table 2.2 Advantages and disadvantages of the *in vitro* fermentation methods.

Advantages	Disadvantages
Rapid	Difficulty in maintenance of critical conditions
Cheap	No absorptive surface
Manageability for large number of samples	Product inhibition
Good screening tool	Lag phase
Possibility of reproducability and precision (repeatability) check.	
Offers the quantitative measurement of products	

metabolic layers by the bacteria in cultures of bigger volume. Viability of the microorganisms, present in *in vitro* fermentation cultures, depends on the characteristics of original inoculum (McBurney & Thompson, 1987). In this regard, inoculum size is an important characteristic of an *in vitro* fermentation procedure. Since survival of the bacteria and lag phase are critical in the *in vitro* system, large inoculum size provides larger population of the bacteria

initially which allows better bacterial survival and limits the lag phase. A larger inoculum size can also provide the micro-minerals and bile salts for the bacteria to grow in. Therefore, the chemical and physical nature of the culture is closer to that of colonic contents compared to the use of a smaller inoculum size (Edwards et al., 1996). A large inoculum size is reported to increase bacterial survival rate and, to produce a lower redox potential and higher final osmolality in cultures (Edwards, 1984). These parameters serve as the indicators for higher production of SCFA. This *in vitro* system is very helpful in testing and comparing large number of the test materials in a very short period of time without involving complicated expensive systems which, after all, will not be able to cover large number of samples.

2.2 Fermentation procedure in this study

The fermentation of complex carbohydrates *in vitro* was studied according to the fermentation model of Adiotomre et al. (1990), which was modified to our laboratory conditions (Fig. 2.1). The amount of substrate and volume of the fermentation medium was reduced five fold from that reported in the original method, to make the results more comparable to the earlier work and to facilitate the manageability of large numbers of cultures in our laboratory.

A faecal slurry (32%) was used to inoculate the culture, in presence of the pre-reduced basic salt medium in McCartney bottles, containing 100mg (or none in case of control cultures) of the carbohydrate substrate. Each McCartney bottle was fitted with a screw cap containing a hole in the centre and a rubber lining to allow flushing of culture with oxygen free nitrogen (OFN) before incubation for creating an internal anaerobic environment. With this type of bottle, it was possible to sample the gases produced during fermentation.

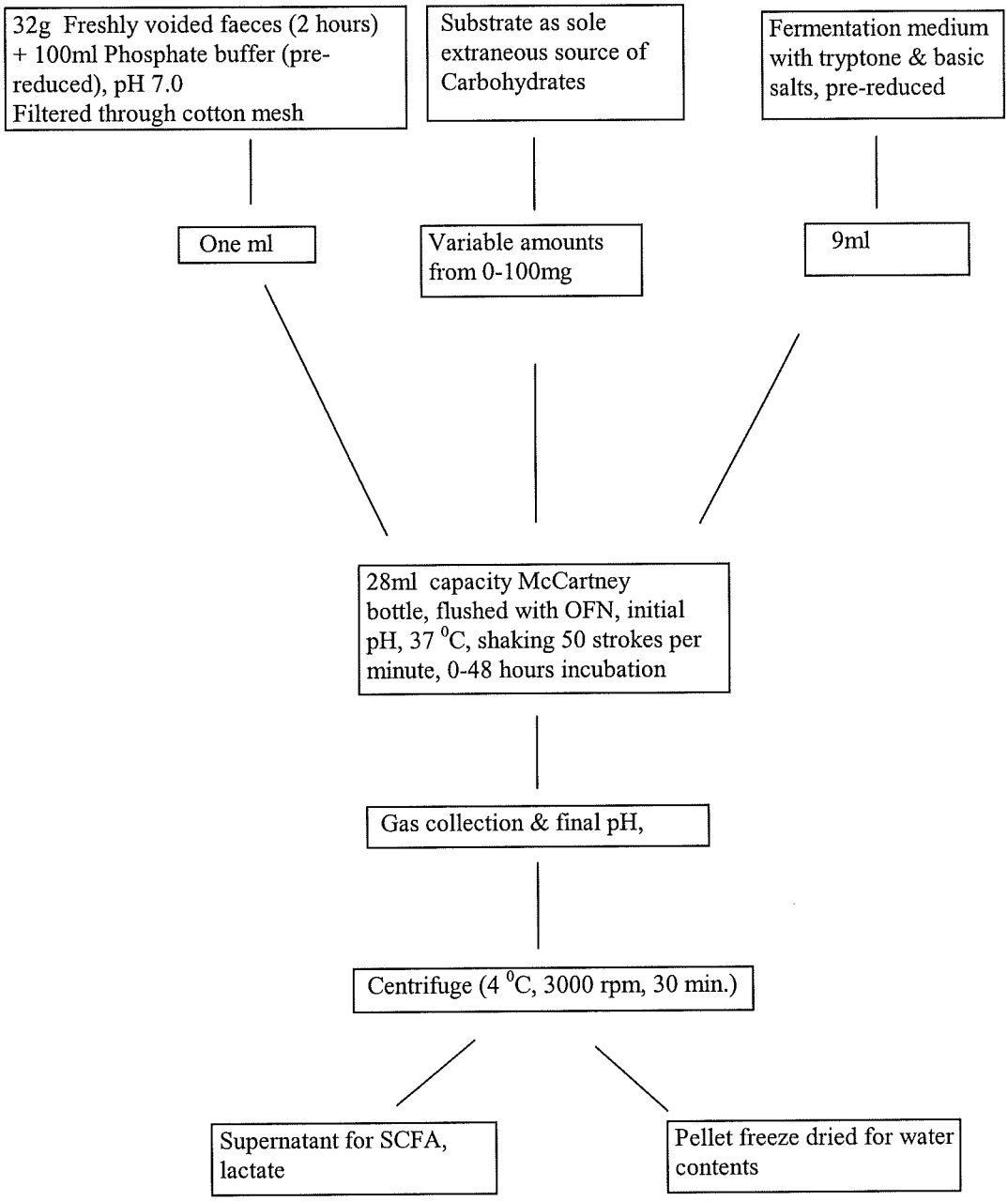


Fig 2.1 Flow chart of the *in vitro* fermentation method.

The cultures were mixed (vortex) mechanically before flushing them with oxygen free nitrogen to create an anaerobic atmosphere. The incubation was carried out at 37 ^{0}C in a shaking water bath at 50 strokes per minute. These bottles, lying horizontally in shaking water bath, were also good for maintaining the optimum surface area/volume ratio of cultures (Stevenson et al., 1997b). The cultures were stopped at different time points from 0-48 hours (Fig. 2.1). Exact details are given in chapters of individual experiments. The fermentation procedure was replicated with faeces from different healthy subjects. The number of replications were different in individual experiments and details are given in the respective chapters, but always at least five subjects were used, the number which was recommended by previous work (Edwards et al., 1992b).

2.3 Substrates

Details of the substrates are given in the table 2.3.

2.4 Reagents & fermentation media

The fermentation medium used in my study was adapted from Adiotomre et al., (1990). It was a complex nutritive growth medium (Table 2.4). This medium contained all the essential mineral salts, and tryptone as the principle source of nitrogen, for further growth of the bacterial population. The phosphate and carbonate buffers were used as part of the medium to delay changes in pH in cultures. Although the pH decreased to pH 5-6 range in some cultures, this was in the range near to that reported in the large gut (Cummings et al., 1987). Use of phosphate buffer negated the need of CO_2 supply, necessary with the bicarbonate buffer, as bicarbonate is a physiological component secreted within the digestive tract.

Table 2.3 Substrate used for *in vitro* fermentation in this study

Substrate	Source	Remarks
Lactulose	Duphalac- Duphar Laboratories Ltd Gaters Hill Southompton	Each 5ml contains; Lactulose 3.35g; lactose ≤0.3g; galactose≤0.55g
Raftilose	Raffinerie Tirlemontoise SA Brussells Belgium	P95; 93.2-95.8% oligofructose
Raftiline	Siber Heghner Ltd 221-241 Beckenham Road Beckenham Kent	ST; 90-94% inulin mixture of oligo- and polysaccharides
Fibruline	JC Dudley & Co Ltd Cheyney House Francis Yard East Street Chesham Bucks	Powdered inulin, 30 (Instant) <50 microns
Raw potato starch	Englyst Carbohydrate Services Ltd Cambridge UK	80.1% total starch contents; 56.7% RS
Gum arabica	KWR Ltd Food Ingredient Division Basingstoke Hants RG24	
Guar gum	Meyhall Chemical AG	Meyprogat 30 MR 4812; low molecular weight
Pectin	Supplied by SHS Liverpool	Low viscosity HM citrus pectin B11503
Oat fibre	Supplied by SHS Liverpool	Specified properties not known
Ispaghula	Madaus AG, Cologne, Germany	

The medium was pre-reduced by addition of a solution of cysteine hydrochloride and sodium sulphide (Na_2S). The cysteine, which contains a sulpha-hydral group, is a reducing agent and plays a protective role to anaerobic microbes against oxygen toxicity. Iron salts are also protective against the toxic substances. Some of the minerals are essential for inducting bacterial enzymes. The medium was adjusted to pH 7.0 with 5M HCl. Sorensen's phosphate buffer (pH 7.0) was made with proportions of solutions of KH_2PO_4.anhydrous and $Na_2HPO_4.2H_2O$. The buffer was used to prepare faecal slurry from freshly voided faeces immediately after defaecation (within two hours). The fermentation medium and phosphate buffer were boiled, cooled and kept under anaerobic conditions by flushing with oxygen free nitrogen before use.

2.5 Subjects used as donors of faecal inocula

Fresh human faeces were used as a source of fermenting bacteria for *in vitro* inoculation. Faecal samples were collected from different healthy individuals with no previous history of serious gastrointestinal disorder and who did not take antibiotics over three months prior to faecal delivery for the study. Faeces were used to inoculate cultures within the shortest possible time not exceeding at any time two hours of defecation, as faeces could be used successfully within two hours (Personal communication - Dr. C A Edwards, Human Nutrition, University of Glasgow). Due to the large number of cultures per subject, two hours was set as the latest start of the incubation, most inoculation of cultures were accomplished well before this target each time.

Table 2.4 General composition of the fermentation media and other reagents used in this study.

Sorensen's phosphate buffer (pH 7.0)	39 parts
Solution A containing KH_2PO_4	
Solution B containing $Na_2HPO_4.2H_20$	61 parts

Van Soest fermentation medium (Adiotomre et al., 1990)

Tryptone as source of nitrogen

Micro-mineral solution	Macro-mineral solution
in distilled water containing;	in distilled water containing;
$CaCl_2.2H_20$	$Na_2HPO_4.2H_20$
$MnCI_2.4H_20$	KH_2PO_4.anhydrous
$CoCl_2.6H_20$	$MgSO_4$.anhydrous
$FeCl_3.6H_20$	
Buffer solution	Reducing solution
in distilled water containing;	in distilled water containing;
Ammonium bicarbonate & Sodium	Cysteine hydrochloride
bicarbonate	Sodium sulphide.$9H_20$
	Sodium hydroxide

McBurney & Thompson (1987) recommended that several donors, at least three, be used to improve the accuracy of *in vitro* estimates of colonic production, thus enhancing the predictive value of *in vitro* fermentation variables for *in vivo* situation. Edwards et al., (1996) reported the greatest intra-laboratory variance in their inter- laboratories study, where inocula were used from only four faecal donors. Therefore, at least five and where possible more than five inocula from different human subjects, were used for the best

estimation of fermentability in my study. Details of the gender and age range is given in the individual chapters of different experiments.

2.6 Detection of un-metabolised sugars in cultures

Since cultures show a flattening curve after 8 hours of incubation, to rule out the possibility of unfermented substrate present in cultures, thin layer chromatography (TLC) was used to identify sugars in culture residues. The presence of an oligosaccharide, raftilose, was determined by TLC to ascertain inhibition of its fermentation. This method was adopted from Humbel & Collart (1975) and modified for our purpose. A standard mixture of raftilose (Sigma Chemical Co. Ltd., Poole, UK), lactulose (Sigma Chemical Co. Ltd., Poole, UK), sucrose (Sigma Chemical Co. Ltd., Poole, UK) and fructose (Sigma Chemical Co. Ltd., Poole, UK), was prepared freshly before the TLC. A 100mg of each carbohydrates was dissolved and made up to 25ml separately with 10% (V/V) isopropanol (AnalaR, BDH, Ltd. Poole, UK). One ml of each sugar solution were pooled together to make a 4 ml standard mixture containing 1μg/1μl of each sugar.

Thin layer chromatography was performed on cultures containing raftilose after 4-24 hours of incubation. From each test culture and standard mixture, samples of 10μl were applied as 1.5cm lines at a distance of 2 cm from each other. Side edges of 2.0 cm were left on a 20cm x 20cm silicagel plate (Camlab-DC Fertigplatten Sil Gel 25- Tenside. Layer: 0.25mm Silica gel G25, $(NH_4)_2SO_4$ impregnated). The cultures were applied at a distance of 2.5cm from lower edge of the TLC plate. After thoroughly drying the plates in air, chromatography was developed overnight for about 24 hours in 200ml of freshly prepared solvent (n-butanol/acetic acid/water; 100:50:50 V/V).

Orcinol developing solution was made freshly by putting 0.2 g orcinol in 100ml of sulphuric acid (11ml conc. H_2SO_4/100ml water). After drying, the chromatographic plate was evenly sprayed with freshly prepared orcinol solution in a fume cupboard and then heated for 10 minutes at 100 ^{0}C and observed for presence of raftilose.

2.7 pH of cultures

A Kent pH meter model EIL 7045/46 was used to measure pH of cultures before and after incubation. Buffer tablets (pH 4.0±0.02 & pH 7.0±0.02) from BDH while buffer tablets (pH 9.2) from Fison Scientific Equipment, England were used for calibration. Before setting up cultures for incubation, the initial pH of individual cultures was noted. Cultures were stopped at different times and after sampling for gases, final pH were noted.

2.8 Preservation of cultures for further measurements

The cultures were centrifuged at 3000 rpm in a refrigerated centrifuge (4 ^{0}C) for 30 minutes. After centrifugation, supernatants and sediments of cultures were stored separately in a freezer at -20 ^{0}C for use in further analysis.

2.9 Percent dry weight of sediments after incubation

The sediments of cultures were freeze dried in Edwards Micromodulyo 1.5 K Freeze Dryer, Edwards High vacuum International, England. The weight of the sediment before and after freeze drying was used to determine the dry weight of the residue after fermentation and the water held in the residue.

Water content in residue (%)={(wet weight of the sediment - dry weight of the sediment) / (wet weight of the sediment)} x 100

2.10 SCFA measurement by GLC

SCFA were measured in supernatants from cultures by GLC.

2.10.1 Internal standard stock solution

β methyl valeric acid (Sigma Chemical Co. Ltd., Poole, UK) was used as the internal standard. One g (1.075ml) of β methyl valeric acid was dissolved and diluted up to 100ml with distilled water. The solution was adjusted to pH 7.0 with 0.34g of NaOH (Merck Loughborough, UK).

2.10.2 External standard stock solution

The external standard stock solution was a mixture of acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid and n-valeric acid (Sigma Chemical Co. Ltd., Poole, UK). A mixture of 1g of each of these acids was dissolved in distilled water and diluted to up to 100ml, after adding 2.9g NaOH (Merck Loughborough, UK), in order to adjust pH 7.0 of the solution. This external standard mixture was used for plotting a calibration curve and calculation of individual short chain fatty acids in the test samples from cultures.

2.10.3 Sample extraction procedure for GLC

A series of six external standard concentrations was set up by putting 10µl, 25µl, 50µl, 100µl, 200µl, 300µl in separate extraction tubes and making up to 800µl each with distilled water. A volume of 800µl of supernatant of each test samples from fermentation cultures, was taken in separate extraction tubes. To all the standards and test samples 100µl of internal standard and 100µl of ortho-phosphoric acid (AnalaR, BDH, Ltd. Poole, UK) were added.

Three ml of diethyl ether (AnalaR, BDH, Ltd. Poole, UK) was added to each tube and vortex mixed for 1 minute. The supernatant layer was collected in separate tubes from each respective extraction tube. This extraction procedure was repeated three times for each external standard and the test sample. The supernatant layers from three repeated extraction, were pooled in a single tube for each sample (Spiller et al., 1980). A sample of 3µl from the pooled ether extract was injected in the GLC to determine concentration of SCFA in each sample (Spiller et al., 1980).

2.10.4 GLC conditions

Short chain fatty acids were measured using a Philips Pye Unicam 304 series GLC (Gas Liquid Chromatograph, Unicam Ltd., Cambridge), this was connected to a Phillips pm257 (Unicam Ltd., Cambridge) chart recorder. A glass packed column 4ft in length with ¼ inches external diameter and 0.19 inches internal diameter, was used in GLC for determination of SCFA. The packing material was 10% SP1000+H_3PO_4 on Chromosorb WAW 80-100 mesh (Phase Separation Ltd., Deeside). A flame ionisation detector was used for the purpose of detection. GLC Conditions were as follow:

Gas flow rates [carrier gas, nitrogen: 60ml/min; flame gases, air: 30ml/min & Hydrogen: 20psi]. Temperature [Column, 200^{0}C; injector, 180^{0}C; detector, 200^{0}C]. GLC running conditions [Column temperature, 125^{0}C; Initial time, 2 minutes; rate 10^{0}C/minute; upper time, 1 minute; Final temperature: 150-155 ^{0}C; Attenuation: 2-16]. After injecting a 3µl ether extracted sample, it took about 10 minutes to complete a single run of each sample.

Most of the chromatogram from each sample consisted of at least three principal SCFA, with an additional peak for the internal standard. Some of the chromatograms, but not all, had peaks of the branched chain fatty acids (iso-butyarte, iso-valerate, n-valerate). The position of the respective peaks were determined by the position of the external standards peaks. The eluent sequence of the short chain fatty acids was acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, n-valerate.

2.10.5 Calculation of SCFA concentration

For quantitative measurement, height of individual peaks were divided by the peak height of internal standard and a peak ratio was calculated for both external standards as well as test samples. A standard curve was plotted using the peak ratio as y-axis against the known concentration of the respective SCFA in the external standards mixture. Using the peak ratio of individual SCFA in chromatogram of each sample, concentration of particular SCFA was calculated.

Concentration of SCFA (μmoles/ml);

$$(Z) \times (1000/\text{mol.wt. of specific SCFA}) \times 1.25$$

The value of 'Z', the x-axis was calculated from the standard curve in InStat and value 1.25 was derived from ratio of liquid sample i.e. 1000μl/800μl.

The repeatability of the method showed 2.7%, 6.3%, 4.2% error for acetic acid, propionic acid and n-butyrate respectively (Lokerse, 1994).

2.11 Measurement of lactate

Prior to analysis on GLC, samples had to be methylated to make the lactate volatile.

2.11.1 Internal standard

Succinic acid (Sigma Chemical Co. Ltd., Poole, UK) was used as the internal standard. One g of succinic acid was dissolved and diluted up to 100ml with distilled water.

2.11.2 External standard

The external standard stock solution was a mixture of 833µl lactic acid (AnalaR, BDH, Ltd. Poole, UK) and 1g phenylacetic acid (Sigma Chemical Co. Ltd., Poole, UK). Mixture of these acids was diluted to up to 100ml with distilled water.

This external standard mixture was used for plotting a calibration curve which was used to calculate lactate concentration in the test samples from cultures.

2.11.3 Lactate sample extraction procedure for GLC

Lactate was measured by GLC using method described by Holdmann & Moore (1973). A series of three external standard concentrations was set up by putting 25µl, 50µl, 100µl and making their volume up to 500µl each with distilled water into separate extraction tubes. A volume of 500µl of supernatant of each test samples from fermentation cultures, was taken in separate extraction tubes. To all standards and test samples 200µl of 50% sulphuric acid (AnalaR, BDH, Ltd. Poole, UK) was added. After adding 1ml methanol (AnalaR, BDH, Ltd. Poole, UK), 100µl internal standard, the tubes were stopped with covers and heated at 55^0C for 30 minute in a water bath. After completion of heating, 100µl of orthophosphoric acid (AnalaR, BDH, Ltd. Poole, UK) was added to each tube.

After addition of 1.5ml of diethyl ether (AnalaR, BDH, Ltd. Poole, UK), each tube was vortex mixed for 1 minute and supernatant was collected. This extraction procedure was repeated three times for each external standard and the test sample. The supernatant layers from three repeated extraction, were pooled in a single separate tube for each sample and standard. A sample of 3µl from the pooled ether extract was injected in GLC to determine concentration of lactate in each sample.

2.11.4 GLC conditions

Lactate was measured using a Philips Pye Unicam PU 4550 Gas Chromatgraph (Unicam Ltd., Cambridge), this was connected to a Phillips PM257 (Unicam Ltd., Cambridge) chart recorder. A glass packed column 4ft., in length with ¼ inches external diameter and 0.19inches internal diameter, was used. The packing material was 10% SP1000+H_3PO_4 on Chromosorb WAW 80-100 mesh (Phase Separation Ltd., Deeside). A flame ionisation detector was used for the purpose of detection. GLC Conditions were as follow:

Gas flow rates [carrier gas, Helium: 60 ml/min; flame gases, air: 30 ml/min & Hydrogen: 20psi]. Temperature [Column, 200^{0}C; injector, 180^{0}C; detector, 200^{0}C]. GLC running conditions [Column temperature, 117 ^{0}C; Initial time, 2minutes; rate, 10^{0}C/minute; upper time, 2minute; Final temperature, 146^{0}C; Attenuation, 2-16]. After injecting a 3µl ether extracted sample, it took about 11 minutes to complete a single run of each sample. Each chromatogram consisted of peaks of the internal standard and lactate, if the sample contained

lactate. The first peak adjacent to the solvent front was lactate, followed by the peak of the internal standard.

2.11.5 Calculation of lactate concentration

For quantitative measurement, the height of lactate peak was divided by the peak height of the internal standard and a peak ratio was calculated for both external standards as well as test samples. A standard curve was plotted using the peak ratio as y-axis against the x-axis which is the known concentration of lactate in external standard mixture. Using the peak ratio of the lactate in chromatogram of each sample, concentration of lactate in the particular sample was calculated.

Concentration of Lactate (μmoles/ml);

$$(Z) \times (1000 / \text{mol. wt. of specific SCFA}) \times 2.0$$

The value of 'Z ', the x-axis, was calculated from the standard curve in InStat and value 2.0 was derived from ratio of liquid sample i.e. 1000 μl/500μl. Repeatability of the method showed 2.5% error (Lokerse, 1994).

2.12 Fermentation rate

The rate of fermentation was estimated by the rate of SCFA accumulation in cultures. Quantitative measurement of the fermentation rate was not practical with this method but by plotting a graph of concentration of SCFA against the time of sampling, it was possible to gain a basic index of the rate of fermentation. In this way, the rate of fermentation was estimated by the rate of accumulation of SCFA in the cultures at different time points.

The pattern of the SCFA accumulation varied between cultures. Sometimes a plateau occurred and sometimes the accumulation continued up to 24 or 48 hours. If two curves reached a plateau at different values of SCFA

concentration, the lower curve was described as representing lower production of SCFA. If one curve had a lower accumulation of SCFA than the other at most time points, but did not plateau, and showed evidence of the possibility of reaching the final SCFA concentration of the other curve with extended fermentation, it was considered to a have a slower rate of fermentation. ,

2.13 Statistical Analysis

The data of SCFAs and lactate were calculated from a calibration curve in a statistical computer software package, InStat 2.01. Concentrations and ratios of SCFA were calculated by the procedure and formula given in section 2.12.5. Ratios were the expression of percentage value of individual SCFA of the total SCFA. Net total SCFA were calculated by subtracting values of total SCFA in control (with no added carbohydrates) cultures from values of substrate cultures for each set of incubation. Mean data of SCFA were calculated in most cases from five to eight subjects for interpretation of the fermentation of different carbohydrates and their mixtures in this study. Concentrations of SCFA that could be expected from fermentation of hypothetical mixtures were calculated by arithmetical addition of the values obtained from fermentation of 25mg (Chapter 3 & 4) or 50mg (Chapter 6) of such individual carbohydrates. Computer application Excel95 was used for general calculation of the results, means and SEM. Minitab 10.5 was used for one way ANOVA, two tailed unpaired t-test and simple regression. Means of different factors were compared by one way ANOVA and then followed by the comparison of individual mean by two tailed t-test.

The differences in the initial and final pH of the respective cultures were converted into a percentage value.

CHAPTER 3

Substrate Concentration and Product Inhibition

3.1 Introduction

In this chapter, an *in vitro* fermentation model is validated which will be used in all subsequent experiments in the present study. The experiments described were designed to explore the limitations of the system and to allow adaptation and corrections where necessary. As discussed in Chapter 1, indigestible carbohydrates are fermented by colonic bacteria into SCFA and gases. The type of substrate has a significant impact on the rate, amount and profile of SCFA produced during the bacterial fermentation (McBurney & Thompson, 1987).

The fermentation rate of a carbohydrate determines the fate of SCFA produced *in vivo*. In the case of rapidly fermenting carbohydrate, for example lactulose, fermentation occurs in the caecum and the proximal colon, and the SCFA are readily absorbed in the proximal colon. In contrast, in the case of a slowly fermenting carbohydrates, for example ispaghula, fermentation occurs throughout the large intestine and SCFA may then either be absorbed at a more distal site or they may escape in faeces (Edwards et al., 1992a). Higher production of SCFA by carbohydrate fermentation lowers the pH of the lumenal environment (Cummings, 1984; MacFarlane & Cummings, 1991). Carbohydrate fermentation and subsequent absorption of SCFA causes the pH of colon to change from the lowest (pH 5.4-5.9) in the caecum with a gradual rise to about pH 6.6-6.9 towards the distal end (Cummings et al., 1987). This low pH plays an important role in changing the gut bacterial population and regulating the bacterial activity in the gut as well as affecting the motility of the gut. A lowered pH may affect the ionisation, thus facilitating the absorption, of metabolites produced during fermentation in the gut (Sakata 1987).

Conversely, *in vivo* fermentation may be affected by host factors such as transit time and the dietary habits of the host. *In vitro* fermentation however may be affected by other factors, not critical *in vivo*, such as changing physiological conditions in cultures.

There are many difficulties in conducting and observing the effects of carbohydrate fermentation in humans. It is possible to determine the extent of degradation of a dietary fibre in the human gut by measuring faecal residues of a particular indigestible carbohydrate (Prynne & Southgate, 1979; Nyman et al., 1986; Phillips et al., 1995). However, it is almost impossible to predict the amount of SCFA produced in human large intestine due to their extensive absorption and metabolism and the difficulty of collecting a sample of colonic contents from the intact gut. The only published data, in this regard, have been obtained from the victims of sudden death (Cummings et al., 1987). In view of these difficulties, *in vitro* fermentation methods are useful tools to predict the fermentation properties of dietary fibre, including quantitative determination of SCFA production (McBurney & Thompson, 1987; McBurney& Thompson, 1989; Adiotomre et al., 1990). When the main objective is to study the extent of SCFA production and other factors related to fermentation, *in vitro* batch systems are generally adopted. *In vitro* methods have many limitations but their usefulness can be enhanced if such limitations have been taken in account (section 2.1.2.1 for detailed discussion).

The experimental conditions, that are usually not controlled critically in *in vitro* batch systems, and may keep changing from start to the end of incubation. This deviation from ideal *in vivo* conditions may affect the growth and

metabolic activities of the bacteria in *in vitro* cultures and the bacterial composition may change continuously throughout the entire period of incubation, resulting in a marked transformation of the metabolic activities of bacteria. For example, higher production and accumulation of organic acids in *in vitro* cultures, results in the depression of pH which may lead to different metabolic pathways such as the production of propionate rather than acetate at pH 6 as seen previously in continuous culture (Edwards et al., 1985). The changing conditions in *in vitro* cultures can affect fermentation reactions in more than one way, but mostly through influencing the intracellular and extracellular enzyme activity.

3.1.1 Relation of pH to the bacterial metabolism

Adverse pH affects at least two aspects of a bacterial cell; the functioning of its enzymes and the transport of nutrients into the cell. Changes in pH alone can affect the physiological activities of faecal bacteria without affecting the numeric values of species (Edwards et al., 1985). These changes in pH may make conditions unfavourable for the optimal activity of certain bacterial species in *in vitro* cultures because of the variable growth requirements of different bacteria. It seems that pH can affect the metabolic reactions in bacterial cell in several ways.

3.1.1.1 Effect of pH on ionisation of active site of an enzyme

The catalytic activity of an enzyme may require a specific ionisation state of its substrate and the enzyme for their interaction. The ionic character of side chains ionisable groups is affected on either side of neutrality, increasing denaturation of membrane and transport enzymes (Jay, 1996), and other

extracellular and intracellular enzymes. For example, it may require an amino group of the enzyme be in the protonated form ($-NH_3^+$). Alkaline pH can deprotonate such groups. Therefore, pH can affect the ionisation of an active site of the enzyme, completely changing the reactivity of the enzyme towards the substrate. Although pH is an indicator of the presence of acidity in the cultures, it is not a true measure of the amount of the organic acids present. The pH is a measure of H^+ concentration and organic acids do not ionise completely because they are weak acids with low pKa. Therefore, the presence of higher amounts of acids, than indicated by pH, can lead to the denaturation of proteins (enzymes) required in metabolic pathways of the bacterial cells. Such extreme conditions may result in metabolic inhibition, causing a decrease or a halt in the bacterial activity in culture.

It seems that the influence of pH is mostly extracellular, affecting the enzymes related to the extracellular degradation of complex substrate molecules. For example, *Lactobacilli*, *Bifidobacteria* and *Eubacteria* species were able to maintain an intracellular pH of 6.5 with decreased caecal pH, even in the presence of an extracellular pH of 3.5 or less (Padan et al., 1981). At such acidic pH these microorganisms remained good producers of β galactosidase (Florent et al., 1985).

3.1.1.2 Optimum pH requirement of different enzymes

The pH changes in cultures can affect the reaction velocity due to differences in the optimum pH for different enzymes in the metabolic reactions. For example, a decreased colonic pH is considered helpful in reducing the synthesis of carcinogens such as conversion of primary bile acids into secondary bile

acids through 7 α de-hydroxylation by the catalytic activity of 7 α de-hydroxlase (Thornton, 1981; Nagengast et al., 1988), is inhibited at acidic pH due to optimal pH requirement (pH 7-8) of this enzyme.

With increasing amounts of intermediary and end products and decreasing amounts of substrate, bacterial cells may induce (see section 3.1.2) enzymes with very different requirements for their activity. This may result in a shift from one metabolic pathway to another producing different end products for example production of lactate. Generally, glucose is converted into pyruvic acid through glycolysis in the bacterial cell. This pyruvic acid is subsequently metabolised into different metabolites like acetate, lactate, propionate, butyrate, by different species depending on the conditions in culture. For example, in an *in vitro* study of colonic bacteria, significantly lower SCFA were found in cultures at pH 5.0 compared with cultures at pH 6 to 7 (Edwards et al., 1985). Acetate was highest at pH 7 and lower at pH 6 and 5, whereas, propionate was higher at pH 6 than at pH 5 or pH 7.

3.1.1.3 The pH and the bacterial growth

Changes in pH may make conditions unfavourable for the optimal growth of certain bacterial species in *in vitro* cultures, because the growth rates of different bacteria change with variations in growth conditions. Some species, such as *lactobacilli*, show an optimal growth at an acidic pH (5.4 - 6.4), whereas, optimal growth of Clostridium species is at higher pH (6.5 - 7.5). A number of Bacteroides species were well maintained at all pH from 5 to 7 in continuous anaerobic cultures (Edwards et al., 1985). In the same experiment, *Lactobacilli* and *Bifidobacteria* species survived in cultures at pH 5 but

disappeared with increasing pH from 5 to 6 and then to pH 7. In contrast, *Cl. perfringens* disappeared with decreasing pH from 7 to 6 and then to 5. On either side of the optimum pH range, microorganisms undergo an increased lag phase.

In another competition experiment (Wang & Gibson, 1993), batch fermenters were inoculated with actively growing cultures of different bacteria and maintained at a pH from 3.5 to 7.0, to determine the effect of pH on bacterial growth. *B. infantis*, *E. coli*, and *Cl. perfringens* showed approximately equal growth rates at neutral pH (7.0), whereas, growth of Bifidobacterium species remained unaffected at lower pH. *B. infantis* demonstrated the ability to withstand the effect of acidic conditions at pH 5.0 and 4.5, but the same pH inhibited the growth of *E. coli* and *Cl. perfringens* completely.

The state and stages of life cycle of bacterial cells may be important regarding their sensitivity to pH changes. Young cells are more susceptible to pH changes than older and resting cells. Similarly an optimal pH range should not be accepted as an absolute determinant of growth since it may depend on a number of growth factors, such as the types of acid and salt present in the medium (Jay, 1996). For example, the growth of certain lactobacilli is permitted at lower pH with citric, hydrochloric, phosphoric and tartaric acids compared with acetic and lactic acids (Juven, 1976). Therefore, it is important to know about the acid that is responsible for the reduction in pH, to determine the rate of subsequent growth and minimal pH for a microorganism to initiate its growth. Chung and Geopfert (1970) reported a minimal pH of 4.05 for salmonella to initiate growth when HCl and citric acids were used, but 5.4 and

5.5 when acetic and propionic acids were used respectively. Similarly, *Alacaligenes faecalis* has been shown to grow over a wider range of pH in the presence of 0.2 M NaCl, than in the absence of NaCl or in the presence of 0.2M sodium citrate (Sherman & Holm, 1922). These authors noted similar effects for *E. coli*. This shows the ability of bacteria to adapt to their environment more easily in the presence of one substance (or acid) compared with another (or acid).

3.1.1.4 Relation of pH to the rate of absorption into the cell

An adverse pH may also affect the transport of nutrients into the cell, as bacterial cells tend to have a residual negative charge. Therefore, non-ionised compounds can enter cells, whereas, ionised compounds cannot (Jay, 1996). Moreover, intracellular reactions require different minerals as prosthetic groups to their enzymes and rates of different biosynthetic pathways may be determined by the rate of diffusion of such minerals through the cell membrane. Thus, transport processes across the cell membrane can also serve as pacemakers in changed physiological conditions (Metzler, 1977). Changes in pH may affect the absorption of various substrates or minerals and a lowered lumenal pH has been shown to play an effective role in mineral absorption, probably resulting from increased solubility. A higher solubility of calcium was reported in caecal contents of rats in the presence of a low pH (Younes et al., 1996). These authors reported a 5 to 6 fold increase in absorption of calcium and increased caecal absorption of magnesium after feeding the resistant starch. Such effects on the absorption of colonocytes may also be relevant to bacterial cells.

3.1.2 De novo enzymes synthesis or repression

The inhibitory modifications of enzyme activity by different mechanisms, as discussed above (sections 3.1.1), are concerned with existing enzymes participating in the metabolic reactions. In certain situations, the bacterial cells may regulate reactions by controlling the availability of enzymes for particular reactions under the stress of changed physiological conditions. This is usually accomplished by altering the rate of enzyme synthesis, as the rate of the reaction is directly proportional to the enzyme concentration at all substrate concentrations (Champe & Harvey, 1994).

The production of some enzymes is referred to as constitutive, implying that the enzyme is formed irrespective of the environmental conditions of the cell. For example, bacterial cells synthesise the enzymes required to catabolise glucose under all conditions of growth. Another group of enzymes, known as inducible enzymes, are often produced only in trace amounts and their synthesis is regulated by their need for a specific substrate. For example, when *E. coli* cells are grown in a culture containing lactose, without prior exposure to this substrate, they exhibit an appreciable lag phase before fermenting the lactose at a high rate. However, if cells are already grown in the presence of lactose, such cells will metabolise this disaccharide at almost the same rate as they degrade glucose (Stanier et al., 1986). When *E. coli* cells are cultivated in the presence of lactose, they induce a series of enzymes required for the catabolism of that disaccharide, otherwise repressed (Metzler, 1977). A comparison of the enzymatic composition of *E. coli* cells grown separately in glucose and lactose, revealed that the enzymes barely detectable in the glucose-grown cultures are present in appreciable concentrations in the lactose

cultures (Stanier et al., 1986). Such enzymes include an extracellular galactoside permease and an intracellular β-galactosidase. The former facilitates the entry of lactose into the cells and the latter hydrolyses the lactose into its constituent monosaccharides. As a result of the release of these monosaccharides, three other enzymes (galactokinase, transferase, epimerase) are induced to convert galactose into glucose derivative to initiate the Embden-Meyerhof pathway. Hence, a single compound lactose, induced two enzymes (one extracellular & other intracellular) directly, and three other intracellular enzymes indirectly as a consequence for further metabolism of galactose (Stanier et al., 1986).

The inductive or repressive status of protein (enzyme) synthesis leads to an alteration in the total number of available active sites, rather than influencing the efficiency of the existing enzyme molecules. The enzymes, which are induced and regulated through synthesis, are often those that are needed at only one stage of development or under selected physiological conditions. Such alterations in the protein (enzyme) synthesis are very slow (hours to days) compared to the instantaneous allosteric changes in the activity (Champe & Harvey, 1994).

The repression of enzymes may be affected through catabolite repression or end product repression. When bacterial cells are grown in cultures containing a rapidly metabolisable energy source, the increasing intracellular concentrations of ATP lead to the repression of enzymes required for the catabolism of less rapidly degrading energy sources. For example, when *E. coli* cells are actively involved in the degradation of glucose, synthesis of the

β-galactosidase, enzyme for the degradation of lactose, is repressed. This phenomenon is called catabolite repression, which is the basis of diauxic growth, a peculiar growth response to substrate mixtures. In many biosynthetic pathways the catalysing enzymes are repressed by the end product of a particular pathway. This phenomenon results in the regulation of the rate of a metabolic pathway by the intracellular concentration of its end products. The rate of such a pathway is higher when the end products are rapidly utilised in the further metabolism and vice versa (Stanier et al., 1986).

3.1.3 Substrate concentration

The present study tested different levels of substrate to evaluate any changes in *in vitro* fermentation due to increasing concentration of substrate. The intracellular and extracellular availability of bacterial enzymes can be influenced by the presence of a particular substrate during *in vitro* fermentation. For example, enzymes are often induced and synthesised in accordance with the saccharides available, enabling the bacteria to switch from one substrate to another as the diet changes (Salyers & Leedle, 1983; Stanier et al., 1986). Gut bacteria are capable of inducing required extracellular enzymes for a particular complex carbohydrate. This has been shown by feeding a particular carbohydrate for one week in human subjects (Daly et al., 1993; section 3.1.2).

Similarly, the reaction velocity of different intracellular and extracellular enzymes in microbial culture will be affected because the reaction rates of most of the enzymes are responsive to changes in substrate concentration. Generally, each biochemical reaction proceeds by forming an enzyme-substrate

(ES) complex, with an intermediate conversion to enzyme-product (EP) complex, which subsequently dissociates to enzyme and product. Although in many enzyme catalysed reactions, the equilibrium lies far to one side, and such reactions are irreversible, some are freely reversible. In this case, when concentrations of substrate and the product are at equilibrium, rates of forward and reverse reactions are about the same. In such a case, a metabolic pathway can keep going in the forward direction, only if the product is being removed rapidly by a subsequent reaction with another enzyme.

Normally the intracellular levels of many substrates are in the range of the K_m (substrate concentration at half maximum reaction velocity), of their respective enzymes. In the case of higher amounts, binding of a substrate molecule at one site on the allosteric enzyme may enhance the catalytic properties of the other substrate-binding sites demonstrating co-operativity between the active sites of a particular enzyme. This mechanism may operate in the initial hours of incubation where higher amounts of substrate may prompt an increase in the reaction velocity, bringing the concentration of substrate towards acceptable levels. However, this increase in the reaction velocity poses another problem in *in vitro* fermentation. It results in the higher accumulation of end products over a short period of time, which may trigger end product feedback inhibition.

3.1.4 End product inhibition

To co-ordinate numerous extracellular and intracellular metabolic processes, a bacterial cell actively regulates enzymatic activity through certain rapid mechanisms, other than those discussed above, without a significant time lapse. Only those bacterial enzymes which hold strategic branch points in the

complex network of intermediary metabolic pathways and affect flow rate of the metabolites through the entire pathway at the first step, are controlled through these immediate regulatory mechanisms (Stanier et al., 1986). Many enzymes are inhibited by compounds which have sufficiently close steric similarity to their respective substrates. Enzymes which have the ability to be inhibited or activated by compounds of low molecular weight with no close steric resemblance to their substrate are termed allosteric. In metabolic pathways, the enzyme that catalyses the first step in a particular series of reaction are characteristically allosteric in their nature. Most often the activity of the first enzyme unique to a particular metabolic pathway, is checked by end-product inhibition or in general terms by feedback inhibition. End product inhibition of a particular biochemical pathway is more common than any other effect on an enzyme. The end product of a metabolic sequence accumulates and turns off the enzyme needed for its own formation (Metzler, 1977). For example, in *E. coli* the conversion of fructose-6-phosphate into fructose-1,6-phosphate is subjected to allosteric inhibition of the enzyme, phosphofructokinase by the phospho-enol pyruvic acid, blocking the biosynthetic pathway to its fate of producing the pyruvic acid (Stanier et al., 1986).

The enzymatic activity in a chemical reaction may be slowed down or stopped by an intermediate product, exerting inhibitory force on the reaction. A product often inhibits more than one enzyme in a biosynthetic sequence. When the velocity of the reverse reaction is much less than that of the forward reaction, to maintain the equilibrium of the reaction, the K_m of the product has to be much lower than that of the forward reaction. In an equilibrated reverse

reaction, the product will have higher affinity, i.e. be more strongly bound, for the enzyme due to its low K_m value, in order to maintain the equilibrium. In such a situation, the product will remain tightly bound to the enzyme and since velocity of the reverse reaction is low, it will tend to clog the enzyme. Such product inhibition may sometimes slow down a whole metabolic pathway. This is a kind of 'one way safety valve' that turns off the flow in a metabolic pathway when the concentration of its product rises (Metzler, 1977). Thus, inhibition by the intermediate product or end product serves to coordinate the flow of substrate or intermediate molecules through a series of reactions with the needs of cell for the product of that particular pathway.

In summary, there are a number of the possible mechanisms regulating enzyme activity in closed *in vitro* batch cultures. Those with immediate effect include the amount of substrate, product and end product in cultures with large amounts of substrates. The control of synthesis or degradation of the particular enzymes may take effect after prolonged fermentation (Champe & Harvey, 1994).

At present very little is known about the effects of substrate levels (Mortensen et al., 1991; Barry et al., 1995; Stevenson et al., 1997b) and the possible effect of end product on *in vitro* fermentation of carbohydrates (Edwards et al., 1985). Therefore, it is important to develop a fermentation model for complex carbohydrates, which can evaluate the practicable amounts of substrates and their potential impact on SCFA production. The present study was designed to examine the *in vitro* effect of different amounts of substrate of a rapidly and a slowly fermenting carbohydrate. A rapidly fermenting carbohydrate, lactulose and slowly fermenting ispaghula were used in these experiments to model for different types of carbohydrates used in subsequent

studies in the present thesis. The impact of different levels of carbohydrates on *in vitro* fermentation of carbohydrates were noted.

3.1.3 Lactulose

Lactulose is a semi synthetic disaccharide, which is highly soluble in water. It is resistant to the digestive enzymes in the human small intestine, however, lactulose is readily fermented by bacteria in the large intestine. Lactulose differs from other indigestible carbohydrates due to its simple disaccharide structure, resulting in great variability of its fermentative properties. Bacterial fermentation of lactulose yielded higher ratio of n-butyrate compared with pectin and guar gum (Rémésy & Demigné, 1989). Although lactulose produced higher concentrations of acetate, another *in vitro* study confirmed that lactulose was rapidly fermented and produced a SCFA profile with n-butyrate predominance (Vince et al., 1990). Due to its rapid fermentation in caecum, lactulose produced high amounts of SCFA and, as a result significantly lowers caecal pH from 7.0 to well below 5.0 after 8 days of lactulose maintenance (Florent et al., 1985). Similar low pH was noted in *in vitro* study (Vince et al., 1990).

3.1.4 Ispaghula

Ispaghula (psyllium) is the husk (epidermis) of seeds from Plantago ovatum, and in lesser amounts from Plantago psyllium. Ispaghula is a concentrated mucilage made of hemicellulose in the form of a highly branched acidic

arabinoxylan (Sandhu et al., 1981). Ispaghula is a good source of 85 percent water-soluble viscous fibre. Ispaghula is slowly fermented and although, *in vitro* molar ratios of the SCFA for ispaghula vary, a consistent profile of the propionate predominance was demonstrated in different laboratories (Mortensen et al., 1991; Stevenson et al., 1997a).

3.2 Hypothesis

This study tested the hypothesis that with increasing amount of substrate, the increase in *in vitro* production of SCFA is not linear due to inhibition of fermentation.

3.3 Objectives

The objectives of this study were to determine;

1) the properties of a rapidly and slowly fermenting carbohydrate in an *in vitro* fermentation system.
2) the SCFA produced *in vitro* by different amounts of carbohydrates.
3) the inhibitory effect in *in vitro* fermentation with increasing amounts of substrates.

3.4 Methods

The specific procedures for this experiment are described in this chapter, whereas, general methods are detailed in the Chapter 2.

3.4.1 *In vitro* measurement of fermentation

1) A general description of the *in vitro* incubation system used in this study is given in the section 2.2. Metabolic inhibition or bacterio-static conditions may be

expected in *in vitro* batch cultures. Inhibition of fermentation with increasing amounts of substrate can be judged by two methods;

2) stopping the cultures at different time points.
3) using the increasing amounts of individual carbohydrates in cultures.

I have used both procedures in my study, to investigate this phenomenon.

3.4.2 Subjects used as donors of faecal inocula

Fresh faeces from five different healthy human subjects were used as source of fermenting bacteria for *in vitro* inoculation (aged between 30-56, all female). Although the target was to get a mix of subjects, only women subjects could provide faecal samples on the days of the study. Incremental portions of each substrate were fermented with faeces from subjects aged between 27-58 years, three men and two women, in the second part of the study (section 2.5 for a detailed criterion of the donors).

3.4.3 Fermentation procedure

Detailed general fermentation procedures are given in Chapter 2 (section 2.2). Lactulose and ispaghula (isolated portions 25, 50, 75 and 100mg) were used as the sole extraneous source of carbohydrate in these experiments. One control culture without any added substrate was used separately with each set of incubations to allow for the fermentation of the indigestible substrates in the original inoculum.

Incubation times of 0, 4, 8, 12 and 24 hours were used for the cultures with 100mg of the substrates. Since the 12 hour point was not practicable and also

did not provide any different information about the fermentation pattern in initial studies, beyond that provided by 8 & 24 hours points of incubation, 12 hours incubation cultures were excluded from further experimentation.
A 2 hours incubation time was substituted for further investigation to allow better characterisation of early events. Cultures of small portions of individual carbohydrates were stopped at 0, 2, 4, 8 and 24 hours to determine the initial rates of production, the extent of production at each time point and the maximum production of SCFA. This was to allow the prediction about the site of SCFA production in the large bowel.

The details of the analytical procedure and statistical analysis are the same as described in Chapter 2.

3.4.4 Data presentation

The values of net total SCFA from the fermentation of small isolated portions of lactulose and ispaghula were corrected to 100mg by multiplying with respective fractions (i.e. 25mg x4, 50mg x2, 75mg x4/3) to achieve theoretical values of SCFA that could be expected from a hypothetical 100mg of the substrate. The percent inhibition was calculated with values of SCFA from 25mg of the two substrates at 8 hours, assuming zero inhibition at the 25mg level of substrates.

3.5 Results

3.5.1 The *in vitro* fermentation of lactulose and ispaghula

Lactulose (100mg) was fermented rapidly, producing higher concentrations of SCFA in initial hours, whereas, ispaghula (100mg) was very slowly fermented, producing lower concentrations of SCFA even at 24 hours. Ispaghula did not

show any noticeable variation in the amount of SCFA with increasing incubation from 4 to 24 hours (Fig. 3.1).

Although higher concentrations of SCFA were produced by 100mg lactulose with increasing incubation from 8 to 24 hours, the gradient became progressively less linear after 8 hours of incubation (Fig. 3.1). The rate of fermentation in cultures of 100mg lactulose ($p < 0.02$, between 4 and 8 hours of incubation of lactulose) was significantly more rapid than in the case of 100mg ispaghula, where there was no significant increase with time. The increasing concentrations of the SCFA produced by the fermentation of 100mg lactulose at 24 hours were significantly higher from that at 8 hours ($p < 0.05$), but there was no significant difference between 8 and 12 hours of fermentation or between 12 and 24 hours of fermentation.

3.5.2 Fermentation of the incremental portions of lactulose

Since values of net total SCFA produced by fermentation of small portions of ispaghula did not vary appreciably, only values from fermentation of lactulose are reported (Fig. 3.2). There was no significant difference in SCFA production with increasing amounts 25mg to 75mg of lactulose at 2 and 4 hours (Fig. 3.2). In general, fermentation of different amounts of lactulose started losing linearity in production of SCFA with increasing amounts of the substrate after 8 hours of fermentation. There was no significant increase between the SCFA produced by the fermentation of different amounts of lactulose at 24 hours compared with that at 8 hour but there was a significant

difference between concentrations of SCFA at 8 hours and 24 hours in cultures

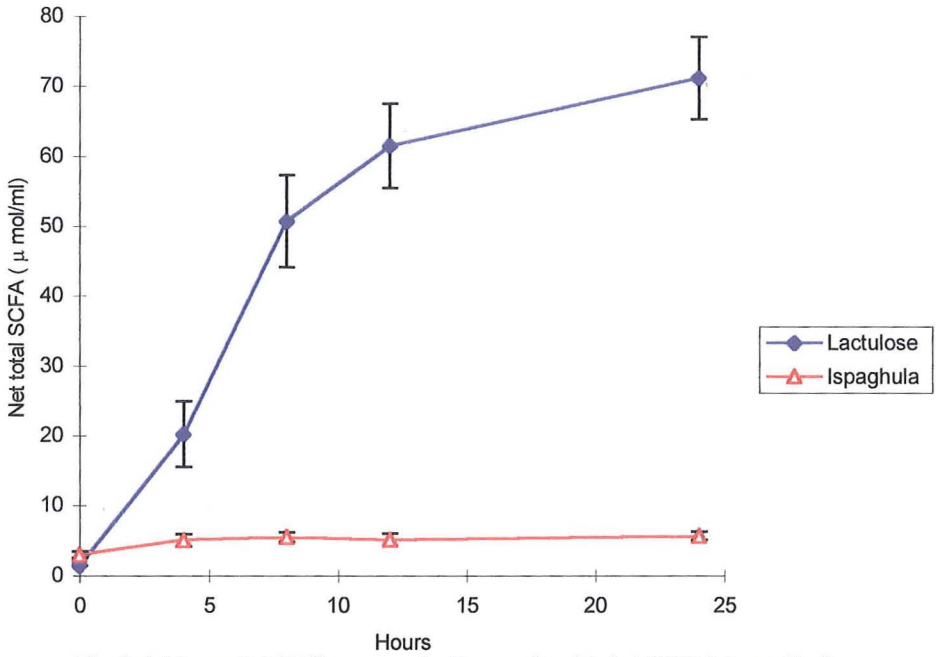


Fig 3.1 Mean (±SEM) concentrations of net total SCFA (μmol/ml) produced by the fermentation of 100 mg of ispaghula and lactulose with the human faecal bacteria.

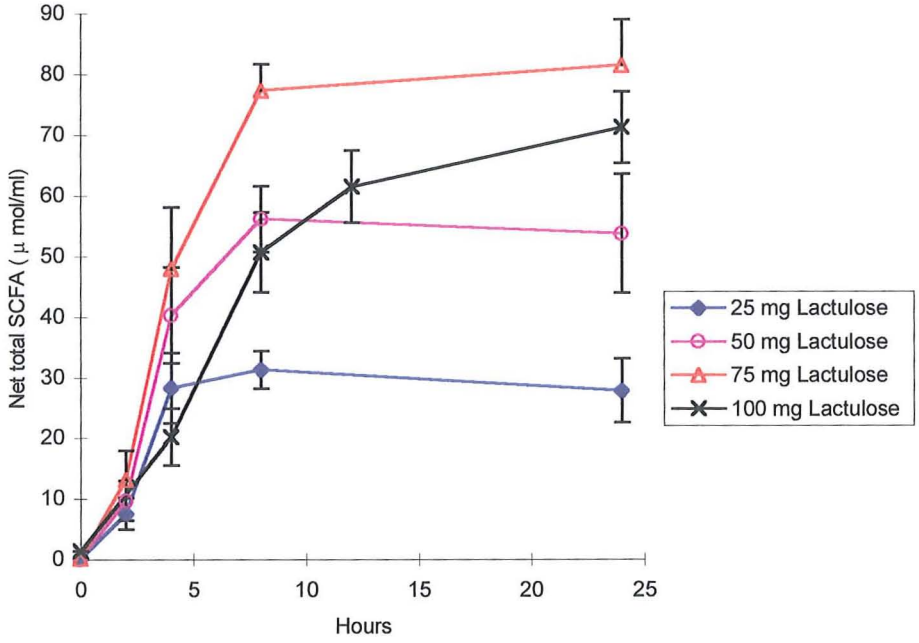


Fig. 3.2 Mean (±SEM) concentrations of net total SCFA (μmol/ml) produced by the fermentation of different amounts of lactulose with the human faecal bacteria.

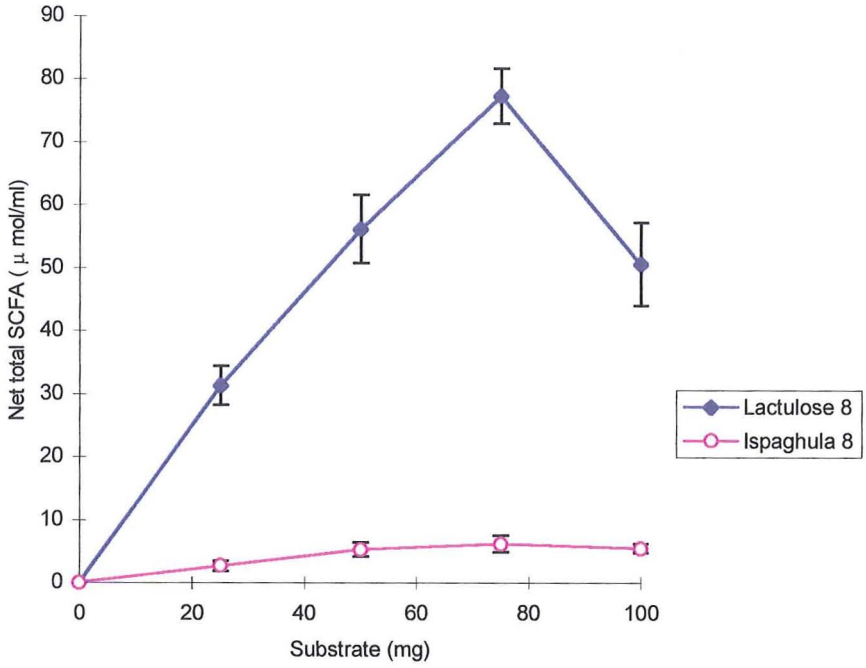


Fig. 3.3 Mean (±SEM) concentrations of net total SCFA (µmol/ml) produced by the fermentation of different amounts of lactulose and ispaghula with the human faecal bacteria at 8 hours.

of 100mg lactulose ($p < 0.05$). This increase in SCFA continued until the end of incubation, however, the rate of fermentation was not linear after 4 hours of incubation (Fig. 3.2), showing a flattening of the slope from 8 to 24 hours (Fig. 3.2). Therefore, net total SCFA produced at 8 hours point of incubation were used for further interpretation of the fermentation of two carbohydrates (Fig. 3.3). A rapid increase was noted in production of SCFA by the fermentation of different amounts of lactulose at 8 hours. There was a highly significant difference between the values of SCFA produced by the fermentation of incremental amounts of lactulose at 8 hours of fermentation ($p < 0.02$). Fermentation of 75mg lactulose produced significantly higher net total SCFA compared with that produced by the fermentation of 100mg lactulose ($p < 0.02$). However, production of SCFA continued in the 100mg lactulose culture and there was no significant difference between the cultures

of 75mg and 100mg of lactulose at 24 hours of fermentation. There was no significant difference between the SCFA produced by 50mg and 100mg lactulose at 8 and 24 hours of fermentation.

3.5.4 Inhibition of SCFA production by increasing amounts of substrate

Net total SCFA from the fermentation of incremental isolated portions of the lactulose and ispaghula were made up to values that could be expected from a hypothetical 100mg substrate by multiplying with respective fractions (25mgx4, 50mgx2, 75mgx4/3) to achieve theoretical values of SCFA that could be expected from fermentation of 100mg of the respective substrate, for comparison with the actual values of SCFA from 100mg of the substrate. Such theoretical values for the fermentation of lactulose showed that production of SCFA progressively decreased with the increasing amounts of lactulose at 4 to 24 hours of incubation (Fig. 3.4).

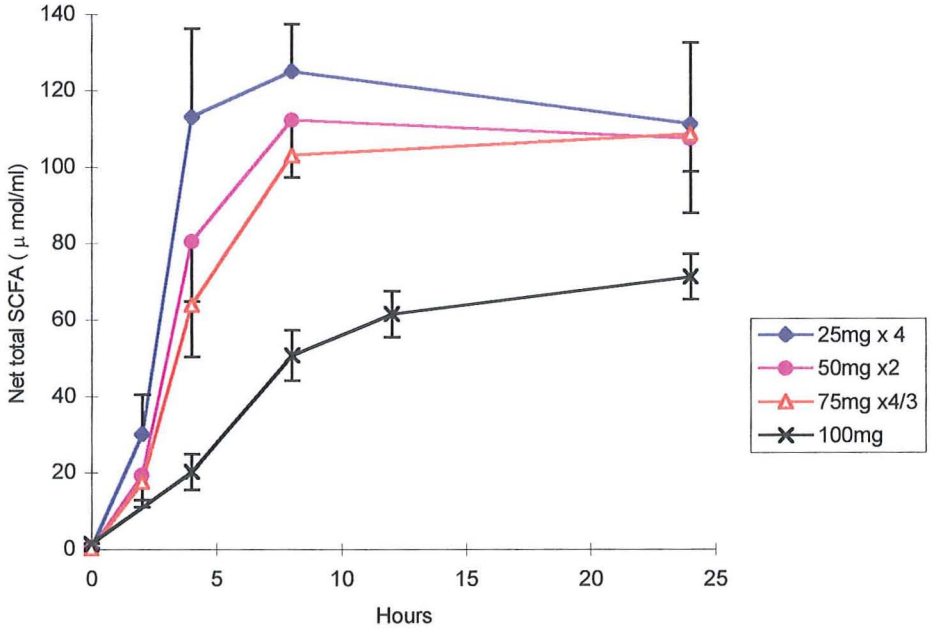


Fig. 3.4 Mean (±SEM) concentrations of net total SCFA (μmol/ml) produced by the fermentation of different amounts of lactulose (corrected for 100mg by multiplying with respective factors) with the human faecal bacteria.

The smallest quantity (25mg) of lactulose tested produced SCFA concentration (mean value 125.1μmoles/ml) at 8 hours, apparently reaching the maximum extent of fermentation at this time point (Fig. 3.2), showing no significant increase in net total SCFA with extended incubation. Thus, value of SCFA produced by fermentation of 25mg of lactulose at 8 hours of incubation, was selected for further interpretation, using it as the base level data for determination of any reduced SCFA accumulation in cultures of lactulose.

The impact of substrate concentration was judged by using theoretical values of SCFA that could be expected from a hypothetical culture of such composition. Theoretical concentrations of SCFA calculated from incremental portions of the two substrates, decreased progressively with the increasing amounts of substrate at 8 hours (Fig. 3.5).

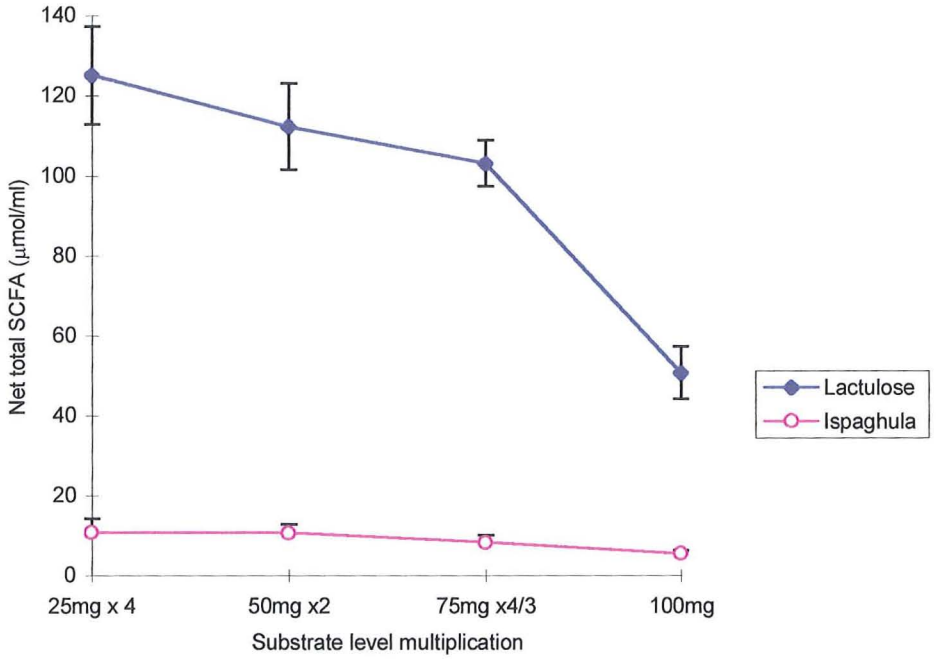


Figure 3.5 Mean (±SEM) concentrations of net total SCFA (μmol/ml) produced by the fermentation of small portions of lactulose and ispaghula (corrected to 100mg) at 8 hours with the human faecal bacteria.

The increase in production of SCFA was not linear with increasing amounts of substrate. SCFA production was significantly lower in culture of 100mg of these carbohydrates compared with the theoretical concentrations of SCFA (value from 25mgx4) of the respective substrates ($p < 0.02$; Fig. 3.5). Although there was no significant difference between the incremental portions from 25mg to 75mg made up to 100mg at 8 hours of fermentation, there was a significant difference 25mgx4 vs 100mg ($p < 0.02$), 50mgx2 vs 100mg ($p < 0.02$) and 75mgx4/3 vs 100mg ($p < 0.02$) at 8 hours of fermentation. A percentage inhibition in the production of net total SCFA was calculated for the increasing amounts of lactulose at 8 hours (Table 3.1).

Table 3.1 The concentrations expected from the fermentation of different amounts of lactulose at 8 hours and inhibition (%) of the fermentation.

Substrate (mg)	Net total SCFA (μmol/ml)	Inhibition (%)
25mgx4	125.1	-
50mgx2	112.3	10.2
75mgx4/3	103.1	22.6
100mg	50.7	60.2

The production of SCFA was significantly inhibited in cultures of increasing amounts of lactulose ($p < 0.02$). Fermentation of 100mg lactulose produced 60.2% less SCFA compared with the theoretically expected value for 100mg (25mg x4) at 8 hours (Table 3.1). A highly significant difference was noted between the percent inhibition at 50mg x2 vs 75mg x4/3 ($p < 0.02$), 50mg x2 vs 100mg ($p < 0.02$) and 75mg x4/3 vs 100mg ($p < 0.02$) at 8 hours (Table 3.1). Although a similar pattern was seen with ispaghula, showing 51.4% inhibition in production of SCFA by the fermentation of 100mg of ispaghula

compared with theoretical values (25mg x4) at 8 hours, the difference was not significant.

The fermentation of 100mg lactulose, although partially recovered from the inhibitory effects with the extended incubation at 24 hours, was still inhibited (42.2 %) compared with the theoretical values at 24 hours.

3.5.3 Fermentation of the two carbohydrates and pH

The pH markedly decreased with increasing amounts of lactulose as incubation progressed. A highly significant difference was noted in the reduction of pH between the cultures containing different amounts of lactulose at 8 and 24 hours ($p < 0.02$). There were significant differences between the pH in cultures containing 25mg to 100mg lactulose ($p < 0.05$) at 8 hours. However, the percent reduction in pH in cultures containing 50 and 75mg lactulose was not significantly different from that in cultures containing 100mg at 8 hours. Fermentation of 25 to 100mg lactulose did not show any significant difference in decreasing the pH within cultures between 8 and 24 hours of incubation (Fig. 3.6). Cultures containing different amounts of ispaghula showed no appreciable differences in the final pH from initial pH. Therefore, the pH in cultures of ispaghula was not further reported.

The reduction in pH increased linearly with increasing amounts of lactulose but linearity was lost at 100mg (Fig. 3.7)

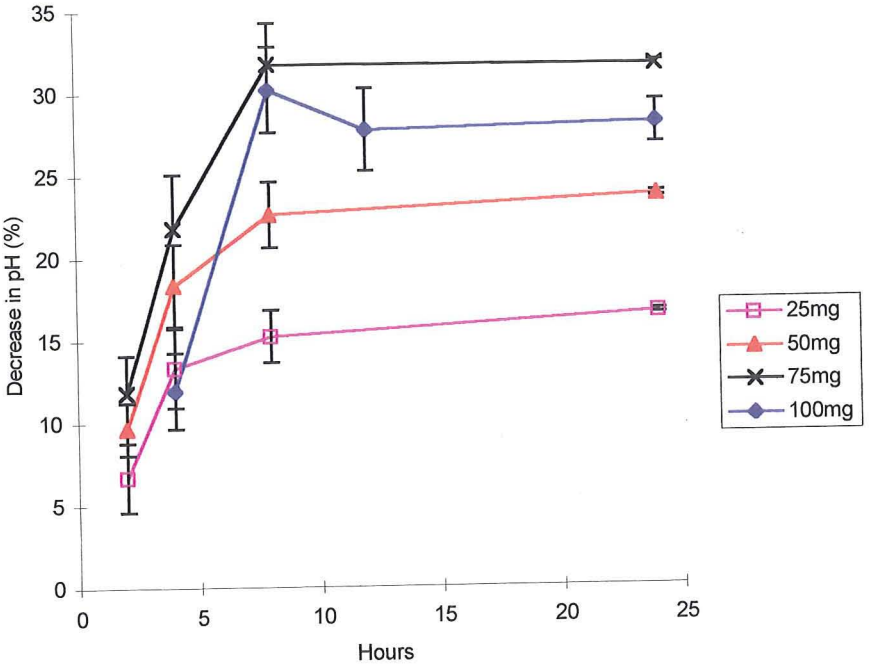


Fig. 3.6 The decrease in pH (%) by the fermentation of different amounts of lactulose with the human faecal bacteria.

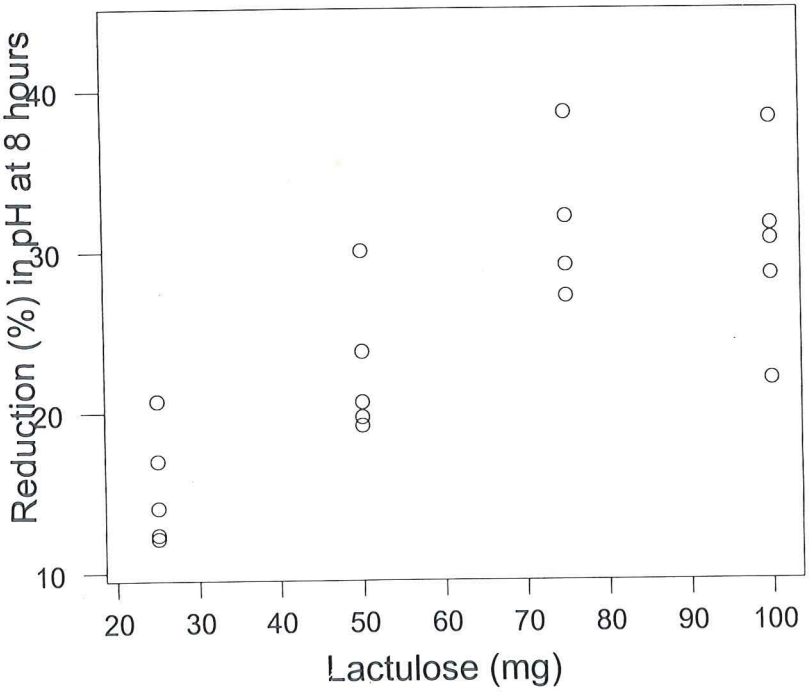


Fig. 3.7 Reduction in pH (%) with the increasing amounts of lactulose at 8 hours of *in vitro* fermentation with the human faecal bacteria.

3.6 Discussion

In this experiment, lactulose and ispaghula were fermented in accordance with earlier studies (Vince et al., 1990; Edwards & Rowland, 1992). Lactulose was fermented rapidly producing higher concentrations of SCFA with increasing hours of incubation from 0 to 24 hours. However, this increase in the production of SCFA was less linear from 4 to 24 hours of incubation for rapidly fermenting carbohydrate. In contrast, ispaghula was fermented slowly, producing lower concentrations of SCFA than lactulose and showing no appreciable change with increasing hours of incubation from 0 to 24 hours (Fig. 3.1). There may be important factors such as initial substrate concentration, pH changes and accumulation of fermentation products, influencing the rate of *in vitro* fermentation in these cultures.

3.6.1 The *in vitro* fermentation of the two carbohydrates

The fermentation activity was under the influence of the characteristics of the original inoculum in *in vitro* cultures in the initial hours in our study. However, it appeared that amount of substrate was the dominant influence on the fermentation profile in later hours until substrate became limiting or physiological conditions in cultures are changed for the bacterial activity. Of course, this would depend on the amount and fermentability of the substrate used. In the present study, almost all the substrate seemed exhausted with the fermentation of the 25mg of the rapidly fermenting lactulose, apparently reaching the maximum extent of fermentation by 8 hours of incubation (Fig. 3.2). In the case of smaller amounts such as 50mg & 75mg of the substrate, some substrate may be left for further fermentation after 8 hours of incubation. In a previous study of guar gum increased SCFA which occurred after

exhaustion of the guar gum was explained by the lysis and re-fermentation after 12 hours (McBurney & Thompson, 1987).

In the present study, the linearity in the rate of fermentation was lost with increasing amounts of the respective substrate at 8 hours of incubation. The increase in production of SCFA with increasing amount of substrate was not linear compared with the expected concentrations of SCFA of the respective substrates (Fig. 3.4). Accumulation of SCFA in cultures of 100mg lactulose was reduced by 60.2 % at 8 hours compared with the expected production of net total SCFA. Accumulation of SCFA in cultures of 100mg ispaghula was also inhibited at 8 hours, however, the effect was not statistically significant. In our study, although less linear rates of fermentation for lactulose were noted even with the longest incubation time (24 hours), the percent inhibition was reduced at 24 hours and it was not significant when the incremental portions were compared. In this context, 8 hours incubation seemed logical to be used for further interpretation.

3.6.2 Reduced SCFA production from the fermentation of lactulose

Small portions, 25mg, of lactulose were nearly completely fermented at 8 hours due to the rapid fermenting nature of the substrate. However, larger amounts showed an inhibition in the rate of fermentation (Fig. 3.2). This may be due to the occurrence of extreme changes (Edwards & Rowland, 1992) such as increase of acidity and lower pH, in cultures containing higher amounts of substrate. The decrease in rate of fermentation with the increasing amounts of lactulose may be an indication of an inhibition exerted by the changed physiological conditions in cultures of rapidly fermenting carbohydrates. Such changed physiological conditions may result in a complete transformation of

the microbial population in cultures of rapidly fermenting lactulose after 8 hours of incubation (Wang & Gibson, 1993; Edwards et al., 1985).

The production of SCFA progressively declined in our study with increasing amounts of lactulose, showing an extreme fall in the concentration of SCFA with the fermentation of 100mg lactulose (Table 3.1). The significantly higher net total SCFA in cultures containing 75mg compared with 100mg of lactulose at 8 hours of fermentation, showed that the fermentation of 100mg lactulose was slowed down and did not reach its full potential. On the other hand, net total SCFA in cultures of 100mg was not significantly different from that in cultures of 75mg lactulose at 24 hours of fermentation. This showed that the fermentation of 100mg lactulose continued slowly and approached the values for 75mg lactulose after 24 hours of fermentation. This finding is in agreement with previously reported studies (Mortensen et al.,1991; Barry et al., 1995; Stevenson et al., 1997b).

There was no significant difference in the SCFA produced by 50mg compared with 100mg lactulose at 8 and 24 hours of fermentation. Thus, we do not lose any SCFA, even using 50mg instead of 100mg lactulose. This suggested that use of 50mg substrate in *in vitro* could be an ideal amount in the case of rapidly fermenting carbohydrate. In another *in vitro* study, the production of SCFA was almost identical whether the substrate, monosaccharides or lactulose, was added in concentrations of 100 or 300mmol/l (Mortensen et al., 1988). This showed that amount of substrate is an important determinant in the concentrations of SCFA produced by the fermentation of a particular substrate. A linear increase in the SCFA production was reported with the

substrate concentration up to 30mg/ml of culture in 24 hours incubation, but this linearity was lost with higher concentration of substrates (Mortensen et al., 1991).

In another *in vitro* study, relatively less SCFA were produced with increasing amounts of substrate (Barry et al., 1995). This was a complimentary experiment during an inter-laboratories study, using 50, 100 and 200mg of pectin per 10 ml of the culture. These authors found higher but less linear production of SCFA with the increasing amounts of substrates. In addition, these authors noted that an increased amount of substrate led to a reduced rate of fibre degradation. Similarly, a recent study showed that the rate of fermentation is inversely dependent upon the substrate mass (Stevenson et al., 1997b). These authors used 25, 50, and 100mg of pectin and reported a proportional increase in SCFA production after 24 hours of incubation when less substrate was added to culture (Stevenson et al., 1997b). This indicated an inverse relation between the substrate mass and the SCFA production. Although these authors suggested accessibility of the substrate, as the likely cause, this may not be important with highly soluble and rapidly fermenting pectin, and changing physiological conditions may be more important. The accessibility of the substrate, however, may be a factor with higher amounts of slowly fermenting carbohydrates such as ispaghula, as noted in our experiment.

The amount (Barry et al., 1995; Stevenson et al., 1997b) and consequently, the rate of fermentation of a particular substrate appeared to be important in *in vitro* fermentation procedures. In the present study, a loss of linearity was noted with much smaller amounts than those reported by Mortensen et al., (1991). This may be due to some difference in the fermentation method which is not very clear.

The lack of an absorptive epithelial surface in *in vitro* fermentation models leads to the accumulation of excessive amount of SCFA and other end products. The accumulating organic acids may change the physiological conditions in cultures and may lead to a transformation in the bacterial activity. For example, their accumulation may cause a depression in the pH of cultures. Such a depression in pH was noted also in the present study with the increasing amount of rapidly fermenting lactulose (Fig. 3.6). Due to extreme changes in cultures of lactulose the bacterial population may undergo a corresponding change in the metabolic pathways, or it may lead to bacteriolysis with prolonged time.

3.6.2.1 Feed back Inhibition

In the case of high amounts, binding of a substrate molecule on the allosteric site of the respective enzyme (refer to section 3.1.3) may enhance the rapid degradation of the substrate. Probably this may be true in the initial hours of incubation in the present study where higher amounts of the substrates may prompt an increase in the reaction velocity, bringing the concentration of substrate towards acceptable levels. But this increase in the reaction velocity leads to the high accumulation of end products in the fermentation vessels in a very short period of time. The only way that an enzymatic sequence could keep going in the forward direction, if an intermediate product or end product, was rapidly removed by a subsequent reaction with a second enzyme.

In contrast to in vivo fermentation, there was no absorptive epithelial surface in this *in vitro* fermentation models, resulting in the accumulation of excessive amount of SCFA and other end products. Such accumulating organic acids and other end

products, may exert the end product feedback inhibition in an allosteric fashion (Champe & Harvey, 1994; Metzler, 1977) on the intracellular and/or extracellular enzymes, thus affecting the different fermentation reactions in *in vitro* cultures (for details section 3.1.4). The changed physiological conditions and accumulating organic acids would reduce the subsequent SCFA production in *in vitro* cultures. However, it is not clear how such changes would inhibit carbohydrate degradation. Indeed the normal problem with rapid fermentation is the excessive accumulation of intermediary products such as lactate. Therefore, it is not very likely that SCFA accumulation will inhibit carbohydrate fermentation directly.

3.6.2.2 Low pH and other regulatory mechanisms

Higher concentrations of SCFA were noted in cultures of lactulose in the present study and there was a corresponding decline in the pH with the increasing amounts of lactulose in cultures (Fig. 3.6). The greatest decrease was noted in pH at 8 hours in all cultures. There was no significant difference in the reduction of pH between 8 and 24 hours in cultures containing 25 to 100mg of lactulose (Fig. 3.6).

The bacterial population may undergo a corresponding change, such as bacteriolysis and re-fermentation (McBurney & Thompson, 1987), due to such extreme changes in pH of cultures containing rapidly fermenting lactulose. Such low pH has been implicated in different metabolic pathways or change in microbial populations (Edwards et al., 1985; Wang & Gibson, 1993). Although apparently low pH seemed the main effector in the inhibition of lactulose fermentation in the present study, it is difficult to separate the effect of pH from other factors, as other factors at the same pH may be very important (Sherman & Holm, 1922; Chung & Geopfert, 1970; Juven, 1976) and such factors may play a significant role in the varying abilities of organic acids as growth inhibitors. A low pH may have favoured other metabolic routes

through requirements of optimal pH for different necessary enzymes, thus re-routing the reactions in cultures. In this way, different metabolic pathways may have utilised the substrate or organic acids already present in cultures, producing different metabolites (Jay, 1996). Consequently, there may be a decrease in the SCFA concentration, resulting in an increased pH with extended incubation (Fig. 3.6). Therefore, the increase in pH noted at 24 hours of incubation may be a function of re-fermentation of SCFA in other metabolites. Other effects of low pH such as effects on the metabolic pathways and cell transport are discussed in the introduction to this chapter. Such effects could not be examined in this simple *in vitro* model.

3.6.3 Fermentation of slowly fermenting ispaghula

Ispaghula showed an insignificant reduction in the production of SCFA at 8 hours. There was no marked difference in fermentation with increasing amounts of the substrate up to 4 hours of fermentation and a linear increase was noted in the SCFA production with 25-75mg ispaghula. Although there was no apparent inhibition with 25-75mg substrate at 24 hours of incubation, the rate of fermentation of 100mg ispaghula was not linear even at 24 hours of incubation. The final pH was also not appreciably changed from initial pH. The bulking properties of higher amounts of ispaghula may hinder the accessibility of the fibre to fermenting bacteria.

3.7 Conclusion

In this study of the *in vitro* model, there was an accumulation of SCFA and organic acids in culture vessels through rapid fermentation of large amounts of lactulose, resulting in a lowered pH. A reduced SCFA production was noted with the increasing amounts of lactulose. No evidence for the exact mechanism was identified but decreasing pH may have a possible role. The impact of carbohydrate level and catabolic repression could not be separated in this study. However, this study allows

a proposal for the best substrate to be used in such models to avoid any artefactual decrease in SCFA production. There was no significant difference in the SCFA produced by 50mg compared with 100mg lactulose at 8 and 24 hours of fermentation, showing the possibility of obtaining similar results, using 50 to 100mg lactulose. As previous studies have indicated that lower amounts do not give reproducible results (Mortensen et al., 1991), 50mg of substrate would therefore be the most appropriate amount for such rapidly fermented materials in *in vitro* models such as this.

Although all these experiments were performed concurrently and information from one experiment could not be taken in account in the next experiment, in the next chapter mixture of lactulose and ispaghula is described taking the reduced production of SCFA into account.

CHAPTER 4

In vitro interaction of two indigestible carbohydrates

4.1 Introduction

In this chapter, I will discuss a study of the *in vitro* fermentation of mixtures of two indigestible carbohydrates with different fermentation properties, and their interactive effect in combination. Most studies of fermentation have considered a single source of carbohydrate. However, it is likely that when two carbohydrates of different physical properties are combined, the final fermentation characteristics will result not simply as an addition of the fermentation of each but from an interaction of the properties of the two component carbohydrates. Indigestible carbohydrates differ in their physicochemical characteristics, which can result in great variability in their fermentative properties. This difference in their physicochemical characteristics is due to the different sugar components, the bonds between individual sugar residues and the final three dimensional structure.

The rates of production, amounts and types of SCFA are dependent on the type and chemical nature of the substrate fermented by colonic bacteria. For example, starch (Englyst et al., 1987), resistant starch (Englyst & MacFarlane, 1986), oat bran (McBurney & Thompson, 1987) and lactulose (Vince et al., 1990) produced proportionally more n butyrate during fermentation, whereas ispaghula (Edwards et al., 1992a), gum arabic, tragacanth, xanthan, Gellan (Adiotomre et al., 1990), guar gum (Adiotomre et al., 1990; McBurney & Thompson, 1987), arabinogalactan (Englyst et al., 1987) produced proportionally more propionate.

Some carbohydrates, such as ispaghula, are slowly fermented, resisting bacterial degradation in the human gut (Edwards & Eastwood, 1992; Morteau

et al., 1994), whereas others including lactulose and gum arabic, are rapidly fermented (Vince et al., 1990). The rate of fermentation determines the fate of SCFA *in vivo*. In the case of rapidly fermenting carbohydrate, for example lactulose which is fermented in the caecum, SCFA are readily absorbed in the proximal colon and become available to the body. In the case of slowly fermenting carbohydrates, for example ispaghula which is fermented throughout the large intestine, SCFA may either be absorbed at a more distal site or they may escape in faeces (Edwards et al., 1992a).

In Chapter 1, the health benefits of fermentation were discussed. It is unlikely that any one indigestible carbohydrate will in isolation provide optimal conditions for health such as higher SCFA and low pH at more distal sites in the human gut. Rather a more logical approach is to study the effects of combining different carbohydrates. It is important to evaluate the fermentation of mixtures of complex carbohydrates and their potential impact on SCFA production. At present very little is known about the effects of mixtures of carbohydrates during fermentation (Washington et al., 1998). The present study was designed to examine the effect of a slowly fermenting carbohydrate on the fermentation of rapidly fermenting carbohydrate *in vitro*. I selected lactulose, a very rapidly fermenting carbohydrate, in combination with slowly fermenting ispaghula to determine the impact on fermentation of carbohydrates in mixtures. This choice of carbohydrates would also provide a butyric acid predominant carbohydrate and a propionic acid predominant carbohydrate. The known knowledge of the fermentation of these individual carbohydrates is discussed below;

4.1.1 Ispaghula

Ispaghula husk, the epidermis of seeds of Plantago species, is a concentrated mucilage made of hemicellulose in the form of a highly branched acidic arabinoxylan (Sandhu et al., 1981). Ispaghula husk is a good source of soluble viscous fibre. The component polysaccharides make viscous solutions over a wide range of concentrations. Being a structural component of the plant, ispaghula forms a matrix, when hydrated, and this matrix resists hydrolysis (Washington, 1998). Similarly, Ispaghula, being a very slowly fermenting substrate, resists the degradation by colonic bacteria in the human gut (Edwards & Eastwood, 1992; Marteau et al., 1994). In rats, ispaghula was fermented throughout the colon and increased the volume of colonic contents, resulting in increased stool weight (Edwards & Eastwood, 1992) and faster transit of digesta through the gut. Degradation of ispaghula is usually 85-100% (Hill 1995b; Prynne & Southgate, 1979). Ispaghula showed paradoxical properties of treating constipation (Kumar et al., 1987) and improving chronic diarrhoea (Qvitzau et al., 1988). These contrasting effects are due to its property of increasing the volume and viscosity of gut contents, and ultimately increasing stool output because of its high hemicellulose content (Williams & Olmsted, 1936) which holds a lot of water, approximately 7g per g fibre (Personal communication-Dr C A Edwards). As discussed previously, ispaghula has a consistent SCFA profile of propionate predominance (Bovlquin et al., 1993; McBurney & Thompson, 1987; Mortensen et al., 1988; Mortensen et al., 1991).

4.1.2 Lactulose

Highly water soluble lactulose, although resistant to the digestive enzymes in the human small intestine, is rapidly fermented by the colonic bacteria. Lactulose, compared with pectin and guar gum, yielded a higher ratio of n-butyrate after bacterial fermentation (Rémésy & Demigné, 1989; Vince et al., 1990), although Wang & Gibson (1993) showed a higher acetate profile. Due to rapid fermentation of lactulose in the caecum, high production of SCFA significantly lowered caecal pH (Florent et al., 1985). Rapid fermentation of lactulose also produces gases which may speed up intestinal transit. Another factor, which contributes to the speed of transit through the gut, is the retention of fluid by lactulose in the small intestine. Lactulose acts as an osmotic laxative if intake exceeds the fermentation capacity of the colonic flora (Read et al., 1980). This limits the use of large amounts of lactulose *in vivo*. Rapid fermentation in *in vitro* cultures increases the production of gases, improves anaerobic conditions in cultures and also stimulates the growth of new bio-mass if the fermentation is taking place in the presence of an abundant source of nitrogen. Lactulose was used as a marker of mouth to caecum transit time because of its rapid fermentation in the caecum (Levitt et al., 1987; Anderson et al., 1981; Zavoshy, 1998).

4.1.3 Expected effects of carbohydrate interaction

The physical properties of carbohydrates in a mixture can be very important under *in vitro* and *in vivo* conditions. Although influence of the changed physiological conditions on SCFA production is a very important factor in *in vitro* cultures (Chapter 3), it may not be as effective *in vivo*. In the normal gut, the organic acids and other end products are rapidly cleared by the colonic

mucosa and normally there is no accumulation of the end products. Although lactulose significantly reduced colonic pH due to accumulation of acidic end products through its rapid fermentation (Bown et al., 1974), the changed physiological conditions are unlikely to a sufficient extent to reduce the rate of colonic fermentation over a prolonged time period. Therefore, *in vivo* conditions we expect that the physical properties of the component carbohydrates will be more effective in the interaction of the carbohydrates in mixtures. In this regard, the most important factor is the physical accessibility of the substrate to the enzymes.

A slowly fermenting carbohydrate such as ispaghula could interfere with the fermentation of a rapidly fermenting carbohydrate such as lactulose in a mixture by hindering its accessibility for bacterial enzymes. In contrast, a rapidly fermenting carbohydrate may inhibit or stimulate the bacteria which could ferment a slowly fermenting carbohydrate. For example, the rapid fermentation of lactulose stimulates the growth of new bacterial mass which may result in an increase of bacterial cells with more efficient fermentative action on the slowly fermenting ispaghula.

4.1.4 The fermentation and interaction of carbohydrates in mixtures

As there are many difficulties in measuring fermentation, especially the quantitative determination of SCFA production in humans (Chapters 2), I adopted the *in vitro* fermentation model which is described in detail in previous two chapters. Similar *in vitro* fermentation models have been used in different studies for quantitative determination of SCFA produced, and

prediction of other fermentation characteristics of different indigestible carbohydrates (McBurney & Thompson, 1987; McBurney & Thompson, 1989; Adiotmre et al., 1990). However, such an *in vitro* fermentation model has its own limitations which may have an impact on the fermentation reactions in cultures (detail in Chapter 3). Despite this limitation, such model is useful for the quantitative prediction of the SCFA produced by the indigestible carbohydrates and its usefulness could be enhanced if such limitations have been taken in account. This is now possible because of the study presented in the previous chapter.

4.2 Hypothesis

In this study, I tested the hypothesis that a slowly fermenting carbohydrate affects the fermentation of a rapidly fermenting carbohydrate using two carbohydrates, ispaghula and lactulose, with extremely different fermentation characteristics. Both carbohydrates were tested in isolation and in combinations of different proportions.

4.3 Objectives

The objectives of this study were to determine;

1) the fermentation properties of a rapidly and a slowly fermenting carbohydrate in an *in vitro* fermentation system.
2) the SCFA produced *in vitro* by mixtures of these two carbohydrates
3) the additive or interactive effect of one carbohydrate on the other in mixture during *in vitro* fermentation.

4.4 Methods

The specific procedures for this experiment are described in this chapter, whereas, general methods are detailed in Chapter 2.

4.4.1 *In vitro* measurement of fermentation

A general description of the *in vitro* incubation system used in this study is given in Section 2.2. The impact of the interaction of the fermentation of two carbohydrates can be judged by two methods;

1) stopping cultures at different time points.
2) using mixtures containing the component carbohydrates in different ratios in cultures.

I have used both procedures in my study, to investigate this interaction between the two carbohydrates (detailed discussion of the method is given in Chapter 2).

4.4.2 Subjects used as donors of faecal inocula

Fresh faeces from human subjects were used as a source of fermenting bacteria for *in vitro* inoculation (age and gender details are given in section 3.4.2).

4.4.3 Fermentation of mixtures of substrates in the cultures

Details of the fermentation procedure are the same as described in Chapter 2. Lactulose and ispaghula were used as the sole extraneous source of carbohydrate in these experiments. Ispaghula and lactulose were incubated individually on their own and in combinations of 25:75, 50:50 and 75:25 (Lactulose: Ispaghula) making a total 100mg of substrate in each culture. A control culture, without carbohydrate substrate, was incubated with each set of cultures to allow for fermentation of endogenous carbohydrates in faeces.

Cultures were stopped at different time points of 0, 4, 8, 12 and 24 hours to determine the initial rates of production, the extent of production at each time point and the maximum production of SCFA.

Data from fermentation studies of smaller portions of the two substrates (refer to section 3.4.3) were used in this study. In this second set of experiment separate 25, 50 and 75mg portions of ispaghula and lactulose were fermented. Values of net total SCFA from the fermentation of 25mg portions of lactulose and ispaghula were used to calculate the theoretical values of SCFA that could be expected from a hypothetical 100mg mixture of such composition. Cultures were stopped at 0, 2, 4, 8 and 24 hours (section 3.4.3). The procedure and calculation for determination of SCFA by GLC were same as described in the Chapter 2 of general methods. Statistical analysis were performed as detailed in section 2.14.

4.5 Results

4.5.1 Fermentation of individual carbohydrates

4.5.1.1 Net total SCFA

Values of net total SCFA were used to characterise the fermentation pattern of the two individual carbohydrates in this experiment. Higher concentrations of net total SCFA showed that 100mg lactulose was fermented significantly more rapidly than ispaghula. Fermentation of lactulose showed a linear increase in the production of net total SCFA up to 8 hours, but then the production progressively lost its linearity (Fig.4.1). The increasing concentration of SCFA produced by the fermentation of 100mg lactulose were significantly higher at 8

hours compared with that at 4 hours ($p < 0.02$) and similarly at 24 hours compared with that at 8 hours ($p < 0.05$). There was no significant difference in the SCFA in cultures containing 100mg lactulose at 8 and 12 hours, and at 12 and 24 hours of fermentation (Fig. 4.1). In contrast, ispaghula did not show appreciable change in total SCFA with increasing incubation, exhibiting the property of very slow fermentation over 24 hours. Production of SCFA by ispaghula between 4 and 24 hours of faecal incubation were practically the same (Fig. 4.1). Patterns of fermentation were described by plotting net total SCFA produced at different time points, against the amount of the lactulose in the substrate mixture (Fig. 4.2).

Although production of net total SCFA increased at 4 hours compared with 0 hours of incubation, there were no differences with increasing amounts of lactulose in the substrate mixtures at 4 hours of incubation. All the substrates produced the same level of net total SCFA irrespective of the composition of substrate mixture at 4 hours (Fig. 4.2). These cultures started showing increased production of net total SCFA with increasing amounts of lactulose in substrate mixtures after 4 hours of incubation. Thus the differentiating changes in net total SCFA, corresponding to the composition of the substrate mixtures, were seen at 8 hours of incubation for the first time.

4.5.1.2 Acetate

Lactulose was rapidly fermented producing significantly higher concentrations of acetate than ispaghula and control cultures at 4 to 24 hours of incubation (p

< 0.02 at 8 and 24 hours; Fig. 4.3). Ispaghula showed

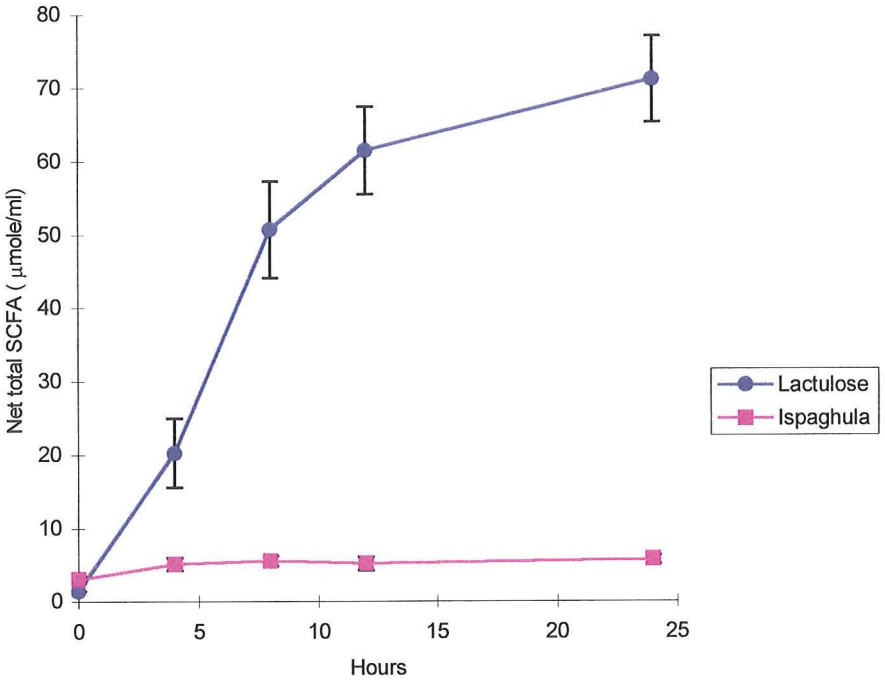


Figure 4.1 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of lactulose and ispaghula with the human faecal bacteria.

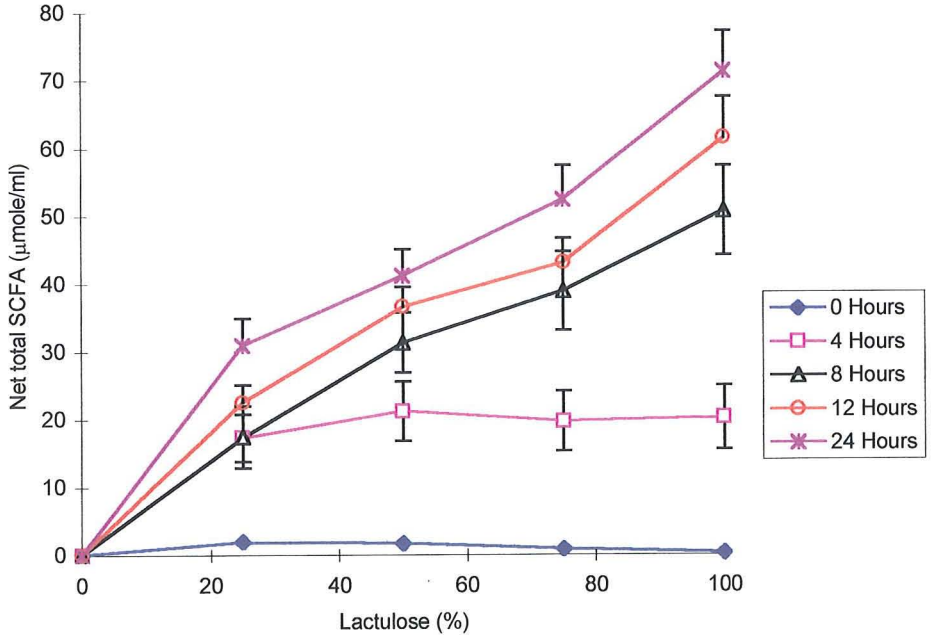


Fig 4.2 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of lactulose in different combinations with ispaghula with the human faecal bacteria.

higher concentrations of acetate at 0 hours because ispaghula was found to have a small amount of acetate present before fermentation. Lactulose produced significantly higher acetate ratios compared with ispaghula at 24 hours ($p < 0.02$). The acetate ratios by lactulose peaked at 8 hours and then declined until 24 hours. Acetate ratios for ispaghula showed a gradual decline with increasing fermentation (Fig.4.4).

4.5.1.3 Propionate

The amount of propionate was significantly higher in cultures containing lactulose compared with cultures of ispaghula at 8 and 24 hours ($p < 0.02$), however, ispaghula did not produce markedly different propionate compared with the control cultures at any time (Fig. 4.5).

There was no significant difference in the ratio of propionate between ispaghula and lactulose at 8 and 24 hours. The ratios of propionate produced by lactulose decreased progressively with increasing incubation, these ratios were not significantly different at 24 hours compared with that at 8 hours (Fig. 4.6).

4.5.1.4 n-Butyrate

Fermentation of lactulose produced significantly higher concentrations of n-butyrate than cultures of ispaghula ($p < 0.02$) at 24 hours. Ispaghula did not produce appreciably different concentrations of n-butyrate from control cultures at any time (Fig. 4.7).

There was a corresponding increase in the ratio of n-butyrate (Fig. 4.8) with declining ratio of acetate (Fig. 4.4)in cultures of lactulose from 8 to 24 hours.

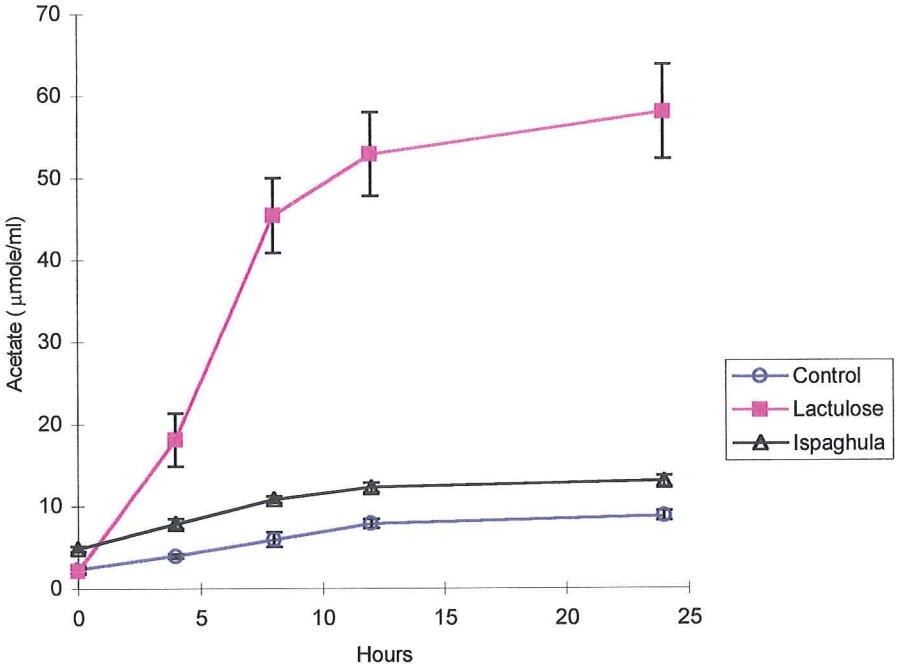


Fig 4.3 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the separate fermentation of 100 mg of lactulose and ispaghula with the human faecal bacteria.

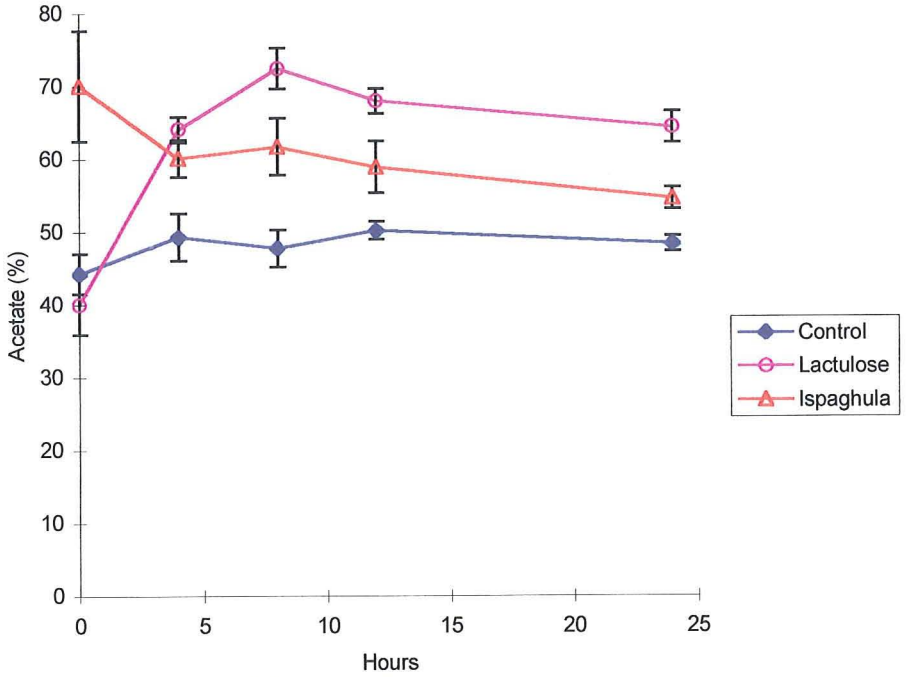


Fig 4.4 Mean (±SEM) acetate percent of total SCFA produced by the separate fermentation of 100mg lactulose and ispaghula with the human faecal bacteria.

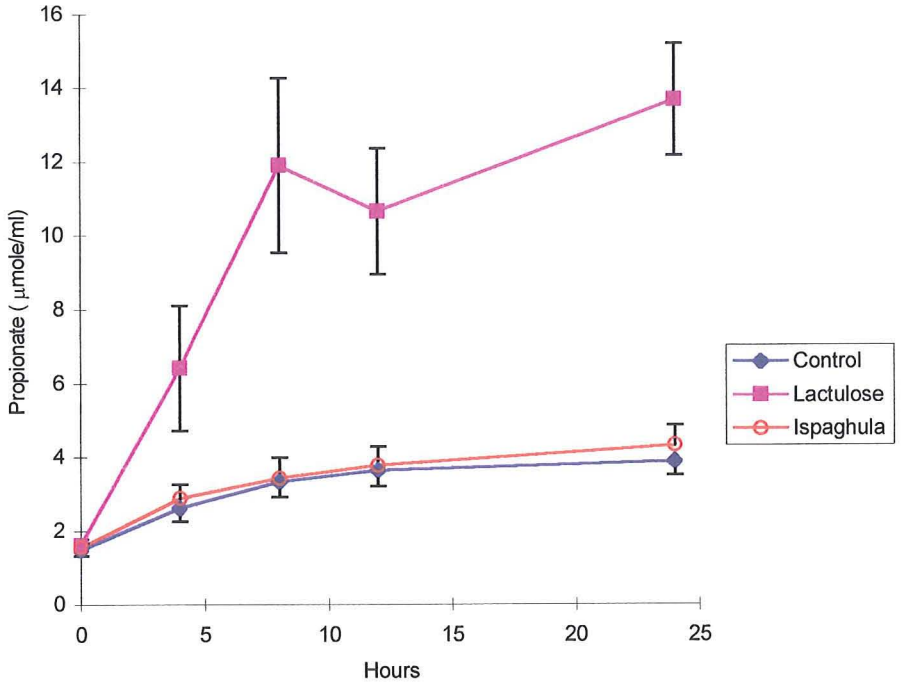


Fig 4.5 Mean (±SEM) concentrations of propionate(µmole/ml) produced by the separate fermentation of 100 mg of lactulose and ispaghula with the human faecal bacteria.

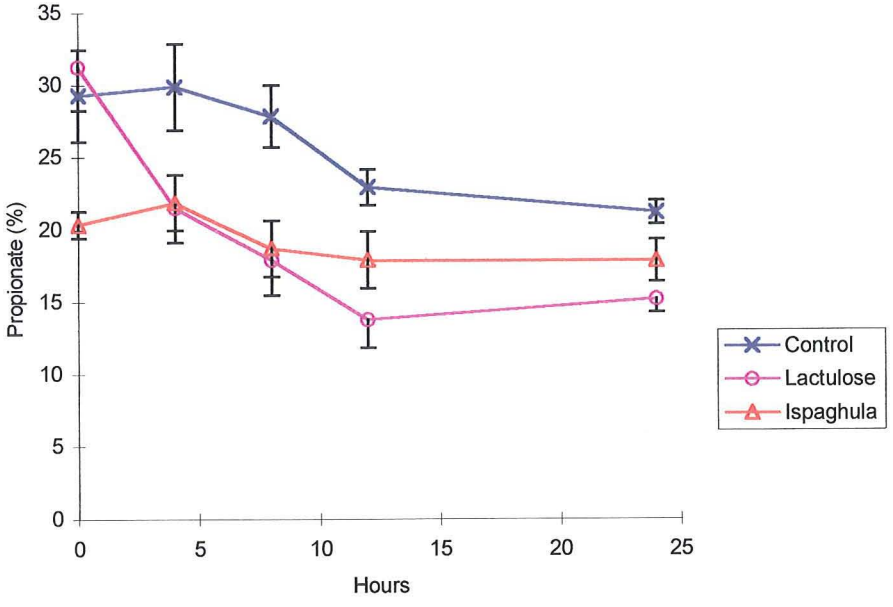


Fig 4.6 Mean (±SEM) propionate percent of total SCFA produced by the separate fermentation of 100mg lactulose and ispaghula with the human faecal bacteria.

Fermentation of lactulose produced significantly lower ratio of n-butyrate compared with propionate at 8 hours ($p < 0.05$), this fermentation produced

ratio of n-butyrate which was higher but not significantly different from the ratio of propionate at 24 hours of fermentation (Fig. 4.6, 4.8). Ratios of n-butyrate were significantly higher in cultures containing lactulose compared with ispaghula at 24 hours of fermentation ($p < 0.02$). Ispaghula did not show an appreciable change in the ratios of n-butyrate with increasing fermentation, rather this ratio of n-butyrate was significantly lower compared with the control cultures at 24 hours of fermentation ($p < 0.02$). Ispaghula produced significantly lower ratios of butyrate compared with propionate at 8 and 24 hours ($p < 0.02$; Fig. 4.6 & 4.8)

4.5.2 Fermentation of lactulose in combination with ispaghula

4.6.2.1 Net total SCFA

All substrates produced similar net total SCFA irrespective of the composition of the substrate mixtures at the 4 hours time point. Significant differences were noted in mixtures at 8 and 24 hours of fermentation ($p < 0.05$ & $p < 0.02$ respectively) and a corresponding increase was noted in the production of net total SCFA with the increasing amounts of lactulose in substrate mixtures for the first time (Fig. 4.2 & 4.9).

Mixture with 50mg of each substrate produced net total SCFA which were not significantly different compared with 25mg and 75mg lactulose in combination with ispaghula, these values in mixture containing 50mg of each component carbohydrates were significantly different from 100mg of ispaghula ($p<0.02$) and 100mg of lactulose at 8 hours ($p<0.05$). Mixtures containing either 75mg lactulose or 75mg ispaghula in combination with 25mg complimentary

carbohydrate did not produce significantly different net total SCFA from 100mg of the respective major constituting carbohydrate.

4.5.2.2 Acetate

Acetate production was significantly lower ($p < 0.02$) in mixtures containing 25mg ($p < 0.02$) and 50mg ($p < 0.05$) of lactulose than cultures containing 100mg lactulose but there was no significant difference between the concentrations of acetate produced by mixture of 75mg lactulose and 25mg ispaghula compared culture containing 100mg lactulose at 8 hours of fermentation. In contrast, the mixtures containing 25mg lactulose and 75mg ispaghula produced significantly higher acetate than 100mg ispaghula at 8 hours ($p < 0.02$; Fig. 4.10).

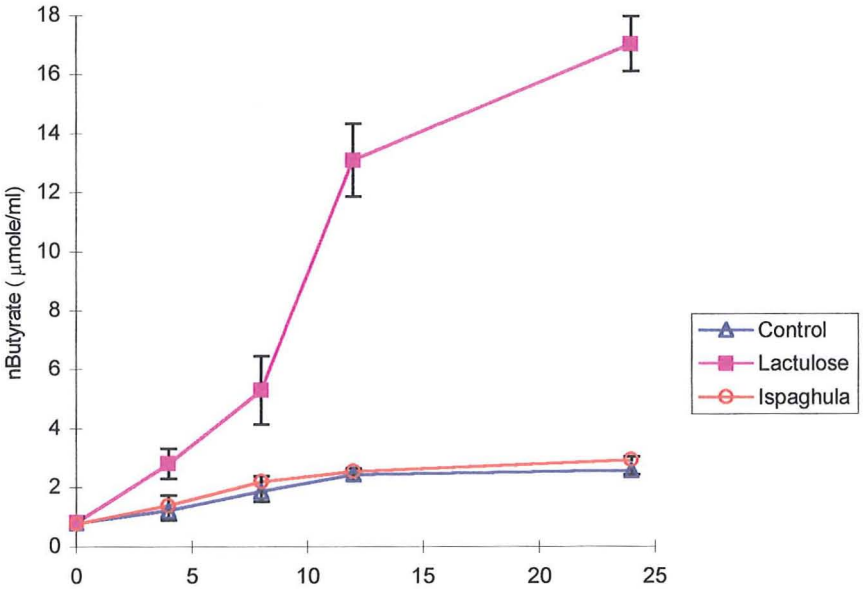


Fig 4.7 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the separate fermentation of 100 mg of lactulose and ispaghula with the human faecal bacteria.

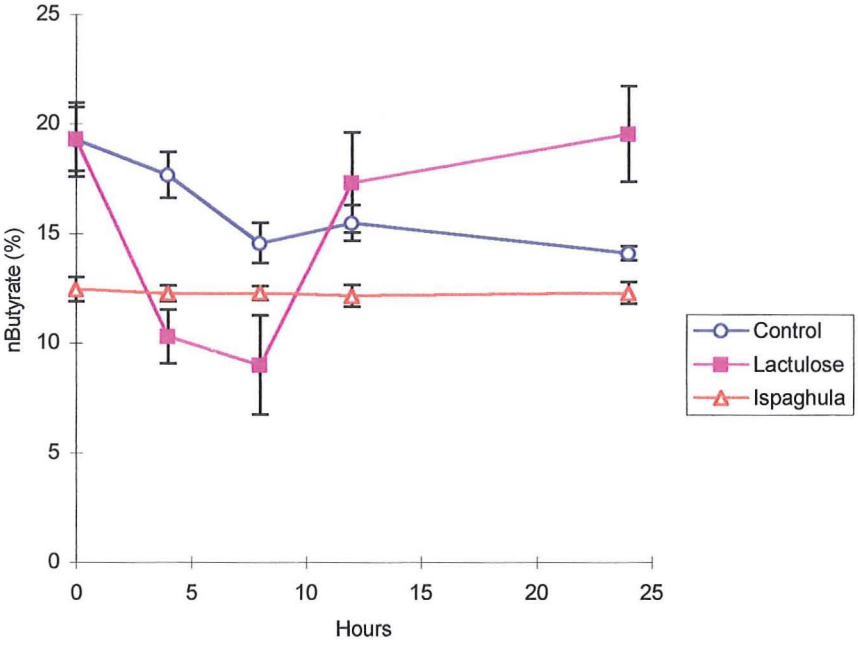


Fig 4.8 Mean (±SEM) nbutyrate percent of total SCFA produced by the separate fermentation of 100mg lactulose and ispaghula with the human faecal bacteria.

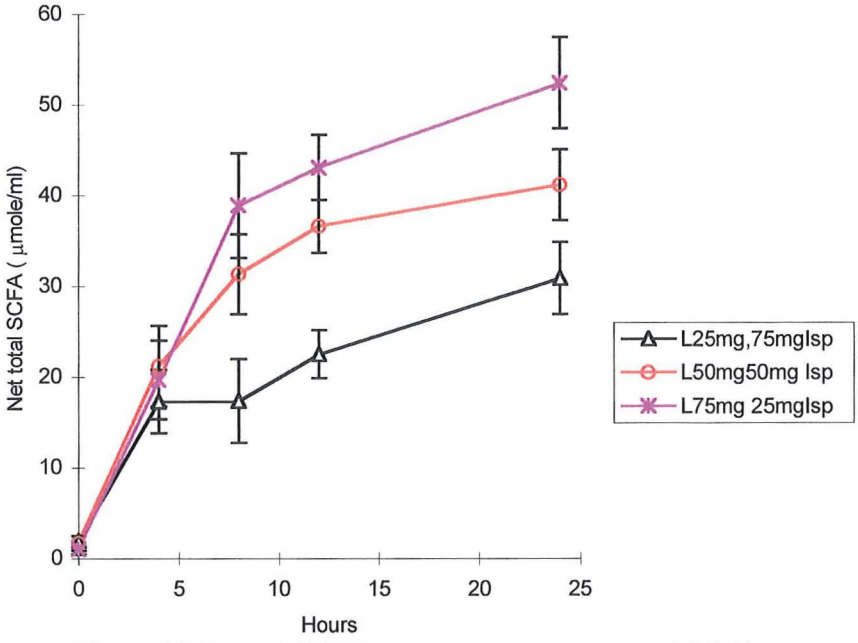


Figure 4.9 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

The concentration of acetate was not appreciably different at 4 hours with increasing amounts of lactulose but this concentration was significantly different at 8 hours ($p < 0.02$). Acetate concentrations produced by mixture

containing 25mg lactulose and 50mg lactulose, and similarly acetate concentrations in mixed substrate cultures of 50mg and 75mg lactulose in combination with ispaghula were not significantly different from each other. However, mixture containing 75mg lactulose produced significantly higher acetate concentrations than mixture containing 25mg lactulose in combination with ispaghula ($p < 0.02$) after 24 hours fermentation (Fig. 4.10). Mixture containing 25mg lactulose and 75mg ispaghula produced significantly higher acetate compared with 100mg ispaghula at 24 hours ($p < 0.02$). Cultures containing 50mg of each carbohydrate did not produce significantly different acetate from cultures of 100mg lactulose ($p < 0.05$).

There was no significant difference in acetate ratio in the three mixtures and the individual component carbohydrates at 8 hours of fermentation. These ratios were slightly higher at 8 hours compared with 12 and 24 hours, except for mixture containing 50mg of each carbohydrate (Fig. 4.11). Ratios of acetate were significantly higher in mixtures compared with cultures of 100mg ispaghula ($p < 0.02$).

4.5.2.3 Propionate

Considering the pattern of fermentation in Figures 4.5 and 4.12, the concentration of propionate produced by different substrate mixtures showed that fermentation patterns were more under the influence of amount of lactulose in mixtures (Fig. 4.12). The concentration of propionate was significantly different in mixtures from that in cultures containing 100mg component carbohydrates at 8 hours ($p < 0.02$). Cultures containing 100mg lactulose produced similar propionate to that produced by mixtures containing

50mg of each carbohydrate at 8 hours. In contrast, the same combination (50mg of each) produced significantly higher propionate than 100mg ispaghula at 8 hours of fermentation ($p < 0.02$). Significant differences were also noted between mixtures and the individual component carbohydrates at 24 hours of fermentation.

There was no significant difference in the ratio of propionate in cultures of mixed substrates and individual component carbohydrates (Fig. 4.13).

4.5.2.4 n-Butyrate

The concentrations of n-butyrate, produced by the fermentation of different mixtures, were more differentiating according to the composition of mixtures after 8 hours (Fig. 4.14). There was no significant difference in the concentrations of n-butyrate in different cultures at 8 hours of fermentation, however, this difference was significant in different cultures at 24 hours ($p < 0.02$). Mixture containing 75mg of lactulose and 25mg ispaghula produced significantly lower n-butyrate than 100mg lactulose at 24 hours ($p < 0.02$). This mixture produced significantly higher n-butyrate at 24 hours than at 8 hours ($p < 0.02$). Similarly, mixtures containing 25 and 50mg of lactulose in combination with ispaghula produced significantly higher n-butyrate at 24 hours than at 8 hours ($p < 0.02$). Mixture of 25mg of lactulose and 75mg ispaghula produced significantly higher n-butyrate compared with 100mg ispaghula at 24 hours ($p < 0.02$). In contrast, mixture of 50mg of each carbohydrate produced significantly lower n-butyrate than 100mg lactulose alone at 24 hours.

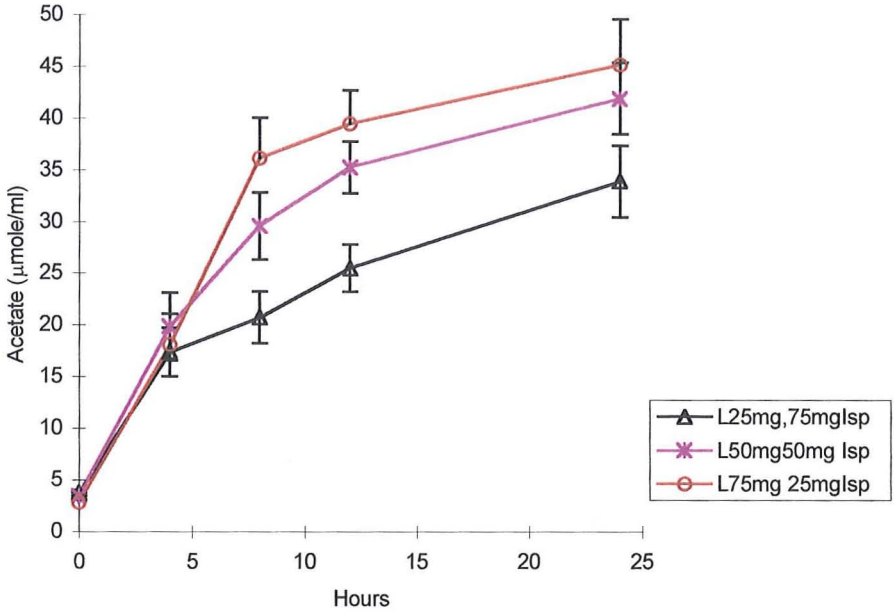


Fig 4.10 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

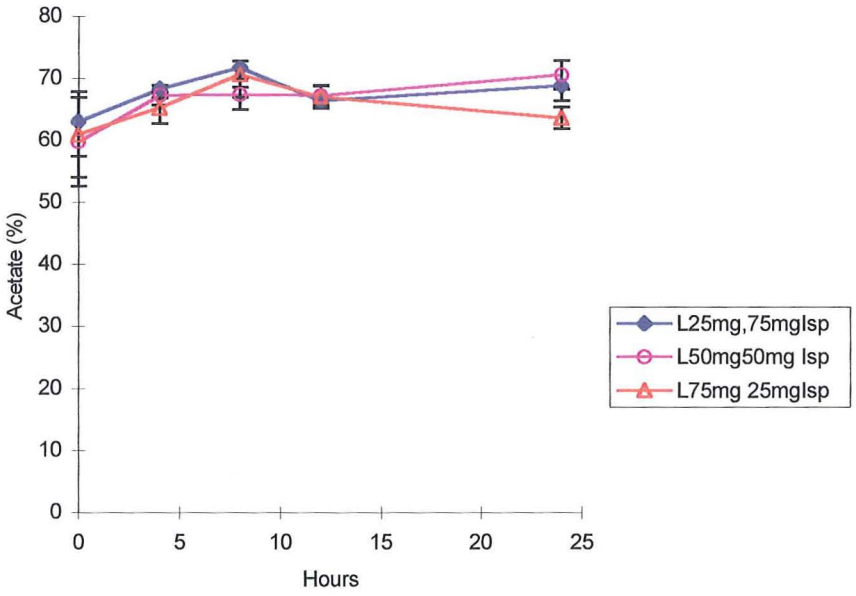


Fig 4.11 Mean (±SEM) acetate percent of total SCFA produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

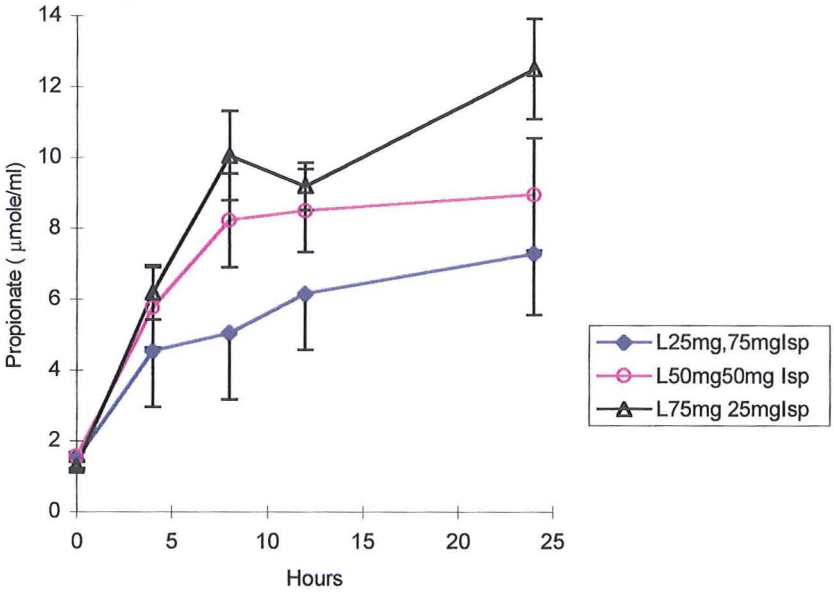


Fig 4.12 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

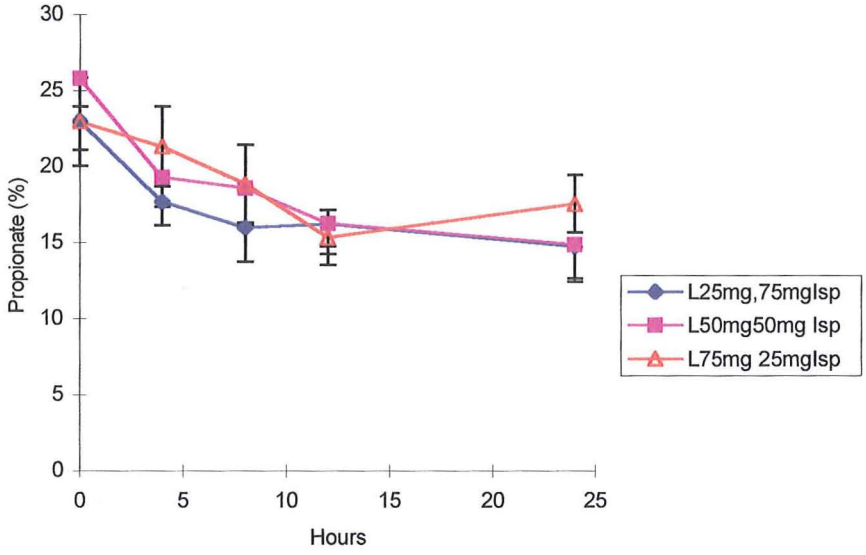


Fig 4.13 Mean (±SEM) propionate percent of total SCFA produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

There was no significant difference in the ratio of n-butyrate in cultures of mixed substrate and of individual carbohydrates at 8 hours of fermentation. However this difference was significant at 24 hours ($p < 0.02$). Significantly

higher n-butyrate ratios were produced in cultures containing 100mg of lactulose compared with mixture of 50mg each carbohydrate ($p < 0.02$). There was no significant difference in the ratio of n-butyrate in cultures containing 25 to 75mg lactulose in mixtures at 24 hours and 8 hours (Fig. 4.15). When the pattern of fermentation was compared (Figs 4.8 and 4.15), the ratio of n-butyrate and the pattern of production were seen to be more under the influence of increasing amounts of lactulose than ispaghula.

4.5.3 Production of branched-chain SCFA

The ratios of branched iso-butyrate, iso-valerate and n-valerate, produced by the fermentation of nitrogenous substances, increased from the lowest amount in lactulose to higher amounts in ispaghula and control cultures. Lactulose produced negligible ratios (1%) of branched SCFA, whereas, ispaghula produced higher ratios (nearly 15%) of these SCFA after 24 hours of incubation. Similarly, mixtures showed an increased production of branched SCFA with increasing amounts of ispaghula.

4.5.4 Lactate

The levels of lactate produced by different substrates in this study are reported in Table 4.1. Production of lactate was high in cultures containing high amounts of lactulose at 4 and 8 hours.

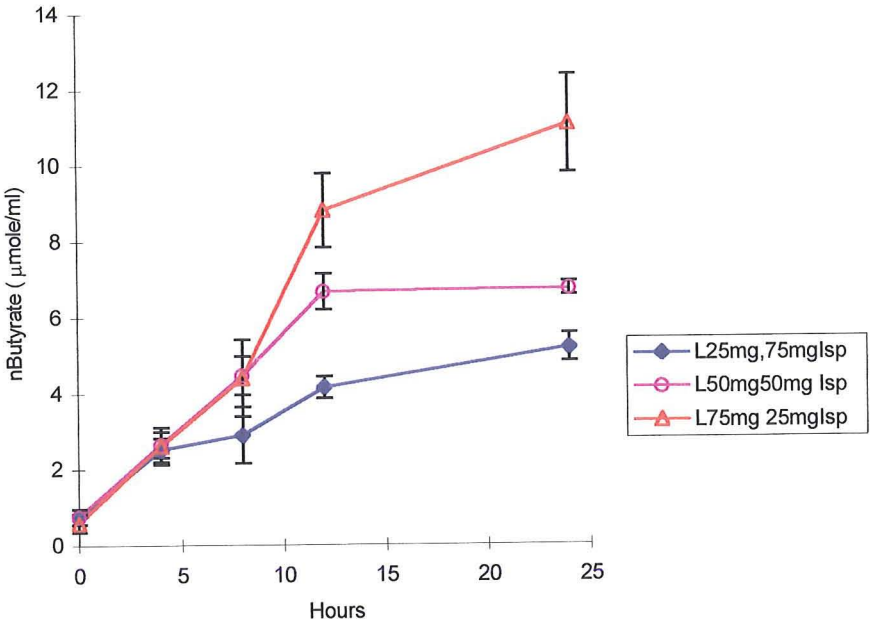


Fig 4.14 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

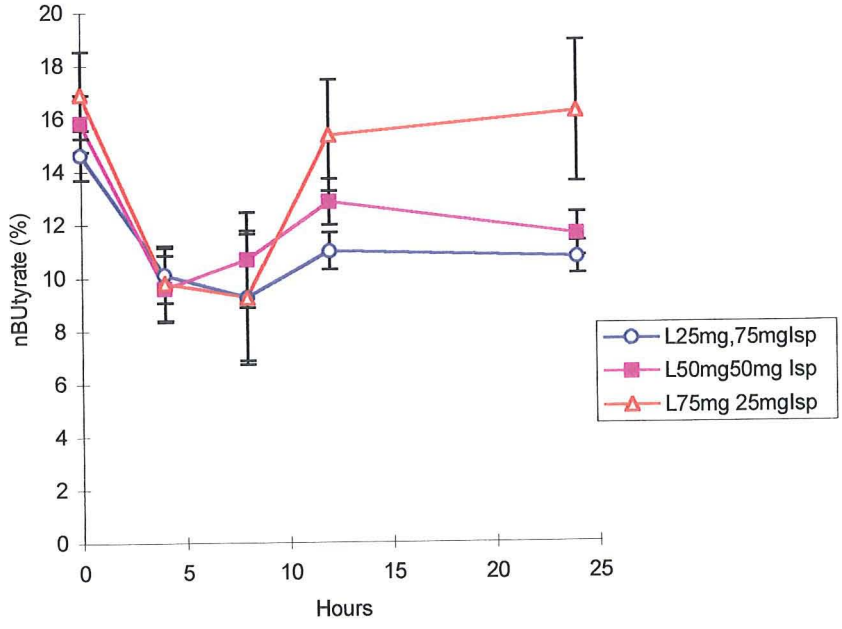


Fig 4.15 Mean (±SEM) nbutyrate percent of total SCFA produced by the fermentation of different combinations of lactulose (L) and ispaghula (Isp) with the human faecal bacteria.

Table 4.1 Concentrations Lactate (μmol/ml) produced by the *in vitro* fermentation of lactulose, ispaghula and their combinations with the human faecal bacteria (**Medians and ranges**).

Substrates	0 hours	4 hours	8 hours	12 hours	24 hours
Control	0 (0 - 2.2)	0	0 (0 - 4.6)	1.3 (0 - 2.6)	0 (0)
Lactulose	0 (0 - 2.5)	1.8 (0 - 6.7)	6.4 (0.8-12.4)	0 (0 - 4.6)	0 (0)
Ispaghula	2.8 (0 - 7.6)	0 (0 - 4.2)	0 (0 - 4)	0 (0 - 0.6)	0 (0 - 1.8)
LI 2575	0 (0 - 1.3)	0	0 (0 - 0.7)	0 (0 - 1.5)	0 (0)
LI 5050	0 (0 - 2.6)	0 (0 - 2.6)	0 (0 - 3.3)	0	0 (0 - 1.9)
LI 7525	0 (0 - 2.8)	1.5 (0 - 6.3)	5.0 (0 - 9.2)	0 (0 - 1.5)	0 (0 - 3.9)

LI- represents mixtures of lactulose and ispaghula with different compositions.

4.5.5 Changes in pH of cultures

Cultures of ispaghula did not show any noticeable decrease in pH even after 8 & 24 hours of fermentation, therefore, these results are not reported (Fig. 4.16).

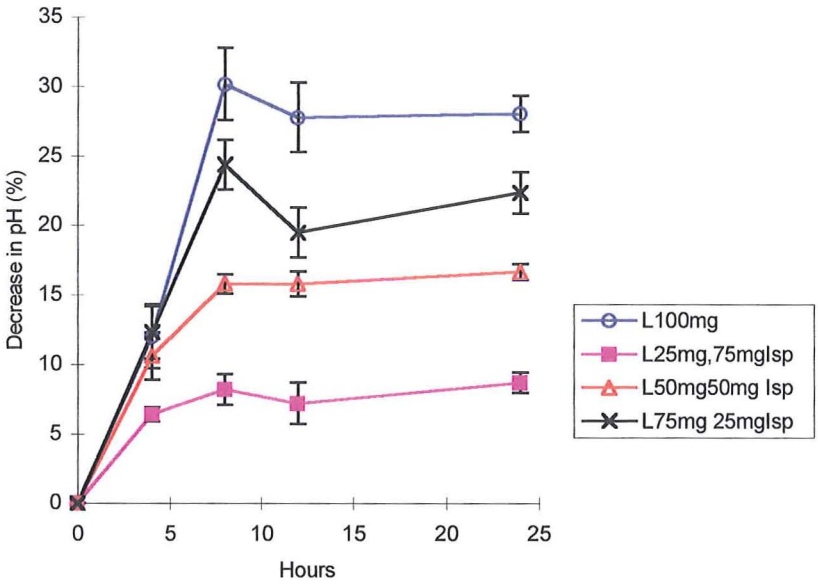


Fig. 4.16 The decrease in pH (%) by the fermentation of different amounts of lactulose (L) in combination with ispaghula (Isp) with the human faecal bacteria.

A corresponding decrease in pH was noted with increasing amounts of lactulose in cultures at all incubation times. This decrease was significantly different between incremental portions of lactulose in cultures at 8 and at 24 hours ($p < 0.02$). The percent pH reduction was significantly greater, with the increasing amounts of lactulose in cultures from 25mg to 75mg lactulose in combination with ispaghula at 8 hours of fermentation ($p < 0.02$). There was no significant difference in the pH between cultures of 75mg lactulose and 25mg ispaghula and cultures of 100mg lactulose at 8 hours.

Mixtures of 25mg lactulose and 75mg ispaghula did not show any marked decrease in pH after 4 hours of incubation, whereas, cultures with 75mg and

100mg lactulose in mixtures, showed a greater decrease in pH at 8 hours of incubation but no significant change thereafter. The decrease in pH of mixtures containing 50mg of each carbohydrate remained consistent in the subsequent incubation compared with that in other mixtures (Fig. 4.16).

4.5.6 Interaction of lactulose and ispaghula in mixture

In view of the observations in the previous and current chapters, results at 8 hours of incubation will be used for comparison and interpretation of different combinations of the carbohydrates. In the previous chapter, fermentation rates of the two carbohydrates were inhibited with increasing amounts of substrate (Fig. 3.6; Section 3.5.3). Further observations and comparison of actual mixtures with theoretical mixtures of the two carbohydrates revealed that there was more inhibition than could be explained by rapid fermentation of increasing amounts of substrate or low pH.

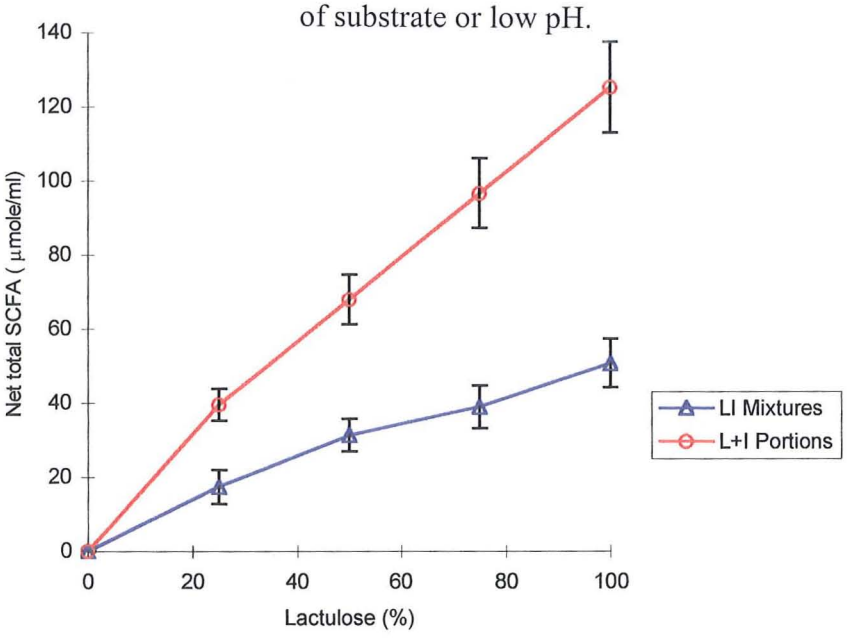


Fig 4.17 Concentrations of net total SCFA (µmole/ml) from the fermentation of actual mixtures and that expected from theoretical mixtures of lactulose and ispaghula at 8 hours (LI mixtures-actual mixtures; L+I portions- theoretical mixtures).

In the current chapter, we tried to determine any change in the fermentation rate of one carbohydrate under the influence of a second carbohydrate present in the mixture. Such interaction was judged by using values of net total SCFA from 8 hours fermentation of the two carbohydrates. Net total SCFA derived from 8 hours fermentation of 25mg of each carbohydrate were used to calculate theoretically expected values of net total SCFA for 100mg of mixed substrates (refer to section 3.5.4). These expected values of net total SCFA in theoretical mixtures, were significantly higher ($p < 0.02$) than the actual values of SCFA in cultures containing corresponding amounts of lactulose in actual mixtures (Fig. 4.17).

The net total SCFA produced by the actual mixtures lost their linearity progressively, whereas, theoretical values of such mixtures were linear with the increasing amounts of lactulose. The pattern of increasing net total SCFA produced by the actual mixtures was not the same as that shown by the expected values of SCFA. The gradient of production of SCFA, became less sharp progressively with increasing amounts of lactulose in actual mixtures of the two carbohydrates. The difference between the actual and the expected values of SCFA gradually increased with increasing amounts of lactulose, especially at 75mg and 100mg of lactulose, (Fig. 4.17). This situation revealed an inhibition of the fermentation in the actual mixtures of the two carbohydrates.

The actual values of net total SCFA produced by the fermentation of lactulose in different combinations with ispaghula were corrected for the percent inhibition that may be expected resulting from the inhibitory effects of

increasing amounts of lactulose (Fig. 3.6; Section 3.5.3). Such corrected values of net total SCFA in mixtures of lactulose and ispaghula were significantly lower ($p<0.05$) than the actual values from individual incremental portions of lactulose in cultures (Fig. 4.18), revealing the existence of another inhibitory force affecting the rate of lactulose fermentation in mixtures.

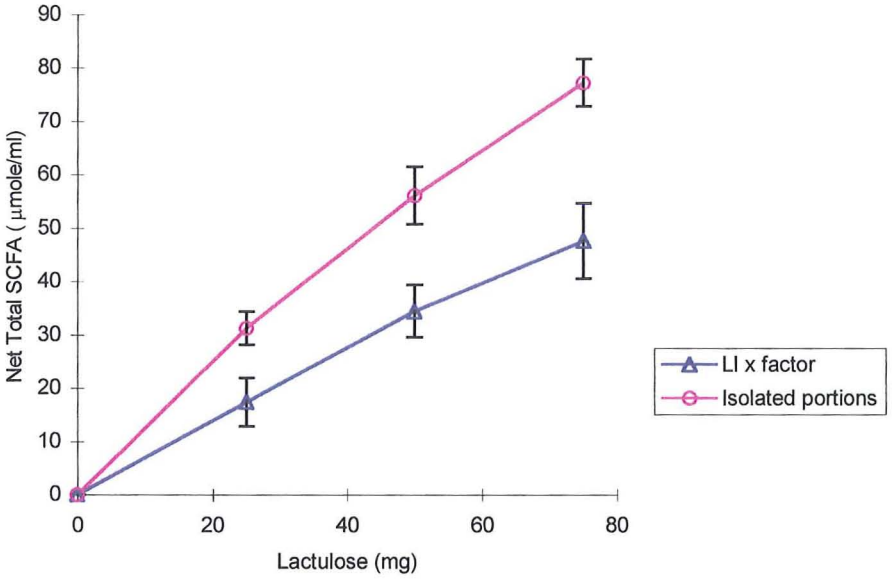


Fig. 4.18 Net total SCFA (µmole/ml) produced by the fermentation of mixtures of lactulose and ispaghula at 8 hours [LIxfactor- actual mixtures corrected for the percent inhibition; isolated portions of lactulose-uncorrected values of SCFA].

The percentage inhibition after substrate concentration had been taken into account revealed inhibition of lactulose fermentation with increasing amounts of ispaghula in mixtures, showing an influence due to the physical presence of ispaghula (Fig. 4.19).

There was a progressive decrease in the production of net total SCFA by the fermentation of lactulose with increasing amounts of ispaghula in mixtures. This SCFA lowering effect is more noticeable with 75mg ispaghula in mixtures

(44.4 % inhibition) compared with the 38.4% inhibition exerted by the presence of 25mg ispaghula.

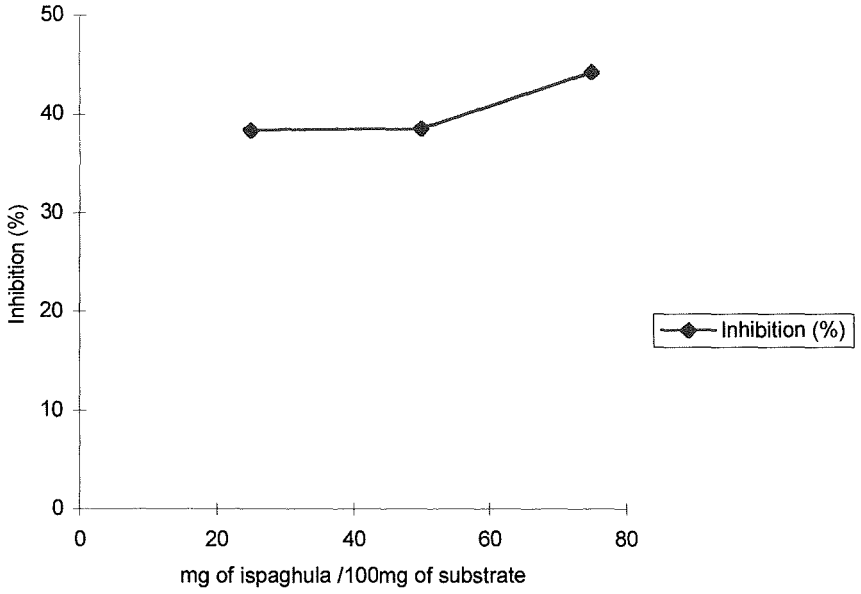


Fig. 4.19 Percent inhibition of latulose fermentation by the presence of ispaghula in mixed substrate cultures incubated with faecal inoculum for 8 hours.

In summary, the fermentation of highly fermented lactulose was inhibited by increasing amount of lactulose (refer to section 3.6.3) as well as by the physical presence of slowly fermenting ispaghula.

4.6 Discussion

In this study 100mg substrate was used by combining rapidly fermenting lactulose and the slowly fermenting ispaghula in different proportions.

4.6.1 Fermentation of individual carbohydrates

4.6.1.1 Net total SCFA

This study confirmed earlier reports about the general fermentation characteristics and SCFA profile of these two individual indigestible carbohydrates (Vince et al., 1990; Edwards et al., 1992a; Edwards &

Eastwood, 1992; Morteau et al., 1994). Lactulose was fermented rapidly producing higher concentrations of SCFA at all times. Ispaghula was fermented very slowly, producing small concentrations of SCFA even at 24 hours of fermentation. This slowly fermenting property of ispaghula is in agreement with the other studies (Edwards & Eastwood, 1992; Morteau et al., 1994). Lactulose produced a higher proportion of n-butyrate in culture of 24 hours (Fig. 4.8), whereas, ispaghula produced significantly higher ratios of propionate compared with butyrate at 8 and 24 hours.

Fermentation of lactulose was in two phases. The first phase was up to 4 hours where fermentation started in cultures but it was at the same level in cultures of all substrates including mixtures of the two carbohydrates. Mixed substrates with different composition could not be differentiated (Fig. 4.1 & 4.2). The second phase of fermentation started at 8 hours of incubation when the composition of mixed substrates in cultures started to influence the SCFA profiles.

4.6.1.2 Individual short chain fatty acids

Lactulose produced significantly higher concentrations of acetate compared with ispaghula at 8 and 24 hours. Similarly, higher acetate ratios were noted in cultures containing 100mg lactulose compared with 100mg ispaghula at 8 to 24 hours. The acetate ratio was the highest at 8 hours of fermentation in cultures containing 100mg lactulose. These ratios subsequently declined from 12 to 24 hours (Fig. 4.4), with a corresponding increase in the ratio of n-butyrate. It may be that the decrease in acetate ratios with corresponding increase in the ratio of n-butyrate is due to the utilisation of acetate in the

formation of ketone bodies, hindering the path of n-butyrate in this direction. This may result in the accumulation of more n-butyrate at later stages of fermentation (Rémésy et al., 1992).

Ispaghula did not produce high concentrations of SCFA and did not show any marked increase in the ratios of the three principal SCFA. Rather, there was progressive decrease in the ratios of acetate and propionate, and no change in ratio of n-butyrate with increasing fermentation time. The only explanation for this could be the increasing ratios of branched chain SCFA (refer to 4.7.3). There was an increase in the ratio of n-butyrate in cultures containing 100mg lactulose so that the n-butyrate ratio was higher than the propionate ratio at 24 hours (Fig. 4.6, 4.8). This n-butyrate predominance of lactulose is in agreement with the earlier studies. Lactulose was reported to yield a higher ratio of n-butyrate than pectin and guar gum in rat caecal contents after bacterial fermentation (Rémésy & Demigné, 1989). In another *in vitro* study lactulose produced very high acetate (81%), with higher propionate (12%) than n-butyrate (7%) (Wang & Gibson 1993). This may have been because the fermenter in this latter study was maintained at pH 7 throughout and, therefore, higher pH did not favour the production of the propionate and n-butyrate as noted in an earlier study (Edwards et al., 1985). The SCFA profile with higher ratio of propionate compared with n-butyrate produced by ispaghula, is the same as reported by the earlier studies (Vince et al., 1990; Edwards et al., 1992a).

4.6.2 Fermentation of lactulose in combination with ispaghula

There was a corresponding increase in the production of SCFA with increasing amounts of lactulose in mixtures at 8 and 24 hours.

4.6.2.1 Net total SCFA

The differentiating changes in the net total SCFA corresponding to the composition of the substrate mixtures were noted at 8 hours of incubation for the first time (Fig. 4.2). The initial hours of incubation may be passed in lag phase by the bacteria regaining fermentative activity after 4 hours.

4.6.2.2 Individual SCFA

Acetate concentrations were also similar in all cultures at 4 hours but increased with increasing lactulose in mixtures at 8 hours of fermentation (Fig. 4.10). There was some indication of interaction between the two carbohydrates in mixtures with respect to acetate production (Fig. 4.3). However, no significant difference was noted in the concentrations of propionate in culture containing 100mg lactulose compared with that produced in mixture containing 50mg each carbohydrate. At the same time this combination (50mg of each) produced significantly higher propionate compared with 100mg ispaghula (Fig. 4.6 & 4.13). If combinations of carbohydrates, such as lactulose and ispaghula, are to be used therapeutically, it is important that the amounts of beneficial SCFA such as propionate and n-butyrate are equivalent to that produced from fermentation of 100mg lactulose alone. In this way, the benefit of the higher SCFA production by the fermentation of 100mg lactulose will be maintained without the unwanted side effects of using 100mg of either constituent carbohydrate.

In this study, the SCFA profiles at 4 hours for different substrates were more under the influence of original inoculum than the substrate composition (Figs. 4.11, 4.13, 4.15). The mixed substrates showed increasing influence related to their composition on the fermentation only after 8 hours incubation.

Propionate ratios were higher at 8 hours compared with that at 24 hours of incubation in all mixtures. A corresponding delayed production of n-butyrate was noted in these cultures. In addition, the actual ratios of propionate and n-butyrate were lower than those expected from such mixtures when calculated theoretically. It is possible that the physiological conditions were not favourable for the pathway producing propionate as an end product at 24 hours, resulting in increasing ratios of n-butyrate. This delay in the production of SCFA, especially of n-butyrate may be beneficial in the human colon, as with delayed production n-butyrate may be produced at a distal colonic site, which would show positive implications in health.

4.6.3 Branched chain SCFA

The branched SCFA (iso-butyrate, iso-valerate and n-valerate), which are derived from the fermentation of nitrogenous substances, were produced in these cultures when highly fermentable carbohydrate was limited. The differences of branched SCFA concentrations in different cultures depend on the availability of nitrogenous compounds for fermentation, which in these cultures would be provided by the tryptone in the media, from sources in the faecal inoculum and possibly from dead and lysed bacteria. In cultures with large amounts of fermentable carbohydrate the available sources of nitrogen are used for bacterial cell growth. Ispaghula was a slowly fermenting

carbohydrate and bacterial growth was presumably lower allowing more fermentation of amino acids.

4.6.2 Changes in pH and the SCFA

The effects of pH on bacterial fermentation have been discussed at length in the previous chapter. The pH fell to the greatest extent in 100mg lactulose cultures and very little decrease was seen in cultures containing high amounts of ispaghula. In some cultures (containing 75mg and 100mg lactulose), pH appeared to rise a little after 8 hours. The greater decrease in pH at 8 hours could be due to the higher production of lactate and acetate. Some organisms may re-ferment (Gibson et al., 1990; Jay, 1996) these acids to other metabolites at 12 and 24 hours of incubation, resulting in a slight increase in pH.

The initial lowering of pH may make the conditions in cultures favourable for production of propionate (Edwards et al., 1985) or butyrate depending on the specific nature of substrates. Other factors, in addition to pH, such as the presence of a particular acid, may be important in this regard (Chung & Geopfert, 1970; for detail Chapter 3). In the present study, lactulose produced more propionate compared with n-butyrate up to 8 hours of fermentation. The increased production of propionate at lower pH may be related to changes favouring propionate producing bacteria. However, with increasing time, lactulose produced a SCFA profile with n-butyrate predominance. This indicated that re-fermentation of acetate during prolonged fermentation, may make the physiological conditions in cultures unfavourable for producing propionate but favourable for n-butyrate production. Propionate production

was reduced at pH 5 in an earlier study (Edwards et al., 1985). Propionate and n-butyrate are produced by different bacterial species. Eubacteria, fusobacteria, peptococci produce n-butyrate, whereas, bacteroides, propionibacteria, veillonella produce propionate. The accumulating SCFA may have further reduced the pH and changed the physiological conditions in cultures and instead of inhibiting, actually facilitated the production of n-butyrate with increasing fermentation.

Although the fermentation pattern in this study indicated that production of these three principal SCFA corresponded to the prevailing pH in cultures, pH could not be the exclusive factor in this regard. Similar microorganisms have different optimal pH under different physiological conditions. The real optimal pH range for a particular bacteria may depend on a number of growth factors such as type of acid, types of salt present in the medium of growth (Sherman & Holm, 1922; Juven, 1976; Jay, 1996; detail in Chapter 3). Therefore, not only pH, but pH in conjunction with changing physiological conditions may become progressively more favourable for the production of one SCFA compared with the other. In this way, the low pH, although not exclusive, is an important driving factor in the production of different SCFA, as reported by earlier study (Edwards et al., 1985; detail was discussed previously).

4.6.3 Interaction between the two carbohydrates

In this study, mixtures contained rapidly fermenting lactulose in combination with slowly fermenting ispaghula as the second carbohydrate. Lactulose is highly soluble in water and it loses its viscosity very rapidly in the solution.

Whereas, ispaghula is fermented very slowly and makes a viscous matrix when hydrated, maintaining its viscosity over a longer period (Tomlin & Read, 1988). An important interaction was noted between these two carbohydrates because of their contrasting physico-chemical characteristics.

Apparently two inhibitory forces were working during the *in vitro* fermentation of mixtures of these carbohydrates. The inhibitory effect due to decreased pH and other changes in the physiological conditions of cultures with increasing amounts of lactulose was discussed in the previous chapter (Section 3.6.3). Previous studies have also noted that the amount of rapidly fermenting substrate affected *in vitro* fermentation (Barry et al., 1995; Stevenson et al., 1997b), where end products are accumulated due to the lack of absorptive epithelial surface (Section 3.1). As discussed in the previous chapter, an inhibition in the SCFA production with increasing amounts of lactulose was noted. However, in the normal colon, such high concentrations of SCFA from fermentation are rapidly cleared by the colonic epithelium and physiological conditions in the colon are not changed to such an extent.
In this chapter, another type of inhibition was described (Fig. 4.19).

Fermentation of smaller portions of rapidly fermenting lactulose in mixtures with ispaghula did not reach their maximum potential. When net total SCFA produced by mixtures were corrected for the expected inhibition due to the increasing amounts of lactulose, SCFA production was still lower than that may be expected (Fig. 4.18). This additional inhibition of the fermentation of lactulose may be because of the presence of slowly fermenting ispaghula (Fig. 4.18). Net total SCFA produced from lactulose in combination with ispaghula

progressively decreased with increasing amounts of ispaghula (Fig. 4.19). This highest inhibition was seen with 75mg ispaghula (44.4 %) compared with the inhibition (38.4 %) in presence of 25mg ispaghula in mixture. However, this difference is small. Unfortunately, we were unable to measure the final viscosity of these cultures so the inhibition could not be compared with the viscosity.

SCFA and other metabolites from fermentation in the colon are rapidly absorbed and so the physical properties of slowly fermenting carbohydrates such as ispaghula, may become more important than changes in physiological conditions and resulting influence on the SCFA production. Three effects of ispaghula in the human gut, diluting the colonic contents, viscosity and stimulation of propulsion, could delay the fermentation of lactulose, making it available for bacterial fermentation at more distal site of the colon. The viscosity of ispaghula may trap the smaller molecules of lactulose in a complex three dimensional viscous matrix which may make the molecules of lactulose less accessible to bacteria.

The viscous and bulking properties of ispaghula may also inhibit absorption of metabolites from fermentation as well as promoting propulsion. These effects could take the production and absorption of SCFA towards more distal sites before they are available for absorption and/or further metabolism where they would be of more use in preventing colonic diseases. In a recent study in which a mixture of lactulose and ispaghula was fed to human subjects the fermentation of lactulose was delayed and its effects on transit time were reduced by ispaghula (Washington 1998).

4.7 Conclusion

The fermentation of lactulose was inhibited by the presence of increasing amounts of ispaghula *in vitro*. This was probably related to the increased viscosity of these cultures. Such effects *in vivo* may help to move SCFA production and absorption to more distal sites in the large intestine where most disease occurs.

The impact of increasing amounts of substrate can be corrected for in an *in vitro* system. To reduce this effect, it is recommended that substrate concentration of 50mg of rapidly fermented carbohydrate per 10ml incubation volume is used *in vitro*.

CHAPTER 5

Indigestible carbohydrates and their fermentation properties

5.1 Introduction

Indigestible carbohydrates belong to all the different classes of saccharides, including disaccharides, such as lactulose, and polysaccharides such as resistant starch and polysaccharide gums. Interest in the physiological action of these unabsorbable carbohydrates was initiated in the early 70s (Chapter 1). In the wake of this interest in their metabolic importance and potential health benefits, an immediate consideration was to provide these carbohydrates without disturbing the balance of the other constituents in the human diet. Many researchers considered supplementation with isolated carbohydrates as a solution to the problem and isolated polysaccharides such as guar gum and locust bean gum were tested and approved for use. Studies of guar gum showed a reduction in postprandial glucose levels (Jenkins et al., 1978) and cholesterol (Blake et al., 1997).

As discussed in previous chapters, there are difficulties in the *in vivo* as well as *in vitro* investigation, of these carbohydrates. Despite these difficulties, it is possible to predict some of the effects of these carbohydrates on the basis of *in vitro* studies, using the *in vitro* model discussed in Chapter 2.

As discussed in Chapter 1, these un-absorbable carbohydrates have potential therapeutic properties which need to be carefully considered and explored before they can be used in mixtures to promote health and prevent diseases. It is unlikely that any single carbohydrate will provide the optimal conditions in the gut which could be considered ideal in major colonic problems. The logical approach would be to combine different indigestible carbohydrates in mixtures of two or more carbohydrates. In the previous chapter, it was noted that a slowly fermented carbohydrate could alter the fermentation of a rapidly

fermented carbohydrate and vice versa. Similarly, carbohydrates with different SCFA profile interacted in their ability to produce a particular SCFA. At present very little study has been performed on this aspect of indigestible carbohydrates. Before embarking on studies of combining different carbohydrates, it is necessary to evaluate the fermentation characteristics of individual carbohydrates.

In the present study, individual carbohydrates were evaluated for their properties, reflected in the end products of their fermentation cultures, before speculating on their interactive influences and on the results that may be expected from different combinations of these carbohydrates. Some of the important indigestible carbohydrates, used in this experiments are described below;

5.1.1 Oligosaccharides

Some oligosaccharides are indigestible carbohydrates but are readily soluble in water (Cummings & Englyst, 1995) and *in vitro* studies showed that these carbohydrates are fermented by the colonic bacteria (Wang &Gibson, 1993). The oligosaccharides are mostly produced synthetically or by the processing of higher molecular weight carbohydrates, for example, maltodextrins are produced from partial hydrolysis of the starch. In nature oligosaccharides mainly originate from legumes, onions, artichokes and root vegetables (Wiggins, 1984).

The importance of oligosaccharides emerged in late 1980s as a result of suggestions that they may affect microbial function in the human large intestine in a beneficial way (Hidaka, 1986). There is a growing interest since then in

these indigestible carbohydrates which stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli (pro-biotics), and suppress harmful bacteria, such as clostridia, in the human gut. Oligosaccharides with such beneficial effects have been recently termed as pre-biotics (Gibson & Roberfroid, 1995). The fructo- and galacto-oligosaccharides and some inulins are common examples of these oligosaccharides (Cummings & Englyst, 1995). Among such oligosaccharides, fructo-oligosaccharides meet all the criteria of being ideal candidate for the classification as pre-biotics. The fructo-oligosaccharides include an oligofructose commercially known as raftilose (Gibson & Roberfroid, 1995).

5.1.1.1 Raftilose

This is a food ingredient which is composed of oligofructose, fructose, glucose and sucrose. The oligofructose (DP 2 - 8) is a mixture of oligosaccharides which are composed of fructose units linked together by β(2-1) linkages. These molecules are terminated by a glucose unit. Raftilose (fructo-oligosaccahrides) is produced by partial hydrolysis of chicory inulin by using endoglycosidases (Cummings & Englyst, 1995). In batch cultures, both oligofructose and inulin specifically stimulated bifidobacterial growth (Yazawa & Tamura, 1982; Hidaka et al., 1986; Wang & Gibson, 1993), an effect that has been confirmed in humans (Gibson et al., 1993). In contrast, *B. infantis* inhibited the growth of *Cl. perfringens* and *E. coli* (Wang & Gibson, 1993). The mechanism for this inhibition was thought to be related to the lowering of intestinal pH by bifidobacterial fermentation (Cummings & Englyst 1995) but Gibson and Wang (1994) showed that factors other than pH, lead to repression

of the growth of Clostridial species. A number of factors may be relevant in this regard. For example, Bifidobacterial species may compete with the Clostridial species for the substrate or for binding sites for adhesion on to the mucosa. In addition, the beneficial bacteria may produce proteases to destroy biotoxins produced by harmful bacteria.

Raftilose promotes butyrate production, which, as previously discussed, is essential for colonic health. However, large amounts of small molecules have a potential osmotic effect in the small intestine, by retaining larger quantities of fluids in the intestine. This effect may increase the bulk of intestinal contents and, thus faecal output after ingestion of moderate amounts and higher amounts may result in osmotic diarrhoea. Despite this, raftilose is a larger molecule than lactulose, used in Chapters 3 and 4, and is less likely to induce diarrhoea.

5.1.2 Raftiline & Fibruline

The raftiline used in this study is a chicory inulin which is a mixture of oligo- and polysaccharides which are composed of fructose units linked together by β(2 - 1) linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units ranges mainly between 2 and 60. Fibruline is also a polysaccharide inulin .

5.1.2 Indigestible polysaccharides

The polysaccharides resistant to digestion in the human small intestine, belong to two categories;

Resistant Starch

Non-starch Polysaccharides.

In this experiment, raw potato starch and different types of non starch polysaccharides, such as pectin, guar gum, gum arabica and oat fibre were tested for their fermentation properties.

5.1.2.1 Raw potato starch

Raw potato starch has large starch granules with type B crystalline structure. It is resistant to digestion in the small intestine because of the intact structure and specific crystalline pattern of granules and is generally referred to as resistant starch 2 (see detailed description in section 1.2.4.2.3.1). Fermentation of resistant starch is much slower than most dietary fibre (Achour et al ., 1996). A recent study reported an estimated 78 to 82 % fermentation of resistant starch in human subjects, using cooked and uncooked flour supplements from green banana, wheat grains and corn (Phillips et al., 1995). These researchers also found a significant inverse relation between dietary intake of resistant starch and faecal pH. Significantly higher faecal SCFA were reported with diets containing high resistant starch contents compared to diets with low resistant starch contents. Faecal n-butyrate was increased after starch ingestion in the diet (Scheppach et al., 1988; Phillips et al., 1995).

In another study, breath H_2 and total serum acetate were significantly higher with a diet containing high amounts of resistant starch compared with a low resistant starch diet (Muir et al., 1995). Similar results were reported *in vitro* where a semi-purified retrograded amylose (resistant starch 3) from potatoes, was less fermentable, producing the lowest SCFA, than raw potato starch (Edwards et al., 1996) which, in turn, was less fermentable than cooked starch.

This study showed that resistant starch may resist not only amylolytic action in the small intestine, but also degradation by bacteria in the large intestine. Starches from different sources may be fermented differently in the colon. Although both amylomaize and potato starch decreased caecal pH in rats, there was marked variation in the production of SCFA between resistant starches from the two sources. Amylomaize increased SCFA but potato starch did not (Mallet et al., 1988), suggesting that the substantial decline in pH was due to the production of another organic acid such as lactate. Resistant starch yielded a profile of SCFA with a higher ratio of n-butyrate in *in vitro* fermentation studies with human faecal slurries (Englyst et al., 1987b; Weaver et al., 1992). Production of n butyrate was 29 % compared with only 2 % n-butyrate produced by pectin during *in vitro* fermentation with human faeces (Englyst et al., 1987b; Wang & Gibson, 1993).

5.1.2.2 Gum arabica

Gum arabica is the sap exudate from the Acacia species. Gum arabic is a water-soluble, rapidly fermented complex heteropolysaccharide with the unique property of dissolving up to 50 percent concentration. At low concentrations, it provides little viscosity, making itself a preferred choice for use in combinations with other stabilisers (Chinachoti, 1995). It produced a propionate predominant SCFA profile *in vitro* system using human faecal inoculum (Adiotomre et al., 1990). Gum arabic is a highly branched arabinogalactan. Gum arabic ingestion decreased serum cholesterol significantly but did not affect excretion of bile acids. It increased the H_2 excretion but had no effect on faecal weight or faecal constituents (McLean Ross et al., 1983).

5.1.2.3 Guar gum

Guar gum, a storage polysaccharide derived from seeds of the cluster bean (Cyamopsis tetragonoloba), is frequently used as a model indigestible polysaccharide in physiological and nutritional studies for several reasons. It is possible to isolate guar gum in a relatively pure form and it has well characterised physico-chemical properties. In addition, de-polymerisation of guar gum is easy to achieve. Guar gum is composed of galactomannans with single unit galactose side chains and has a mannose to galactose ratio of 2:1. It is cold water soluble, giving a highly viscous medium even at a low concentration (Chinachoti, 1995). Guar has no significant osmotic effect in the small intestine but forms viscous solutions in the stomach and small intestine. This viscous property delays the absorption of the glucose and other nutrients from the small intestine by several mechanisms (Blackburn et al., 1984; Ellis et al., 1995). These included delayed gastric emptying, reduction of mixing in the small intestine, and delayed transit through the small intestine. Guar gum fermentation produced a propionate predominant profile *in vitro* (McBurney & Thompson, 1987; Adiotomre et al., 1990), and in the caecum in rats *in vivo* (Rémésy & Demigné, 1989). In rats, guar gum produced higher SCFA and lower caecal pH compared with lactulose and pectin. The acetate/propionate ratio is 2:1 for guar gum (Wolever, 1991b).

Guar gum is used clinically to treat the diabetes mellitus (Jenkins et al., 1977). The effects of guar gum on postprandial glucose and insulin levels were not influenced by large variations in the molecular weight or particle size of guar gum (Ellis et al., 1991) suggesting that viscosity was not important, as these

parameters are important determinants of viscosity. However, in another study by the same group, different concentrations of guar gum significantly reduced glucose absorption and insulin secretion in the growing pigs and this was related to the viscosity of jejunal digesta (Ellis et al., 1995).

Blake et al., (1997) reported a significant reduction (10%) in total plasma cholesterol concentration by feeding a de-polymerised guar gum in wheat bread in human hypercholesterolaemic subjects. Guar gum is reported to alter significantly endogenous cholesterol metabolism in guinea pigs by different mechanisms (Fernandez et al., 1995). These included decreasing the cholesterol absorption and the release of cholesterol to the liver by the chylomicron remnant or by trapping bile acids, resulting in the mobilisation of the hepatic cholesterol pools because of an increased need for bile acid synthesis.

5.1.2.4 Pectin

After cellulose, pectin is one of the most abundant carbohydrates on Earth. In pure form pectin can readily make viscous solutions in water. Pectin is a non cellulosic polysaccharide in the plant cell wall with a backbone of galacturonic acid derived from carbohydrates. Pectin has common side chains including arabinose, xylose, rhamnose and fucose constructed from various monomers (Whitney et al., 1998). Fruits and vegetables contain approximately 1% pectin, mainly in the cell walls and it is extracted commercially. There are two types of pectin; on each residue of galacturonic acid in the long chains of pectin molecules, is a carboxyl (–COOH) group. Sometimes these groups are modified by the addition of methyl (–CH3) groups. The result is a methoxyl

(–COOCH3) group. Pectin in which half or more of the acid residues have methoxyl groups tagged onto them is called high methoxyl pectin; where there are fewer, they are termed low methoxyl pectin. In this way, pectins from different sources may be different in their chemical and metabolic actions. For example, apple pectin is highly methylated compared with pectin from oranges which is only partly methylated. This is because orange juice naturally contains large amounts of pectin esterase; an enzyme which strips methoxyl groups from the pectin molecules.

Pectin is a propionate predominant carbohydrate, although it also produced very high levels of acetate (Englyst et al., 1987; Adiotomre et al., 1990). A similar SCFA profile was noted in rat caecum (Rémésy & Demigné, 1989). In one study however, pectin produced higher n-butyrate (Vince et al., 1990). This may be due to the increased time (48 hours) of incubation. Pectin molecules, unlike guar gum, carry a charge due to the presence of polygalacturonic acid residues. This charge will aid binding of bile acids and may be one of the mechanisms involved in the reduction of blood cholesterol by the soluble fibres such as pectin (Anderson et al., 1990). Fernandez et al., (1994) reported that dietary citrus pectin showed modest effects on plasma LDL level and hepatic cholesterol equilibrium in presence of low dietary cholesterol content. However, these researchers noted significant effects of high doses of citrus pectin on the regulatory enzymes of cholesterol synthesis and esterification, and expression of the hepatic apolipoprotein (apo) B/E receptors in guinea pigs, fed high dietary cholesterol.

The acetate/propionate ratio for pectin is 5:1 (Wolver, 1991b). Vince et al., (1990) reported very high acetate production by pectin after *in vitro* fermentation and supported the 5:1 acetate/propionate ratio for pectin, but this ratio was 3:1 in rat caecum (Rémésy & Demigné, 1989). These studies suggested that since pectin from various sources contain different chemical groups associated in side chains or different degree of methyl groups, the levels of individual SCFA will be affected, as the chemical and physico-chemical properties of the fibre source are important in this regard (Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993).

5.1.2.5 Oat fibre

Although there has been an extensive amount of research to establish the cholesterol lowering effect of oats or its components (Swain et al., 1990; Kestin et al., 1990; Anderson et al., 1991; Keenan et al., 1991; Van Horn et al. 1991; Uusitupa et al., 1992; Zhang et al., 1992; Cara et al., 1992; Braaten et al., 1994), little research work, with less consistent results, is reported about its effects on glycaemic response (Braaten et al., 1991; Kestin et al., 1990; Cara et al., 1992). The highly soluble β-glucan contents of oats have been shown to reduce serum cholesterol in several studies (Topping, 1991; Uusitupa et al., 1992; Braaten et al., 1994). A recent study, however, reported no consistent lipid lowering effects consistently but did show reduced glycaemic response (Hallfrisch, 1995).

5.2 Aim

This study was designed to identify the SCFA profiles of potentially beneficial indigestible carbohydrates for identifying the most suitable for developing mixtures for supplementation and for tube feeding.

5.3 Objectives

The objectives of this study were to determine;

1) the properties of indigestible carbohydrates with different fermentability in an *in vitro* fermentation system, thus comparing the utilisation of these carbohydrates by faecal bacteria.
2) the SCFA profiles produced *in vitro* by different indigestible carbohydrates in our faecal incubation model.

5.4 Methods

The specific procedures for this experiment are described in this chapter, whereas, general methods are detailed in the Chapter 2.

5.4.1 *In vitro* measurement of fermentation

A general description of an *in vitro* incubation systems used in this study is given in the section 2.2, of fermentation procedure. The rates of fermentation of different indigestible carbohydrates were determined by stopping the cultures at different time points from 0 to 24 hours.

5.4.2 Subjects used as donors of faecal inocula

Faecal samples were collected from five different healthy individuals (age range 26-57 years; 1 male, 4 females). The criterion for the faecal donors is described in Chapter 2.

5.4.3 Fermentation procedure

The details of fermentation procedure are same as described in Chapter 2. All substrates (100mg each) were used separately in culture. Raftilose, raftiline, raw potato starch, guar gum, gum arabica, pectin, oat fibres and fibruline were used as the sole source of carbohydrate in these experiments. The cultures were stopped at time points of 0, 2, 4, 8 and 24 hours of incubation. The analytical procedures were the same as described in Chapter 2. Statistical analyses were performed as detailed in section 2.14. Details of reagents and fermentation media are given in Chapter 2.

5.5 Results

Different carbohydrates showed different patterns of fermentability. Raftilose, raftiline, fibruline and pectin were rapidly fermented, whereas, oat fibre showed a negligible degree of fermentation. Other carbohydrates were intermediate in their rate of fermentation and fermentability.

5.5.1 *In vitro* fermentation of individual carbohydrates

Net total SCFA, produced during *in vitro* fermentation were used to characterise the individual carbohydrates in this study (Fig. 5.1). There was no significant difference in the net total SCFA produced in these cultures at 0 and 2 hours of incubation. It was noted that the SCFA produced from different substrates started differentiating between carbohydrates at 4 hours, as the first significant differences were seen after this time point ($p<0.02$). However, the slowly fermenting carbohydrates such as raw potato starch, gum arabica were not differentiated significantly from the very slowly fermenting oat fibre even at 4 hours fermentation. Even rapidly fermenting fibruline, did not produce

significantly different net total SCFA compared with oat fibre at 4 hours. There was no significant difference in net total SCFA between fibruline, raw potato starch and gum arabica at 8 hours, whereas, guar gum produced significantly higher net total SCFA compared with raw potato starch ($p<0.02$) and gum arabica ($p<0.02$) at 8 hours. Net total SCFA in cultures containing raftilose, raftiline, fibruline and pectin were not significantly different from each other at 8 hours. Raftilose produced significantly higher SCFA compared with raw potato starch ($p<0.02$), gum arabica ($p<0.02$), guar gum ($p<0.02$) at 8 hours. Oat fibre produced significantly lower net total SCFA compared with other carbohydrates at 8 hours.

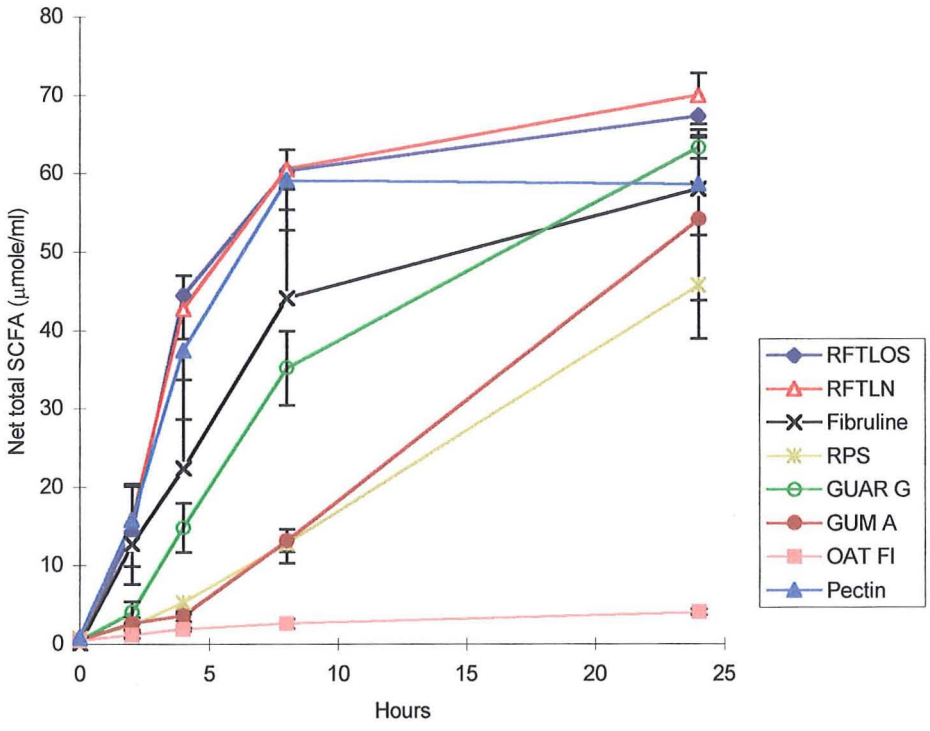


Fig 5.1 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of different carbohydrates with the human faecal bacteria.

Raftilose and raftiline produced almost the same concentrations of net total SCFA at all times. The fermentation patterns of the two carbohydrates were also similar, showing an initial rapid fermentation and then a slight plateau with

increased incubation after 8 hours. Pectin produced a net total SCFA of the same order as that noted in cultures containing raftilose but this reached a complete plateau after 8 hours of fermentation. There was no significant difference in net total SCFA at 24 hours compared with 8 hours in cultures containing oat fibre, fibruline and pectin. All other cultures produced significantly higher net total SCFA at 24 hours compared with that at 8 hours of fermentation ($p<0.05$). Oat fibre produced negligible concentrations of SCFA even after 24 hours of fermentation (Fig. 5.1).

Guar gum produced significantly higher net total SCFA than gum arabica and raw potato starch at 8 hours ($p<0.02$). The latter two indigestible carbohydrates showed similar fermentation patterns to each other, producing almost the same concentrations of SCFA with no significant difference at 8 and 24 hours (Fig. 5.1).

5.5.2 Concentrations and ratios of SCFA produced by individual carbohydrates

5.5.2.1 Acetate

Fermentation of raftilose, raftiline, fibruline and pectin produced acetate very rapidly but this production showed a plateau after 8 hours. There was no significant difference in acetate concentration at 8 and 24 hours of fermentation in these cultures. Fermentation of raftilose, raftiline and pectin showed the same pattern of acetate production. There was a consistent but not very rapid increase in acetate in cultures containing guar gum, gum arabica and raw potato starch with significantly higher acetate ($p<0.02$) at 24 hours compared with that at 8 hours (Fig. 5.2).

Fermentation of oat fibre produced significantly higher concentrations of acetate at 24 hours compared with 8 hours ($p<0.05$). The rate of acetate production was very slow. Acetate concentrations were not significantly higher than that produced in control cultures up to 8 hours but the increase above control levels did reach statistical significance at 24 hours ($p<0.05$; Fig. 5.2).

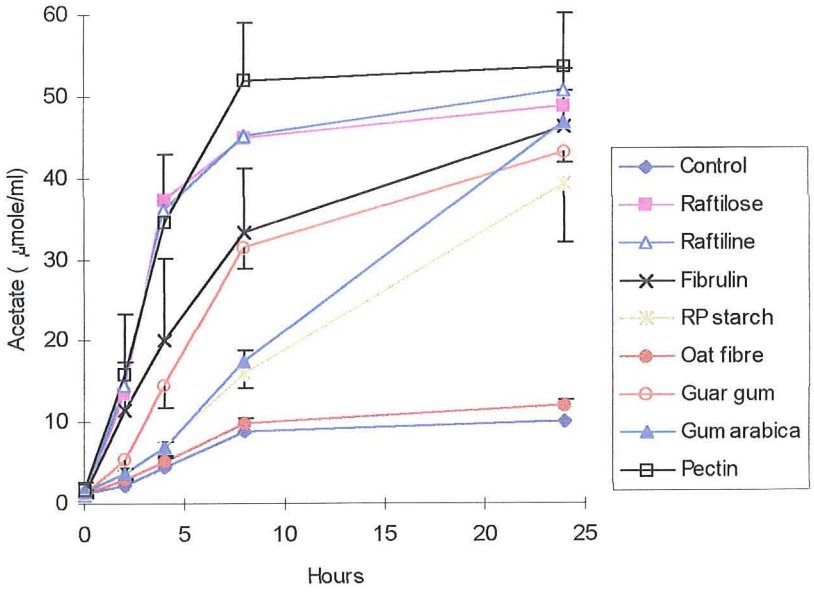


Fig 5.2 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of different carbohydrates with the human faecal bacteria.

In cultures containing pectin, the acetate ratio remained appreciably higher, whereas, acetate ratios produced by the fermentation of guar gum, gum arabica and raw potato starch were markedly lowered compared with pectin, raftilose, raftiline and fibruline. Fermentation of pectin produced a significantly higher acetate ratio compared with raftilose ($p < 0.05$), gum arabica ($p < 0.05$) and raw potato starch ($p < 0.02$). Raftilose, raftiline and fibruline produced almost similar acetate ratios to each other. The acetate ratio in cultures containing these carbohydrates increased rapidly initially then declined with the prolonged fermentation (Fig. 5.3).

In cultures of the other carbohydrates, there was a slow increase in acetate ratio initially which declined similarly in all cultures after 8 hours of incubation, with the exception of cultures containing pectin and guar gum. There was no significant difference in acetate ratios in cultures containing raftiline, fibruline, pectin and guar gum at 8 hours. Fermentation of pectin produced a significantly higher acetate ratio compared with that in cultures containing raftilose ($p < 0.02$), raftiline ($p < 0.02$), guar gum ($p < 0.02$), whereas, gum arabica produced significantly higher acetate ratios compared with cultures containing raftilose ($p < 0.05$), fibruline ($p < 0.02$) and guar gum ($p < 0.02$) at 24 hours of fermentation. Oat fibre produced a significantly lower acetate ratio compared with all the other carbohydrates except that in cultures containing raw potato starch, raftiline and guar gum at 24 hours.

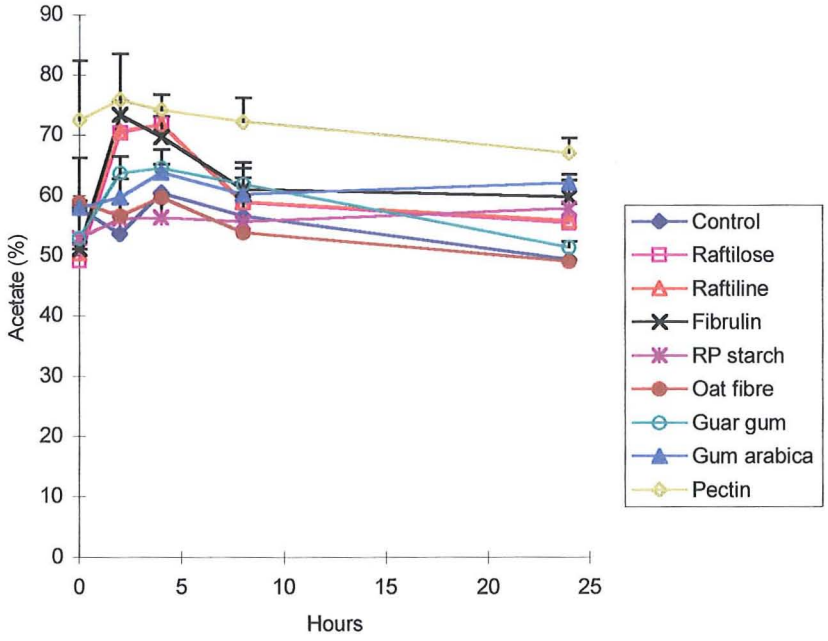


Fig 5.3 Mean (±SEM) acetate percent of total SCFA produced by the fermentation of different carbohydrates incubated with the human faecal bacteria.

There was no significant difference in the acetate ratio at 8 and 24 hours within cultures, except for guar gum cultures in which the acetate ratio at 8 hours was significantly higher than that at 24 hours ($p<0.02$).

5.5.2.2 Propionate

Propionate production was very slow in the initial hours of fermentation, even in cultures containing propionate predominant carbohydrates, such as pectin, guar gum. In later stages of fermentation, raftilose, raftiline, fibruline and guar gum, produced rapidly higher propionate compared with gum arabica and raw potato starch (raftilose vs raw potato starch or vs gum arabica $p<0.02$) at 24 hours. There was no significant difference in the propionate production in cultures of raftilose, raftiline and fibruline at 24 hours. Gum arabica and raw potato starch produced similar propionate at 24 hours of fermentation to each other.

Guar gum was ranked the highest in propionate production at 24 hours, which was significantly higher compared with that in cultures of raftilose and raw potato starch at 24 hours ($p < 0.02$). There was no significant difference between propionate production in cultures of guar gum, raftiline and fibruline at 24 hours (Fig. 5.4). The culture containing guar gum produced significantly higher propionate than pectin ($p<0.00$), which produced similar propionate to gum arabica at 24 hours.

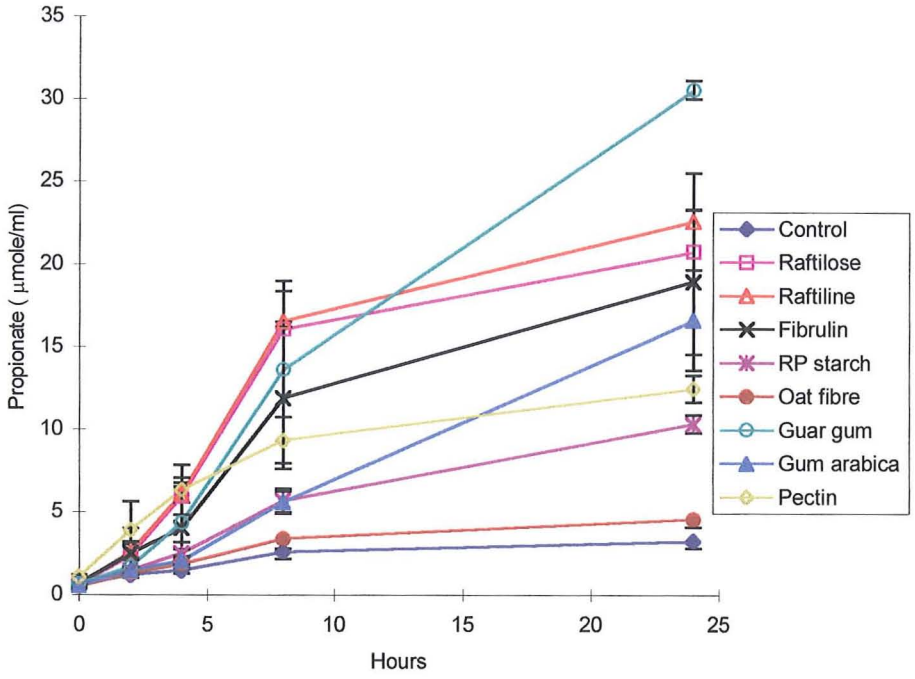


Fig 5.4 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of different carbohydrates with the human faecal bacteria.

Fermentation of guar gum showed a consistent increase in propionate production with increasing incubation. Concentrations of propionate in cultures of guar gum, gum arabica and raw potato starch were significantly higher at 24 hours than that at 8 hours ($p<0.02$). There was no significant difference in the concentrations of propionate within cultures of rapidly fermenting raftilose, raftiline, fibrulin and pectin at 8 and 24 hours. Propionate production rate was very slow in cultures containing oat fibre. Concentrations were not appreciably higher than that produced in control cultures and there was no significant difference of concentrations between 8 and 24 hours of fermentation (Fig. 5.4).

Propionate ratios declined in cultures of all indigestible carbohydrates during the initial hours of incubation. There was a rapid increase in the ratio of propionate in cultures containing raftilose, raftiline, fibruline and guar gum after 4 hours. Fermentation of guar gum yielded significantly higher

propionate ratios compared with all the other carbohydrates tested in this experiment at 24 hours of incubation (Fig. 5.5). Gum arabica produced a significantly higher propionate ratio compared with raw potato starch ($p= 0.025$) and pectin ($p= 0.026$) at 24 hours. Similarly, raftiline yielded significantly higher propionate ratio compared with raw potato starch and pectin at 24 hours ($p < 0.05$). All the other cultures had similar ratio of propionate at 24 hours. Guar gum was the only carbohydrate to have a significantly higher propionate ratio at 24 hours than at 8 hours.

5.5.2.3 n-Butyrate

Fermentation of guar gum showed a consistent increase in n-butyrate production with the increasing incubation. Fermentation of gum arabica and

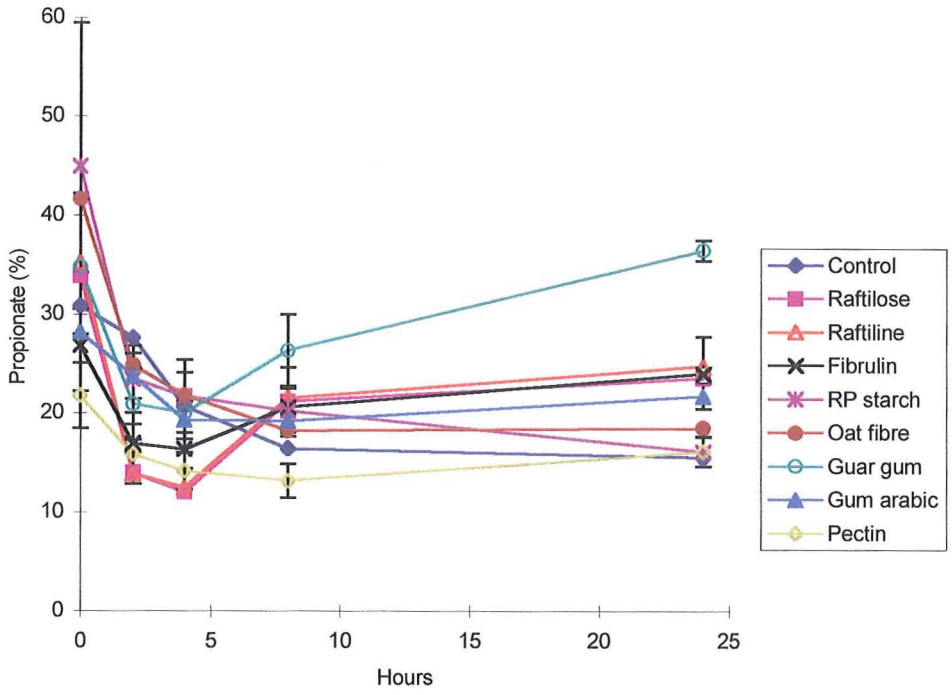


Fig 5.5 Mean (±SEM) propionate percent of total SCFA produced by the fermentation of different carbohydrates with the human faecal bacteria.

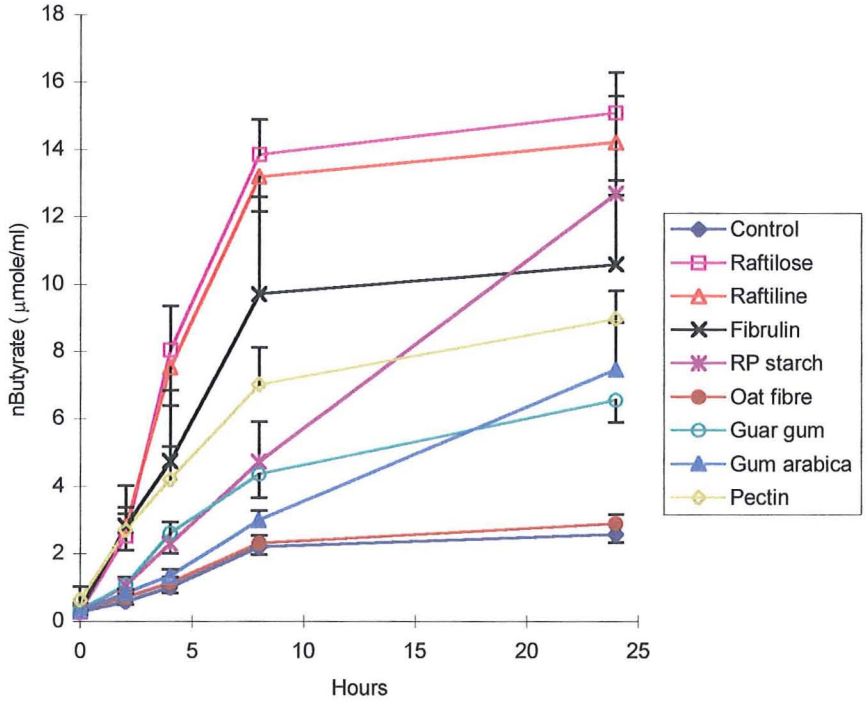


Fig 5.6 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of different carbohydrates with the human faecal bacteria.

pectin also showed a substantial butyrate production with the increasing incubation. The rate of production of n-butyrate was very slow in cultures of

oat fibre (Fig. 5.6). Fermentation of raftilose produced the highest concentrations of n-butyrate. Raftiline showed similar very rapid n-butyrate production. Raw potato starch, although only slowly fermented, was ranked next in the production of n-butyrate at 24 hours (Fig. 5.6). In terms of n-butyrate concentration, two distinct groups emerged; the lower concentrations of n-butyrate were produced by gum arabica, guar gum and pectin. Higher concentrations were produced by raftilose, raftiline and raw potato starch. There was no significant difference in the production of n-butyrate between carbohydrates in each of these groups at 24 hours of fermentation. When the two groups were compared, raftilose and raftiline produced significantly higher n-butyrate ($p < 0.02$) compared with gum arabica, guar gum and pectin at 24 hours. However there was no significant difference in n-butyrate concentration in cultures containing raw potato starch, guar gum, gum arabica or pectin at 24 hours.

Rapidly fermenting carbohydrates such as raftilose, raftiline, fibruline and pectin had no further increase in concentrations of n-butyrate in cultures after 8 hours. Cultures containing raw potato starch, guar gum and gum arabica produced significantly higher n-butyrate at 24 hours than at 8 hours ($p < 0.05$).

The increase in n-butyrate ratio was not rapid in any culture. There was a gradual increase in n-butyrate ratios in cultures containing raftilose, raftiline, fibruline and raw potato starch. Only cultures containing raw potato starch continued an increasing trend in n-butyrate ratio, whereas, fermentation of the other indigestible carbohydrates showed a decline in n-butyrate ratios.

Cultures containing pectin showed a rapid decline in ratio of n-butyrate initially but these ratio increased later. Cultures containing gum arabica and guar gum showed a slight but progressive decrease in n-butyrate ratios with increasing hours of fermentation (Fig. 5.7).

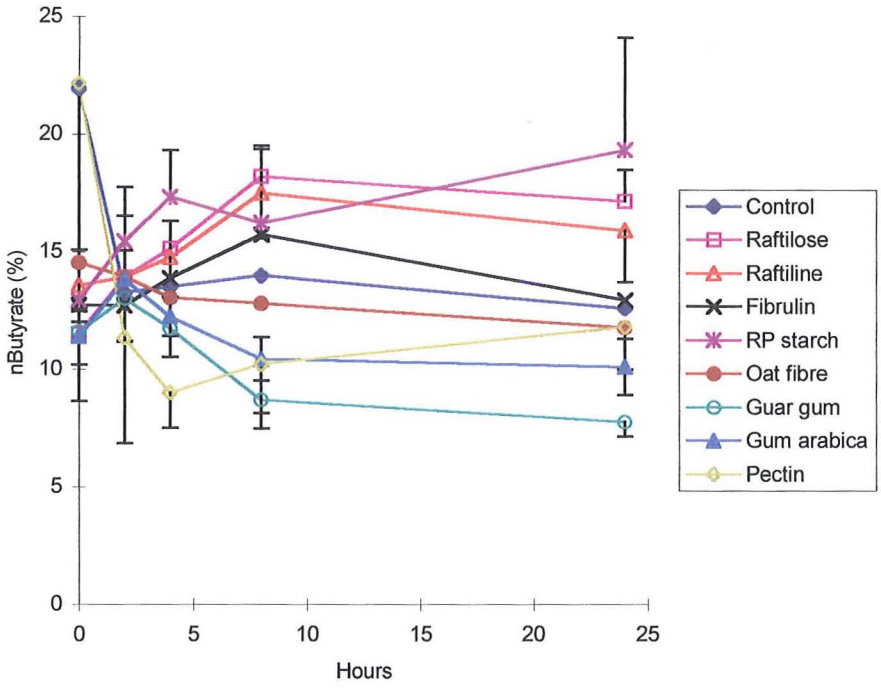


Fig 5.7 Mean (±SEM) nbutyrate percent of total SCFA produced by the fermentation of different carbohydrates with the human faecal bacteria.

At 24 hours, two distinct groups again emerged; those which yielded lower n-butyrate ratios included oat fibre, fibruline, guar gum, gum arabica and pectin, whereas, those which produced higher n-butyrate ratios included raftiline, raftilose and raw potato starch. Within these two groups there was no significant difference in the ratio of n-butyrate. Comparing the two groups raftilose yielded significantly higher n-butyrate ratio ($p < 0.02$) than oat fibre, guar gum, gum arabica and pectin at 24 hours. Raw potato starch did not produce significantly higher n-butyrate ratios than any of these carbohydrates. Raftiline produced significantly higher n-butyrate ratios than guar gum ($p <$

0.02) but not than the other carbohydrates. There was no significant difference in n-butyrate ratio between cultures containing fibruline and other carbohydrate at 24 hours. No carbohydrate culture showed a greater ratio of n-butyrate at 24 hours than at 8 hours.

5.5.3 SCFA predominance profiles

Ratios of the three principal SCFA produced by the fermentation of different indigestible carbohydrates are described in relation to the branched SCFA produced by the fermentation of nitrogenous compounds at 8 and 24 hours (Table 5.1). Oat fibre, raftiline, fibruline, guar gum and gum arabica produced significantly higher propionate ratios ($p < 0.05$) compared with n-butyrate ratios at 24 hours. There were no significant differences in these two ratios within cultures containing raftilose, raw potato starch and pectin. Pectin produced significantly higher acetate ratios compared with oat fibre, raftilose, raftiline and guar gum ($p < 0.02$) at 24 hours (Table 5.1). The sum of the ratios of the three principal SCFA produced by carbohydrates were in increasing order from raftilose, guar gum, pectin, gum arabica and raw potato starch at 24 hours of fermentation. Raw potato starch fermented slowly but produced a significantly higher total ratio of these three SCFA than cultures containing oat fibre and control cultures (with no added carbohydrate) at 24 hours of fermentation (Table 5.1). The ratios of branched SCFA (isobutyrate, iso-valerate, n-valerate) were higher in cultures containing slowly fermenting carbohydrates. After oat fibre, raw potato starch produced the highest branched SCFA at 24 hours of fermentation (Table 5.1).

5.5.4 Lactate

Only raftilose produced lactate (mean value 5.9 ± 1.0) after fermentation at 4 hours with faecal inoculum from all five subjects. At 2 hours of fermentation the inoculum from only one subject produced lactate in cultures containing raftilose and raftiline.

5.5.5 *In vitro* carbohydrate fermentation and pH changes

The pH decreased rapidly during the fermentation of raftilose, raftiline, fibruline, guar gum and pectin up to 8 hours. The pH in cultures of pectin progressively decreased up to 4 hours, then it did not change appreciably during prolonged incubation. A progressive fall in pH from 2 hours to 24 hours of fermentation was seen only in cultures containing raw potato starch and gum arabica. The final pH (24 hours) was significantly lower than at 8 hours only for these two substrates ($p<0.02$; Fig. 5.8).

Fermentation of oat fibre did not show any appreciable change in pH even after 24 hours of fermentation with no difference from that of control cultures (Fig. 5.8). The carbohydrates in this study can be grouped in three categories on the basis of pH reduction at 8 hours of fermentation;

Table 5.1 The mean ratios of individual SCFA (% of total SCFA) produced by the fermentation of different carbohydrates with the human faecal bacteria for 8 and 24 hours (n = 5), (see SEM in the figures).

	8 hours				24 hours			
Substrates	Acetate	Propionate	n-Butyrate	Branched SCFA	Acetate	Propionate	n-Butyrate	Branched SCFA
Raftilose	59	21	18	2	55	24	17	4
Raftiline	59	22	18	1	56	25	16	3
Fibruline	61	21	16	2	60	24	13	3
RP starch	56	20	16	8	58	16	19	7
Guar gum	62	26	9	3	51	37	8	4
Gum arabica	60	19	10	11	62	22	10	6
Pectin	72	13	10	5	67	16	12	5
Oat fibres	54	18	13	15	49	19	12	20

lesser order than the first group. In this group the pH progressively decreased throughout fermentation with a significantly greater reduction ($p < 0.02$) than with gum arabica and raw potato starch after 8 hours (Fig. 5.8). In the third

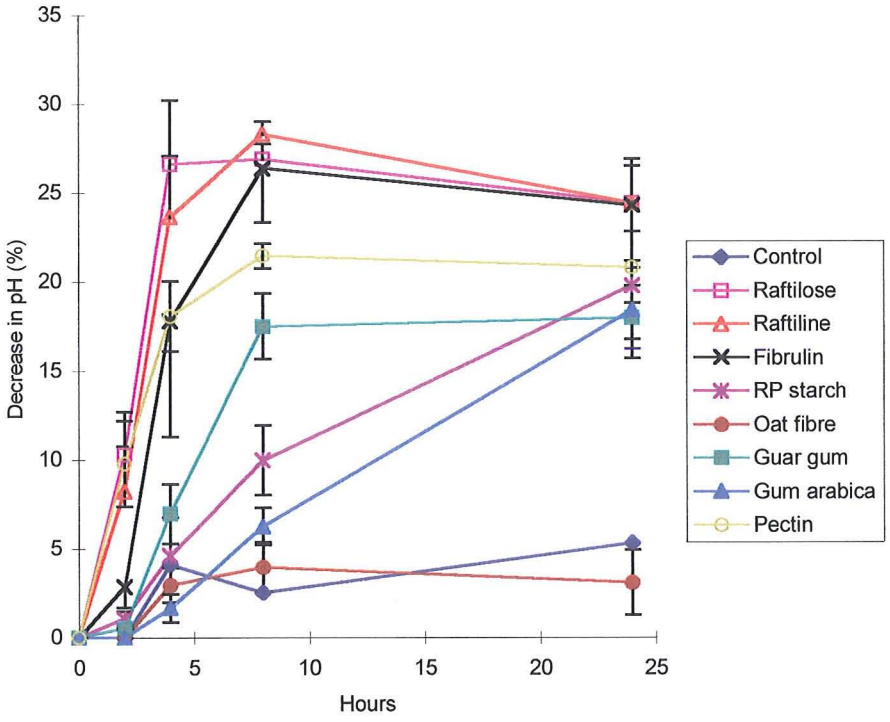


Fig. 5.8 The decrease in pH (%) by the fermentation of different carbohydrates incubated with the human faecal bacteria.

group, oat fibre, gum arabica and raw potato starch, a very slow reduction in pH was noted (Fig. 5.8). Fibruline was intermediate between groups I and II, and the fall in pH in this culture was not significantly different from cultures of guar gum, pectin, raftilose and raftiline at 8 hours.

5.5.6 Dry weight of the residue and water held in cultures

The percent water held by residue was highest in culture of ispaghula (from experiment of the previous chapter) with significantly more water held than raw potato starch and oat fibre at 24 hours ($p<0.02$). The final dry weight of the residue was also higher in culture of ispaghula than other carbohydrates ($p<0.02$; Table 5.2).

5.6 Discussion

5.6.1 Patterns & rates of fermentation

The indigestible carbohydrates used in this study showed different rates of fermentation and on this basis, can be loosely be grouped in three categories. Raftilose, raftiline and pectin were rapidly fermented, whereas fibruline and guar gum showed an intermediate rate of fermentation. Finally, oat fibre, gum arabica and raw potato starch were fermented slowly.

These results were in general agreement with earlier studies (Vince et al., 1990; Adiotomre et al., 1990; Weaver et al., 1992; Achour et al., 1996; Edwards et al., 1996). In an *in vitro* study raw potato starch was fermented slowly with incomplete fermentation and residual starch in the culture and low

Table 5.2 Dry weight of residues and water held (%) in cultures of different carbohydrates after fermentation with the human faecal bacteria at 24 hours.

	Dry weight of residues (g)	Water held in residue (%)
Lactulose	0.064	1.22
Raftilose	0.035	0.66
Raw potato starch	0.043	0.72
Guar gum	0.044	0.82
Gum arabic	0.032	0.69
Pectin	0.046	0.76
Oat fibres	0.111	1.7
Ispaghula	0.149	4.73

SCFA production (Edwards et al., 1996). Fermentation of resistant starch is much slower than most dietary fibres. The peak of fibre fermentation, measured by breath H_2, was reached within 6 hours compared with 10-13 hours of fermentation of resistant starch (Achour et al., 1996). Significantly higher SCFA in faeces of subjects with high resistant starch in their diet have also been recorded (Scheppach et al., 1988; Phillips et al., 1995). This slow fermentation may be either due to slower transit of resistant starch through small intestine or to a greater resistance to bacterial fermentation than most dietary fibre. Oat fibre was fermented very slowly, was not characteristic of oat fibre and it was not in accordance with the earlier reports (McBurney & Thompson, 1987; Fig. 5.1).

5.6.2 Concentrations of SCFA

5.6.2.1 Acetate

Fermentation of raftilose, raftiline, fibruline and pectin produced acetate very rapidly up to 4 hours of fermentation but this production showed a plateau after 8 hours. This increased production of acetate in cultures containing pectin is in accordance with the other studies (Englyst et al., 1987; Adiotomre et al., 1990; Vince et al., 1990). Fermentation of raftilose, raftiline and pectin showed the same pattern of acetate production. It indicated that there was accumulation of the end product due to rapid fermentation in these cultures and the changing physiological conditions may have slowed down the fermentation of these cultures, as discussed earlier in Chapter 3.

Guar gum, gum arabica and raw potato starch showed appreciably linear increase in the SCFA and these cultures did not show any significant difference

in production of net total SCFA and acetate from each other at 24 hours (Fig. 5.1).

5.6.2.2 Propionate

Propionate production was initially very slow, then rapidly increased in cultures of all carbohydrates in this study. Fermentation of guar gum showed a consistent increase in propionate production with increased incubation. Such delayed production of propionate was noted also in the previous chapter (section 4.6.1.2). This is probably due to the pH changes induced in cultures resulting from the production of different organic acids. Fermentation reactions producing propionate, may be favoured by the initial lowering of pH (Edwards et al., 1985).

There was a rapid increase in the ratio of propionate in cultures containing raftilose, raftiline, fibruline and guar gum after 4 hours. Fermentation of guar gum yielded the highest propionate ratio at 24 hours of incubation (Fig. 5.5).

5.6.2.3 n-Butyrate

The highest n-butyrate producing carbohydrates included raftilose, raftiline and raw potato starch. Intermediate producers of n-butyrate included gum arabica, guar gum and pectin. Raftilose produced significantly higher n-butyrate ($p<0.02$) compared with gum arabica, guar gum and pectin, whereas, there was no significant difference in n-butyrate between raw potato starch and these carbohydrates at 24 hours. Raw potato starch produced n-butyrate very slowly compared with all the other carbohydrates (Fig 5.17 8 hours vs 24 hours $p<0.02$).

5.6.3 SCFA predominance profiles

The SCFA profiles yielded by the fermentation of starch, guar gum and gum arabica were in agreement with the earlier studies (McBurney & Thompson, 1987; Adiotomre et al., 1990). Pectin produced very high acetate ratios (Mortensen et al., 1991). Guar gum and gum arabica, although not better n-butyrate producers, had a higher production of propionate, which is another important SCFA, which may have a role in reducing cholesterol (Chen et al., 1984). Raftilose, guar gum and pectin have all been shown to reduce the plasma cholesterol in man (Ellis et al., 1991; Gibson et al., 1993; Wang & Gibson 1993; Ellis et al., 1995; Fernandez et al., 1995; Blake et al., 1997).

In most of the cultures acetate ratios peaked at 8 hours of fermentation, then showed a decline with prolonged fermentation. The acetate ratio was the highest in cultures containing pectin and the lowest in cultures containing guar gum, the other carbohydrates were in between these two extremes. Fermentation of pectin produced higher ratios of acetate with increasing incubation (Fig. 5.3), in accordance with other *in vitro* studies (Vince et al., 1990; Adiotomre et al., 1990; Weaver et al., 1992; Wang & Gibson, 1993). Pectin was reported as a n-butyrate predominant carbohydrate (Vince et al., 1990), in contrast to the other studies in which this was noted as propionate predominant carbohydrate (Englyst et al., 1987b; Adiotomre et al., 1990). Our findings are in agreement with these latter studies, showing a higher yield of acetate as well. In study of Vince et al., (1990) probably prolonged fermentation (48 hours) favoured the production of n-butyrate more.

All these carbohydrates showed SCFA profile with propionate predominance in this experiment at 8 and 24 hours of fermentation, except the culture containing raw potato starch which showed a SCFA profile with n-butyrate predominance at 24 hours of incubation. Our results of guar gum and gum arabica yielding propionate predominant SCFA profiles, were in agreement with the findings of other studies (McBurney & Thompson, 1987; Adiotomre et al., 1990). Similarly SCFA profile with n-butyrate predominance produced by starch were also observed by earlier *in vitro* studies (Englyst et al., 1987b; Weaver et al., 1992; Wang & Gibson, 1993). The fermentation of all indigestible carbohydrates, except starch and pectin, showed a decline in the ratio of n-butyrate with increasing fermentation from 8 to 24 hours time point (Fig. 5.7). Pectin yielded higher ratios of n-butyrate with prolonged (48 hours) incubation. Raw potato starch, however, produced a higher ratio of n-butyrate than pectin in agreement with other *in vitro* studies (Englyst et al., 1987b, Wang & Gibson, 1993; Weaver et al., 1992).

The profile characteristic of each carbohydrate was seen mainly at 24 hours and not before. This finding is in accordance with our previous results reported in Chapter 4 (Section 4.6.1.2), where combinations of different compositions showed corresponding SCFA profiles only after 8 hours. Cultures containing gum arabica and guar gum, showed a slight but progressive decrease in n-butyrate ratios (Fig. 5.7), with a corresponding increase in propionate ratios with increasing fermentation (Fig. 5.5). The final profile was that usually reported for these fibres (McBurney & Thompson, 1987; Adiotomre et al., 1990).

In general, the ratios of acetate, propionate and n-butyrate, were higher at 8 hours than at 24 hours of fermentation in cultures of all carbohydrates, except starch and pectin. This may be an indication of re-fermentation (McBurney & Thompson, 1987) in these cultures. The ratios of the branched chain SCFA showed a similar pattern to previous study (Chapter 4) with more being produced when fermentation of carbohydrate was limited. This probably reflects the low growth of biomass and increased cell death.

5.6.4 SCFA production and pH in cultures

The patterns of decreasing pH in these cultures were as expected in view of their SCFA production. The same groups of carbohydrates as in section 5.6.1 could be identified on the basis of pH. Decreased colonic pH from fermentation of carbohydrates, has been shown to stimulate growth of beneficial bacteria (Edwards et al., 1985; Wang &Gibson 1993). The pH decreased progressively only in cultures containing raw potato starch and gum arabica from 2 hours to 24 hours of fermentation (8 hours vs 24 hours: $p < 0.02$; Fig. 5.8). No other culture showed a significant reduction in pH after 8 hours of fermentation. This was related to the profile of SCFA.

5.6.5 Chemical composition and fermentation of carbohydrates

Several *in vitro* and *in vivo* studies have shown that the intermediate products and end products of fermentation are related to the chemical and physicochemical characteristics of the fibre sources (Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993). Salvador et al., (1993) suggested that chemical nature and physical arrangement of sugars in the fibre matrix controlled the rate and the extent of fermentation as well as the SCFA profiles

produced. The chemical bonds between the constituent monosaccharides may also be important. However, the chemical nature of the monosaccharides may be more important but no general rule can be applied. Fermentation of individual monosaccharides showed that all promoted acetate production. Propionate production was promoted by rhamnose, arabinose xylose, ribose galacturonic acid and glucuronic acid and butyrate was promoted by sorbitol, galacturonic acid and glucuronic acid (Mortensen et al 1988). Salvador et al., (1993) measured the disappearance of sugars from fibre during fermentation and found that uronic acid promoted acetate, glucose, xylose and arabinose promoted propionate and xylose had the greatest impact on butyrate, with some promotion by glucose and uronic acid.. In support of this, ispaghula, an arabinoxylan, promoted propionic acid in our study but starch, a polyglucan, promoted butyrate rather than propionate. Pectin which is mainly polygalacturonic acid, on the other hand, produced more acetate, but in our study, produced more propionate than n-butyrate as previously reported (Englyst et al., 1987b; Adiotomre et al., 1990). Moreover , Salvador et al., (1993) showed completely different fermentability of D-glucose and L-glucose, which means the arrangement of the bonds in molecule of a monosaccharide is also important.

5.7 Conclusion

In this study, the aim was to identify carbohydrate to be used in mixtures which gave the best actions of individual carbohydrates while diluting the potential side effects. In summary, raftilose, raftiline and pectin were rapidly fermented, fibruline and guar gum showed an intermediate rate of

fermentation, whereas, gum arabica and raw potato starch were fermented slowly.

Generally, two categories of carbohydrates emerged on basis of SCFA profile predominance at 24 hours of fermentation; Good producers of propionate included raftilose; raftiline; fibruline; guar gum and gum arabica. Good producers of n-butyrate were raftilose, raftiline and raw potato starch. Raftilose emerged as the most suitable carbohydrate for the production of SCFA, especially n-butyrate. A delay in the rapid fermentation of raftilose will be beneficial in producing more SCFA, especially n-butyrate, at a more distal colonic site in the human. Raftilose has higher water solubility and can be easily used in water based liquid food supplements for tube feeding. On the other hand, raftilose, being a small molecule (oligosaccharide), may have a higher osmotic effect. Ingesting large amounts of raftilose may result in osmotic diarrhoea, thus it will not be possible to use very large amounts of raftilose. Therefore, it would be better to mix raftilose with another suitable carbohydrate to develop a mixed supplement.

For this purpose, an ideal situation might be to combine raftilose with raw potato starch, as both carbohydrates are higher n-butyrate producers. However, raw potato starch, although a good producer of n-butyrate and a slowly fermenting carbohydrate, is not an ideal substrate for tube feeding due to its insolubility in the water, as it will sediment at the bottom of any solution.

Good alternatives include guar gum, gum arabica and pectin which give the additional benefits of their action in the small intestine (Jenkins et al., 1977; McLean Ross et al., 1982; Blackburn et al., 1984; Ellis et al., 1991; Fernandez et al., 1994; Ellis et al., 1995; Fernandez et al., 1995; Blake et al., 1997). These three carbohydrates are soluble in water and could be easily incorporated into the water based liquid food

supplements for tube feeding. In case of the guar gum, high viscosity may become a problem in tube feeding, but in our study we used lower molecular weight guar gum. This may not be a factor in its effects on plasma glucose and cholesterol (Ellis et al., 1991; Blake et al., 1997). Mixing raftilose with guar gum would also reduce the poor organoleptic properties of a guar gum only preparation.

Ispaghula, studied in Chapter 4, should also be considered as it has already been shown to increase the SCFA in the distal colon and to promote propulsion in the large intestine.

CHAPTER 6

Fermentation of mixtures of indigestible carbohydrates

6.1 Introduction

As discussed in previous chapters, indigestible carbohydrates have therapeutic properties which could be exploited in the treatment and prevention of several diseases. Ideally, a carbohydrate should provide higher amounts of SCFA, especially n-butyrate, while promoting gut transit so that the production of these SCFA occurs more at a distal colonic site. On the other hand, such carbohydrates should have good organoleptic properties, and should not have undesirable effects such as bloating, and osmotic diarrhoea. Some carbohydrates are not suitable for naso-gastric tube feeding due to their insolubility and sedimentation in water-based liquid supplements. Considering the idiosyncratic actions of individual indigestible carbohydrates, it is unlikely that any single carbohydrate will provide therapeutically optimal conditions in major human colonic pathologies.

To demonstrate the potential of mixtures of indigestible carbohydrates, I will consider the case of the oligosaccharide raftilose. Raftilose has lower osmotic action than lactulose due to its higher molecular weight. The pre-biotic action of raftilose, stimulating bifidobacterial growth, is especially characteristic (Gibson et al., 1993). Raftilose is readily soluble in water, however, ingesting large amounts may result in osmotic diarrhoea and, as it is rapidly fermented, is likely to promote flatulence and bloating. Therefore, smaller amounts of raftilose used in mixture with another suitable carbohydrate, if this can be achieved without affecting its beneficial actions, will be more effective and practicable for therapeutic supplements. The high solubility of raftilose, makes it ideal for water based naso-gastric tube feeding.

Raftilose, ispaghula, pectin, guar gum and gum arabica were selected from previous experiments (Chapters 4-5). Most of these indigestible carbohydrates are good SCFA producers, showing potential therapeutic effects in different human pathologies, however, these carbohydrates have some undesirable side effects, if ingested in excess (Table 6.1).

Therefore, a logical approach would be to combine different indigestible carbohydrates in mixtures of two or more carbohydrates in order to harvest their potential therapeutic benefits and simultaneously avoid their undesirable side effects.

6.1.1 Potential actions of carbohydrate mixtures.

Tables 6.2A & B show the potential effects of carbohydrates and their mixtures in this chapter based on their known actions and properties from previous studies. It would appear ideal to combine a rapidly fermenting carbohydrate with a slowly fermenting carbohydrate. Rapidly fermenting carbohydrate could produce higher SCFA, especially n-butyrate, in mixtures with a complementary carbohydrate. The other physical properties of carbohydrates such as their viscosity, bulking of colonic contents could have an important role in the interaction of two or more carbohydrates. Such interactions in cultures containing mixtures of carbohydrates with contrasting properties, may result in delayed fermentation of rapidly fermenting carbohydrates such as raftilose or pectin by slowly fermenting carbohydrates such as gums or ispaghula.

In this experiment, mixtures of individual carbohydrates were evaluated for their fermentative properties. First the results of the last chapter will be used

to predict the results that may be expected from different combinations of these carbohydrates in human intestine. The speculated and observed effects

Table 6.1 Advantages and disadvantages of indigestible carbohydrates *in vivo*

Advantage	Disadvantages
i) Delay in gastric emptying & increased satiety, e.g. **guar gum**.	i) Organoleptic un-acceptability, e.g. higher amounts of higher molecular weight **guar gum**.
ii) Lowering of plasma glucose, e.g. **guar gum**.	ii)Bloating, e.g. guar gum.
iii) Reduced plasma lipids and cholesterol, e.g. **pectin**.	iii) Probable osmotic action with higher amounts, in acute case may result in osmotic diarrhoea, e.g. **lactulose**, **raftilose**.
iv) Accelerated intestinal transit, e.g. **ispaghula**.	iv) Abdominal pain, e.g. higher amounts of **lactulose**.
v) Higher fermentability, producing higher concentration of SCFA, consequently may prevent certain colonic diseases, which may be cost effective, e.g. **lactulose, raftilose**.	v) Distension & higher volume of colonic gases and consequently increased flatulence, e.g. higher amounts of **lactulose and raftilose**.
vi) Enhanced the selective bacterial growth, e.g. **raftilose**.	vi) Very slow fermentation may result in loss of un-degraded fibre and un-absorbed SCFA in faeces. e.g. resistant starch
vii) Higher stool frequency and in some cases increased stool output; softer and wetter stool texture, consequently ease of stool passage and reduced risk of constipation, e.g. **ispaghula**.	

of these carbohydrates and their mixtures are given (Table 6.2A & B).

Table 6.2A Observed and expected effects of the fermentation of individual carbohydrates.

Raftilose	Gum arabica	Guar gum	Pectin	Ispaghula
Highly soluble; potential osmotic effect; very rapid fermentation in proximal colon; butyrate production; pre-biotic; very low pH.	Non viscous even at high concentration; slower fermentation; acetate predominant.	Intermediate fermentation; propionate production[2]; high viscosity[8]; low palatability; lower digestion and absorption in the small intestinal resulting in lower plasma glucose and cholesterol[9].	Intermediate palatability; rapid fermentation; acetate predominant[6]. Viscous; and charged molecule; slower digestion and absorption in the small intestinal resulting in lower plasma glucose and cholesterol.	Intermediate palatability and viscosity; very slow fermentation[7]; propionate predominance[5]; high water holding capacity[4] (7g/g); improved plasma cholesterol; increased stool output; faster colonic transit[3].

(1) Hidaka, 1986; Mitsuoka et al., 1987; Gibson et al., 1993; Wang & Gibson, 1993 (2) Wolver et al. 1991; McBurney & Thompson, 1987; Adiotomre et al., 1990; Vince et al., 1990 (3) Edwards et al., 1992a (4) personal communication- Dr. Edwards (5) McBurney & Thompson, 1987; Mortensen et al., 1988; Mortensen et al., 1991; Bourquin et al., 1993 (6) (Englyst et al., 1987; Adiotomre et al., 1990; Vince et al., 1990; Weaver et al., 1992; Wang & Gibson, 1993 (7) Edwards & Rowland, 1992 (8) Chinachoti, 1995 (9) Holt et al., 1979; Johnson & Gee 1980; Blackburn et al., 1984; Jenkins et al 1977; Aro et al., 1984.

Table 6.2B Expected effects of the fermentation of mixtures of carbohydrates.

Substrates	Gum arabica	Guar gum	Pectin	Ispaghula
Raftilose in mixture with	Reduced osmotic load Slower fermentation; increased propionate; bi-fidogenic.	Reduced osmotic load; higher viscosity but much less than GG alone; butyrate and propionate predominance; effects on nutrient absorption in small intestine; Still pre-biotic effect.	Lower osmotic load, increased viscosity (but lower than pectin), slightly slower fermentation, increased acetate production, as well as maintained butyrate; pre-biotic.	No probable osmotic effect; improved palatability of ispaghula; less rapid fermentation of raftilose; high n-butyrate at more distal site; bifidogenic; high solubility; improved bulking and water holding capacity; metabolic effects.
Pectin in mixture with	not done	not done	not done	Less rapid fermentation; delayed high propionate production; reduced viscosity; improved bulking and water holding capacity; improved metabolic effects.
Raftilose and pectin in mixture with	not done	not done	not done	less rapid fermentation; no less SCFA especially high n-butyrate; less rapid fermentation; no osmotic effect; improved palatability & metabolic effect.

6.2 Objectives

The objectives of this study were to determine;

1) the SCFA produced by mixtures of two or three carbohydrates, using carbohydrates with different fermentation properties in an *in vitro* fermentation system.
2) the additive or interactive effect of one carbohydrate on another carbohydrate in mixtures during the *in vitro* fermentation.
3) evaluation of mixtures of carbohydrates with different properties, to speculate on their potential benefit in the human gut.

This may enable us to develop mixtures of carbohydrates with different properties to achieve an optimised beneficial effect in the human gut.

6.3 Methods

The specific procedures for this experiment are described in this chapter, whereas, general methods are detailed in the Chapter 2.

6.3.1 *In vitro* measurement of fermentation

A general description of the *in vitro* system used in this study is given in section 2.2.

6.3.2 Subjects used as donors of faecal inocula

Fresh faeces from human subjects were used as source of inoculum for *in vitro* fermentation, age ranged from 26 years to 57 years, with four males and four females. Faecal samples were collected from eight (five in some cases) different healthy individuals. The criterion for the faecal donors was the same described in Chapter 2.

6.3.3 Fermentation procedure

General details of the fermentation procedure are described in Chapter 2. Three experiments are described in this chapter. In general, guar gum, gum arabica, pectin, ispaghula, and their respective mixtures with raftilose, were used as the sole source of carbohydrate. In each experiment, a control culture without any substrate was incubated to allow for the fermentation of the indigestible substrates in the original inoculum. The procedure and calculation for determination of SCFA by GLC are described in Chapter 2. Statistical analyses were performed as detailed in section 2.14.

6.3.3.1 Two-carbohydrate mixtures of raftilose with gum arabica or guar gum

Raftilose was tested in combination with either gum arabica or guar gum. In each mixture, 50mg of each carbohydrate was used, making 100mg of total amount of the substrate in each culture (n=8). Since cultures showed a flattened curve after 8 hours of incubation in previous experiments and in the initial five subjects of this experiment, cultures from the last three subjects (part of experiment) were incubated for extended period of 48 hours in this experiment, to rule out the possibility of unfermented residual substrate and to determine the full extent of fermentation.

6.3.3.2 Small amounts of the respective substrates

In the second experiment, isolated portions of 50mg of raftilose, gum arabica and guar gum were tested to provide predicted values of SCFA production for hypothetical mixtures of these carbohydrates (n=5).

6.3.3.3 Raftilose in combination with ispaghula and pectin

In the third experiment (n=5), pectin and ispaghula were tested individually and in mixtures with each other and with raftilose, 50mg from each carbohydrate, thus making the total amount of the substrate 100mg. In the same experiment, three-carbohydrates mixture was also tested to determine the interaction of three carbohydrates in mixture. In this experiment, all the three carbohydrates were in equal amounts, making the total amount of substrate 100mg.

6.3.4 Detection of residual sugars in cultures by thin layer chromatography

Since cultures containing raftilose showed a flattening curve after 8 hours of incubation in previous studies and during the initial stage of this experiment, TLC was used to follow the disappearance of raftilose in these cultures from 4-24 hours. Raftilose, was determined by comparison with standards. This method was adopted from Humbel & Collart (1975) and modified for our purpose. Details are given in section 2.6.

6.4 Results

6.4.1 Fermentation of raftilose in combination with gum arabica

Raftilose was fermented very rapidly and gum arabica was fermented slowly. The rate of fermentation in mixtures was not significantly different from that in cultures of rapidly fermenting raftilose but were significantly higher compared with gum arabica ($p< 0.02$). There was no significant difference in the production of net total SCFA between three cultures at 24 hours. SCFA

production declined in mixture as well in culture containing raftilose after 24 hours (Fig. 6.1).

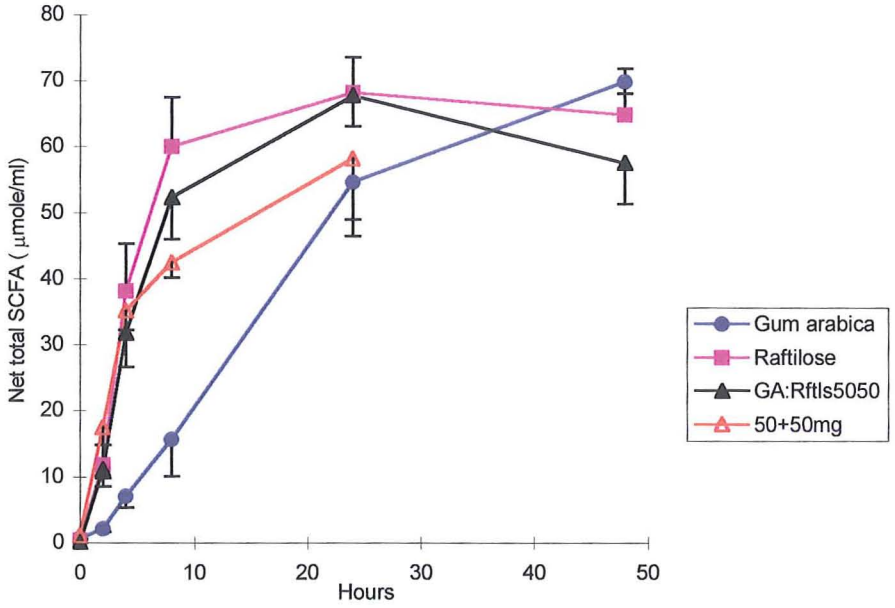


Fig 6.1 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the faecal inoculum [50+50mg represents value of hypothetical mixture; GA:Rftls5050 represents values of actual mixture].

SCFA production was not significantly different in cultures of actual mixtures of two carbohydrates from a hypothetical mixture of such composition at 8 and 24 hours of fermentation (Fig. 6.1).

6.4.1.1 Acetate production

The concentrations of acetate was not significantly different in cultures of all three substrates at 24 hours. Acetate was significantly higher in cultures containing mixtures compared with cultures of gum arabica up to 8 hours ($p < 0.00$). This mixture did not produce significantly different acetate from that predicted with the hypothetical mixture (Fig. 6.2). Cultures containing

mixtures of these carbohydrates did not produce significantly different ratios

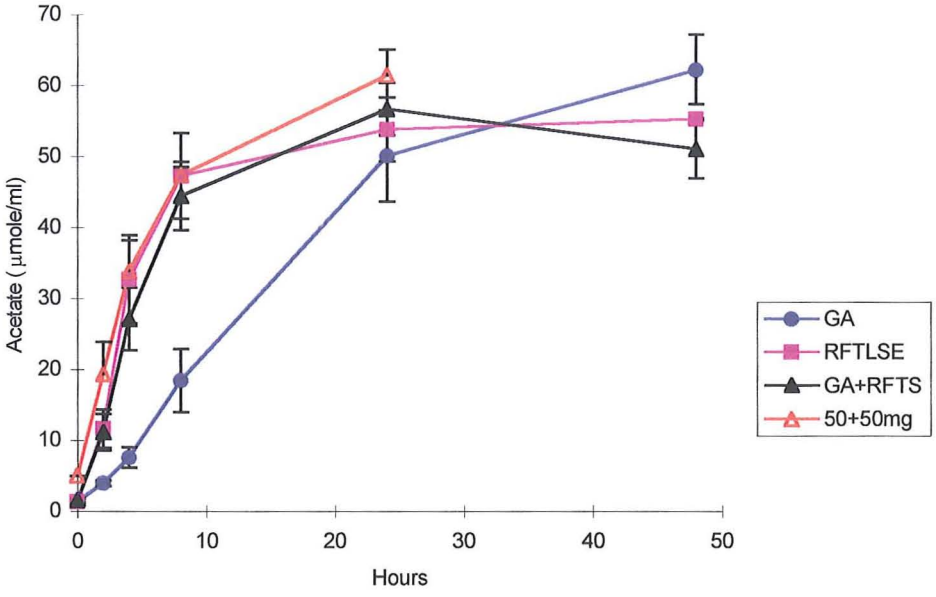


Fig 6.2 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture, with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GA+RFTS represents values of actual mixture].

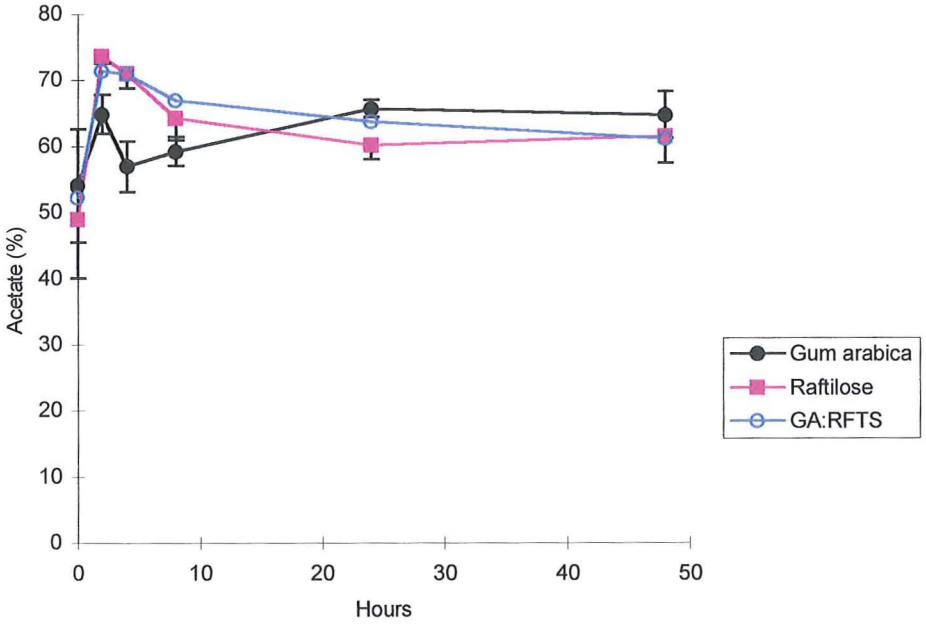


Fig 6.3 Mean (±SEM) acetate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GA:RFTS represents values of actual mixture].

of acetate from cultures of individual carbohydrates at 8 and 24 hours (Fig. 6.3).

6.4.1.2 Propionate production

Mixture of raftilose and gum arabica did not differ in concentration of propionate from those containing raftilose alone but had significantly higher propionate compared with culture of gum arabica alone at 8 hours ($p < 0.018$). There was no difference between any of these cultures at 24 hours. Similarly, the actual mixture did not produce significantly different propionate from a hypothetical mixture of such composition at 8 and 24 hours (Fig. 6.4). There was no significant difference in the propionate ratios in any of these cultures (Fig. 6.5).

6.4.1.3 n-Butyrate production

There was significantly more n-butyrate in mixed carbohydrate cultures than in cultures of only gum arabica at 8 and 24 hours ($p< 0.02$), but there was no significant difference compared with that in cultures of raftilose at 8 and 24 hours. n-Butyrate was produced less rapidly in mixture of these two carbohydrates compared with raftilose alone. There was no difference between real and hypothetical mixtures (Fig. 6.6). n-Butyrate production in mixture was more linear compared with individual component carbohydrates after 24 hours.

Although raftilose produced significantly higher ratios of n-butyrate than gum arabica ($p<0.05$), mixture of these two carbohydrates did not produce significantly different ratios compared with cultures of component carbohydrates at 8 hours. Interestingly, this mixture produced significantly

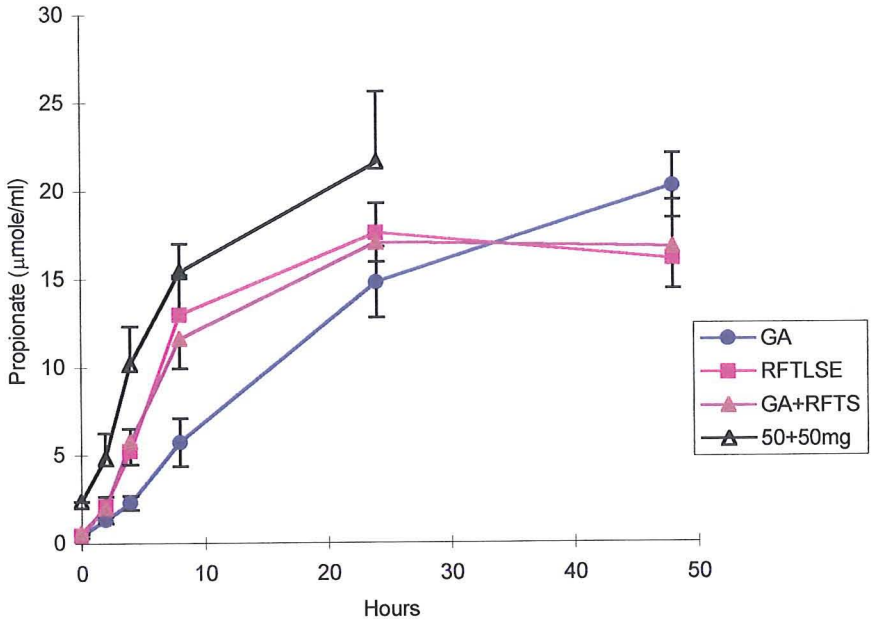


Fig 6.4 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture, with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GA+RFTS represents values of actual mixture].

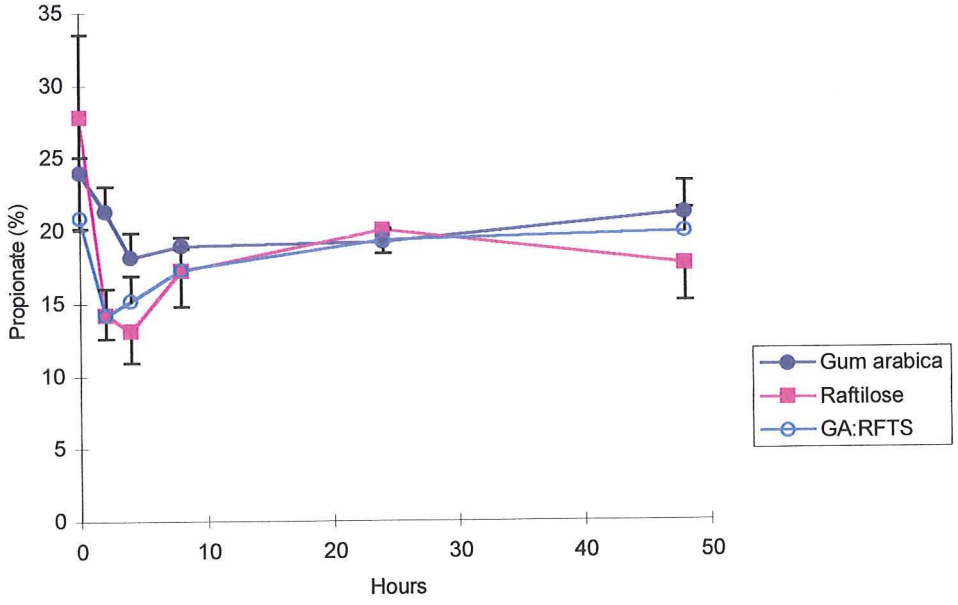


Fig 6.5 Mean (±SEM) propionate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GA:RFTS represents values of actual mixture].

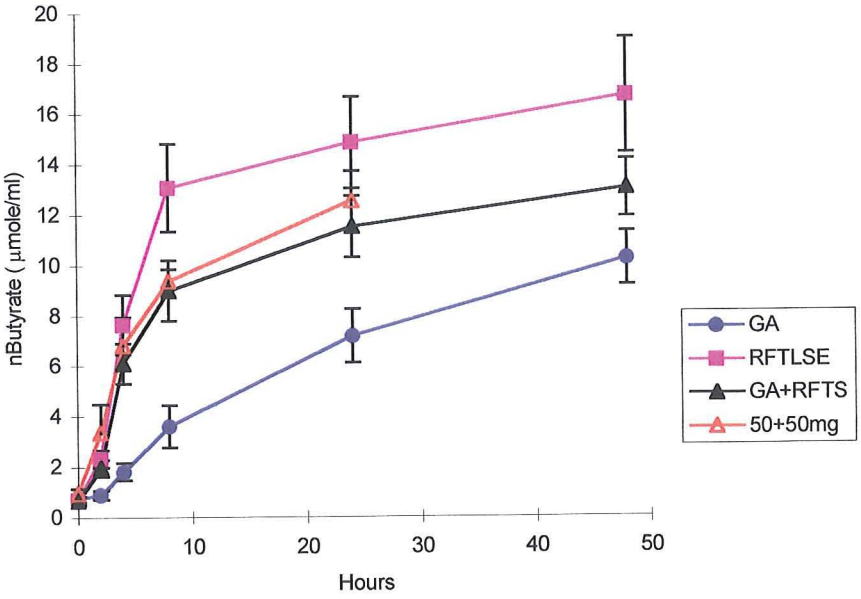


Fig 6.6 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GA+RFTS represents values of actual mixture].

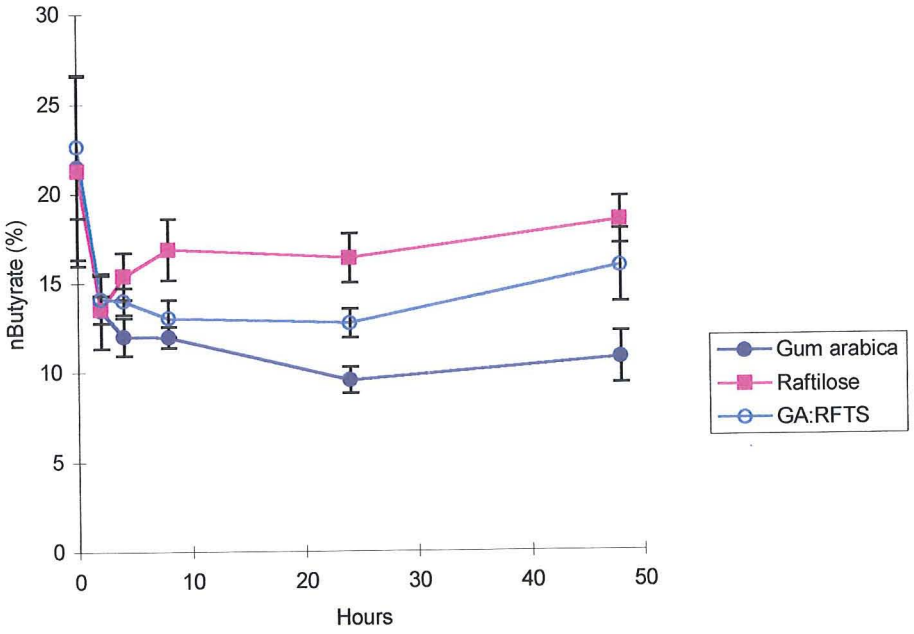


Fig 6.7 Mean (±SEM) nbutyrate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GA:RFTS represents values of actual mixture].

higher n-butyrate ratios than gum arabica ($p < 0.02$) and significantly lower than culture of raftilose at 24 hours. Consequently n-butyrate ratio in mixture

was not significantly lower than in cultures of raftilose at 48 hours of fermentation (Fig. 6.7).

6.4.2 Fermentation of raftilose in combination with guar gum

Cultures containing mixtures of raftilose and guar gum produced significantly higher net total SCFA compared with cultures containing 100mg guar gum ($p<0.02$), but were not significantly different from cultures containing 100mg raftilose at 4 and 8 hours. The rate of fermentation was less rapid in cultures containing mixed substrate compared with cultures of raftilose alone (Fig. 6.8).

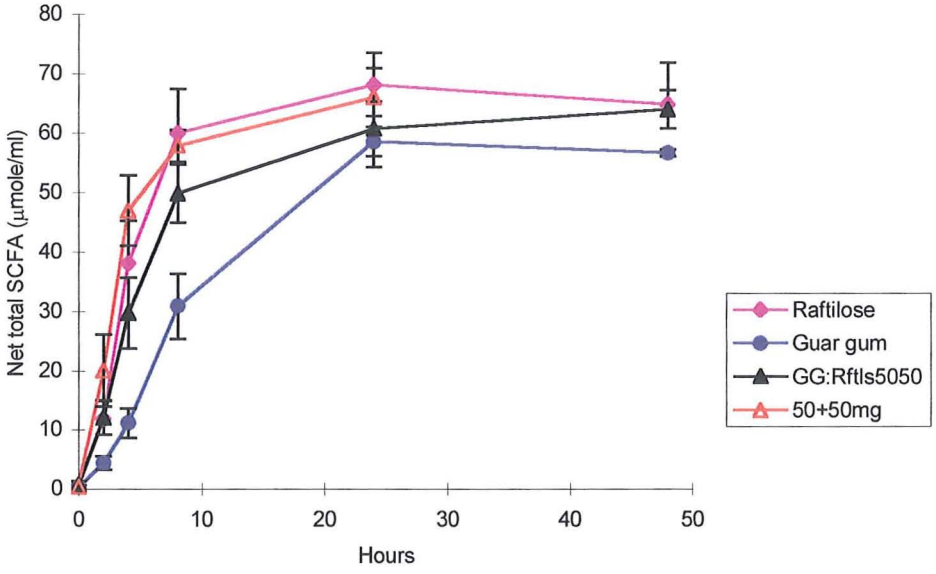


Fig 6.8 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with faecal inoculum [50+50mg represents value of hypothetical mixture; GG:Rftls5050 represents values of actual mixture].

Cultures containing either raftilose or guar gum (100mg), showed a decline in SCFA production at 48 hours, but production in mixtures continued to increase between 8 and 24 hours. The net total SCFA in mixture was similar to that in culture containing 100mg raftilose at 48 hours (Fig. 6.8). The production of net total SCFA in actual mixture was not significantly different

from values that expected from mixture of the same composition at 8 and 24 hours.

6.4.2.1 Acetate production

Acetate production was less rapid in mixtures than in cultures of raftilose alone. The mixture, in contrast to cultures containing individual carbohydrates, showed a continuous increase in acetate even after 24 hours. Acetate concentration was significantly higher in mixture compared with guar gum ($p< 0.05$), but not with raftilose at 8 hours (Fig. 6.9). Acetate concentrations were significantly lower in actual mixtures compared with hypothetical mixtures of such composition at 8 and 24 hours ($p< 0.02$).

Ratios of acetate were not significantly different in mixtures compared with culture of raftilose or guar gum at 8 and 24 hours alone (Fig. 6.10).

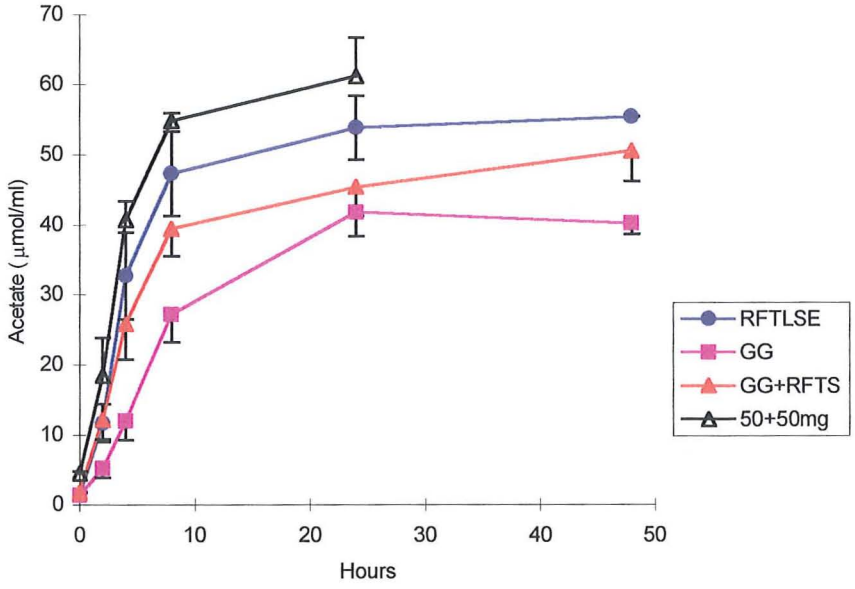


Fig 6.9 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GG+RFTS represents values of actual mixture].

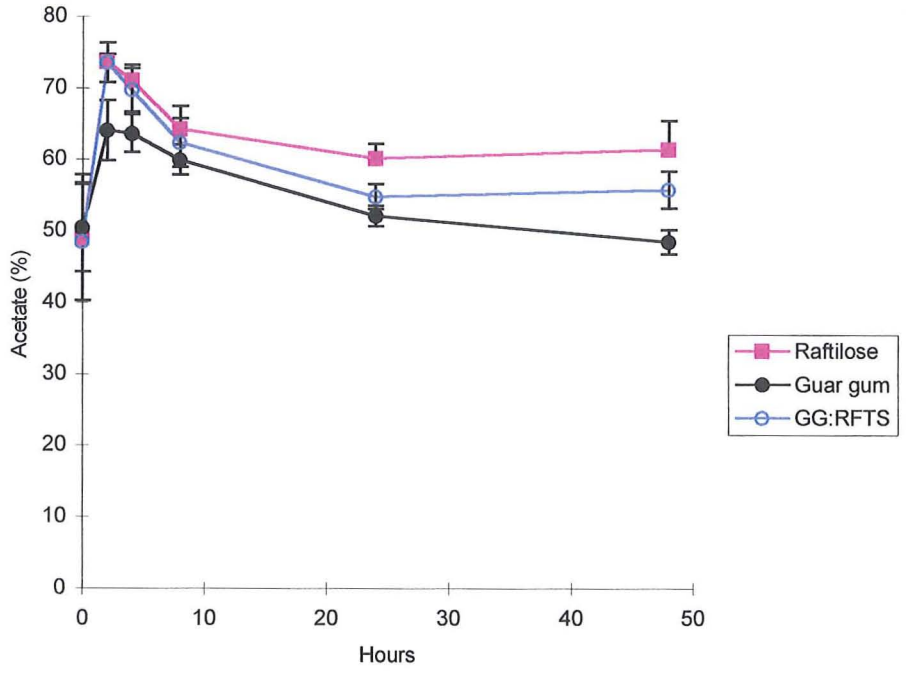


Fig 6.10 Mean (±SEM) acetate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GG+RFTS represents values of actual mixture].

6.4.2.2 Propionate production

Propionate concentration was not significantly different in mixture from that in culture of 100mg raftilose or guar gum at 8 and 24 hours. The production of propionate was less rapid in mixture than culture of guar gum alone. Actual mixtures, in contrast to cultures of raftilose, showed a continuous increase in propionate concentrations even after 24 hours. The rate of propionate production was significantly lower in the actual mixture than that in a hypothetical mixture of such composition at 8 and 24 hours ($p < 0.05$; Fig. 6.11).

Ratios of propionate were not significantly different in mixture of raftilose and guar gum compared with culture of 100mg raftilose or guar gum at 8 hours. Ratios of propionate were intermediate and significantly different ($p<0.02$) in

mixture compared with that in cultures of individual component carbohydrates at 24 hours (Fig. 6.12).

6.4.2.3 n-Butyrate

Mixtures produced significantly higher concentrations of n-butyrate than cultures of guar gum alone ($p< 0.02$). Production of n-butyrate in mixtures was not significantly different from that in cultures of raftilose alone at 8 and 24 hours. Production of n-butyrate in mixtures, however, was less rapid than in cultures of raftilose. n-Butyrate concentration was not significantly different from that in a hypothetical mixture (Fig. 6.13).

Ratios of n-butyrate were significantly higher ($p<0.05$) in mixture than in cultures of guar gum alone but not in cultures of 100mg raftilose at 8 and 24 hours (Fig. 6.14).

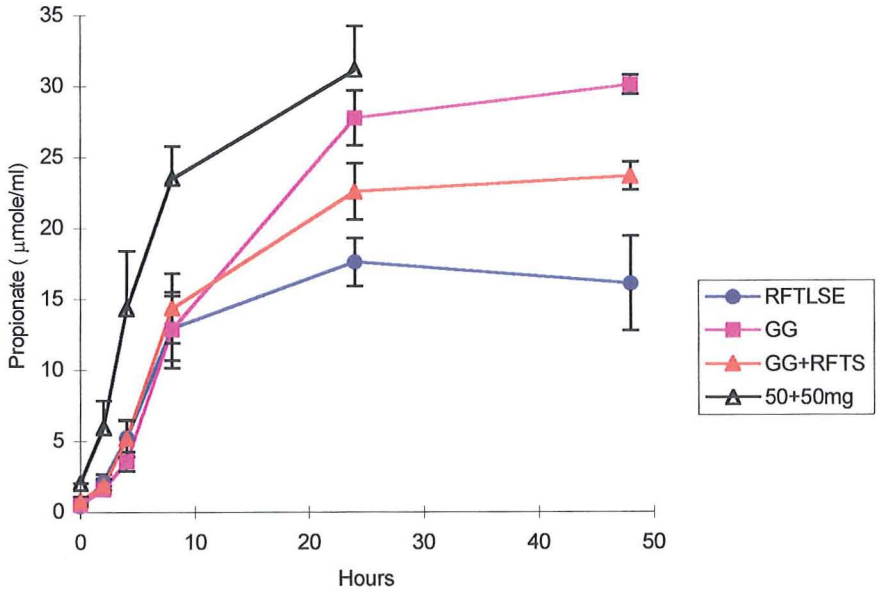


Fig 6.11 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GG+RFTS represents values of actual mixture].

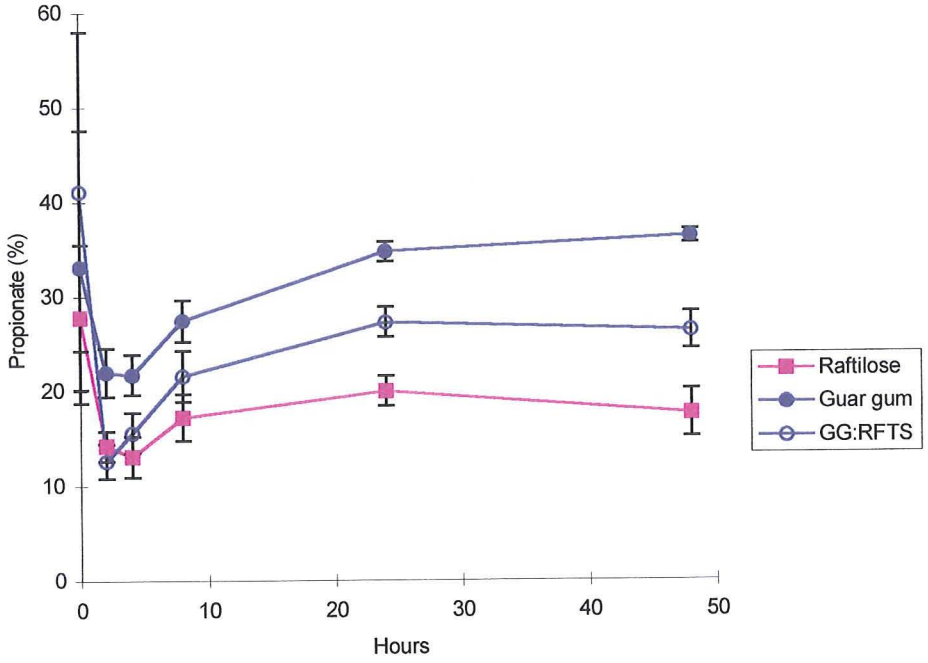


Fig 6.12 Mean (±SEM) propionate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GG+RFTS represents values of actual mixture].

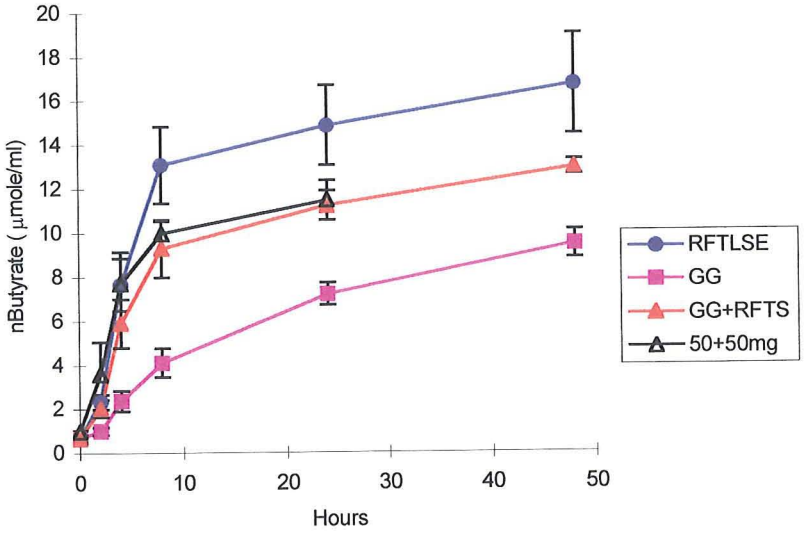


Fig 6.13 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50mg represents values of hypothetical mixture; GG+RFTS represents values of actual mixture].

6.4.3 Fermentation of raftilose in combination with ispaghula

The rate of fermentation of mixed substrates was markedly slower than cultures of 100mg raftilose. Net total SCFA were significantly higher in mixtures than in cultures of 100mg ispaghula at 8 and 24 hours ($p< 0.02$), but not significantly different from culture of 100mg raftilose at 8 hours. Net total SCFA in mixed substrates were significantly lower than in culture of 100mg raftilose at 24 hours ($p<0.05$). Net total SCFA produced in the actual mixtures were not significantly different from that expected from hypothetical mixture of such composition at 8 and 24 hours (Fig. 6.15).

6.4.3.1 Acetate production

Acetate was produced less rapidly in mixture compared with cultures containing 100mg raftilose. Fermentation of mixtures produced significantly higher acetate concentrations than cultures of 100mg ispaghula at 8 and 24 hours ($p< 0.05$ & $p< 0.02$ respectively), but not significantly different from cultures of 100mg raftilose (Fig. 6.16). Production of acetate in actual mixture was not significantly different from a hypothetical mixture at 8 hours (Fig. 6.16).

Acetate ratios were not significantly different in mixture from that in cultures of the constituent carbohydrates (Fig. 6.17).

6.4.3.2 Propionate production

Mixtures produced significantly higher propionate concentrations than cultures of 100mg ispaghula at 8 and 24 hours ($p<0.02$). The rate of production was less rapid with significantly lower propionate concentrations in mixture than

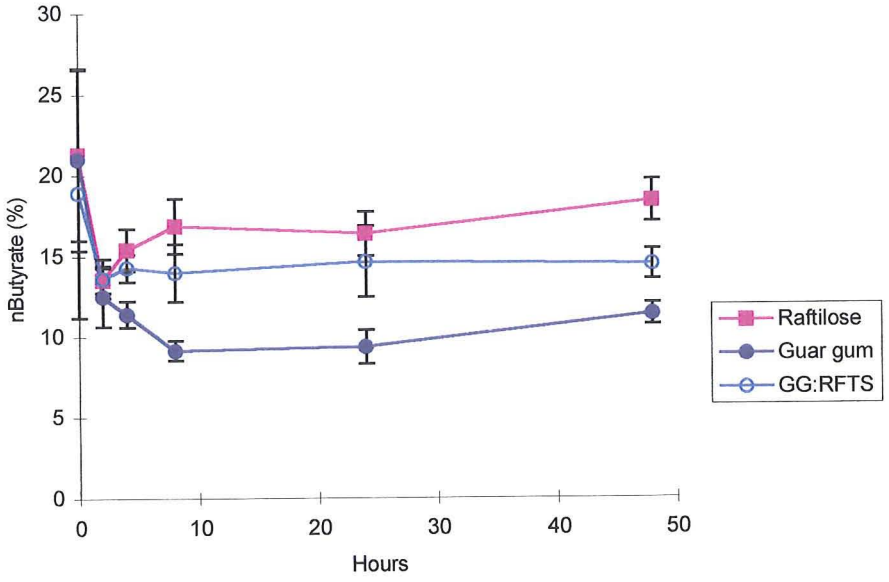


Fig 6.14 Mean (±SEM) nbutyrate percent of total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [GG+RFTS represents values of actual mixture].

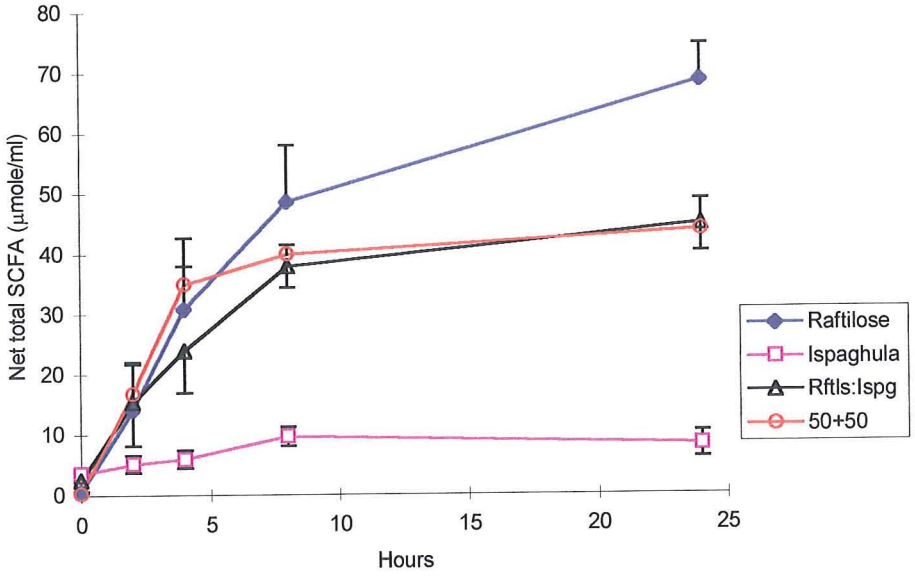


Fig 6.15 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with human faecal bacteria [50+50 represents value of hypothetical mixture; Rftls:Ispg represents values of actual mixture].

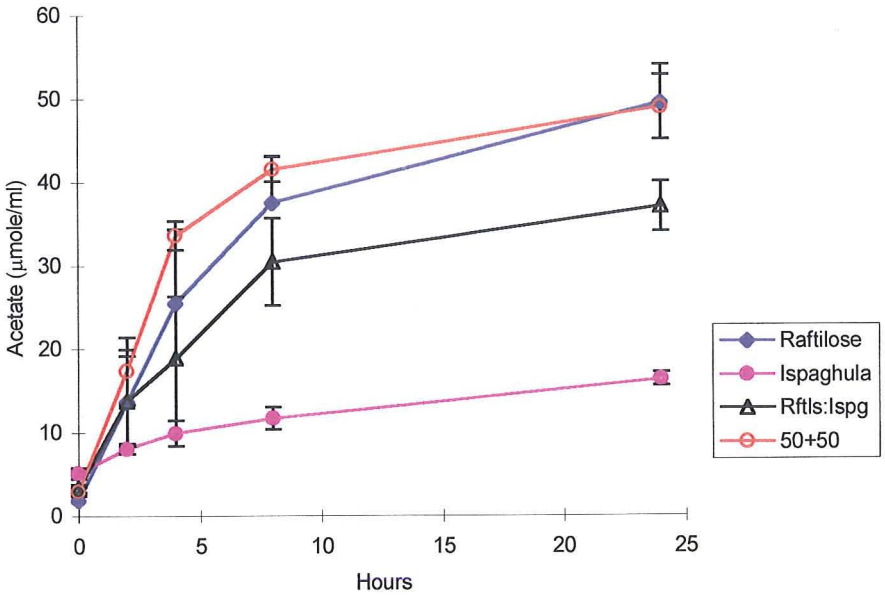


Fig. 6.16 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50 represents values of hypothetical mixture; Rftls:Ispg represents values of actual mixture].

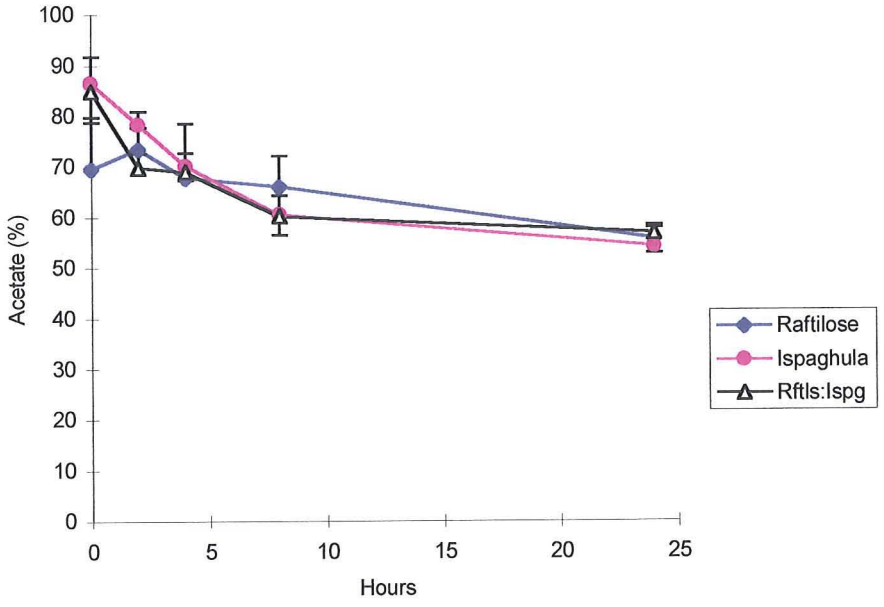


Fig. 6.17 Mean (±SEM) acetate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg represents values of mixture].

culture of raftilose at 24 hours ($p<0.05$), but not significantly different at 8 hours (Fig. 6.18). Production of propionate was not significantly different in

actual mixture from hypothetical mixture of such combination at 8 and 24 hours (Fig. 6.18).

Propionate ratios were not significantly different in mixtures from cultures of the constituent carbohydrates at 8 and 24 hours (Fig. 6.19).

6.4.3.3 n-Butyrate production

Significantly higher n-butyrate concentrations were produced in mixtures than cultures of 100mg ispaghula ($p < 0.02$ & $p < 0.05$) at 8 and 24 hours respectively. n-Butyrate production was less rapid, however, n-butyrate concentrations were not significantly different in mixtures than cultures of 100mg raftilose at 8 and 24 hours. n-Butyrate concentrations was not significantly different in mixtures from hypothetical mixtures (Fig. 6.20).

There was no significant difference in the ratios of n-butyrate in cultures of 100mg ispaghula, raftilose and their mixtures at 8 and 24 hours (Fig. 6.21).

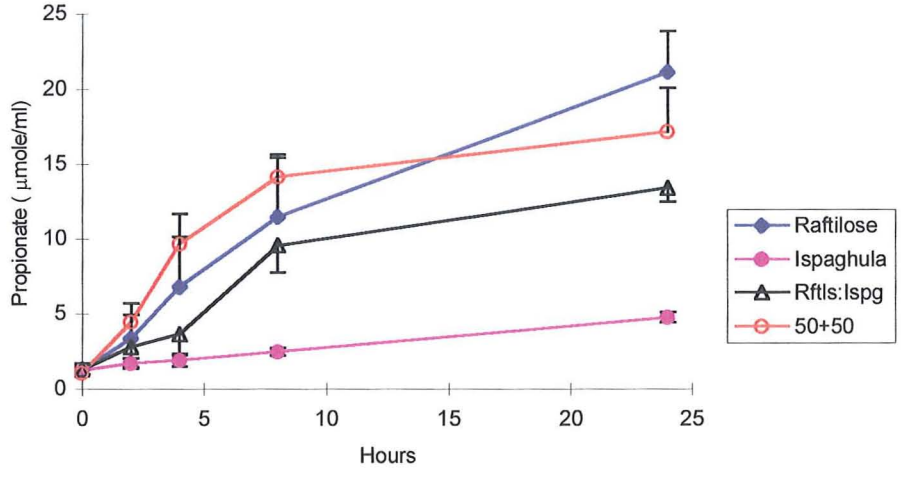


Fig. 6.18 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50 represents values of hypothetical mixture; Rftls:Ispg represents values of actual mixture].

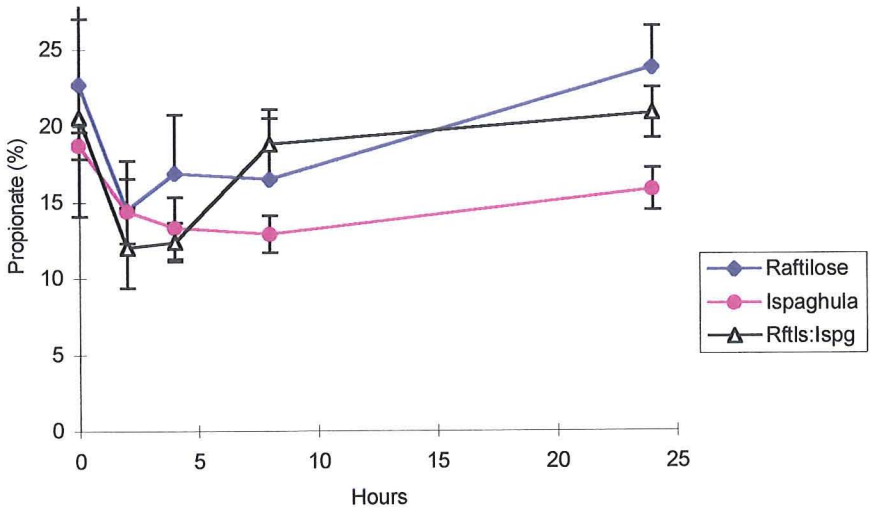


Fig. 6.19 Mean (±SEM) propionate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg represents values of mixture].

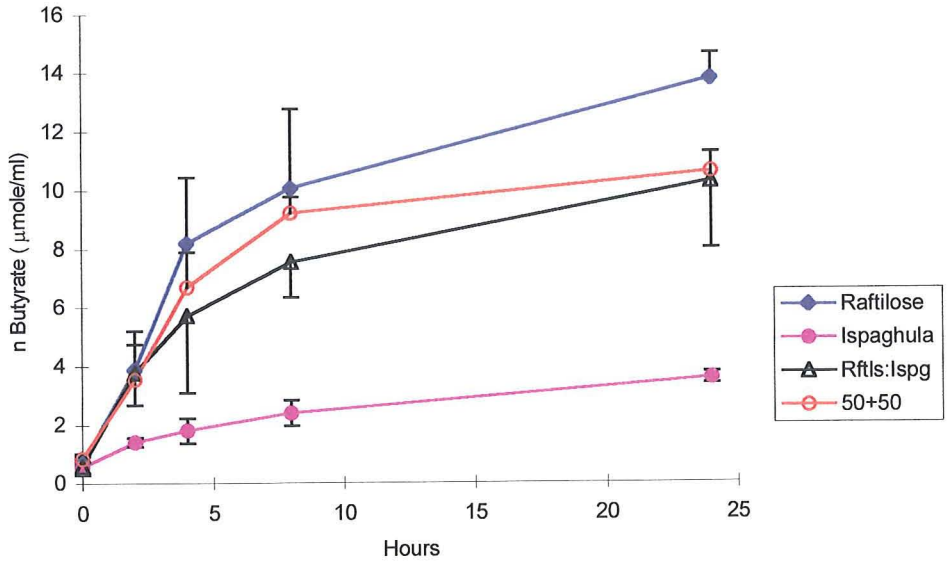


Fig. 6.20 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [50+50 represents values of hypothetical mixture;Rftls:Ispg represents values of actual mixture].

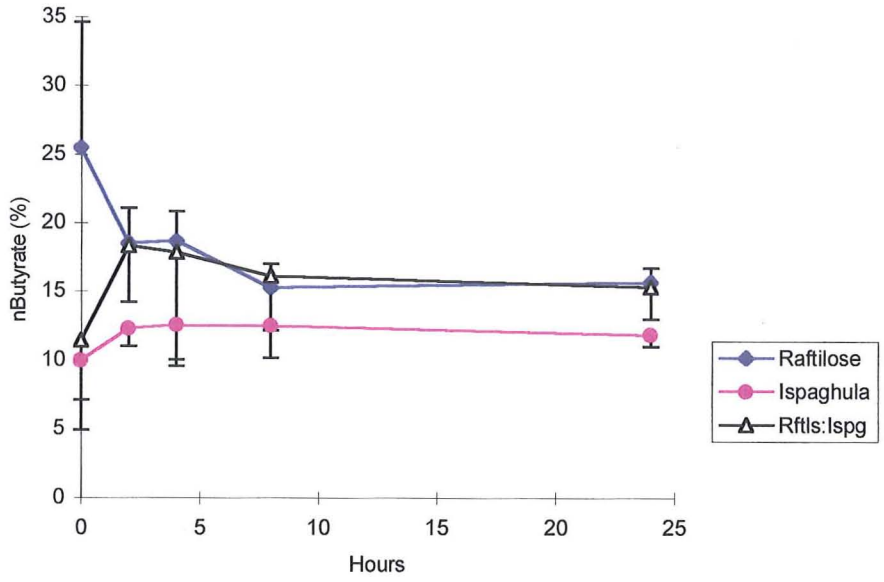


Fig. 6.21 Mean (±SEM) nbutyrate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg represents values of mixture].

6.4.4 Fermentation of raftilose in combination with pectin

The rate of fermentation was slightly more rapid in mixtures compared with the constituent carbohydrates. Mixed substrate cultures showed a linear increase in concentrations of net total SCFA, unlike the plateau effect noted in cultures of 100mg pectin,. The fermentation pattern of raftilose was dominant in cultures of mixed substrates. Net total SCFA were not significantly different in mixtures from cultures of the constituent carbohydrates (Fig. 6.22).

6.4.4.1 Acetate production

Acetate concentrations were not significantly different in mixtures from cultures of the individual constituent carbohydrates at 8 and 24 hours. The plateau effect observed in cultures of pectin, was not noted in mixtures (Fig. 6.23).

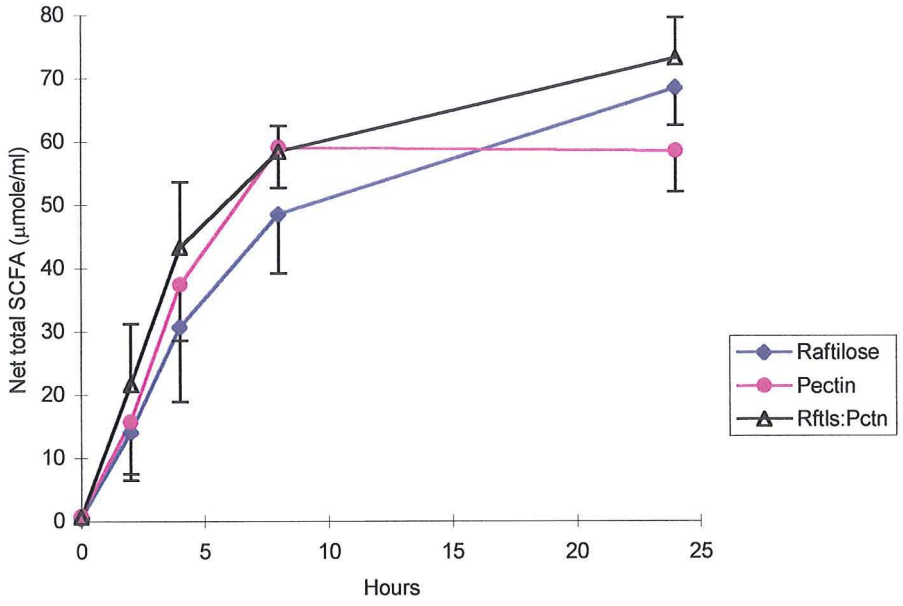


Fig 6.22 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn represents values of mixture].

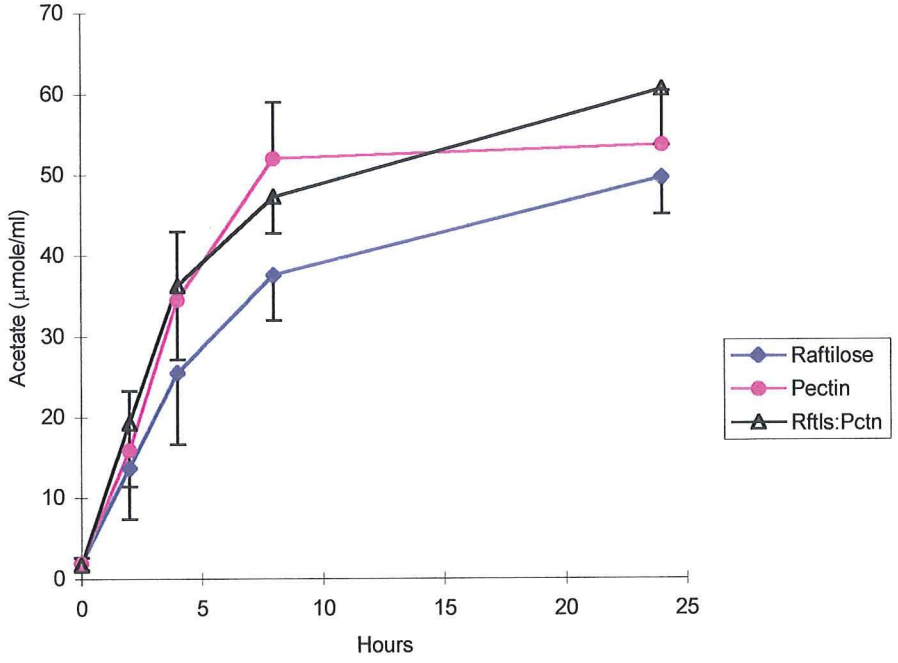


Fig. 6.23 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:pectin represents values of actual mixture].

Acetate ratios in mixtures of raftilose and pectin were not significantly different from that in cultures of the constituent carbohydrates at 8 and 24 hours (Fig. 6.24).

6.4.4.2 Propionate production

The rate of propionate production was not different in mixtures of raftilose and pectin from cultures of the constituent carbohydrates, except at 24 hours, where production of propionate was significantly higher in the mixture compared with the culture of 100mg pectin ($p < 0.05$). Propionate concentration was not significantly different in mixtures compared with cultures of 100mg raftilose (Fig. 6.25).

Ratios of propionate were not significantly different in mixtures of raftilose and pectin from that in cultures of the constituent carbohydrates at 8 and 24 hours (Fig. 6.26).

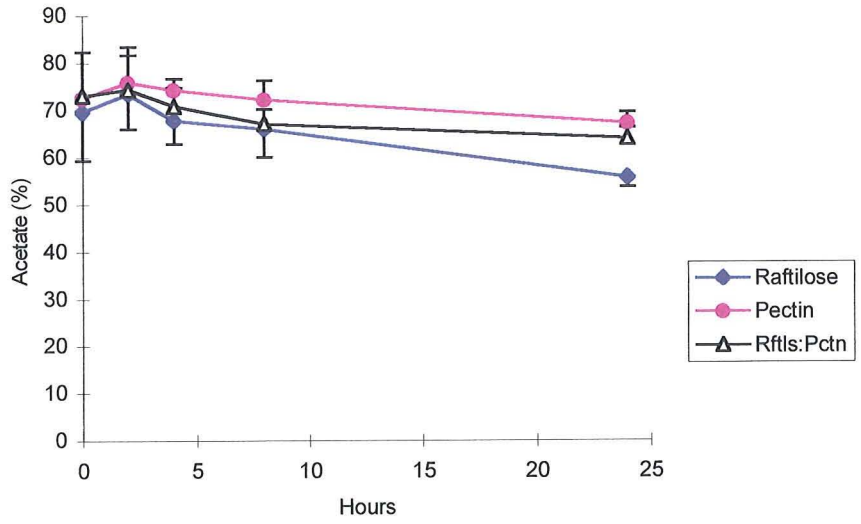


Fig. 6.24 Mean (±SEM) acetate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn represents values of mixture].

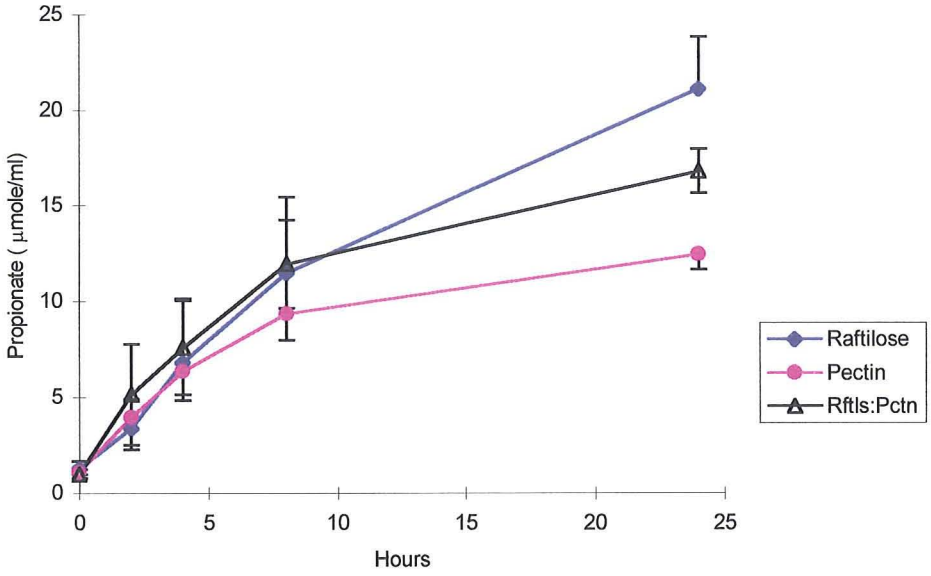


Fig. 6.25 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn represents values of mixture].

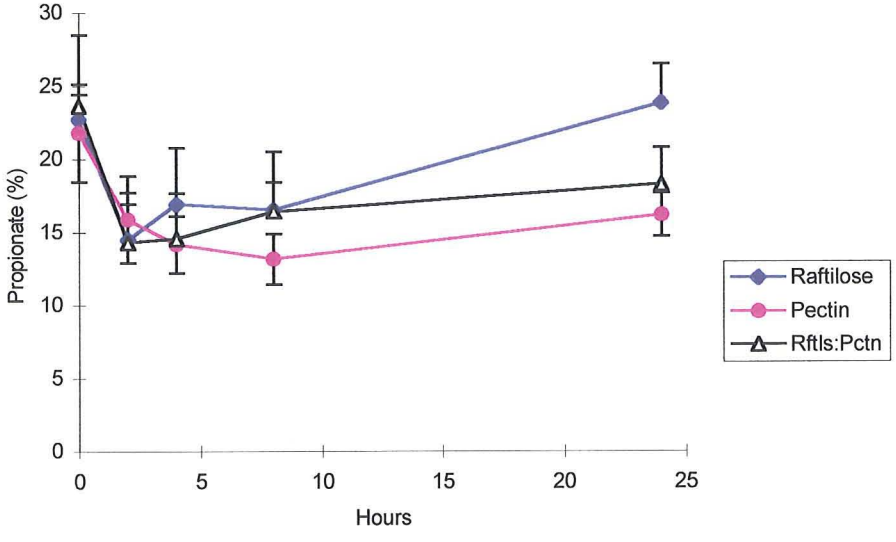


Fig. 6.26 Mean (±SEM) propionate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn represents values of mixture].

6.4.4.3 n-Butyrate production

Mixtures of raftilose and pectin did not produce significantly different n-butyrate concentrations from cultures of the constituent carbohydrates. Production of n-butyrate was less rapid in mixtures compared with cultures of 100mg raftilose (Fig. 6.27).

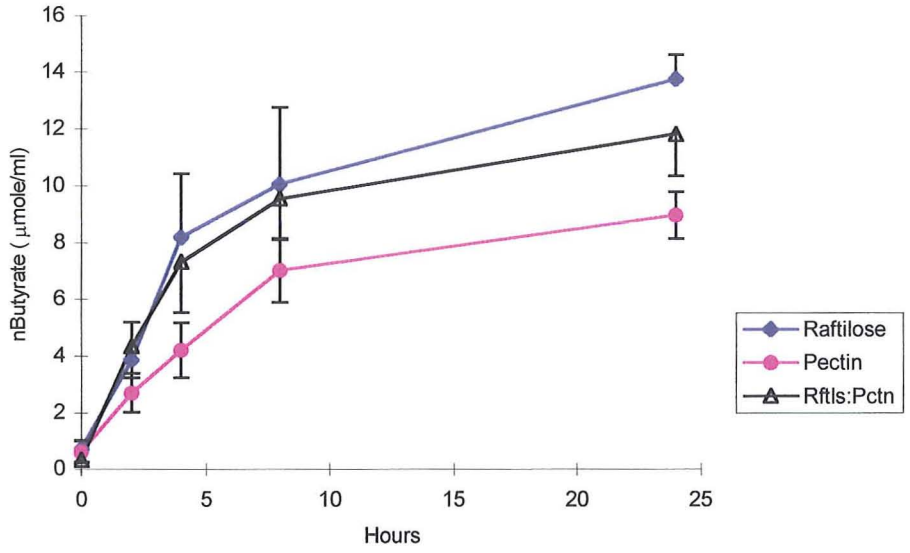


Fig. 6.27 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn - represents values of mixture].

Ratios of n-butyrate were not significantly different in mixtures from cultures of the constituent carbohydrates. Ratios of n-butyrate in mixtures gradually decreased as in culture of 100mg raftilose. These ratios declined to the similar level of those in cultures of 100mg pectin at 24 hours of fermentation (Fig. 6.28).

6.4.5 Fermentation of ispaghula in combination with pectin

Mixtures containing ispaghula and pectin performed similarly to cultures containing 100mg pectin. Net total SCFA were significantly higher in mixtures compared with 100mg ispaghula at 8 and 24 hours of fermentation ($p<0.02$),

but significantly lower compared with 100mg pectin at 8 and 24 hours ($p<0.05$ & $p<0.02$). Ispaghula significantly slowed the rate of fermentation in mixture (Fig. 6.29).

6.4.5.1 Acetate production

Acetate production was less rapid in mixtures containing pectin and ispaghula than cultures of 100mg of pectin. Acetate concentration was significantly higher than culture of ispaghula ($p< 0.02$), but not significantly different from cultures of 100mg pectin at 8 and 24 hours (Fig. 6.30).

Acetate ratios were not significantly different in mixture from that in culture of ispaghula at 8 hours, but were significantly higher in mixture than in culture of ispaghula at 24 hours ($p<0.02$). Similarly acetate ratios in mixtures were not significantly different form cultures of 100mg pectin (Fig. 6.31).

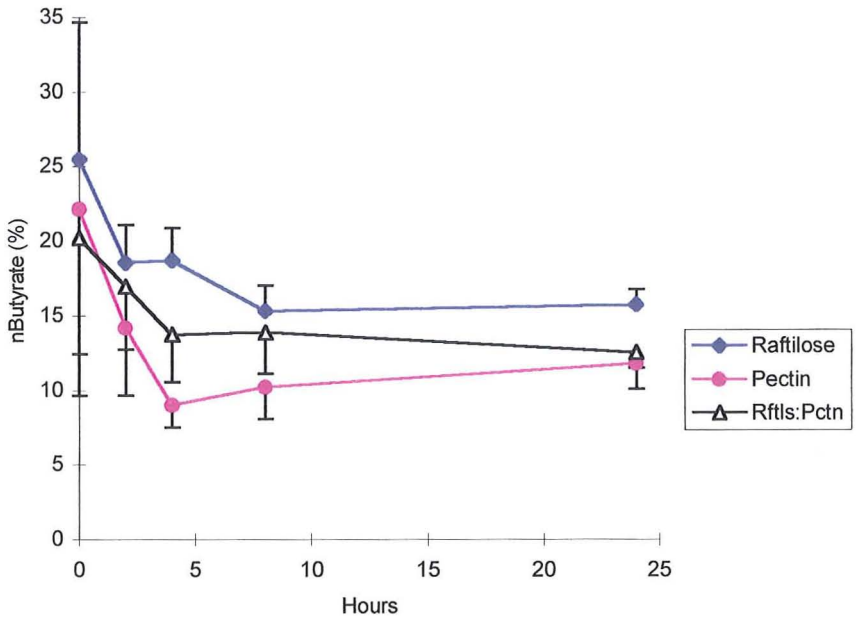


Fig. 6.28 Mean (±SEM) nbutyrate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Pctn represents values of mixture].

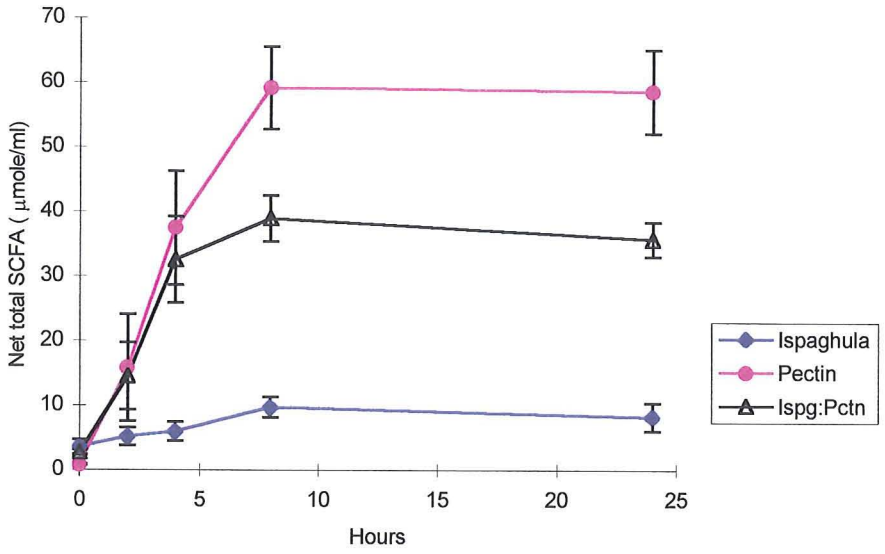


Fig. 6.29 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of mixture].

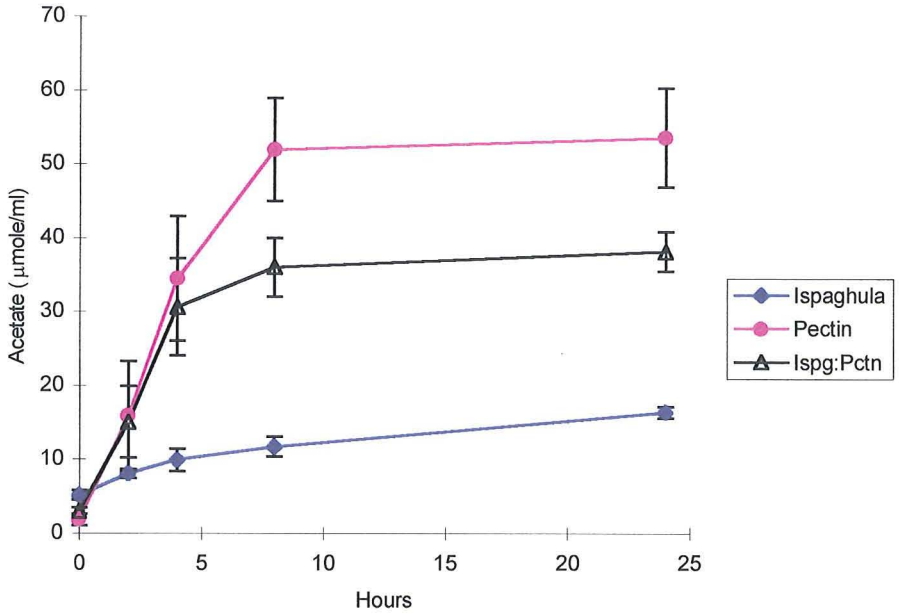


Fig. 6.30 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of actual mixture].

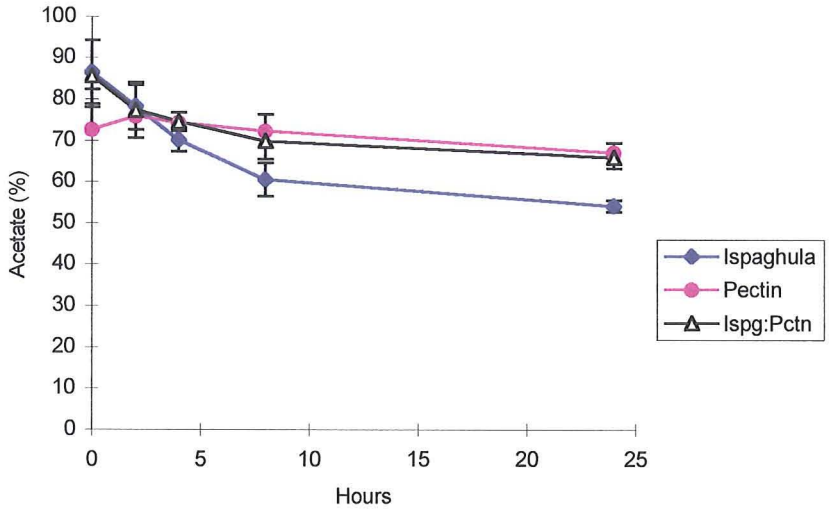


Fig. 6.31 Mean (±SEM) acetate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of mixture].

6.4.5.2 Propionate production

Propionate concentration was significantly higher in mixtures than in cultures of 100mg ispaghula at 8 and 24 hours ($p < 0.02$). Propionate concentration was significantly lower in mixture compared with culture of 100mg pectin at 24 hours ($p < 0.02$). The rate of propionate production was less rapid in mixtures compared with cultures of 100mg pectin (Fig. 6.32). Propionate ratios in mixtures were not significantly different from that in cultures of the constituent carbohydrates (Fig. 6.33).

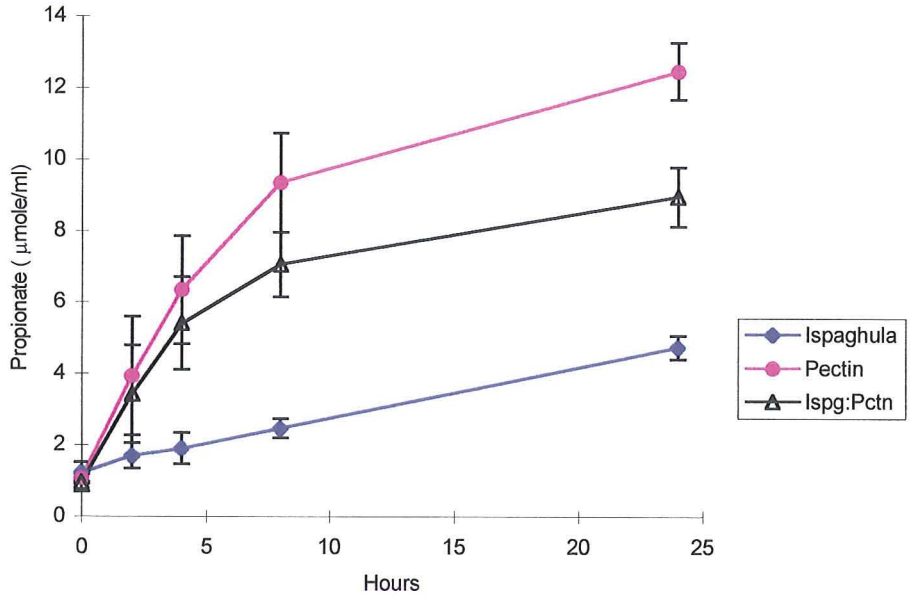


Fig. 6.32 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of mixture].

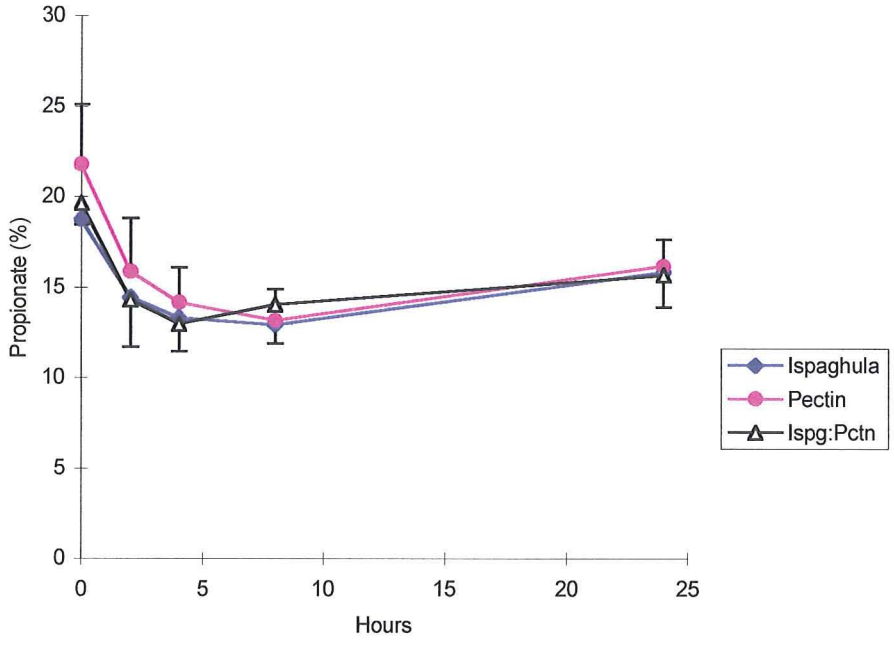


Fig. 6.33 Mean (±SEM) propionate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of actual mixture].

6.4.5.3 n-Butyrate production

The rate of n-butyrate production was less rapid in mixture compared with culture of 100mg pectin. n-Butyrate concentration in mixture was not

significantly different from culture of 100mg pectin at 8 hours, but significantly lower at 24 hours ($p<0.02$). n-Butyrate concentrations were significantly higher in cultures of mixed substrate than cultures of 100mg ispaghula ($p<0.05$; $p<0.02$) at 8 and 24 hours (Fig. 6.34).

Ratios of n-butyrate were not significantly different in cultures of constituent carbohydrates and their mixtures at 8 and 24 hours (Fig. 6.35).

6.4.6 Fermentation of raftilose in combination with ispaghula and pectin in mixture of three carbohydrates

Fermentation was slower in mixtures compared with cultures of raftilose or pectin alone. Net total SCFA were significantly higher in mixtures than in cultures of 100mg ispaghula ($p< 0.02$). SCFA production was not significantly different in three-carbohydrates mixture compared with cultures of raftilose or pectin alone (Fig. 6.36).

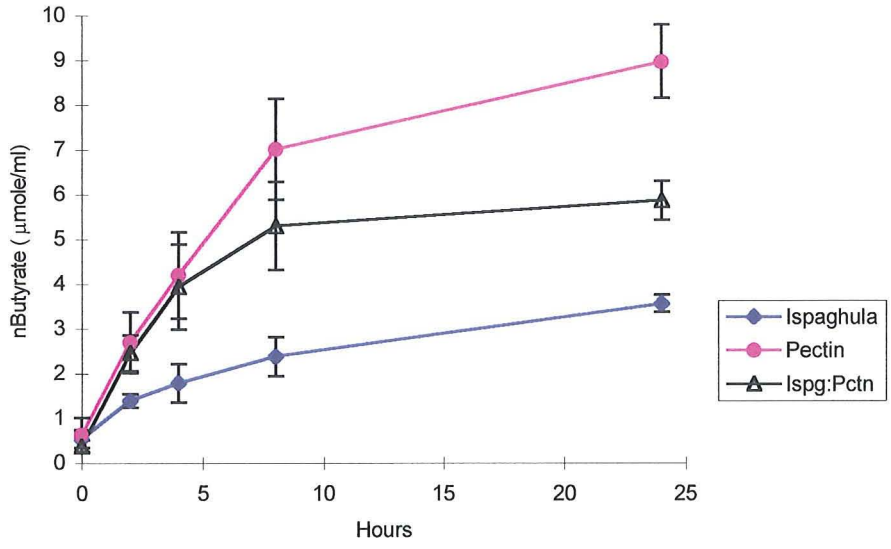


Fig. 6.34 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn- represents values of mixture].

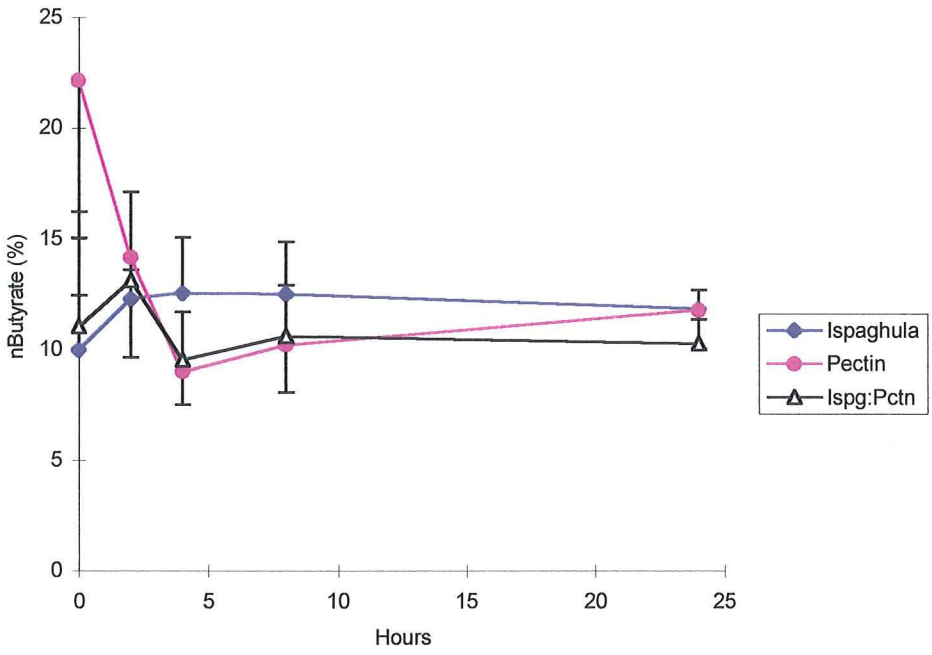


Fig. 6.35 Mean (±SEM) nbutyrate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Ispg:Pctn represents values of actual mixture].

6.4.6.1 Acetate production

Production of acetate in mixtures was more linear compared with the cultures of pectin alone, showing the rate of production was almost similar as in cultures of 100mg of raftilose. Fermentation of mixture produced significantly higher acetate concentrations than that in culture of ispaghula ($p < 0.02$). There was no significant difference in the acetate production in mixtures compared with cultures containing 100mg raftilose or pectin (Fig. 6.37).

Acetate ratios in mixtures were significantly higher than cultures of 100mg raftilose or ispaghula ($p < 0.02$) at 24 hours, but not significantly different from that in cultures of 100mg pectin at 8 and 24 hours (Fig. 6.38).

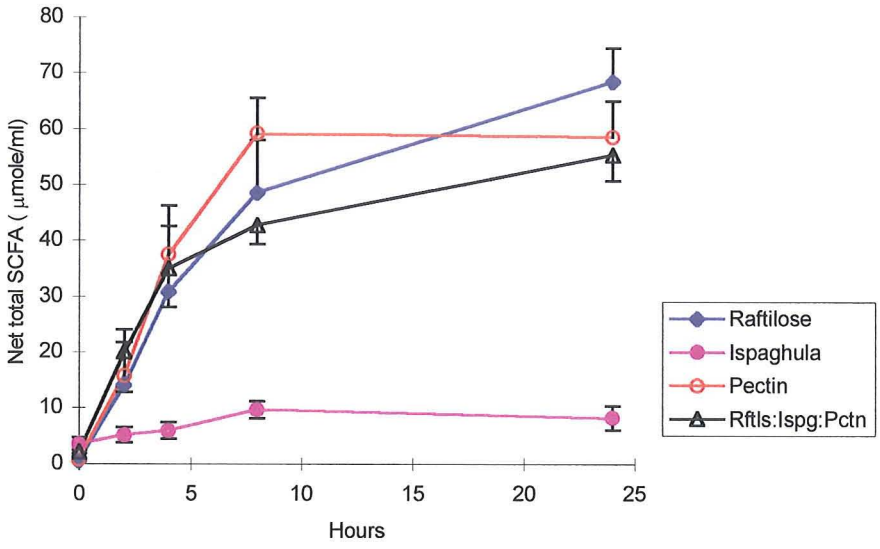


Fig. 6.36 Mean (±SEM) concentrations of net total SCFA (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn represents values of the three-carbohydrates mixture].

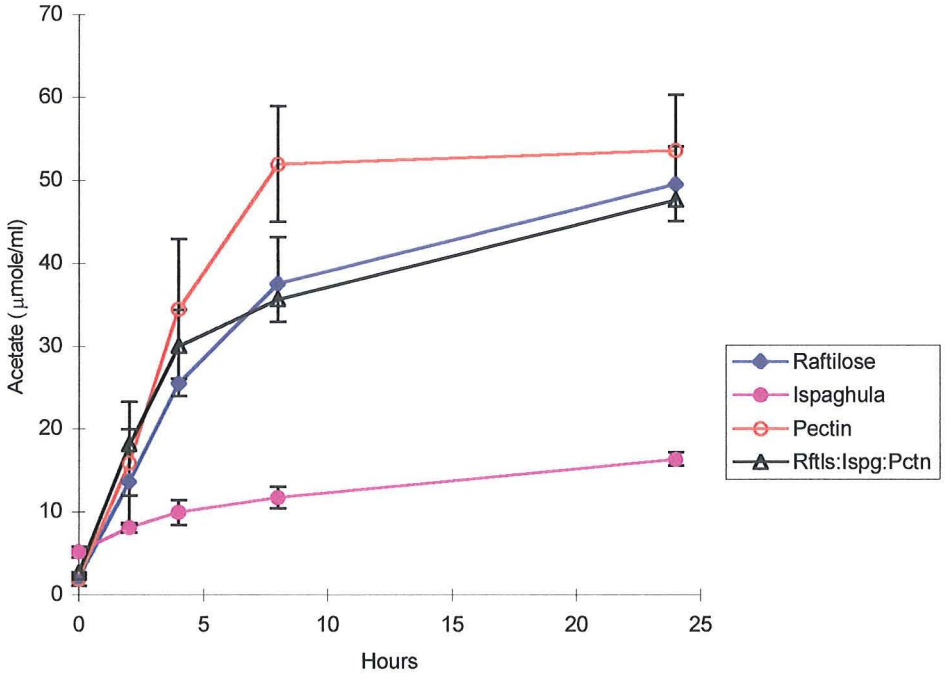


Fig. 6.37 Mean (±SEM) concentrations of acetate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Raftls:Ispg:pctn represents values of the mixture].

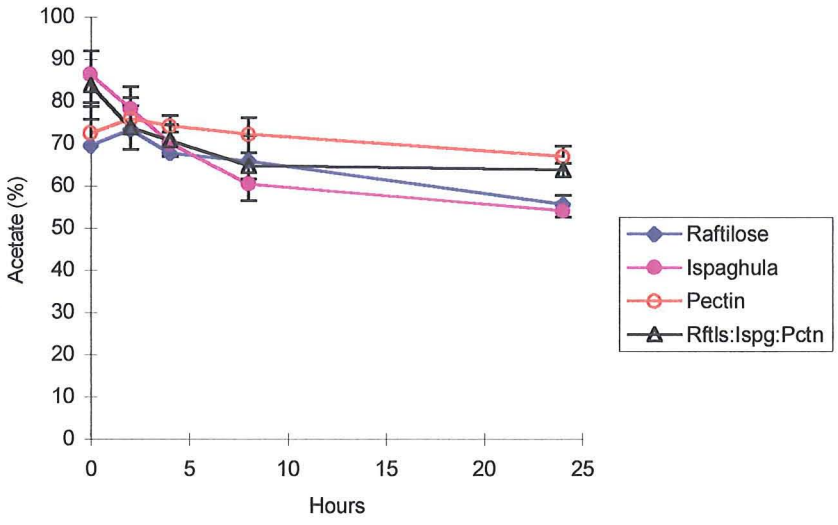


Fig. 6.38 Mean (±SEM) acetate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn represents values of the mixture].

6.4.6.2 Propionate production

Propionate production was less rapid in three-carbohydrates mixture than in culture of 100mg raftilose, but was similar to cultures of pectin alone. Propionate concentration was significantly higher in mixture than in culture of ispaghula ($p < 0.02$), but not significantly different than in cultures of 100mg raftilose or pectin at 8 hours. Mixture produced significantly lower propionate compared with culture of raftilose at 24 hours (Fig. 6.39). Fermentation of three-carbohydrates mixture yielded similar ratios of propionate to cultures of individual constituent carbohydrates at 8 and 24 hours (Fig. 6.40).

6.4.6.3 n-Butyrate production

n-Butyrate production was less rapid in three-carbohydrates mixture than in culture of 100mg raftilose. n-Butyrate concentration was not significantly

different in mixture from culture of raftilose or pectin, but significantly higher than in cultures of ispaghula ($p<0.02$ & $p< 0.05$) at 8 and 24 hours (Fig. 6.41). Ratios of n-butyrate were not significantly different in mixtures from cultures containing 100mg of the three constituent carbohydrates (Fig. 6.42).

6.4.7 Ratios of branched SCFA

Ratios of branched chain SCFA were highest in cultures of gum arabica, at 8 and 24 hours. Much lower ratios of branched chain SCFA were observed in mixtures of gum arabica and raftilose (Table 6.4). Similarly the highest ratios of branched SCFA were noted in cultures of ispaghula but not in two-carbohydrates mixture containing ispaghula with raftilose or pectin or in three-carbohydrates mixture (Table 6.4).

6.4.8 Fermentation and pH changes in cultures

In most cultures, the pH was not significantly different at 24 hours from that at 8 hours. The pH decreased gradually in mixtures of raftilose and gum arabica in comparison to rapidly decreasing pH in mixtures of raftilose and guar gum. The pH was not significantly different in these two mixtures from their constituent carbohydrates at 24 hours (Fig. 6.43).

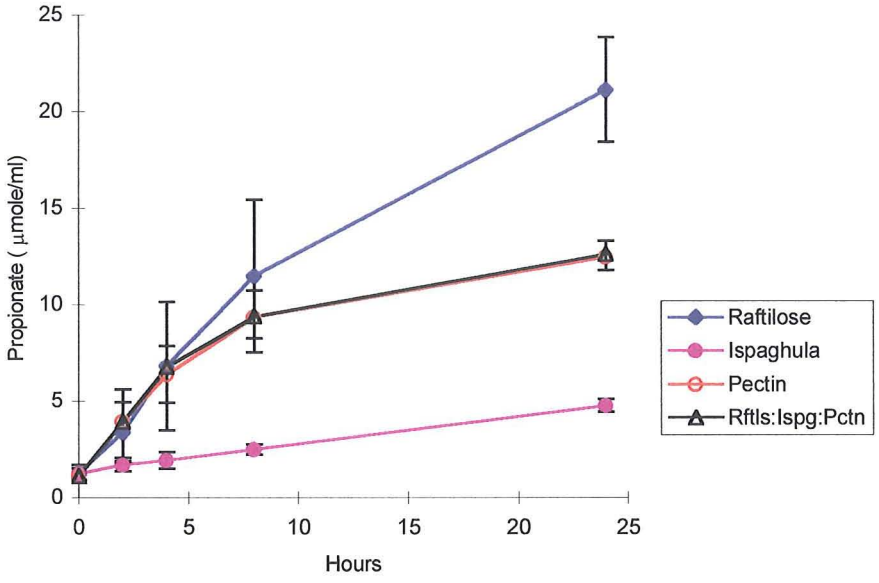


Fig. 6.39 Mean (±SEM) concentrations of propionate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn represents values of mixture].

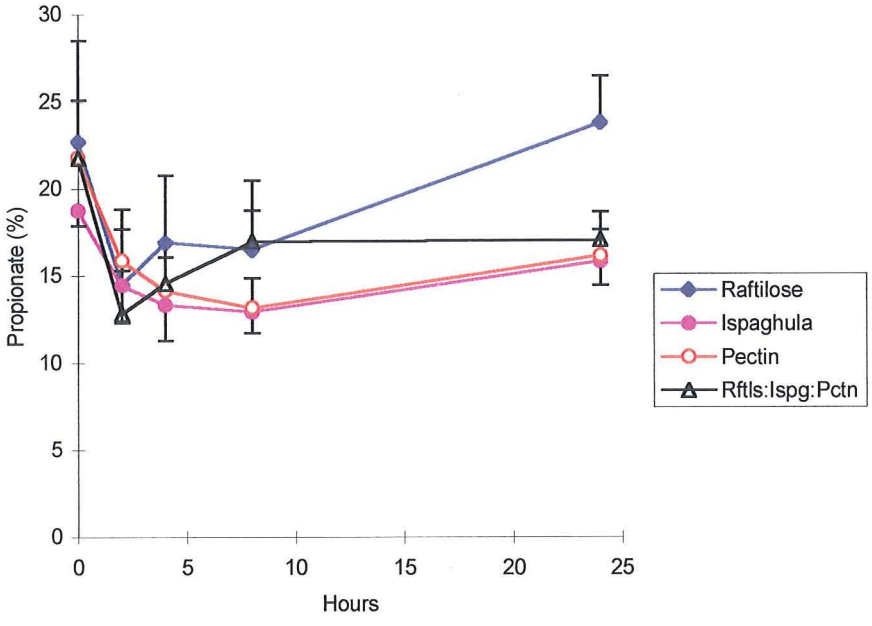


Fig. 6.40 Mean (±SEM) propionate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn- represents values of mixture].

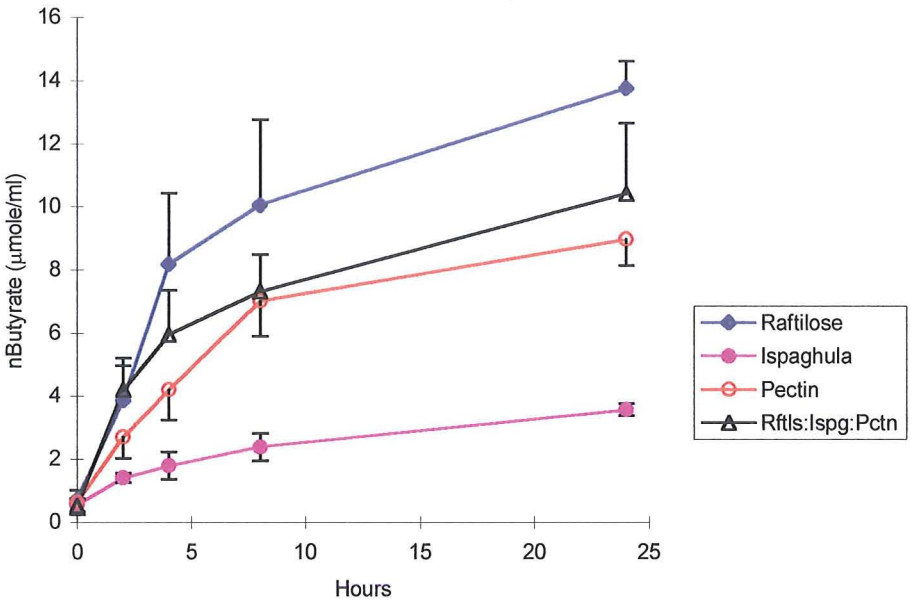


Fig. 6.41 Mean (±SEM) concentrations of nbutyrate (µmole/ml) produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn represents values of the three-carbohydrates mixture].

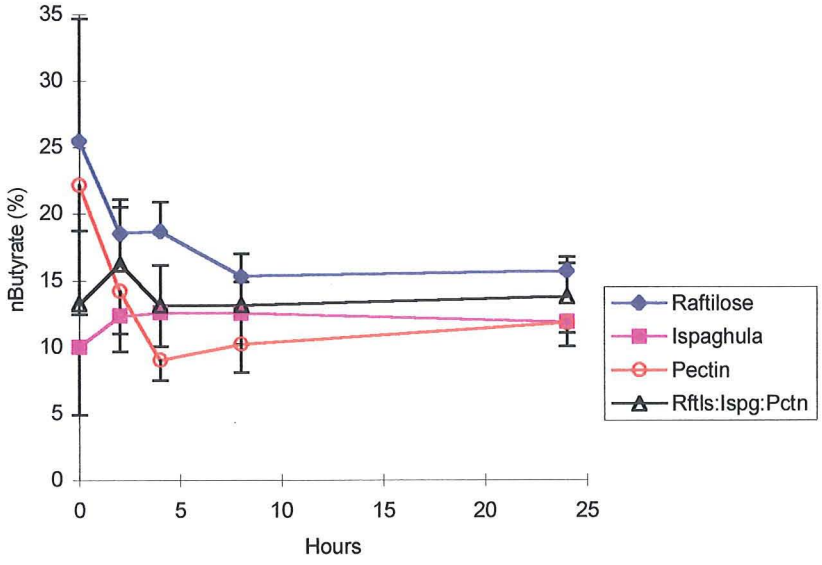


Fig. 6.42 Mean (±SEM) nbutyrate percent of the total SCFA produced by the fermentation of carbohydrates and their mixture with the human faecal bacteria [Rftls:Ispg:Pctn - represents values of mixture].

Table 6.3 Summary of observed (*in vitro*) and possible effects (*in vivo*) on basis of results from the fermentation of mixtures of carbohydrates.

Carbohydrate	Raftilose	Gum arabica	Guar gum	Ispaghula	Pectin & ispaghula
Raftilose in mixture with		no change in solubility; less rapid fermentation; no less SCFA, especially n-butyrate; lower pH than in culture of gum arabica.	no change in solubility; less rapid fermentation; no less SCFA, especially n-butyrate; lower pH than in culture of guar gum.	less rapid fermentation; no less of SCFA especially n-butyrate; lower pH than in ispaghula.	Less rapid fermentation; no less SCFA, especially n-butyrate.
Pectin in mixture with	more rapid fermentation; no effect on SCFA.	Not done	Not done	Less propionate and n-butyrate compared with pectin; apparently no interaction with ispaghula.	Not done

Table 6.4 Mean ratios of SCFA (% of total SCFA) produced by the fermentation of different carbohydrates and their mixtures with human faeces (n=5-8).

	8 hours				24 hours			
Substrates	Acetate	Propionate	n-Butyrate	Branched SCFA	Acetate	Propionate	n-Butyrate	Branched SCFA
Raftilose	65	17	16	2	58	22	16	4
Guar gum	60	27	9	4	52	35	9	4
Ispaghula	61	13	13	14	54	16	12	18
Pectin	72	13	10	4	67	16	12	5
Gum arabica	59	19	12	10	66	19	10	5
GARaftls 50:50	67	17	13	3	64	19	13	4
GGRaftls 50:50	62	22	14	2	55	27	15	3
RftlsIspg 50:50	60	19	16	5	57	21	15	7
RftlsPctn 50:50	67	16	14	3	64	18	13	5
IspgPctn 50:50	70	14	11	6	66	16	10	8
RftlsIspgPctn 34:33:33	65	17	13	5	64	17	14	5

At 8 hours, pH was significantly lower in culture of raftilose than in mixture of raftilose and gum arabica but there was no significant difference between the pH in culture of raftilose and in mixture of raftilose and guar gum at 8 hours. The pH, in general, increased with extended hours of fermentation in these cultures (Fig. 6.43).

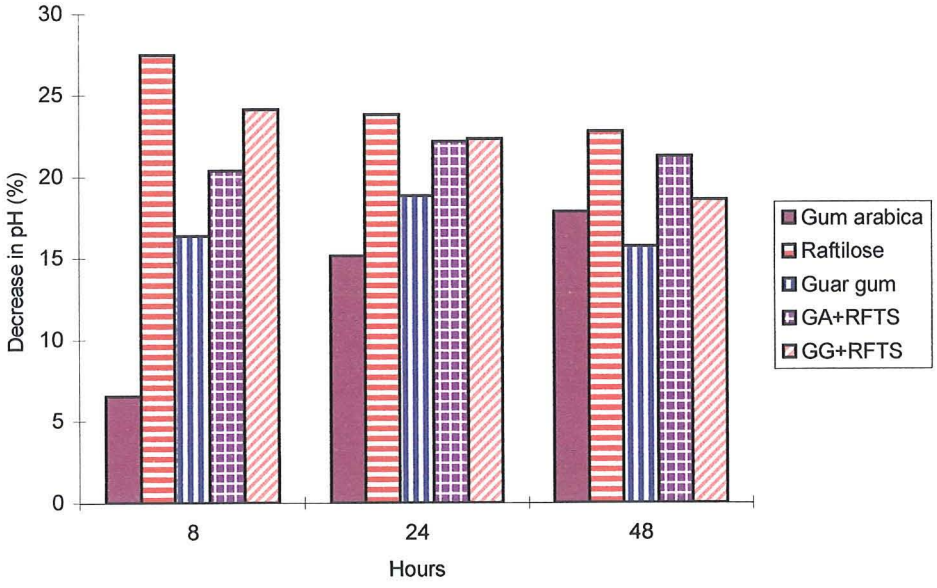


Fig 6.43 The decrease in pH (%) produced by the fermentation of carbohydrates and their mixtures with the human faecal bacteria.

The decrease in pH was significantly more rapid in cultures of 100mg pectin and raftilose than in culture of 100mg ispaghula. The lowest pH was in culture of raftilose at 8 hours, but this did not change with extended incubation. The pH in cultures of raftilose was not significantly different from cultures of pectin. The pH rapidly decreased in mixtures of raftilose and pectin, showing a significant difference from the pH in mixtures of ispaghula at 8 hours. On the other hand, the pH changed gradually in two-carbohydrates mixtures of ispaghula with pectin or raftilose or three-carbohydrates mixture (Fig. 6.44).

The pH in mixtures of raftilose and ispaghula was not significantly different from cultures of ispaghula at 8 hours, but decreased more significantly than in cultures of ispaghula at 24hours. There was no significant difference in the pH in different mixtures at 24 hours of fermentation (Fig. 6.44).

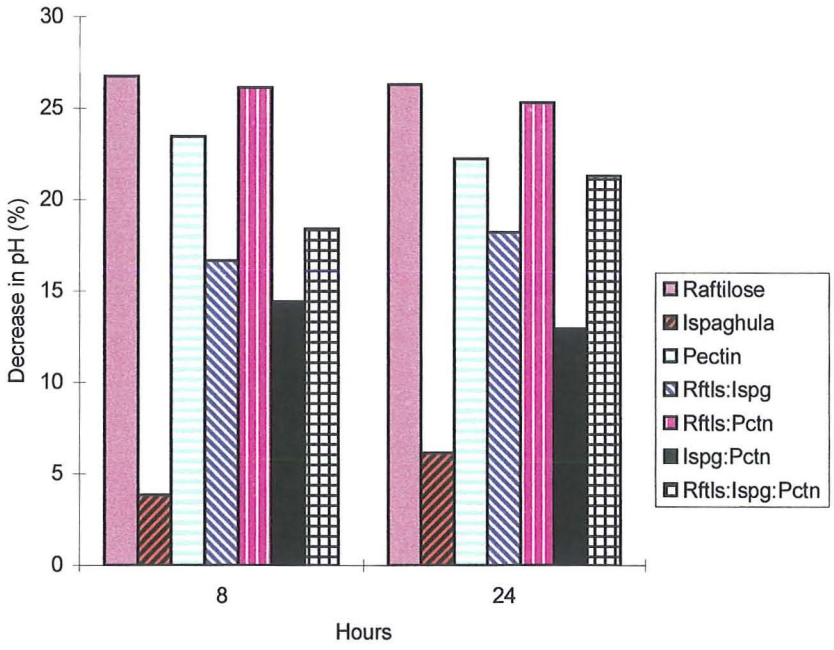


Fig 6.44 The decrease in pH (%) produced by the fermentation of carbohydrates and their mixtures with the human faecal bacteria.

6.4.9 Residual raftilose in single and mixed carbohydrate cultures

Cultures of raftilose and its mixtures with gum arabica and guar gum were tested for residual raftilose in cultures. Cultures from 8 and 24 hours of fermentation did not show any residual raftilose but very small amounts of raftilose were noted in cultures of 100mg raftilose at 4 hours of incubation. There was no detectable residual raftilose in cultures containing 50mg of raftilose in combination with 50mg of guar gum or gum arabica or in cultures containing only 50mg of raftilose at 4 hours of fermentation.

6.5 Discussion

In this study, we were trying to identify a mixture of carbohydrates which would delay but not reduce the production of n-butyrate by raftilose. In this way we hoped to achieve the maximum benefit by using smaller amounts of raftilose, which would reduce the risk of osmotic load and symptoms associated with very rapidly fermented carbohydrates, but which would also, by using smaller amounts of viscous polysaccharides, avoid poor organoleptic values and high viscosity.

6.5.1 Fermentation of raftilose in combination with guar gum and gum arabica

Raftilose dominated the fermentation patterns in mixtures of raftilose with guar gum or gum arabica, showing a general decrease in the rate of fermentation. However, guar gum was more effective in influencing the fermentation of raftilose (Fig. 6.8) than gum arabica (Fig. 6.1). Fermentation in these mixtures was delayed. However, production of SCFA was not affected (Table 6.3; Fig. 6.1 & 6.8). This effect could be of great use, as the same amount of net total SCFA would be produced with only 50mg of raftilose in mixture avoiding the unwanted effects of larger amounts of raftilose. Fermentation continued in mixtures of guar and raftilose (Fig. 6.8), in contrast, there was no further fermentation in mixtures of gum arabica and raftilose after 24 hours (Fig.6.1). Such different performance of the two mixtures after 24 hours may be an indication of difference of interaction in the two mixtures. Since guar gum was not as slowly fermenting as gum arabica, it was expected that the substrate in mixtures of guar gum and raftilose would be exhausted comparatively earlier than in mixtures of raftilose and gum arabica, apparently the reverse was true.

This may be due to the viscous property of guar gum resulting in a delay of fermentation. Decreased SCFA in mixtures of gum arabica and raftilose at 48 hours, may be due to re-fermentation of SCFA into other metabolites (McBurney & Thompson, 1987; Jay, 1996). Thus, not all carbohydrates can be expected to interact in similar manner in mixtures.

The initial very rapid production of SCFA seen in cultures of 100mg raftilose was preserved in mixtures with the gums although these contained only 50mg raftilose but slowed after 4 to 8 hours. This may reflect a phenomenon called catabolite repression, which is the basis of *diauxic growth,* a peculiar growth response to substrate mixtures. When the bacterial cells are grown in cultures contain a rapidly metabolisable energy source, the increasing intracellular concentrations of ATP leads to the repression of enzymes required for the catabolism of the less rapidly degrading energy sources. For example, when E.coli cells are actively involved in the degradation of glucose, synthesis of the *β-galactosidase*, enzyme for the degradation of lactose, is repressed (Stanier et al., 1986).

Net total SCFA in the actual mixtures were similar to that predicted by their respective hypothetical mixtures which the higher amount of substrate did not affect the production of SCFA (Fig. 6.1 & 6.8) and no loss of SCFA (table 6.3). This is an interesting finding, in comparison with the results from Chapter 3.

The pattern of propionate production was completely different in these two mixtures of guar/raftilose and gum arabica/raftilose suggesting that the two gums interacted with raftilose in different ways. Gum arabica did not affect the rate of propionate

production, whereas, propionate was produced less rapidly in mixtures than cultures of guar gum. This may be due to their different SCFA profiles, as guar gum is propionate predominant compared with the acetate predominance of gum arabica. Both raftilose and guar gum when fermented are propionate predominant and as expected that propionate production was an additive activity of the two constituent carbohydrates and the propionate concentration of mixture was intermediate between the two constituent carbohydrates. In the case of the mixture of raftilose and gum arabica, the rate and pattern of propionate production was the same as in cultures of raftilose alone. Moreover, the production of propionate in these mixtures was not similar in comparison with their respective hypothetical mixtures. It seemed that there was an inhibition and delay in the production of propionate in mixture of guar gum and raftilose, an indication of different responses of the mixtures to propionate production.

The concentrations and patterns of n-butyrate production were important features of these mixtures. Production of n-butyrate in both mixtures showed a gradual increase even after 24 hours of fermentation (Fig. 6.6 & 6.13), which was an indication of less rapid and delayed production of n-butyrate. Thus the presence of gums lowered the rate of n-butyrate production from that in cultures of 100mg raftilose (Table 6.3). This was a useful effect of these mixtures of raftilose with guar gum or gum arabica, as replacing 50% of raftilose with a comparatively slower fermenting gum did not reduce butyrate production but may delay the release of butyrate. Producing such an effect *in vivo* may be helpful in prolonging n-butyrate production and perhaps move butyrate further round the colon, at the same time reducing the potential adverse effects of raftilose. Moreover, the addition of guar gum would add the therapeutic

effects of delaying nutrient absorption in the small intestine as well (Jenkins et al., 1977; Aro et al., 1984; Blackburn et al., 1984; Fernandez et al., 1995).

6.5.2 Fermentation of rapidly fermenting carbohydrates in combination with slowly fermenting carbohydrate

As discussed in earlier chapters two inhibitory forces may be working during the *in vitro* fermentation of mixtures of a rapidly fermenting and a slowly fermenting carbohydrates; An inhibitory effect due to the decreased pH and changes in the physiological conditions of cultures, with increasing amounts of lactulose (Chapter 3) and inhibition of fermentation of rapidly fermenting lactulose due to the physical presence of slowly fermenting ispaghula in mixed substrate culture (Chapter 4). The amount of rapidly fermenting substrate appeared to be important in the *in vitro* fermentation procedures (Barry et al., 1995; Stevenson et al., 1997b). On the contrary, in normal healthy human colon such high amounts of SCFA from fermentation reactions are rapidly cleared by the colonic epithelium and these do not accumulate to have a negative effect on physiological conditions in the normal state of colonic health. Therefore, the physical properties of slowly fermenting carbohydrates such as ispaghula, may become more important, compared with the extreme changes in pH and other conditions in the colonic fermentation as discussed in earlier chapters. The specific properties of ispaghula (bulking of gut contents, increased stool out put, increased butyrate in the distal colon: Edwards & Eastwood, 1992; Morteau et al., 1994; Edwards et al., 1992a; Washington et al., 1998) should make it an ideal complimentary carbohydrate

in mixture with rapidly fermenting carbohydrates (Chapter 4). This would be particularly useful in the case of carbohydrates with osmotic properties such as lactulose and raftilose.

In view of such an expected role, ispaghula was mixed, in this study, with other rapidly fermenting carbohydrates raftilose (an oligosaccharide) and pectin (a polysaccharide). In general, the pattern of fermentation in both mixtures was under the influence of rapidly fermenting carbohydrates, and apparently ispaghula in these mixtures of two carbohydrates did not affect the pattern of fermentation of rapidly fermenting carbohydrates. Ispaghula lowered the rate of fermentation in mixture with raftilose (Table 6.3; Fig. 6.15) but it did not affect the rate in mixture with pectin (Table 6.3; Fig. 6.29). In both mixtures, the pattern of fermentation was under the influence of the rapidly fermenting component in mixtures. Net total SCFA in mixture of raftilose and ispaghula were significantly lower than that in culture of raftilose. Similar results were noted with mixtures of lactulose and ispaghula (Chapter 4). Ispaghula probably delayed the fermentation of the mixture due to its viscous property and by entrapping the molecules of raftilose in its bulking matrix. This effect of ispaghula may play an important role in delaying the fermentation of rapidly fermenting raftilose and making it available for bacterial fermentation at more distal site of the colon.

Cultures of raftilose or pectin alone did not produce significantly different acetate from their respective mixtures with ispaghula. These results were similar to those noted with mixtures of lactulose and ispaghula (Chapter 4). All three mixtures showed similar acetate production, despite the differences of

rapidly fermenting carbohydrates in mixed substrates. Acetate concentrations were not as expected in mixtures of ispaghula and pectin. Pectin is an acetate predominant carbohydrate (Chapter 5; Vince et al., 1990). It would be predicted that acetate production in mixtures would be less than that in cultures of pectin. In contrast, acetate concentrations were not significantly different from cultures of pectin (Fig.6.30).

The higher propionate concentrations in both mixtures than that found in cultures of ispaghula alone is a positive effect of mixing these carbohydrates (Fig. 6.18 & 6.32). However, comparing the propionate concentrations in mixtures with that produced by the rapidly fermenting component of respective mixtures, revealed no apparent interaction between the constituent carbohydrates regarding the production of propionate in mixtures. In both mixtures, the rapidly fermenting component may have become limited in the initial hours and ispaghula did not add any significant propionate to the cultures. In contrast, propionate was not significantly different in cultures of 100mg lactulose from that produced in mixture containing 50mg lactulose in combination with ispaghula (Chapter 4). It showed that all these three rapidly fermenting carbohydrates behaved differently in the production of propionate when combined with ispaghula in a mixture. In using mixture of such composition, we could take the advantage of the presence of ispaghula and avoid the undesirable side effects of rapidly fermenting carbohydrates, particularly raftilose and lactulose.

Both mixtures produced significantly more n-butyrate than culture of ispaghula alone. However, these mixtures behaved differently in comparison with the

rapidly fermenting component in cultures. Mixture of ispaghula and raftilose had similar concentrations of butyrate as raftilose alone but a delayed release of n-butyrate. Rate of n-butyrate production was less rapid compared with cultures of rapidly fermenting 100mg raftilose (Table 6.3; Fig 6.20). The slow rate of production indicated that it is possible to delay the production of n-butyrate by using mixture of such combination. Mixture of pectin and ispaghula had lower n-butyrate than pectin alone (Table 6.3; Fig. 6.34), which was the same as mixtures of lactulose and ispaghula. In contrast to the mixtures of lactulose and ispaghula (Chapter 4), and mixtures of pectin and ispaghula (Chapter 6), the mixture of raftilose and ispaghula did not produce significantly different n-butyrate compared with the cultures of rapidly fermenting raftilose alone. This difference between mixtures of different rapidly fermenting carbohydrates (lactulose, raftilose and pectin) with slowly fermenting ispaghula showed that different carbohydrates interacted in different ways. This is probably dependent on their chemistry or some physical factors other than viscosity, but is not dependent solely on their rate of fermentation. n-Butyrate concentration in mixture of raftilose and ispaghula was not significantly different from a hypothetical mixture (Table 6.3; Fig. 6.20), in contrast to mixture of lactulose and ispaghula where n-butyrate was significantly lower in actual mixture than predicted from hypothetical mixture.

In general, ispaghula reduced the rate of fermentation but did not affect the level of SCFA production adversely in mixture with raftilose. Whereas, ispaghula did not show any appreciable interaction with pectin and showed a significant reduction in SCFA in mixture with pectin.

6.5.3 Fermentation of two rapidly fermenting carbohydrates in a mixture

The fermentation pattern of raftilose was dominant in mixtures of raftilose and pectin. The net total SCFA, unlike in cultures of pectin, increased linearly in mixtures with no plateau, despite, the rapid rate of fermentation, showing a positive interaction between the constituent carbohydrates (Table 6.3; Fig. 6.22). The two rapidly fermenting carbohydrates (raftilose and pectin) appeared to affect the rate of fermentation in an additive manner. The production of SFCA was not inhibited in mixtures even with a rapid fermentation, suggesting that fermentation of 100mg mixed substrate of two or more different carbohydrates in *in vitro* cultures, may not necessarily inhibit the rate of fermentation. Therefore, using such mixtures may be helpful in overcoming the inhibitory effect of larger amounts of a single substrate and may produce higher SCFA concentrations.

Acetate concentrations in mixtures followed the same pattern as that of net total SCFA (Fig. 6.23). The pattern of propionate production in mixtures showed a gradual increase from 8 to 24 hours, suggesting that production of propionate was slowed in mixtures (Table 6.3; Fig. 6.25). Production of n-butyrate, unlike in mixtures of raftilose and guar, and raftilose and gum arabica, did not show any appreciable interaction in mixtures of raftilose and pectin (Fig. 6.27). The viscosity of pectin did not appear to affect the level of production, however, a less rapid rate of fermentation was noted in mixture than culture of raftilose alone.

6.5.4 Fermentation of three-carbohydrates mixture containing two rapidly fermenting carbohydrates with slowly fermenting ispaghula

Mixture of three carbohydrates was fermented less rapidly than the rapidly fermenting carbohydrates constituents in mixture (Table 6.3; Fig. 6.36). On the other hand, net total SCFA were significantly higher in mixture than in culture of 100mg ispaghula. The more linear production of net total SCFA in mixture than culture of pectin, suggested a reduced inhibition in mixture. No loss of SCFA in mixture was evident. This suggested that SCFA production may occur over a more prolonged period compared with rapidly fermenting raftilose and pectin.

The more linear and gradual increase in acetate concentrations showed that acetate production was not under the influence of pectin in mixtures, showing less rapid production (Fig. 6.37). On the other hand, propionate production in mixture was exactly the same as in culture of pectin, and less rapid than in cultures of raftilose (Fig. 6.39). Significantly lower propionate concentrations in mixture than in culture of raftilose, suggested that raftilose became limited first, and then pectin continued to contribute to propionate concentrations in the latter stages of fermentation. Interaction between the three carbohydrates affected the rate of propionate production in this mixture.

The rate of n-butyrate production was less rapid in mixture of the three carbohydrates than culture of 100mg raftilose (Table 6.3). Similarly comparing the n-butyrate concentrations in mixture to that in cultures of 100mg pectin or raftilose, showed that the presence of ispaghula did not affect

the concentrations of n-butyrate produced but influenced the rate of production (Table 6.3; Fig. 6.41). The 3-carbohydrates mixtures produced higher n-butyrate than mixed two-carbohydrate cultures of lactulose and ispaghula (Chapter 4), and pectin and ispaghula (Chapter 6). On the other hand, levels of n-butyrate were similar as noted in mixture of raftilose and ispaghula, but lower than two-carbohydrate mixtures of raftilose with gums. In this way, there was no loss of n-butyrate in this three-carbohydrate mixture. A delayed production of n-butyrate could be of potential benefit to human health.

6.5.5 Ratios of branched SCFA

The concentrations of branched SCFA were highest in cultures of slowly fermenting carbohydrates. This may be due to a lower rate of bacterial growth as well as a higher incidence of bacteriolysis. An earlier study suggested that fermentation at 24 hours may be a product of re-fermentation and lysis (McBurney & Thompson, 1987).

6.5.6 Reduction in pH

Low colonic pH may be beneficial in certain pathological conditions such as cancer in the human gut (Chapter 1). In this study, carbohydrates affected the pH in cultures to different degrees (Table 6.3; Figs. 6.43 & 6.44). In most cultures, the pH dropped rapidly and remained low for the duration of culture incubation. However, in some cultures there was a recovery towards neutrality. In slowly fermenting carbohydrate cultures, such as gum arabica, the reduction in pH was gradual but continued falling for the duration of 24 hour. This effect, in general, was maintained in mixtures. This suggested that

combining a slowly fermenting carbohydrate with a rapidly fermenting carbohydrate in a mixture may prolong the impact of reduced pH. This may not be as relevant *in vivo* where the colon is more efficient at buffering pH changes.

6.5.7 Disappearance of raftilose from mixed substrate cultures

Very little raftilose was found in single carbohydrate cultures after 4 hours of incubation. When included in mixtures residual raftilose was not detected at any time. This indicates that raftilose is fermented very rapidly in all cultures and presumably before the more complex carbohydrates in mixtures. This might have implications for the site of production of SCFA from raftilose *in vivo* i.e. butyrate from raftilose would be more likely to be produced in the proximal colon even in mixtures. However, it should be noted that these mixtures continued to produce butyrate beyond 4 hours and to the same level as cultures of raftilose alone. The fermentation of raftilose alone produced much lactate at 4 hours which was then further metabolised to the SCFA after all the raftilose had been fermented.

6.6 Conclusion

In summary, mixtures of carbohydrates including raftilose were fermented more slowly than raftilose alone or raftilose in combination with pectin. Overall, it seemed that ispaghula was the most effective in slowing the rate of fermentation when compared with pectin or gums. Mixing raftilose with ispaghula or guar gum gave the best preservation of butyrate and propionate production. Three-carbohydrates mixture was also effective in preserving butyrate production.

The following conclusion can be drawn from these experiments discussed in this chapter;

1) Combination of carbohydrates of different fermentability, especially ispaghula, may allow a slower fermentation but may preserve the production of important SCFA such as n-butyrate.
2) Carbohydrates with similar rates of fermentation may not necessarily interact with other carbohydrates in the same way. Every combination of different carbohydrates will have unique patterns of fermentation.
3) The decline in SCFA production after 24 hours suggests that 8 and 24 hours are ideal time points to determine the maximum possible concentrations of SCFA in *in vitro* models for describing the fermentability of carbohydrates.

CHAPTER 7

General Discussion

General Discussion

The metabolic and specific therapeutic effects of indigestible carbohydrates in the human colon have attracted much attention from researchers. In view of the metabolic importance of these carbohydrates, many researchers have considered supplementation with isolated carbohydrates without disturbing the balance of other dietary contents (Jenkins et al., 1977; Jenkins et al., 1976; Cummings et al., 1978). Previously, complex carbohydrates were studied extensively for their *in vitro* fermentation and *in vivo* metabolic implications (Englyst et al., 1987b; Adiotomre et al., 1990; Wang & Gibson, 1993; Younes et al., 1996). Most of this work was carried out *in vitro*, using carbohydrates in isolation. However, the potential benefits of these carbohydrates, especially if large amounts are required to be metabolically effective, will be of little value if they have poor organoleptic properties. In addition, they may have adverse gastrointestinal effects such as bloating and osmotic diarrhoea. In view of the idiosyncratic actions of these individual carbohydrates, it is unlikely that any single carbohydrate will provide therapeutic conditions without having some undesirable effects. Therefore, a logical approach would be to combine different indigestible carbohydrates in mixtures of two or more carbohydrates in order to harvest the potential therapeutic benefits but simultaneously avoid their undesirable side effects. However, there is very little information of about the fermentation of carbohydrates mixture (Washington et al., 1998). In this thesis, therefore, the fermentation and interaction of these carbohydrates in mixtures in *in vitro* cultures were investigated to provide a basis for predicting their action *in vivo*.

The main aspect of the interaction of carbohydrates studied was their fermentability and their SCFA profile produced. The aim of this study, therefore, was to develop a carbohydrate mixture, which would probably contain a slowly fermenting carbohydrate and a rapidly fermenting carbohydrate and which would be a good source of n-butyrate. In this way, we were trying to identify a mixture of carbohydrates which would delay but not reduce the production of n-butyrate by a very fermentable carbohydrate such as raftilose. Such mixture would be of maximum benefit using smaller amounts of raftilose which would reduce the risk of osmotic load and other symptoms associated with large amounts of very rapidly fermented carbohydrates. This approach would, at the same time, avoid poor organoleptic values and high viscosity by using smaller amounts of viscous polysaccharides in mixtures. To develop such a mixture for tube fed patients, specific characteristics of the constituent carbohydrates, such as their solubility, viscosity, and ease of feeding down a tube were considered. There are currently some commercial products available containing mixtures of complex carbohydrate including Jevity® (Ross laboratories, UK), containing a mixture of soy polysaccharides, oat hull fibre, gum arabica, carboxymethylcellulose and fructo-oligosaccharide. This product is used for tube feeding but use of such mixtures is not based on published scientific information about the potential interactions and benefits of the specific carbohydrates used.

In this thesis, it was hypothesised that a slowly fermenting carbohydrate would alter the fermentation of a rapidly fermenting carbohydrate and vice versa. This would result in a delay in the fermentation of a rapidly fermenting

carbohydrate, which are generally high SCFA producers. In addition, it was hypothesised that carbohydrates of different SCFA profile may affect the ability of each other to produce a particular SCFA, especially n-butyrate. Therefore, indigestible carbohydrates with different fermentability, rate of fermentation and other related characteristics, were used in mixtures in this study.

To evaluate the potential use of mixtures of indigestible carbohydrates, I have used a simple *in vitro* model. This model is not designed to mimic all aspects of the human colon and will only give an indication of the effects of mixtures *in vivo*. However, the ability to screen many carbohydrates and to investigate in more detail the possible interactions of these carbohydrates makes the *in vitro* system useful in this context. Because of the limitations of the model, some aspects of the model system had to be evaluated to allow better interpretation of the results and better understanding of how they may relate to the effects of mixtures *in vivo*.

7.1 Investigation of the *in vitro* model

The most important limitation of such *in vitro* models is the absence of an absorptive epithelium resulting in the accumulation of end products. This may cause changes in *in vitro* cultures and inhibit the fermentation of carbohydrate and the production of SCFA. Only a few studies have recognised the importance of the amount of test substrate in *in vitro* fermentation procedures (Mortensen et al., 1988; Mortensen et al., 1991; Barry et al., 1995; Stevenson et al., 1997b). These studies agreed on 50 to 100g substrate per litre of media as the optimal amount of a particular carbohydrate to be used in an *in vitro*

model. However, the most suitable amount of substrate to avoid inhibition of fermentation may depend on its fermentability. I, therefore, investigated this limitation using a highly fermentable and a poorly fermentable substrate to test the hypothesis that with increasing amounts of substrate, the increase in *in vitro* fermentation is not linear (Chapter 3).

Rapid *in vitro* fermentation of carbohydrate produces higher amounts of SCFA which, in contrast to *in vivo* conditions, are not absorbed. Consequently, such rapid fermentation results in a rapid decline in pH, increased production of gases and other metabolites. Such rapidly changing conditions in *in vitro* cultures may cause environmental selection resulting in a change in the bacterial activity from that present in the initial stages of the fermentation. In this way, a rapidly fermenting carbohydrate may create conditions that are favourable for the growth and subsequent metabolic activity of bacteria, which are more effective in the fermentation of a slowly fermenting carbohydrate. Of the known factors, which would change the physiological conditions, influencing the bacterial activity, the most important are the accumulating amounts of SCFA and reduced pH in *in vitro* cultures.

In the present study, these two factors were addressed. The fermentation of incremental portions of lactulose demonstrated that fermentation of 100mg lactulose was inhibited significantly compared with the fermentation of 25mg lactulose. Almost all the substrate was fermented in cultures of 25mg of lactulose but when larger amounts were included, an inhibition in the rate of fermentation was apparent (Chapter 3). It can be speculated that factors such as substrate concentration, pH changes, and the accumulation of intermediary and end products, influence the rate of *in vitro* fermentation in these cultures.

Such changed physiological conditions may result in a transformation of the microbial population in cultures of lactulose after 8 hours of incubation. Low pH has been implicated in inducing different metabolic pathways or a change in microbial populations (Edwards et al., 1985; Edwards & Rowland, 1992; Wang & Gibson, 1993).

In our study, a loss of linearity in SCFA production was noted with much smaller amounts of substrate compared with other studies (Mortensen et al., 1991). This may be due to the differences in the fermentation methods of the two studies. Lactulose was very rapidly fermented in the present study and produced higher concentrations of SCFA quickly and caused a rapid decrease in pH (Chapter 3).

7.2 Is pH the main factor regulating the fermentation *in vitro*?

Although the low pH seemed to be the main effector in the inhibition of the *in vitro* fermentation of lactulose in the present study, it is difficult to separate the effect of pH from other factors. The effects of pH may not be related to its absolute measurement but may depend on a number of growth conditions such as type of acid, types of salt present in the medium of growth (Jay, 1996). For example, the growth of certain lactobacilli is permitted at lower pH with citric, hydrochloric, phosphoric and tartaric acids compared with acetic and lactic acids (Juven, 1976). Therefore, it is important to know about the acids that are responsible for the reduction in pH, to determine the rate of subsequent growth and the minimal pH for a microorganism to initiate its growth. Chung and Geopfert (1970) reported the minimal pH 4.05 for salmonella to initiate

growth when HCl and citric acids were used, but 5.4 and 5.5 when acetic and propionic acids were used respectively. Similarly, *Alacaligenes faecalis* has been shown to grow over a wider range of pH in the presence of 0.2 M NaCl than in the absence of NaCl or in the presence of 0.2M Sodium citrate (Sherman & Holm, 1922). This shows that a low pH may transform the metabolic activity of bacterial cells depending on other conditions in culture. Low pH may be implicated beneficially in certain pathological conditions, such as colon cancer (detail discussion in Chapter 1).

In this study, fermentation of lactulose produced higher concentrations of SCFA with a corresponding decline in pH, whereas, ispaghula did not produce significant concentrations of SCFA or change in pH. When mixtures of lactulose and ispaghula were fermented, concentrations of SCFA increased with higher amounts of lactulose with a corresponding decrease in pH. The greatest decrease in pH was noted at 8 hours in all cultures (Chapter 4-6). Similarly, pH decreased rapidly with increasing production of the SCFA in cultures of other rapidly fermenting carbohydrates such as raftilose and pectin, up to 8 hours. A recovery in pH towards neutrality was then noted with slight decrease in SCFA, indicating that concentrations of the SCFA are one of the major factors influencing the pH in these cultures. However, it is also possible that the bacteria may have used the substrate or organic acids already present in cultures, to produced different metabolites such as amines from amino acids, butanol from butyric acid, and acetoin from pyruvic acid (Jay, 1996). This use of organic acids may decrease the concentration of SCFA and other organic acids, resulting in an increased pH at latter stages of incubation. Therefore,

the increase in pH noted at 24 hours of incubation may be a function of conversion of SCFA to other metabolites.

Low pH may influence the fermentation in different ways (Chapter 3; Metzler, 1977; Edwards et al., 1985). A low pH may favour other metabolic routes. For example, propionic acid may be favoured over acetic and butyric acid. Since fermentation of some carbohydrates, such as raftilose and lactulose produced higher concentrations of acetate, lactate and other organic acids in the initial stages of the cultures, there was a corresponding decrease in pH. In cultures containing these carbohydrates, the initial lowering of pH by the production of acetate and/or lactate, may make the conditions favourable for production of propionate (Edwards et al., 1985) or butyrate. The second major factor may be that SCFA would depend on the specific nature of available substrates or the species of bacteria which could convert these substrates to SCFA. Propionate and n-butyrate are produced by different bacterial species (Macfarlane & Gibson, 1995).

In our study, the accumulating SCFA may have further reduced pH and changed the physiological conditions in cultures so that instead of inhibiting, they actually facilitated and changed the metabolic route for the production of n-butyrate as fermentation progressed. In this way, physiological conditions may become progressively more effective favouring the production of different SCFA particularly n-butyrate compared with the production of propionate.

7.3 Feed back Inhibition

Another factor influencing the fermentation in these cultures may be feed back inhibition of intracellular and/or extracellular enzymes by the accumulation of

organic acids and other end products (Chapter 3; Metzler, 1977; Champ & Harvey, 1994). However, as I was measuring fermentation by the accumulation of SCFA, it was not possible to test this mechanism in my study. If I had measured residual carbohydrate it may have been possible to investigate the impact of accumulating SCFA but these methods were not available to me at that time. Later raftilose only was measured in cultures by TLC. Raftilose was no longer present in cultures after 4 hours (Chapter 6).

7.4 Inhibition of fermentation of a rapidly fermenting carbohydrate due to the presence of a slowly fermenting carbohydrate

In this study, another inhibition was investigated which may originate directly from the interaction of carbohydrates in mixtures. In the first experiment, ispaghula and lactulose were used in mixtures. The specific properties of ispaghula (Edwards & Eastwood, 1992; Edwards et al., 1992a; Morteau et al., 1994; Washington et al., 1998) make it an ideal complimentary carbohydrate in mixture with rapidly fermenting carbohydrates. In this study, ispaghula was combined with rapidly fermenting lactulose. Lactulose, being a small molecule (disaccharide), may result in osmotic diarrhoea if ingested in large quantity. On the other hand, lactulose is good n-butyrate producer. Therefore, it seemed ideal to combine the rapidly fermenting and higher SCFA, especially n-butyrate, producing lactulose in mixtures with slowly fermenting, viscous, and stool bulking ispaghula. It was hypothesised that such combinations would delay fermentation of rapidly fermenting lactulose through the action of slowly fermenting ispaghula. Such combination may result in the production of higher

amounts of SCFA, especially n-butyrate, at more distal sites in the colon. This would fulfil one of the aims of this thesis, to evaluate mixtures of carbohydrates with different properties, to achieve the optimised beneficial effect in the human gut.

Combining carbohydrates with different rates of fermentation may influence the fermentation of both carbohydrates. It was hypothesised that a rapidly fermenting carbohydrate would enhance the rate of fermentation of a slowly fermenting carbohydrate by increasing bacterial mass and changing physiological conditions. On the other hand, a slowly fermenting carbohydrate may delay the fermentation of a rapidly fermenting carbohydrate. In particular, the viscous or bulking properties of ispaghula may hinder the accessibility to fermenting bacteria.

In addition to reduced SCFA production with increasing amounts of lactulose (Chapter 3), the physical presence of ispaghula reduced the rate of lactulose fermentation in mixtures (Chapter 4). When fermentation of such mixtures occurs in the colon, the SCFA are rapidly absorbed and their concentrations may not be sufficient enough to have a negative effect on physiological conditions in the colon. Therefore, the physical properties of slowly fermenting carbohydrates such as ispaghula, may become more important. The bulking of colonic contents and the high viscosity of ispaghula in the human colon (Edwards & Eastwood, 1992), could also promote a delayed fermentation of lactulose, making it available for bacterial fermentation at more distal colonic site. The high viscosity of ispaghula may trap the small molecules of lactulose in a complex three-dimensional matrix making lactulose less accessible to bacteria, thus impeding the fermentation of lactulose. Ispaghula may also reduce the absorption of fermentation products by reducing the diffusion.

Therefore, SCFA or molecules of other substrates, entrapped in the digesta, may be transported to more distal sites for absorption and/or for further metabolism. This hypothesis is supported by the delay in fermentation of lactulose in combination with ispaghula in man (Washington et al., 1998).

7.5 Ideal amount of substrate for *in vitro* fermentation

As inhibition was noted with the increasing amounts of substrate (Chapter 3), it is logical to suggest acceptable amounts for such *in vitro* fermentation systems. Considering the fermentation of incremental portions of lactulose, 50mg produced similar SCFA as cultures of 100mg lactulose at 8 and 24 hours. In this way, there was no loss in SCFA, despite using half of the amount of substrate. This suggested that 50mg substrate would be a more appropriate amount of a rapidly fermenting carbohydrate. This is supported by a previous study which did not achieve reproducible results with 25mg substrate (Mortensen et al., 1991). Higher amounts of carbohydrate may result in the inhibition of fermentation.

When ispaghula and lactulose were combined in different proportions, it was noted that the best substrate ratio of the two carbohydrates was 50mg of each carbohydrate. The mixture of 50mg of each carbohydrate produced significantly more of the three principal SCFA than other mixtures of lactulose and ispaghula at 8 hours of incubation (Chapter 4). Although this mixture, produced less SCFA than 100mg lactulose alone, (ispaghula inhibited the fermentation) mixture of raftilose and guar gum in the same proportion produced similar SCFA as in culture of 100mg raftilose (Chapter 6).

The reduction in pH after fermentation of a mixture of 50mg of lactulose and ispaghula reached a plateau with no further change, suggesting that it may be helpful in preventing a further deterioration of physiological conditions in cultures. Also combining two carbohydrates with contrasting fermentation properties, in such proportions will be more effective, as both carbohydrates in a mixture would have optimum chances for their role in the mutual interaction. Fermentation of such mixtures will reap the benefit of both carbohydrates and, on the other hand, will avoid the adverse effects of the two.

7.6 Actual values compared with expected values from hypothetical mixtures

When values of SCFA in actual mixtures were compared with theoretical values calculated from hypothetical mixtures of the same composition, the trends were not similar in these mixtures. The net total SCFA in actual mixtures of raftilose with gum arabica, guar gum or ispaghula were similar to that predicted for their respective hypothetical mixtures suggesting the absence of end product inhibition (Chapter 6). This contrasts with the results from mixtures of lactulose and ispaghula where influence of ispaghula in reducing the SCFA production by the fermentation of lactulose was seen (Chapter 4).

In mixtures of raftilose and guar gum (both propionate predominant) it was expected that propionate production would be an additive. On the contrary, the propionate concentration in actual mixture was significantly lower than the theoretical expected value. Similar findings were noted in mixture of lactulose

and ispaghula. On the other hand, in mixture of raftilose and gum arabica, the rate and concentrations of propionate were similar to hypothetical mixture (Chapter 6). In all these cultures, except in mixture of lactulose and ispaghula, n-butyrate concentrations were not significantly different from hypothetical value. Mixture of lactulose and ispaghula produced significantly lower n-butyrate in actual mixture than predicted from hypothetical mixture.

In general, it may be speculated that although all carbohydrates responded differently, the inhibition due to large amount of substrates may be controlled by mixing different carbohydrates. With the help of such mixtures, we may achieve a slower rate of n-butyrate production, without affecting the concentrations of n-butyrate.

7.7 Patterns & rates of fermentation

The indigestible carbohydrates used in this thesis showed different rates of fermentation and could be grouped in three categories. Lactulose, raftilose, raftiline and pectin were rapidly fermented, ispaghula, oat fibre, gum arabica and raw potato starch were fermented slowly, and fibruline and guar gum showed an intermediate rate of fermentation (Chapters 4 & 5). The difference in concentrations of propionate in cultures of guar gum, gum arabica and raw potato starch at 8 and 24 hours indicated a slow or less rapid fermentation. Although raftilose produced a SCFA profile with propionate predominance, it still produced significantly higher concentrations of n-butyrate compared with culture of gum arabica, guar gum, pectin and raw potato starch. The pattern and slower rate of n-butyrate production in culture of raw potato starch may be beneficial in individuals with long transit time. However, in individuals with

short transit time, un-fermented raw starch or un-absorbed n-butyrate will be excreted with faeces.

In general, the pattern of fermentation seen in mixtures of carbohydrates was under the influence of the rapidly fermenting component. Ispaghula did not affect the fermentation pattern of rapidly fermenting carbohydrates. Ispaghula lowered the rate of fermentation in mixtures with raftilose but no obvious interaction was noted in mixtures of pectin and ispaghula (Chapter 6).

Different mixtures of carbohydrates showed different rates of fermentation. For example, guar gum was more effective in influencing the fermentation of raftilose (Fig. 6.8) than gum arabica. (Fig. 6.1). Such different performance suggests different interactions in the two mixtures after 24 hours. Guar gum was not as slowly fermenting as gum arabica. It was, therefore, expected that the substrate in mixtures of guar gum and raftilose would be fermented comparatively earlier than in mixtures of raftilose and gum arabica. However, the reverse was true. In addition, these mixtures showed a different pattern of propionate production suggesting that the two gums interacted with raftilose in total different ways. Gum arabica did not affect the rate of propionate production, whereas, propionate was produced less rapidly in mixture of guar gum. This may be due to the different SCFA profile of these carbohydrates. On the other hand, propionate production in mixture of lactulose and ispaghula was similar to that of lactulose alone. All the three rapidly fermenting carbohydrates (lactulose, raftilose, pectin) behaved differently in the production of propionate when combined with ispaghula in a mixture. Therefore, it seems that not all carbohydrates can be expected to interact in

similar manner in mixture. All these mixtures produced significantly higher SCFA than ispaghula alone. Thus, ispaghula can be incorporated in mixtures providing the benefit of its useful properties of delaying fermentation and increasing stool output, at no cost of SCFA.

Similarly, the mixture of three carbohydrates were fermented less rapidly, but with no less SCFA, than the constituent rapidly fermenting carbohydrates in the mixture (Fig. 6.36). Fermentation of pectin on its own produced a plateau in SCFA production after 8 hours. In contrast, the three-carbohydrate mixture did not demonstrate a plateau in SCFA at any time. This indicated that any inhibition occurring in pectin only cultures is prevented in this mixed carbohydrate culture, suggesting the possibility of SCFA production over a more prolonged period than with raftilose and pectin alone.

Interestingly the fermentation pattern of raftilose was dominant in mixtures of raftilose and pectin. The net total SCFA increased linearly in mixtures with no plateau, despite rapid fermentation. This was a positive interaction between the constituent carbohydrates (Fig. 6.22), indicating the absence of inhibition of SFCA production in mixtures even with a rapid fermentation. It may be predicted that fermentation of 100mg mixed substrate of two or more carbohydrates in *in vitro* cultures, may not be inhibited fermentation. Such mixtures may be helpful in overcoming the inhibitory effect of large amounts of single rapidly fermenting substrates and may produce higher SCFA concentrations by combining the two.

In general, fermentation of mixtures helped in achieving a linear production of SCFA. The only exception was the mixtures of ispaghula and pectin. All

other mixtures showed a gradual increase in net total SCFA. It was noted that three-carbohydrate mixtures performed better than two-carbohydrate mixtures of ispaghula with lactulose or with raftilose or with pectin, whereas, the same three-carbohydrate mixture produced lower net total SCFA than mixtures of raftilose with guar gum or with gum arabic (Fig. 7.1).

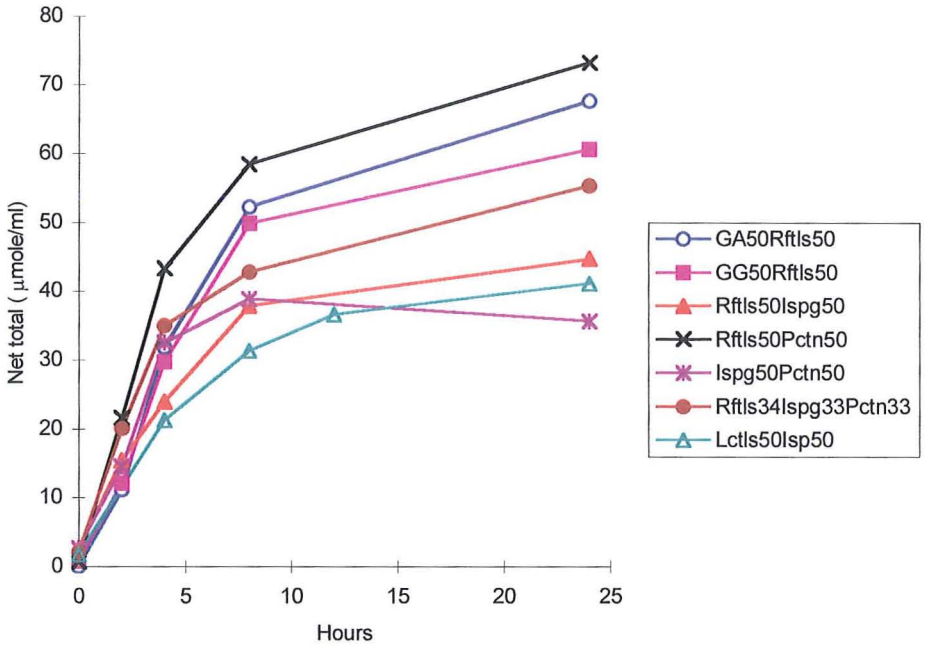


Fig. 7.1 Mean concentraions (µmole/ml) of net total SCFA produced by the fermentation of mixtures of carbohydrates with the human faecal bacteria.

This raised the question of what caused this diversity in the fermentation properties of different carbohydrates.

7.8 Does carbohydrate chemistry determine the fermentation?

Several *in vitro* and *in vivo* studies have shown that the intermediate and end products of fermentation have some relationship with the chemical and physicochemical characteristics of fibres (Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993). For example, starch fermentation produced

29% n butyrate compared with 2 % n butyrate from a more oxidised substrate such as pectin. Similarly, fermentation of pectin produced more acetate than starch (Englyst et al., 1987b; Scheppach et al., 1988b). Our study agreed with SCFA profiles reported in these studies. The two carbohydrates are different in their chemical composition, suggesting a relationship with their chemistry (detailed discussion in Chapter 5). Cherbut showed that with an equal content of soluble fibre, the richer fibres were in acidic polysaccharides the more they were fermented (Cherbut et al., 1991). Fibres richest in pectic substances and uronic acids produced higher ratios of acetate in most *in vitro* studies (Chapter 5; McBurney & Thompson, 1987; Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993). Salvador et al., (1993) suggested that the chemical nature and physical arrangement of sugars in the fibre matrix controlled the rate and extent of fermentation, together with the SCFA profiles produced by fermentation of these fibres. These properties cannot be the exclusive basis for the prediction of end products and other fermentation events. More important may be the chemical bonds between the monosaccharides of the higher molecular weight carbohydrate. It may be speculated that the type of bond between the component monosaccharides has a role, as all these carbohydrates have a diverse range of chemical bonds between their components. These bonds are broken by specific enzymes that may be produced by different bacterial species and, in this way different SCFA. All these species may work at different stages of fermentation, as the task such as the presence of a chemical bond to be hydrolysed, may demand, depending on the prevailing physiological conditions in cultures. A particular bacterial species may hydrolyse one bond, thus stripping the target carbohydrate from

other chemical linkages and exposing certain bonds for another species specific for that linkage. In this way, we may find different SCFA at different stages of fermentation as a result of the synergy between different bacterial cells (Salyers et al., 1977 a, b; Tomlin et al., 1986; Englyst et al., 1987b).

In this study, three rapidly fermenting carbohydrates, lactulose, raftilose and pectin were combined with ispaghula in mixtures of two carbohydrates. Lactulose is a disaccharide composed of galactose and fructose (Chapters 3 & 4). On the other hand, raftilose is an oligosaccharide composed mainly of fructose and some glucose. Pectin is a non-cellulosic polysaccharide in the plant cell wall with a backbone of galacturonic acid. Pectin has common side chains including arabinose, xylose, rhamnose and fucose constructed from various monomers (Whitney et al., 1994). Ispaghula husk is a highly branched arabinoxylan composed mainly of neutral sugars, about 90% of which consisted of arabinose and xylose (Marteau et al., 1994). Starch is α-polyglucan composed of glucose units linked by α 1,4 and α 1,6 glycosidic bonds. Guar gum is galactomannan whereas gum arabica is arabinogalactan. The great structural diversity of these carbohydrates in contrast to the uniformity of their SCFA profiles at 8 hours, indicates that these profiles are not exclusively governed by the chemical composition of these carbohydrates. Even those authors who have specifically studied the effects of individual sugar composition of monosaccharides and polysaccharides could not totally explain the fermentability and SCFA profile on the basis of chemical composition alone (Mortensen et al., 1988; Cherbut et al., 1991; Salvador et al., 1993). It is evident from these studies, that monosaccharide composition is not the major

factor playing a role in this regard, rather intramolecular and intermolecular bond of the individual monosaccharides are more important. For example, L-glucose, in contrast to D-glucose, did not change SCFA significantly compared with the control cultures. Even in the production of n-butyrate the two monosaccharides acted significantly different from each other (Mortesen et al., 1988). In addition, the same monosaccharide can have different fermentabilities depending on the fibre source. Arabinose in sugar beet, cocoa and pea was completely fermented *in vitro* compared with almost undegraded arabinose in wheat bran and maize. A similar situation occurs with xylose in wheat bran compared with that in other fibres (Salvador et al., 1993). These authors also noted that propionic acid could be promoted by the fermentation of glucose, whereas, xylose promoted n-butyrate more than uronic acid and glucose. This suggests that glucose should promote propionic acid over butyrate but starch, which is a polyglucan, has been shown to be n-butyrate predominant (Chapter 5; Phillips et al., 1995; Scheppach et al., 1988b). Thus, fermentability and SCFA profile of the fibres may be predicted to some extent on the basis of their chemical composition and to larger extent on the basis of their structural arrangement of monosaccharides in the complex molecule of fibre but the real determinants in each case are complex and not easily identified or understood.

7.9 Different stages of fermentation and SCFA production

In general, all cultures showed decreasing acetate ratios and corresponding increasing ratios of propionate or butyrate with extended fermentation. Propionate production was initially very slow, then increased rapidly in the

later stages of the fermentation in all cultures in this study. This could be under the influence of decreasing pH. Fermentation reactions that produce propionate may be favoured by the lower pH (Edwards et al., 1985). However, ispaghula produced a significantly higher ratio of propionate compared with n-butyrate at 8 and 24 hours (Chapter 4) without a significant fall in pH. Moreover, lactulose, which produced a significantly higher ratio of propionate than n-butyrate at 8 hours, increased its ratio of n-butyrate as the incubation progressed further, thus the n-butyrate ratio was higher than the propionate ratio at 24 hours of incubation. It seems that SCFA predominance is also governed by the stage of fermentation as well as the type of substrate (Chapters 4 & 5). The profile in the initial stages of the process may be governed by different factors than those determining the profile after several hours. For example, all carbohydrates produced more acetate initially, followed by a higher production of propionate at 8 hours. Even raw potato starch, which is well established for its n-butyrate predominance (Phillips et al., 1995; Scheppach et al., 1988b), produced higher propionate compared with n-butyrate at 8 hours although this did not always achieve statistical significance. With subsequent fermentation, raw potato starch and lactulose produced more n-butyrate than propionate at 24 hours. However, all the other carbohydrates produced more propionate than n-butyrate at 8 and 24 hours. Thus it is not until the later stages of fermentation that the predominant pattern of SCFA, which is characteristic of the carbohydrate, emerges.

It is likely that the bacteria responsible for the carbohydrate specific SCFA profile become dominant only after 8 hours of fermentation. These bacteria are likely to be strict anaerobes such as bacteriodes or clostridia. It may be

that they require some release of substrate in a synergistic attack on the carbohydrate or a drop in pH or redox potential caused by the action of facultative organisms. Once they become active these anaerobes would then expose the characteristic SCFA profile of the carbohydrate, be it propionate or butyrate predominant. Our results suggested that the butyrate predominant profile takes longer to be exposed than propionate predominance. It is also possible that the complex metabolic pathways occurring in individual bacterial cells and between synergistic bacteria may be affected by the accumulation of fermentation products in a way that changes the final end products. Acetate may be utilised in the formation of ketone bodies, instead of n-butyrate. This may result in the accumulation of more n-butyrate at later stages of fermentation (Rémésy et al., 1992) and as a consequence a decreased acetate ratio and an increased n-butyrate ratio.

In mixtures of different proportions of lactulose and ispaghula, the impact of the higher proportions of lactulose was evident only after 8 hours of incubation (Chapter 4). The earlier hours of incubation may represent a lag phase of the fermenting bacteria, which recover fermentative activity after 4 hours. Again, this recovery may be prolonged for those bacteria responsible for the carbohydrate specific SCFA profile. Therefore the ratios of individual SCFA are more under the influence of original inoculum than substrate composition in the initial hours (Chapter 4) or lag phase. However, the substrate becomes the dominant influence on the fermentation profile after 8 and up to 24 hours until substrate becomes limiting or changed physiological conditions inhibit the bacterial activity in cultures. Of course, this will depend on the amount and fermentability of the substrate used. The SCFA profile in the original faecal

inocula showed a high ratio of propionate and butyrate. This could indicate a more rapid absorption of acetate in the colon. It may, however, reflect the dominance of a carbohydrate fermentation that is more representative of the later stages of our cultures, than during the earlier stages at 0, 4 and 8 hours.

The changing pattern of fermentation with time is highlighted by the transient appearance of lactate in cultures of rapidly fermented carbohydrates. Lactate is usually seen only at 4 hours incubations and has decreased substantially or disappeared by 8 hours. This also corresponds to the biggest changes in the pH in cultures. Lactate is an electron sink, which allows regeneration of NAD^+ during rapid fermentation. It is unclear which bacteria are responsible for lactate production at 4 hours but it may be different species which subsequently convert the lactate to the SCFA more characteristic of the carbohydrate fermented.

7.10 Was delayed n-butyrate production achieved?

The main aim of this thesis was to produce a mixture of carbohydrates which would delay but would not reduce butyrate production from rapidly fermenting carbohydrates such as lactulose or raftilose. This was achieved in several mixtures but mostly those containing ispaghula. Mixture of raftilose and ispaghula produced the same n-butyrate as raftilose alone but mixtures of ispaghula with lactulose or with pectin produced lower n-butyrate than pectin or lactulose alone (Chapters 4 & 6). The rate of n-butyrate production was less rapid in three-carbohydrates mixture (raftilose, ispaghula and pectin) than culture of 100mg raftilose but the concentration of n-butyrate was preserved (Fig. 6.41).

Mixtures of ispaghula with lactulose or with pectin produced the lowest concentrations of butyrate and exhibited a plateau at 8 or 12 hours (Fig.7.2).

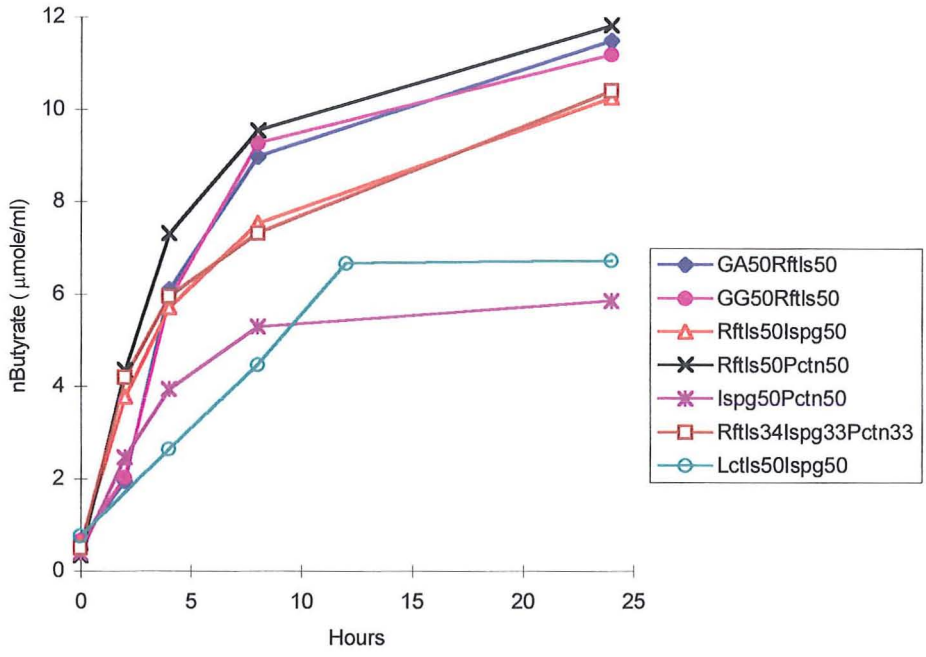


Fig. 7.2 Mean concentraions (µmole/ml) of nbutyrate produced by the fermentation of mixtures of carbohydrates with the human faecal bacteria.

Ispaghula and raftilose in two-carbohydrates mixture and ispaghula, pectin and raftilose in three-carbohydrates mixture would fulfil our requirements for using less raftilose, with delayed release of butyrate but with no loss in butyrate production. Producing such an effect *in vivo* may be helpful in prolonging n-butyrate production and perhaps moving butyrate further round the colon, at the same time reducing the potential adverse effects of raftilose. Moreover, the addition of pectin (or guar gum) may add the therapeutic effects of delaying nutrient absorption in the small intestine as well (Jenkins et al., 1977; Aro et al., 1984; Blackburn et al., 1984; Fernandez et al., 1995).

Support for the delaying effects of ispaghula on fermentation of a rapidly fermentable carbohydrate *in vivo* was supplied by the study of Washington et

al., (1998). They showed that psyllium (ispaghula) delayed the fermentation (breath hydrogen response) of large doses of lactulose with no change in mouth to caecum transit time in 8 human volunteers.

The experiments in this thesis are all based on in vitro fermentation using a very simple batch culture model that had many limitations. In addition, it was very difficult to carry out these studies in large numbers which restricted the power of each experiment. However, the results of these mixtures gave a clear indication of their possible benefits and this series of preliminary screening studies has identified at least two mixtures (Raftilose & Ispaghula; Raftilose Ispaghula & Pectin) which would be worth studying in more detail *in vivo* in man.

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