THE EFFECTS OF RICE FIBRE ON PROBIOTIC FERMENTATION

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ABSTRACT

The role of rice fibre in stimulating the growth and SCFA (Short Chain Fatty Acid) formation by human faecal micro-flora and individual probiotics and co-cultures was investigated. The effects of environmental factors on the adhesion of probiotics on rice fibre were also evaluated.

Fibre fractions of rice enhanced the growth of human colon microflora (*Bifidobacterium* species and *Lactobacillus* species) with a corresponding increase in the quantity of SCFA produced. However, individual microorganisms showed different preferences for different rice varieties and specific fractions of rice fibre. Pure cultures of the genus *Bifidobacterium* and genus *Lactobacillus* fermented rice fibre fractions irrespective of the rice variety. However, the genus *Bifidobacterium* produced more SCFA than genus *Lactobacillus*. Co-cultures of *Bifidobacteria* and *Lactobacilli* showed a greater ability than pure cultures to digest fibre and form SCFA, indicating synergism. Co-cultures used the fibre fractions irrespective of the rice variety. All microflora from mixed faecal inocula, pure and combinations of probiotic cultures showed a preference for total dietary fibre than insoluble and soluble dietary fibre fractions based on fermentation and SCFA production. All cultures tested, including human faecal cultures, pure cultures and co-cultures, produced more acetate than propionate and butyrate.

Pure cultures and co-cultures adhered to rice fibre. Adhesion was influenced by environmental factors and is believed to play a role in the fermentation of rice fibre. Rice fibre is a suitable substrate for probiotic microflora.

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Of course, despite all the assistance provided by my supervisors and others I alone remain responsible for the content of the following, including any errors or omissions, which may unwittingly remain.

PUBLICATIONS

Studies completed during candidature, some of which are reported in this thesis have been presented in books, Journals and in conferences.

Papers

Fernando, W.M.A.D.B., Ranaweera, K.K.D.S., Bamunuarachchi, A., Brennan, S.C. (2007) In vitro fermentative activity of human fecal microflora on rice fiber. **Asia Pacific Journal of Clnical Nutrition** 16 (supplement 3):s83.

Fernando,, W.M.A.D.B., Ranaweera', K.K.D.S., Bamunuarachchi', A., Brennan, C.S. (2008) The influence of fibre fractions in vitro fermentation production of short chain fatty acid using human feacal micro flora. **International journal of food science and technology 43(12): 2237 – 2244.**

Fernando, W.M.A.D.B., Ranaweera, K.K.D.S., Bamunuarachchi, A., Brennan, C.S. (2008) The invitro fermentation of rice dietary fibre: production of short chain fatty acid **In: Proceeding of Nutritional society of New Zealand** (Eds C.S. Brennan), Massey university press ISSN 0110-4187.volume 31:68.

Fernando, W.M.A.D.B., Brennan, C.S., Flint, S.H., Ranaweera, K.K.D.S., Bamunuarachchi, A. (2009) Enhancement of metabolite formation by pure cultures of probiotics on rice fibre. **International Journal of Food Science and Technology 45(4):**690 – 696.

Fernando, W.M.A.D.B., Brennan, C.S., Flint, S.H., Ranaweera, K.K.D.S., Bamunuarachchi, A. (2010) In vitro fermentation of co cultures to rice dietary fibre and its implications in probiotic technologies**. Australasian Medical Journal** 1(1): 107.

Fernando, W.M.A.D.B., Brennan, C.S., Flint, S.H., Ranaweera, K.K.D.S., Bamunuarachchi, A. (2011) The effect of rice fibre fractions on the growth of co-cultures of probiotics. **The journal of food science and technology 48(1):** 14-25.

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ABBREVIATIONS

ADF	Acid Detergent Fibre
DF	Dietary fibre
DP	Degree of polymerization
GC	Gas chromatography
GLC	Gas liquid chromatography
GIT	Gastrointestinal tract
HPLC	High performance liquid chromatography
NDF	Neutral Detergent Fibre
NDO	Non digestible oligosaccharides
NSP	Non starch polyaccharide
BB/B. breve	Bifidobactrea *breve*
BL/B. longum	Bifidobacterea *longum*
h	Hours
IDF	Insoluble dietary fibre
LA/,L. acidophilus	Lactoabcillus *acidophilus*
LR/, L. rhamnosus	Lactoabcillus *rhamnosus*
LAB	Lactic acid bacterea
OD	Optical Density
RS	Resistant starch

CHAPTER 1

General Introduction

Dietary fibre (DF) plays a major role in human nutrition and in human health. The major effect of fibre is to change the nature of the contents of the human gut and to improve the absorption of other nutrients and chemicals. Much evidence favors the fact that the dietary fibre is important to prevent cardiovascular diseases, diverticulosis, diabetes and colon cancer (Marlett *et al.,* 2002; Roberfroid, 2000; Schneeman, 1999; Tabatabai & Li, 2000). Some areas where fibre is known to have biological activity include glucose absorption (Blackwood *et al.,* 2000), stool weight (McBurney *et al.,* 1985), and caecal fermentation (McBurney, *et al.,* 1985). Microbial fermentation of dietary fibre in the large intestines is essential to mediate the health effects of fibre (Chen *et al.,* 1984; Cummings & Englyst, 1987; D'Incà *et al.,*2007). Fibre is an essential part of the daily diet, therefore, to understand the diversity of fibre utilisation in the human body is important.

Dietary fibre is a complex, non starch hetero polysaccharide that varies in chemical composition, structure, and molecular weight depending on its origin (Codex, 2008). Dietary fibre is extracted from the different foods, with cereals being the most common source. The role of dietary fibre from different cereals such as rye, wheat and barley, as a nutrient in human digestion is well known (Casterline *et al.,* 1997; Goni *et al.,* 2002; Lebet *et al.,* 1998a; Wang *et al.,*2004). However, the role of rice fibre is not well reported.

Rice is the staple food in Sri Lanka and popular in Asia. Generally rice contains 4 to 10% crude fibre; however, this changes with the cultivar, variety, environment etc.

Although, rice is the second largest cereal produced in the world (Anonymous, 2009), the role of rice fibre in digestion is not well understood.

Dietary fibre is measured quantitatively in grams, but this measurement does not provide any information about the biological activity of fibre (Adiotomre *et al.,* 1990). Therefore, current study used fermentation to examine the effects of fibre from rice on human health. In the large intestines, fibre is typically fermented by *Bifidobacterium* and *Lactobacilus* species producing beneficial by-products including SCFA (short chain fatty acids) (Cummings, 1981a). Very little is known about the formation of SCFA from rice. The current study focuses on evaluating SCFA formation in rice fibre fermentation.

The ability of probiotic bacteria including genus *Bifidobacterium* and *Lactobacilus* to ferment dietary fibre (Collins & Gibson, 1999; Crittenden, 1999) is important for them to establish as a significant population in the colon. Probiotic foods need to contain high numbers of stable, viable probiotic bacteria to ensure survival during the processing of food and supplements, transit through high acidic conditions of the stomach, enzymes and bile salts in the small intestine as well as compatible prebiotic components. The demand for such foods is increasing (Tannock, 1995). The intake of probiotics at a level of 10^8-10^9 cfu/g per day is a commonly recomended, equating to 100g of a food product with 10^6-10^7 cfu/g (Lee & Salminen, 1995; Kebary,1996). Combinations of *Bifidobacterium* and fructo-oligosaccharides (FOS), *Lactobacillus* GG and inulins, and *Bifidobacterium* and lactobacilli with FOS or inulins are considered good combinations (Kaplan & Hutkins, 2000; Wang & Gibson, 1993). Rice fibre may be a good prebiotic for probiotic foods although this has not been reported. If rice fibre can be fermented by specific strains of *Bifidobacterium* and *Lactobacillus*, then rice fibre can be considered as the potential "prebiotic" for those

strains of probiotic bacteria. In order to determine if this is possible, the growth of probiotics on rice fibre needs to be examined.

Despite the commercial and research interests in dietary fibre and probiotic bacteria, little information is available about the strains of bacteria that actually metabolise fractions of the dietary fibre as insoluble dietary fibre (IDF), soluble dietary fibre (SDF), and total dietary fibre (TDF), in rice or in any other cereal. Most of the commercially available food contains either soluble fibre or total dietary fibre. But most research has focused on the fermentation of individual fibre components such as β-glucan, oligosaccharides, and pectin. Therefore, to bridge this gap the current investigations will examine the fermentation of fibre fractions as TDF, SDF, and IDF.

The human large intestine contains many different types of bacteria (Finegold et *al.,* 1983) responsible for the fermentation of dietary fibre. Studies on the fermentation of dietary fibre with human faeces provide some indication of the effect of the different dietary fibres on human gut microorganisms (Gibson *et al.,* 1995; McBurney & Thomson, 1987). The rate and extent of metabolite formation can be determined under anaerobic conditions, mimicking the conditions of the human gut. However, in the human gut, not all microorganisms ferment dietary fibre. Therefore, studies with human inocula are limited in identifying the most suitable microorganisms for fibre fermentation (Barry *et al.,* 1995; Bird *et al.,* 2000; Lebet *et al.,* 1998a). The current study examined the fermentation of rice fibre using monocultures, co-cultures as well as human faeces as the source of inocula.

Adhesion of probiotics to epithelial cells and to the fermentable substrate enables probiotics to tolerate the environment of the colon, including low oxygen and low pH (Collins & Gibson, 1999). Moreover, adhesion of probiotics to fibre is believed to be important in the digestion of fibre in the rumen (Windham & Akin, 1984).

However, there is little information on the adhesion of probiotics to dietary fibre, relevant to human digestion. There is no information on the adhesion of probiotics to rice fibre, and therefore, this forms another objective of this study.

1.1Research Question

The main issue to be addressed in this thesis is "***What are the effects of rice dietary fibre on probiotic fermentation?"***

1.2 Hypothesis

The main hypothesis of this thesis is the **rice fibre can support the growth and SCFA production by probiotics.**

1.3 Research Objectives

The specific objectives of the work investigated in this thesis are:

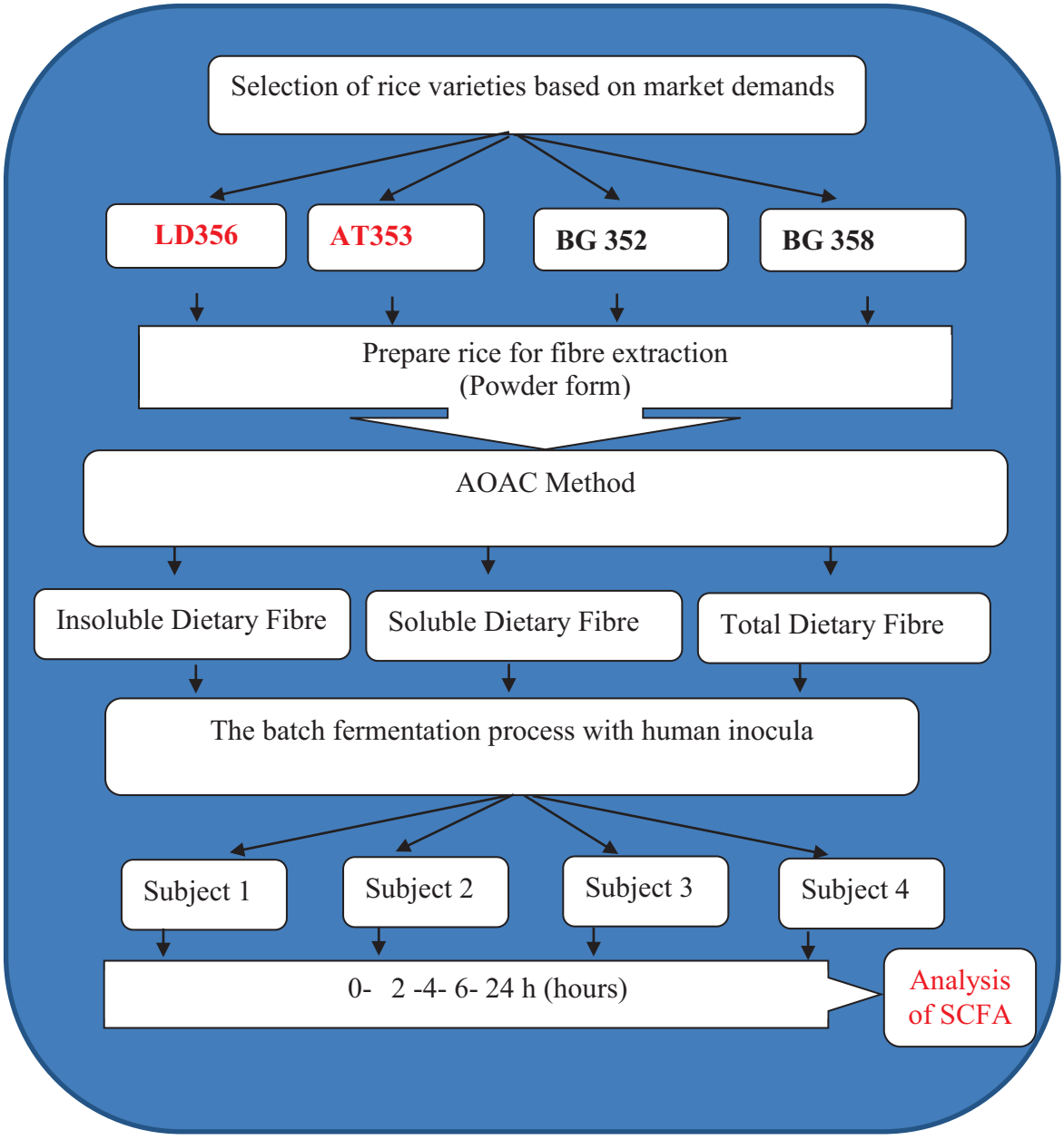
1. ***To characterise the fibre content of rice in terms of total dietary fibre, insoluble dietary fibre, and soluble dietary fibre;***
2. ***To determine the effect of rice fibre on the human faecal micro flora and the formation of SCFA;***
3. ***To evaluate the growth of probiotics on rice fibre fractions;***
4. ***To investigate the formation of SCFA from rice dietaryfibre;***
5. ***To examine the adhesion of probiotics on rice fibre.***

1.4 Research steps

The following diagrams (Fig 1.1) summarise the investigation.

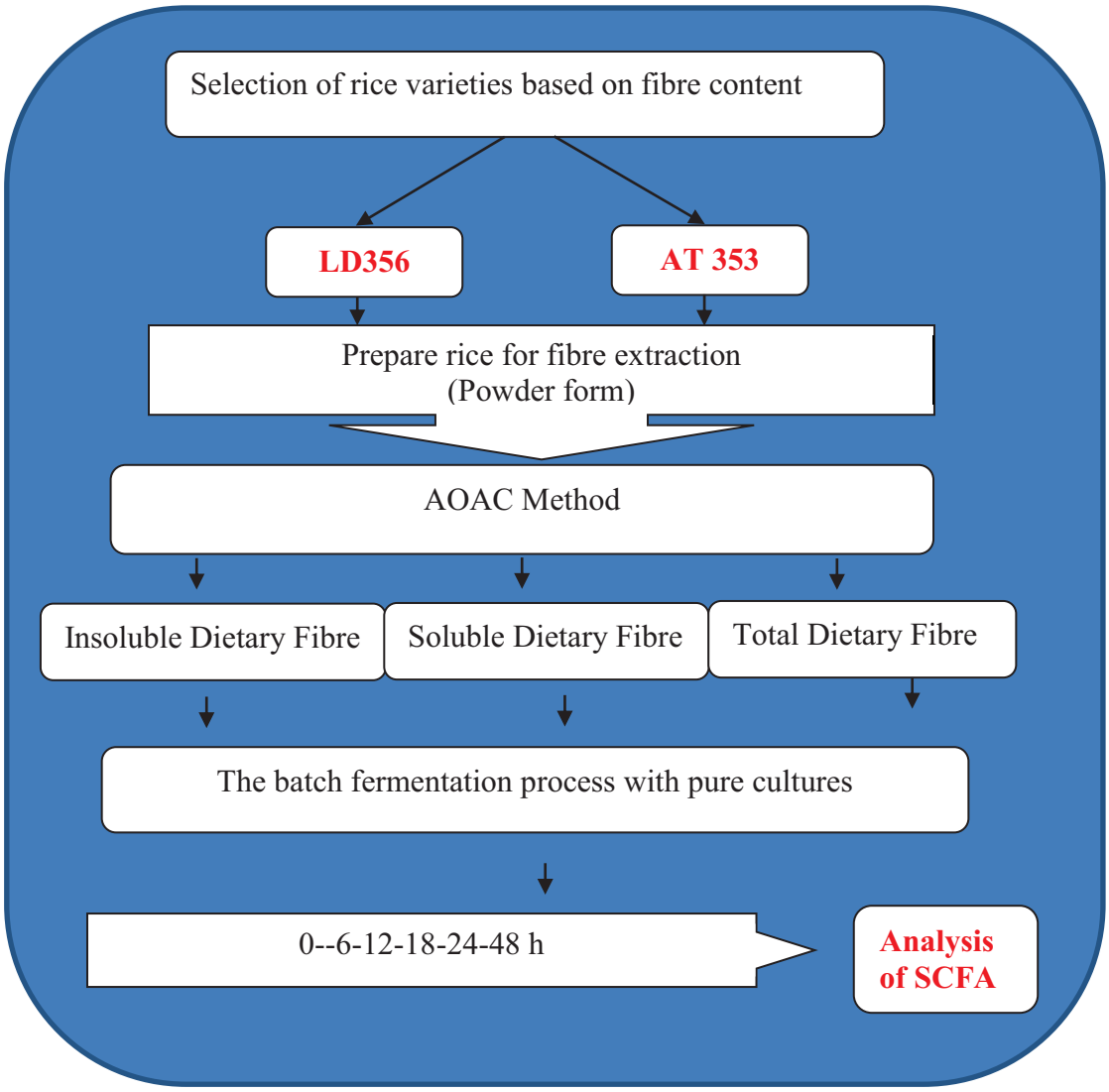
Stage 1

SCFA production from the rice fibre fraction by human faecal microflora



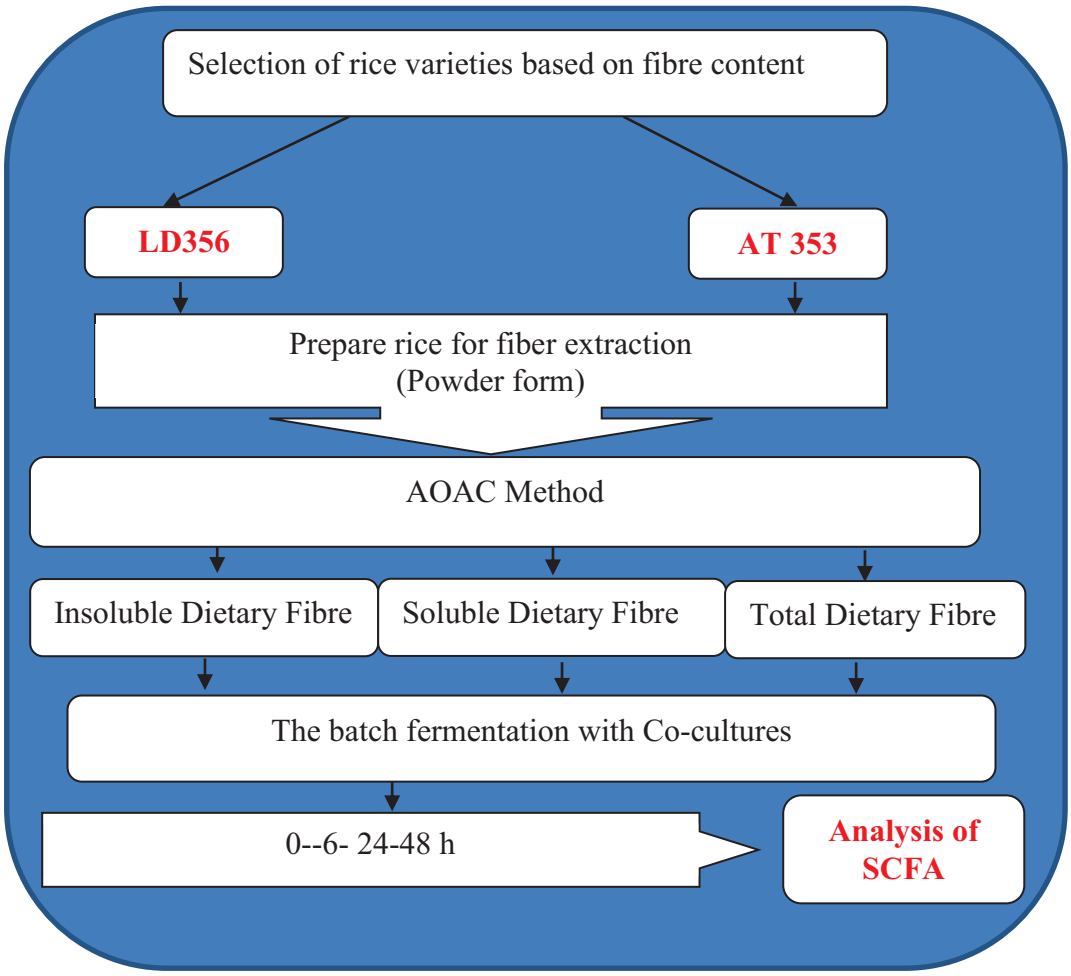
Stage 2

SCFA production from the rice fibre fraction by pure cultures



Stage 3

SCFA production from the rice fibre fraction by co-cultures



Stage 4

Adhesion of pure cultures and co-cultures to rice fibre fractions

Study of the adhesion of probiotics to fibre fractions

Figure 1.1 Research in simple format

Stages 1, 2, 3 and 4 explain the major experiments in this work

CHAPTER 2

Literature Review

2.1 Introduction

The term, dietary fibre, originated in 1953 by Hipsley (Tungland & Meyer, 2002) and is recognized today as an important dietary component with many health benefits including an ability to prevent diseases. Refined cereals and sugar are responsible for several degenerative human diseases (Chatenoud *et al.,* 1999). A fibre-rich diet is believed to be important to control obesity, colon cancer, heart disease, gallstones, irritable bowel syndrome, diverticulosis, and diabetic conditions (Schneeman, 1999).

Fibre is the skeletal system of plants. Without fibre no plant or tree would be able to be rigid. Dietary fibre is commonly defined as group of compounds, which vary in chemical and physical characteristics, and is derived from plant material that resists digestion, through enzymes of the human alimentary tract (Buttriss & Stokes, 2008). Meaningful and reproducible data on the dietary fibre content of the foods in diet is important to understand the physiological effects of fibre. Unfortunately, due to several definitions and methods of analysing of dietary fibre in foods, inconsistent and confusing data have been reported (Butler & Patel, 2000).

The principle actions of fibre are to alter the nature of the human gut and improve the absorption of other nutrients (Phillips *et al.,* 2008).

2.2 Concept of fibre

Dietary fibre has been defined in many ways, however, in broad terms, dietary fibre refers to foods that pass through the stomach and small intestine as undigested components, reaching the large intestine almost unchanged. In the large intestine dietary

fibre is broken down to a certain degree and absorbed by the body with the balance being excreted in the faeces.

Trowell (1972), described dietary fibre as:

"The proportion of food which is derived from the cellular walls of plants which is digested very poorly in human beings".

This definition is based on the physiological, botanical and chemical features of fibre (Eastwood & Kritchevsky, 2005). Only the resistance of dietary fibre to digestion by human enzymes has remained unchanged in terms of defining of dietary fibre by this definition. But the polysaccharides, especially hydrocolloids, would have a similar impact to plant cell walls in terms of impact on the human digestive system. Therefore, dietary fibre is redefined to include polysaccharides and lignin that are not digested in the human small intestine (Tungland & Meyer, 2002).

In 2001, the American Association of Cereal Chemists (AACC), defined the dietary fibre as follows:

"Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (Anonymous, 2001).

This definition shows the connectivity of chemical composition of fibre to its physiological effects. All the non starch polysaccharides resistant to digesion in the small intestine and which are fermentable in the large intestine are included in this new definition. "Analogous carbohydrates" are the carbohydrate-based food ingredients that are non-digestible and non-absorbable. Those carbohydrates are similar to plant dietary

fibre (DeVries, 2003). Few examples of analogous carbohydrates are resistant moltodextrin, resistant potato dextrins, polydextrose, methyl cellulose, and hydroxypropylmethyl cellulose (Anonymous, 2001).

The Food Nutrition Board (FNB, 2002), of The Institute of Medicine (2002), defined fibre as:

"***Dietary fibre consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Added fibre consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans. Total fibre is the sum of dietary fibre and added fibre"*** (FNB, 2002).

This definition had divided the fibre into dietary fibre (added fibre) and functional fibre instead of soluble and insoluble.

Both AACC and FNB definitions are based on the physiological characters of fibre. Added fibre and functional fibre are virtually impossible to distinguish from a mixture of fibre. Pectin is a part of an intact plant tissue but can also be an added fibre (McCleary, 2003) in plant based food products.

In 2008, the European Commission defined the dietary fibre as:

"Carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed

- Edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence;

- Edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence" (European commission, 2008).

In 2008, Codex defined the dietary fibre as:

"Dietary fibre means carbohydrate polymers with ten or more monomeric units,

which are not hydrolysed by the endogenous enzymes in the small intestine

of humans and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed,

- Carbohydrate polymers, which have been obtained from food raw material by physical,

enzymatic or chemical means and which have been shown to have a physiological

effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,

- Synthetic carbohydrate polymers which have been shown to have a physiological

effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities" (Codex Alimentarius Commission, 2008).

Despite much development in dietary fibre research, there is still no universally accepted definition of dietary fibre. Originally, dietary fibre was regarded more as a nutritional component rather than being important for its physical and chemical properties. Some of the recent definitions are based solely on one or more of the analytical methods for isolating fibre, while others are physiologically based. All attempts to define the dietary fibre indicate a lack of a universal definition or analysis method for fibre. Since Codex sets standards for food for the world, the Codex definition is used as the basis for analytical methods, food labeling, setting of nutrient

reference values and health claims (Mann & Cummings, 2009). This definition further describes the intrinsic carbohydrates of the plant cell wall as the major form of fibre, and the importance of the acceptance of the extracted and synthetic carbohydrate polymers as a key fibre component (Mann & Cummings, 2009).

2.3 Development and evolution of fibre determination methods

Despite many scientific attempts to define dietary fibre, many methods have been developed in past 25 years to extract and to analysis the dietary fibre in different foods based on the acceptance of resistant starch (RS) and for non digestible oligosaccharides (NDO) as dietary fibre.

Enzymatic-gravimetric and enzymatic-chemical methods are two methods used for isolating and analyzing dietary fibre. The enzymatic-gravimetric method eradicates starch, protein and fat, and the final result is expressed as a proportion of the initial material after correcting for ash and for protein of extracted material. The enzymatic-chemical method characterises fibre chemically after the removal of available carbohydrate (monosaccharides, disaccharides, and starch) and fat.

The gravimetric approach was first used for the measurement of crude fibre by Henneberg and Stohmann in 1860 (McCleary, 2003). In the 1960s, Van Soest *et al.,* introduced the Acid Detergent Fibre (ADF) method, which utilizes strong acid to hydrolyze all polysaccharides except cellulose and lignin (Robertson & Horvath, 2001). In 1967, Van Soest and Wine developed the Neutral Detergent Fibre (NDF) method, which measures all insoluble cell wall material (Robertson & Horvath, 2001). Based on this early work, a number of analytical methods evolved to extract the "nondigested" fraction of the diet, including soluble material and insoluble components (Asp & Johansson, 1981; Furda, 1977; Hellendoorn *et al.,* 1975; Schweizer & Würsch, 1979). These evolved to form the basis of the Association of Official Analytical Chemists

(AOAC) method 985.29 (AOAC official method 985.29, 1995), developed by Prosky and coworkers (1985). The principle of this method is to digest the food particles using protease and amyloglucosidase to remove the protein and the starch. This is followed by precipitation of soluble dietary fibre by aqueous ethanol (Asp & Johansson, 1984; Asp *et al.,* 1992) leaving insoluble dietary fibre as the residue. The keys to success with the enzymatic-gravimetric methods are enzyme purity, avoiding contaminating enzymes which digest the dietary fibre as well as accurate handling of the digestion steps.

A large number of chemical methods also evolved. In these methods generally polysaccharides are hydrolysed and the resulting monosaccharides are analysed. Hydrolysis of polysaccharides and a colorimetric analysis of monosaccharides were introduced by Southgate in 1969. Again in 1981, gas chromatography (GC) or high-performance liquid chromatography (HPLC) was introduced to provide more information on the components of dietary fibre (Southgate, 1981). Soluble and insoluble fibre components were separated by a chemical method in 1979 by Theander and Åman following a GC method (Theander & Westerlund, 1986). Later this method was modified and measured the fibre-derived monosaccharides by HPLC (Shinnick *et al.,* 1988; Quigley & Englyst, 1992). In 1982, Englyst and coworkers developed a method to analyse of nonstarch polysaccharides using GC. This method was later modified by Englyst and Hudson (1987), to develop a colorimetric method for the measurement of the component of monosaccharides. HPLC methods were further developed for the measurement of uronic acids (Englyst *et al.,* 1994; Quigley & Englyst, 1994).

Both Englyst (1992) and AOAC (1995) methods have been widely used. In either method the initial sample preparation is the same and they identify all non starch polysaccharides that precipitate in 78 to 80 % ethanol. The AOAC method measures

NSP, lignin, and resistant starch (RS) and RS is not included in the Englyst method (only NSP). Therefore, the mean value obtained from the Englyst method for dietary fibre is 20 to25% lower than the AOAC method (Butler & Patel, 2000; Champ *et al.,* 2001). Further results from the Englyst method may have a higher variation than the AOAC method due to large number of steps involved in the Englyst method. Neither the AOAC nor the Englyst methods can determine whether the source of NSP is from the intrinsic cell wall or from the materials added to food (Butler & Patel 2000: Champ *et al.,* 2003).

Compared with previous methods, the AOAC method is reproducible and moderately expensive (Champ *et al.,* 2003). The most extensively used and accepted AOAC method is 991.43 (AOAC, 1995), which is a modification of the 985.29 (Gordon, *et al.,* 2006). These methods determine the soluble, insoluble and total dietary fibre, not as individual compounds of fibre of food (Prosky *et al.,* 1988, 1992, 1994). Lee and co-workers (1992) substituted MES-TRIS buffer of AOAC 985.29 in place of the original phosphate buffer, and formulated the new AOAC method 991.43 (AOAC,1995). Li and Cardozo (1994) introduced AOAC method 993.21 for fruits and some vegetables. The buffer system was improved to minimise the problems with the formation of calcium phosphate (McCleary *et al.,* 2005). The major constraint of this method is the inability to recover the non digestible oligosaccharides (NDO) which have a degree of polymerization (DP) between 3-10 monomer units (Mertens, 2002). The method is applicable to all foods.

The AOAC methods 985.29 and 991.43 are time consuming and labor intensive. The number of sample analyses per time period is limited due to the difficulties of managing a large number of beakers (Mertens, 2003). Filtration of dietary fibre is time consuming, especially with highly viscose food. The AOAC analysis method usually

requires 1.5 to 2 days or more to complete, with some foods requiring longer than others due to difficulties in filtration. With the AOAC method, large amounts of alcohol, acetone and other solvents are generated as waste products resulting in a negative impact on the environment.

To measure polyfructoses (inulin or fructooligosaccharides) or modified starches or dextrins that resist digestion, additional methods are required (Anonymous, 2001). A large number of AOAC methods have been developed to recover the non digestible oligosaccharides which have a DP between 3 to10 monomer units. For example, AOAC Method 997.08 (Hoebregs, 1997) and AOAC Method 999.03 (McCleary *et al.,* 2002) for FOS (fructo oligosaccharides), AOAC Method 2000.11 (Craig *et al.,* 2000) for Polydextrose, and AOAC Method 2001.03 (Gordon & Okuma, 2002) for fibresol. While these methods are useful for the measurement of specific dietary fibres, the problem of choosing the appropriate method arises when an unknown food sample is to be analysed. The development of an integrated total dietary fibre method to measure NDO (nondigestible oligosaccharides) and RS (resistant starch) accurately, may be a good attempt to form a single assay to determine dietary fibre components in food (McCleary, 2010).

The definition and analysis of dietary fibre methods should be strongly linked. Frequently this is not the case due to the demand for quick methods that are practical for food labelling. It is important to separate the different fractions of dietary fibre as IDF, SDF and TDF in this research. The AOAC methods fulfill the requirements of this thesis. The AOAC Official Method 991.43 “Total, soluble and insoluble dietary fibre in foods” is frequently used (Prosky *et al.,* 1988, 1994) and followed in the present study.

Separation of dietary fibre into SDF (soluble dietary fibre) and IDF (insoluble dietary fibre) in humans is believed to take place along the human intestinal tract;

therefore, the conditions for all these methods should represent the conditions in the human intestine. However, the laxative effect of SDF, IDF and NDO in the human intestine, effect of soluble dietary fibre in the viscose nature of the intestinal environment, and the interaction with the muscins, cannot be replicated in a bench method (Gordon *et al.,* 2006). There is no national or international standard to measure the laxative effect of dietary fibre, however, Canada has suggested the new protocol for the laxative effect of cellulose base insoluble sources of dietary fibre (Gordon *et al.,* 2006).

The most attractive methods are low cost and fast with the ability to separate fractions of dietary fibre. The value of analysis method increases when the method is aligned with a specific dietary fibre definition.

2.4 Classification of dietary fibre

Dietary fibre has been classified based on the physiological and metabolic activities in the human body (ÁlvarezGarcía, 2000; Álvarez & Sánchez, 2006; Englyst & Hudson, 1996; Ha *et al.,* 2000) (Fig 2.1, 2.2 and Table 2.2).

The following proposed classification of dietary fibre was based on the following criteria,

- site of the action (upper or lower gut),
- pattern of degradation,
- origin of the fibre (plant cell wall material or not),
- chemical identity of the fibre (Ha, *et al.,* 2000).

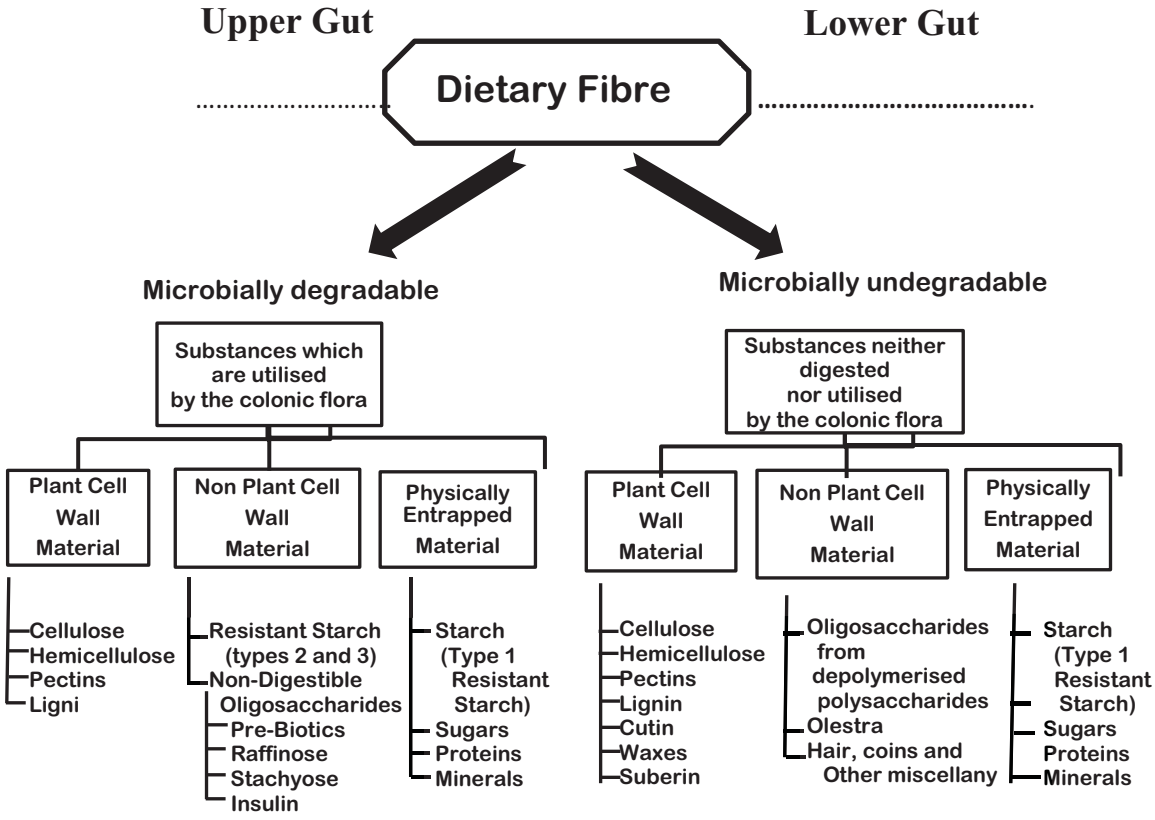


Figure 2.1 Dietary fibre classifications

Source: Ha et al., (2000)

Based on these classifications (Fig 2.1, 2.2 and Table 2.1), fibre can be fermentable, soluble, viscose, or poorly fermentable, insoluble, and non viscous (Álvarez & Sánchez, 2006). However, it is difficult to formulate a general description for the classification of dietary fibres since they are heterogeneous and have been classified based on the interests of the researchers (Englyst & Hudson, 1996). Many researchers have sub classified the NSP (non starch polysaccharides) as soluble and insoluble fibres. This is based on the solubility and extractability characteristics and the physiological functionality in the human gut (Englyst & Cumming, 1990). Cho *et al.,* (1997a), described the soluble fibre as fibre which dissolves in buffers and the enzymes. Soluble fibres are hemicellulose, pectin, β-glucan and galactose, manose and gums. However, hemicelluloses dissolve in alkali solutions and do not dissolve in water. Therefore, hemicelluloses were classified under insoluble fibre too. Insoluble fibre

(cellulose, lignin, and hemicelluloses) does not dissolve in water, alkaline or acids (Smits & Annison, 1996; Cho *et al.,* 1997a).

<table>
<tr><td rowspan="4"></td><td colspan="2">Lignin</td><td rowspan="2">Not water-soluble (“insoluble fibre”)</td></tr>
<tr><td rowspan="3">Non- starch polysaccharides</td><td>Cellulose</td></tr>
<tr><td>Hemicellulose(type B)</td><td rowspan="2">Water-soluble (“insoluble fibre”)</td></tr>
<tr><td>Hemicellulose(type A)
Pectins
Gums
Mucilages
Other polysaccharides</td></tr>
<tr><td rowspan="3">Fibre-analogue substances</td><td colspan="2">Inuline
Frutose-oligosaccharides</td><td rowspan="3">Most of them Water-soluble</td></tr>
<tr><td colspan="2">Resistant starch</td></tr>
<tr><td colspan="2">No digestible sugars</td></tr>
</table>

Figure 2.2 Fibre classifications according to water solubility

Source: Álvarez & Sánchez, (2006)

Table 2.1 Fibre classification by degree of fermentation

Total fermentation in the colon	**Partial fermentation in the colon**
Gums, Pectins, mucilage, Fructooligosaccharides, Hemicellulose	Hemicellulose , cellulose , lignin
GOS, Inulin, Resistant starch	

Source: ÁlvarezGarcía*,* (2000)

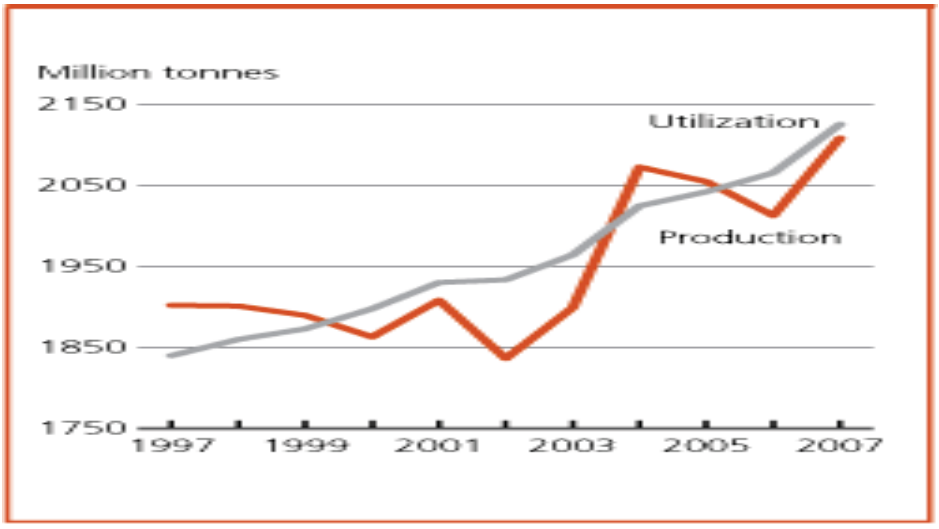


Figure 2.3 World cereal production and utilization

Source: FAO, (2009)

2.5 Rice

Dietary fibre comes from plant food. Cereals are good sources of dietary fibre. The consumption of cereal in the world increases in each year (Fig 2.3). However, total cereal utilization was low in 2002-2003 due to a slowdown in animal feed and industrial sector growth (FAO, 2009). Among the cereals, rice is the second largest produced cereal in the world (FAO, 2009; Hafner, 2003).

Rice (*Oryza sativa*) is one of the most important crops in the world (Souci *et al.,* 1986; Roulin *et al.,* 2010) along with wheat and maize (Anonymous, 2004; Hafner, 2003). Rice is the staple food for a large part of the world's human population and has been a source of food for people from 2500 B.C. Rice production was initiated in China, and spread to countries such as Sri Lanka and India (Chang,2000). Rice is important due to its high digestibility and biological value (Khatoon & Prakash, 2006). The chemical and nutritional quality of rice grain varies significantly and the major factors that contribute to the differences are genetic factors, environmental influences, fertilizer treatments, degree of milling, storage conditions, cultivar, thickness of anatomical

layers, size and shape of grains, and the resistance of grains to breakage and abrasion (Houston, 1972; Luh *et al.,* 1991). The nutritional quality of rice can be further differentiated based on the milling conditions such as processing methods and the machines used for processing (Luh *et al.,* 1991). In addition, rice is an important grain for nutrition (Table 2.2) and caloric intake (Souci *et al.,* 1986).

Freshly harvested rice is called paddy grain or rough rice. The rice grain (rough rice) consists of the hull, and the rice caryopsis (Juliano & Bechtel, 1985). Underneath the hull, bran, germ, and endersperm are present.

Table 2.2 Nutritional composition of selected cereals

Food	Moisture (%)	Protein (g Nx 6.25)	Crude fat (g)	Available carbohydrates (g)	Fibre (g)			Crude ash (g)	Energy (kJ)
					Dietary Total	water soluble	Lignin		
Brown rice	14	7.3	2.2	71.1	4	-2.7	-0.1	1.4	1 610
Wheat	14	10.6	1.9	61.6	10.5	-7.8	-0.6	1.4	1 570
Maize	14	9.8	4.9	60.9	9	-6.8	0	1.4	1 660
Millet	14	11.5	4.7	64.6	37	-2.3	0	1.5	1 650
Sorghum	14	8.3	3.9	57.4	13.8	-12.4	-3	2.6	1 610
Rye	14	8.7	1.5	60.9	13.1	-8.4	(1 4)	1.8	1 570
Oats	14	9.3	5.9	63	5.5	-39	0	2.3	1 640
Potato	77.8	2	0.1	15.4	2.5	-1.9	0	1	294
Cassava	63.1	1	0.2	31.9	2.9	-2.2	0	0.7	559
Yam	71.2	2	0.1	22.4	3.3	-2.6	0	1	411

Source: Souci, et al.,(1986)

Rice is basically in two forms: brown and white rice. Brown rice is produced by dehusking the hull (Fig 2.4). This causes the least damage to the nutritional quality of the rice and prevents excessive loss of nutrients that can arise with further milling.

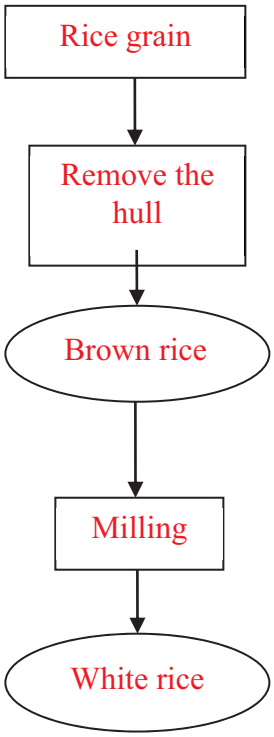


Figure 2.4 Basic steps for the production of white rice and brown rice

When brown rice is further processed to remove the bran and most of the germ layer, the outcome is white rice. At this point, however, cereal is known as unpolished rice, and it takes further milling or polishing to produce polished rice, which is commonly known as white rice. Thus, rice is ground into several milling grades to obtain the many different types of rice flours and grains. This includes: brown rice (hull removed), unpolished rice (hull, bran and most of germ removed), and polished rice (aleurone layer removed from unpolished rice) (Haard *et al.,* 1999). Un-milled rice has more protein and fibre than milled or polished rice and can be considered as more

nutritious (International Rice Commission, 2004). As in Tables 2.3 and 2.4, the major impact of milling on rice is to manipulate the nutritional composition of the grain.

Brown rice consists of the outer layers (pericarp, seed-coat), and the nucleus (bran), the germ or embryo; and the endosperm (Fig 2.5). The endosperm consists of the aleuronic layer and the endosperm proper (subaleurone layer and the starchy or inner endosperm). The aleuronic layer encloses the embryo. Pigment is confined to the pericarp (Juliano & Bechtel, 1985) which gives the colour of brown rice.

Different layers (Fig 2.5) of rice have different quantities of fat, carbohydrate, protein and fibre, and removing the different layers in the milling process alters nutritional quality. The milling of brown rice involves the removal of the pericarp (outer bran layer), seed-coat, aleuronic layer, and embryo. This results in the loss of fat, protein, crude and neutral detergent fibre, ash, thiamine, riboflavin, niacin and α-, (γ+β)-, and δ- tocopherol in milled rice (Table 2.3 and 2.4). The other fraction of the rice grain, rice bran, contains an appreciable quantity of crude fibre (Sera *et al.,* 2005). Rice bran fibre largely consists of non cellulose polysaccharide (24%), cellulose (10.3%), and lignin (10.7%) (Sera *et al.,* 2005).

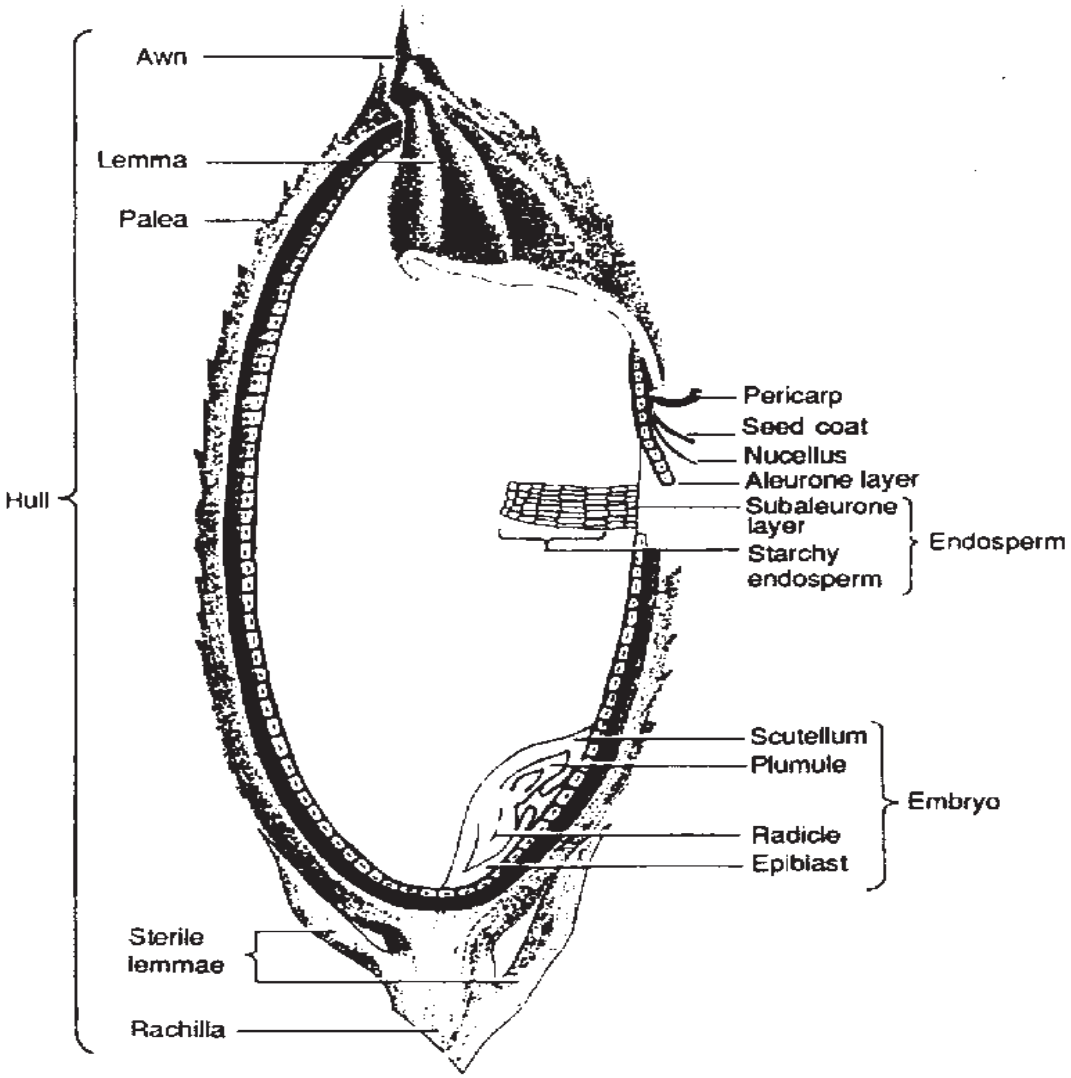


Figure 2.5 Rice grain structure

Source: Juliano, (1993)

The nutritional composition of rice differs with variety, condition of the soil, environmental factors, and fertilizers used (Amissah *et al.,* 2003; Juliano *et al.,* 1964**)**.

Table 2.3 Approximate Composition of Rough Rice

Rice fraction	Crude protein (g N x 5. 95)	Crude fat (g)	Crude fibre (g)	Crude ash (g)	Available carbohydrates (g)	Neutral detergent fibre (g)	Energy content (kJ)
Rough rice	5.8-7.7	1.5-2.3	7.2-10.4	2.9-5.2	64-73	16.4-19.2	1580
Brown rice	7.1-8.3	1.6-2.8	0.6-1.0	1.0-1.5	73-87	2.9-3.9	1520-1 610
Milled rice	6.3-7.1	0.3-0.5	0.2-0.5	0.3-0.8	77-89	0.7-2.3	1460-1 560
Rice bran	11.3-14.9	15.0-19.7	7.0-11.4	6.6-9.9	34-62	24-29	670-1 990
Rice hull	2.0-2.8	0.3-0.8	34.5-45.9	13.2-21.0	22-34	66-74	1110-1 390

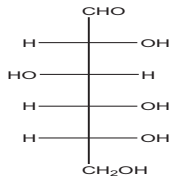
Sources: Eggum, et al., (1982); *Pedersen and Eggum,* (1983)

Table 2.4 Proximate Analyses of Different Rice Types

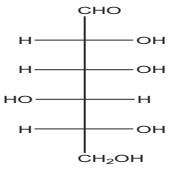
Rice Type / Type of Nutrition	Brown	White -polished	Red	purple	Black	white
Protein(g/100g)	7.9 (*)	6.8(*)	7.0 (*)	8.3 (*)	8.5 (*)	-
Iron(mg/100g)	2.2 (*)	1.2(*)	5.5 (*)	3.9 (*)	3.5 (*))	-
Zinc(mg/100g)	05 (*)	05 (*)	3.3 (*)	2.2 (*)	- (*)	-
Fibre(g/100g)	2.8(*)	0.6(*)	2.0(*)	1.4 (*)	4.9(*)	
Crude Protein (Dry weight%)	7.3 (**)	-	-	-	-	-
Crude Fat (Dry weight%)	2.2 (**)	-	-	-	-	-
Ash (Dry weight%)	1.4 (**)	-	-	-	-	-
Crude Fibre (Dry weight%)	0.8 (**)	-	-	-	-	-
Available carbohydrate (Dry weight%)	64.3 (**)	-	-	-	-	-
Crude fibre% (Dry weight)	1.0%(***)	-	-	-	-	0.3% (***)
Carbohydrate% (Dry weight)	88%(***)	-	-	-	-	91%(***)
Protein% (Dry Weight)	8.5%(***)	-	-	-	-	7.6%(***)
Lipid(crude fat)% (Dry weight)	2.2%(***)	-	-	-	-	0.5%(***)

*Sources: * Data obtained from FAO* 2004 ** *Data obtained from Alais and Linden* (1991) *** *Data obtained from Dendy* (2001)

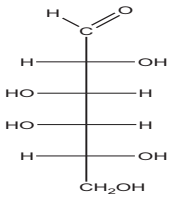
2.6 Soluble and insoluble fibre in rice



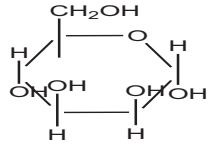
D-Glucose



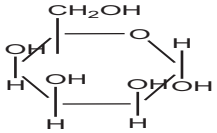
L-Glucose



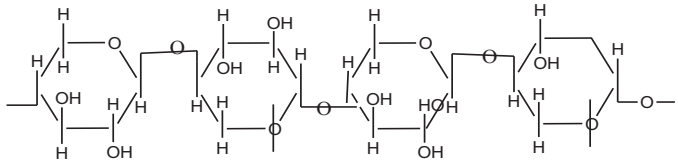
D-Glactose



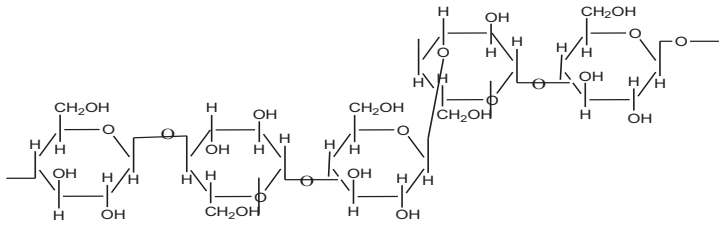
Glactose



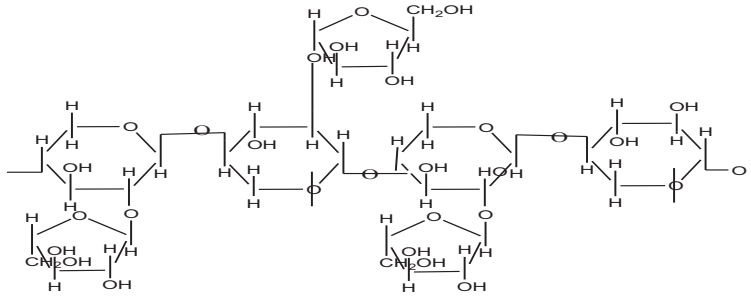
Mannose



Hemicellulose



B-Glucan



Arabinoxylan

Figure 2.6 Structures of sugars present in rice

Source : Gibbons et al., (2002); *Roubroeks et al.,*(2001)

Arabinoxylans (3-4%), along with some amount of β-d-glucans (Fig 2.6), are the major components of SDF (soluble dietary fibre) (Rao & Muralikrishna, 2004) present

in rice. In addition, rhamnose, xylose, fructose, mannose, galactose, and glucose (Fig 2.6) can be found as part of the SDF (Rao & Muralikrishna, 2004). Cellulose, hemicellulose, and insoluble β-glucan and arabinoxylans make up the IDF (insoluble dietary fibre) (Lai *et al.,* 2007). However, the amount and the quantity of these non starch polysaccharides in rice are dependent upon the rice cultivar, degree of milling, and water solubility. In addition, the water solubility of non starch polysaccharides is mainly governed by the rice variety, rather than the degree of milling (Lai *et al.,* 2007).

2.7 Physiological and metabolic effects of dietary fibre on human health

Dietary fibre contains a large variety of physico-chemical properties that are important nutritionally, such as viscosity, water holding capacity, ion exchange (Fleury & Lahaye, 1991), organic acid adsorption, gel filtration, and particle-size distribution (Lai *et al.,* 2007). The direct effect of these properties of dietary fibre can be seen throughout the gastrointestinal tract (Guillon & Champ 2000; Schneeman, 2009).

The particle size of the dietary fibre is important in the transit time, faecal excretion and fermentation. Particle size can be varied mainly by chewing and bacterial degradation in the large intestine. This variation can be influenced by the porosity and surface area of the fibre and by microbial degradation in the colon (Robertson *et al.,* 2006).

In the gastrointestinal tract, fibre can influence gastric emptying by decreasing the glycaemic response to the meal and delaying starvation, without any effect on satiety and the feeling of epigastric fullness (Inui-Yamamoto *et al.,* 2009; Read & Eastwood, 1992). Fibre dilutes enzymes and absorbs compounds in the gut (Dunaif & Schneeman, 1981; Inui-Yamamoto *et al.,* 2009), preventing starch from hydrolyzing, and slowing the diffusion or mobility of enzymes, substrates, and nutrients to the absorptive surface by changing the viscosity of intestinal contents. Fibre traps nutrients and water to

change the physical properties of intestinal content thus increasing the viscosity of the contents (Bach Knudsen, 2001; Cummings, 1993; Serena & Bach Knudsen, 2007a).

The effect of structural and physical properties of dietary fibres on human physiology takes place primarily in the colon. Dietary fibre possesses high water holding capacity that increases the stool output by bulking the stools (Blackwood *et al.,* 2000; Canibe & Bach Knudsen, 2002). In addition, dietary fibre can modulate the contractile activity and water movements in the colon, thus reducing the transit time (Salvador & Cherbut, 1992). On the other hand, purified fibre can reduce the adsorption of some vitamins and minerals by binding with them (Aozasa *et al.,* 2001; Salvador & Cherbut, 1992).

Dietary fibre is important in the controlling of type 2 diabetes. A sufficient quantity of dietary fibre helps to even out the postprandial glycemic and insulinemic excursions and positively influences plasma lipid levels in patients with type 2 diabetes (Hodge *et al.,* 2004; Tabatabai & Li, 2000). Dietary fibre delays glucose absorption, increases hepatic extraction of insulin, increases insulin sensitivity at the cellular level, and binds bile acids (Tabatabai & Li, 2000). Viscocity and the solubility of fibre are important to reduce the postprandial blood glucose. Soluble fibre is more important in this than insoluble fibre (Hodge *et al.,* 2004; Tabatabai & Li, 2000).

Fibre combines with bile acids or cholesterol. This process initiates during the intraluminal formation of micelles and fibre reduces the cholesterol content of liver cells, specially the LDL cholesterol (Brown *et al.,* 1999; Kocyigit *et al.,* 2006; Johnsen *et al.,* 2004; Woollett *et al.,* 2003**),** thereby preventing coronary heart disease. A few studies have reported a significant decrease in cholesterol due to the addition of fibre to the daily diet, but the difference may not be as large as originally expected (Brown *et al.,* 1999; Jenkins *et al.,* 1993, 2002; Knopp *et al.,* 1999; Sola *et al.,* 2007). However,

the mechanism of the reduction of cholesterol by fibre is not well defined (Brown *et al.,* 1999; Jenkins *et al.,* 1993, 2002; Sola *et al.,* 2007). In a study by Sola *et al.,* (2007) soluble fibre had a more positive effect on cholesterol than insoluble fibre. Normand *et al.,* (1987) found that the hemicelluloses of rice bran could bind bile acids, preventing the synthesis of cholesterol. Research on rice bran showed intakes of 100 g/day reduced total plasma cholesterol by 7% (Hegsted *et al.,* 1993). A daily intake of 60 g of rice bran reduced low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol to some extent (Truswell, 2002). In addition, the total HDL/LDL-cholesterol ratio increased significantly and triglycerides reduced (Kestin *et al.,* 1990; Shishehbor *et al.,* 2004).

Dietary fibre may be important in reducing the risk of colorectal cancer. Dietary fibre dilutes the faecal carcinogens and procarcinogens, by reducing the transit time of faeces through the bowel and the formation of short chain fatty acids (butyrate), which promote anticarcinogenic action, and the binding of carcinogenic bile acids (Jensen *et al.,* 1982; Lupton, 2004; McKeown-Eyssen & Bright-See, 1984; Reddy & Mori, 1981). Recent studies have shown that dietary fibre intake was inversely associated with the risk of colorectal cancer in age-adjusted analyses (Park *et al.,* 2005).

Adequate intake of dietary fibre prevents the diverticular diseases, which is a condition in which small pouches, called diverticula, develop in the wall of the colon (Marlett *et al.,* 2002). The insoluble fibre has the capacity to significantly decrease the risk of diverticular disease (Aldoori *et al.,* 1998 ; D'Incà *et al.,* 2007). Dietary fibre is important in reducing the risk of developing gallstones and kidney stones (Pixley *et al.,* 1985). Fermentation of dietary fibre by microorganisms in the colon increases the bacterial mass, thus increasing the faecal bulk resulting in a short transit time that prevents constipation (Chen *et al.,* 2006).

2.8 Daily intake of dietary fibre

Normal intake should be in the range of 20-30 g per day (Sola *et al.,* 2007). However, patients should increase or decrease the quantity of daily intake of dietary fibre, depending on their health. For example patients with type 2 diabetics should increase their dietary fibre intake to 20 to 35 g per day (Tabatabai & Li, 2000). However, high fibre diet can cause gas, diarrhoea and bloating in the colon.

2.9 Fermentation of dietary fibre

Most of the health benefits of dietary fibre are related to the fermentation of fibre in the human gut (vanLoo *et al.,* 1999). Studying fermentation in the gastrointestinal (GI) tract is a challenge (Moughan, 1999), so most studies are done under *in-vitro* conditions.

2.10 Why in-vitro fermentation?

Different methods (batch, fed batch and continuous) have been used to measure factors involved in the fermentation of fibre using blood and faeces (Barry *et al*.,1995; Jeraci & Horvath, 1989; Kim & White, 2009), but these have failed to indicate the true situation in the human gut. Some of the methods involve measuring the remaining quantity of fibre from faeces following fermentation. This method does not indicate the fermentation rate and the quantity of SCFA formed. It is difficult and costly to study digestion and fermentation in the human gut directly, therefore, animal models (rats and pigs) are used to study fermentation of fibre (Glitsø *et al.,* 1998; Nyman *et al.,* 1986; VanSoest, 1995). However, there can be differences in the function of the gut among the different species, so *in-vitro* methods are often favored. Many *in-vitro* models have been developed to simulate the conditions in the human gastrointestinal tract.

2.10.1. Limitations and importance of *in-vitro* fermentation

Some research has found differences in the results between *in-vivo* and *in-vitro* methods (Coles *et al.,* 2005). Under *in-vitro* conditions, digestion and absorption of dietary fibre, the effects of anti-nutritional factors, and the effects of interactions between the host, food, and bacteria cannot be evaluated accurately. Accurate *in-vivo* results are important to benchmark and evaluate the *in-vitro* results. *In-vitro* fermentation systems can be run at a lower cost than *in-vivo* experiments and have the advantage that animal experimentation is not required. The consumption of polysaccharides, the production of SCFA and gases can be monitored as a function of time (McBurney & Thompson, 1987). The flexibility in controlling the conditions of *in-vitro* fermentation facilitates the experiments to be carried out under well defined conditions to determine fermetation of dietary fibre rich substances (Lebet *et al.,* 1988a).

2.11 In-vitro fermentation models

Continuous, semi-continuous and batch (static) systems are the main types of *in-vitro* models used to study fermentation (Coles *et al.,* 2005). Different protocols have been studied to examine the consumption of dietary fibre, formation of SCFA, and the production of gases.

Continuous and semi-continuous methods are most appropriate to understand the fermentation of fibre in human gut. In these systems, artificial environments similar to human gut are created (Coles *et al.,* 2005; Minekus *et al.,* 1999). Some of these technically advanced methods needed for continuous and semi-continuous studies are costly, therefore, batch fermentation is favored (Coles *et al.,* 2005).

Most of the batch systems use freshly prepared faecal samples as the inocula to mimic the microbial composition in the human gut. In human faeces, 60% of the dry

mass of faeces is microflora and the balance is the undigested fibre (Stephen & Cummings, 1980). This fact makes faeces as an ideal source of gut flora for research (Guarner & Malagelada, 2003; Stephen & Cummings, 1980). Faeces are often collected for a fixed period after the consumption of dietary fibre (Fernando *et al.,* 2008; Titegemeyer *et al.,* 1991). Several studies were performed without specifying the diet (Barry *et al.,* 1995; Casterline, *et al.,* 1997; Edwards, *et al.,* 1996; Salvador *et al.,* 1993). These studies differed in terms of the time of fermentation (Titgemeyer *et al.,* 1991; Wisker *et al.,* 1998), concentration of substrate (Casterline *et al.,* 1997; Lebet *et al.,* 1998a) and the type of substrate (Goni *et al.,* 2002; Wang *et al.,* 2004). Previous work indicates that most of the research work on human colonic micro flora has evaluated the microbial content of faeces using standard techniques of taxonomic bacteriology, including bacteriological analyses on selective media. Techniques like DNA profiling (Matsui *et al.,* 2010; Shinkai & Kobayashi, 2007; Wanapat & Cherdthong, 2009) are being used to analyse mostly in rumans. Fat and protein analyses have also been included in these studies. The faeces provide a good source of microbes for fermentation rather than trying to obtain reliable samples from the colonic lumen. However, this source of mixed micro flora in the faeces does not show representative microflora in different parts of the colon. Some studies have examined the flora in different areas of the human colon using victims of sudden death and have found that the differences in microflora in different areas of the colon are not significant (Moore *et al.,* 1978; Croucher *et al.,* 1983).

It is difficult to compare results between the different model systems as they are not standardised. Inter laboratory comparisons are difficult because of the variations in methods and variation in faecal cultures from different subjects (Fernando *et al.,* 2008). Different parameters used in each study make comparisons difficult. Most of the

experiments using *in-vitro* fermentation have not been validated with *in-vivo* fermentation and most of the *in-vitro* fermentation methods have used limited types of substrates.

Some studies have looked at the relationship between *in-vitro* and *in-vivo* fermentation of non starch polysaccharides in humans and in animals (Boisen & Fern´andez, 1997; Christensen *et al.,* 1999; Kikuchi & Yajima, 2006; Pettersson *et al.,* 1996; Wisker *et al.,* 1998). Results from these experiments generally show a good correlation between *in-vitro* and *in-vivo* fermentation, as is the case with the work of Wisker *et al.,* (1998) where there was a good agreement between *in-vitro* and *in-vivo* results from the fermentation of non starch polysaccharides in mixed diets in humans. However, some of the studies in pigs found a poor correlation between *in-vitro* and *in-vivo* experiments (Chen, 1997). The *in-vitro* model used in this dissertation (Goering & VanSoest, 1970) was chosen carefully as one used successfully by many researchers. A buffer solution containing different trace elements (phosphate, sodium) and high pH was used to mimic the physiological conditions found in the colon.

2.12 Products from the fermentation of dietary fibre

The anaerobic breakdown of carbohydrate dietary fibre occurs by enzymatic hydrolysis of bacteria growing in the colon (Crittenden *et al.,* 2002; Cummings & Macfarlane, 1991; Fleming & Floch, 1986). Polymeric substrates are hydrolysed into sugars by these enzymes to produce energy and other metabolic products. The major end products are the SCFA (short chain fatty acids), acetate, propionate and butyrate (Cummings, 1981a), the gases H_2, and CO_2, (Allison & Mac-farlane, 1988; Wolin & Miller, 1983), ammonia (Macfarlane *et al.,* 1986), and energy (Cummings, 1981a). This energy is used by bacteria for their growth and to increase the biomass. However, the rate, extent, and the molar ratio of products formed, are dependent upon the nature of

the substrate. Formation of SCFA and overall stoichiometry (Fig 2.7) has been summarized for a hexose by Levitt *et al.,* (1995),

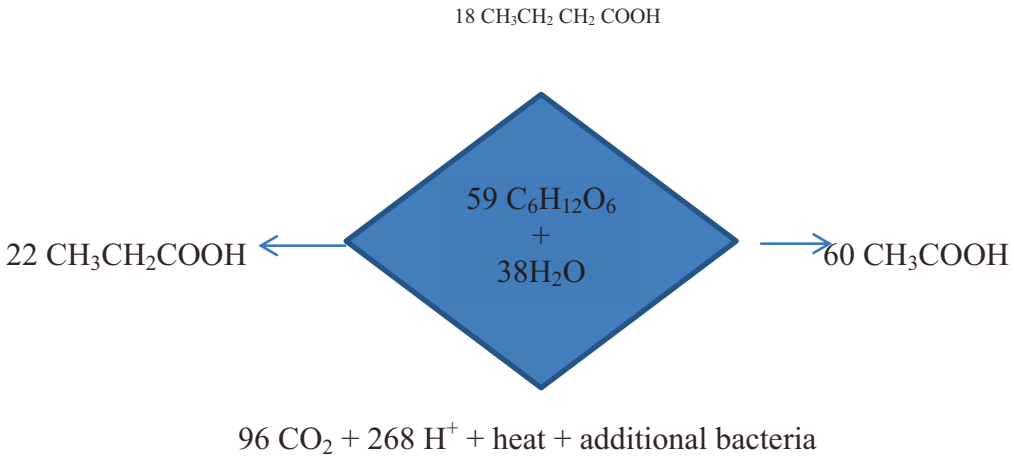


Figure 2.7 Fomation of SCFA and stoichiometry

2.13 Short chain fatty acids and their implications

The molar ratio of acetic, propionic and butyric acids may vary in fermentation depending upon the substrate and the microbial composition. The colonic microflora of humans can change, due to gross nutritional shifts (e.g.weaning), progressive change (such as aging), or differences in the amount of food intake (Mitsuoka, 1996).

Research from various populations shows that faecal SCFA normally exist in the following relative amounts: acetate > propionate > butyrate (Breves & Stuck, 1995; Cummings & MacFarlane, 1991; Hoverstad & Bjorneklett, 1984; Hoverstad, *et al.,* 1984; Moura *et al.,* 2007; Segal *et al.,* 1995). These are the major anions in the colon (Cummings & MacFarlane, 1991) and the SCFA profile may be important in gut development (Norin *et al.,* 2004; Wolin *et al.,* 1998). Short chain fatty acids that form in the gut are absorbed and metabolized in different ways with associated effects on human health. However, less than 5% of bacterially derived SCFA appear in faeces due to the decline in concentrations of SCFA along the large bowel (Peng *et al.,* 2009; Ruppin *et al.,* 1980; Roediger & Moore, 1981).

The SCFA profile may be important in gut development. The colonic mucosa obtains energy by oxidizing SCFAs in the order of butyric> propionic> acetic acid (Clausen & Mortensen, 1994; Henningsson *et al.,* 2002). Acetic acid is transferred into Acetyl-CoA in the liver and used as the precursor for lipogenesis. Acetic acid can stimulate gluconeogenesis (Remesy *et al.,* 1992). Propionic acid has the ability to inhibit the gluconeogenis and hepatic cholesterogenesis (Cameron-Smith *et al.,* 1994; Chen *et al.,* 1984) and increase glycolysis (Andresen *et al.,* 2009; Anderson & Bridges, 1984). Apart from being the main energy source, butyric acid is important in the prevention and treatment of diseases of the colonic mucosa, such as distal ulcerative colitis (Cummings, 1997; Nilsson *et al.,* 2008) and cancer (Clarke *et al.,* 2008; Nkondjock *et al.,* 2003; Scheppach *et al.,* 1995). Some of the effects, such as reducing the pH in the intra-rectal area, may not be due to any particular SCFA (Clarke *et al.,* 2008; Rafter *et al.,* 1986). Lowering of the pH is important to prevent the bacterial conversion of primary to secondary bile acids (Clarke *et al.,* 2008; Nagengast *et al.,* 1988), lowering their carcinogenic potential**.** Secondary bile acids (BA) are associated with adenoma-carcinoma in colorectal cancer (Christl *et al.,* 1997).

2.14 Factors influencing SCFA formation during fermentation

Many factors affect the rate, extent, and type of SCFA produced by the fermentation (Edwards, 1995) of fibre. The three-dimensional arrangement of different components within fibre and the physicochemical properties of dietary fibre (DF) have a major influence on the digestion of different fibres by human faecal bacteria (Cherbut *et al.,* 2003; Guillon *et al.,* 1992). The degree of fermentation and the profile of SCFA depend on the substrate solubility, particle size, lignification, the induction of enzymes, and duration of fermentation (Crater *et al.,* 2007; Edwards, 1995; Salvador *et al.,* 1993).

It has been reported that glucose metabolises faster than xylose (Barry *et al.,* 1989), and glucose-containing soluble polysaccharides ferment faster than xylose-containing polysaccharides (Lebet, *et al.,* 1998a; Monsma, *et al.,* 2000). Non-substituted xylo-oligosaccharides and arabinoxylooligosaccharides ferment more rapidly than the more complex structures of xylo-oligosaccharides (Kabel *et al.,* 2002). Pectin and soybean fibre ferment faster than cellulose and maize bran, and sugar beet fibre ferment at an intermediate rate (Barry *et al.,* 1995; Dongowski *et al.,* 2000; Khan & Edwards, 2002; McBurney & Thompson, 1989). This shows that dietary fibre digestibility differs according to the fibre source.

Previous work found that sugar beet, cocoa and pea, which are the richest in uronic acids and pectins, form predominantly acetic acids following microbial fermentation (Cherbut *et al.,* 1991; Coles *et al.,* 2005; Graham *et al.,* 1989; Khan & Edwards, 2002; McBurney & Thompson, 1987; Stevens & Selvendran, 1988; VanSoest *et al.,* 1983). Xylose and glucose produce little acetic acid (Liu & Yang, 2006; Mortensen *et al.,* 1988). Arabinose, xylose and glucose are important in the production of propionic acid (Barry *et al.,* 1989; Zhao *et al.,* 2010). Studies have found that xylose in wheat bran and sugar beet, and arabinoxylans and β-glucans in wheat bran have a strong ability to produce butyric acid (Cheng, *et al.,* 1987; Mortensen *et al.,* 1988; Högberg & Lindberg, 2004). However, glucose and uronic acid have a lesser ability to form butyric acid (Cheng *et al.,* 1987; Mortensen *et al.,* 1988; Nilsson *et al.,* 2006). These results indicate that the chemical and the physical arrangement of the sugars (such as arabinose, xylose, pectin, β-glucans and uronic acids) in the fibre matrix govern the rate and extent of degradation, together with the nature of the SCFA formation. It is possible to predict which SCFA would be formed during the fermentation of fibre in the human gut if the chemical structure of fibre is known. For

example, arabic and guar gums induce higher levels of propionic and butyric acids more than apple pectin (Phillips *et al.,* 2008; Titgemeyer *et al.,* 1991). Pear, apple, and fig produce low amounts of butyric acid and acetic acid. Propionic and butyric acids are formed by fermentation of dietary fibre fractions from oat and soy (Casterline *et al.,* 1997; Mårtensson *et al.,* 2002).

The chain length of the fibres has an effect on the fermentation rate. For example the fermentation of fructo-oligosaccharides was dependent on the chain length (Roberfroid *et al.,* 1998). Molecules with a degree of polymerization (DP) >10 monomer units were fermented, mostly, only half as fast as molecules with a DP <10 monomer units (Roberfroid *et al.,* 1998; Rossi *et al.,* 2005).

Purity of fibre has an effect on the fermentability and SCFA formation. Soluble DF components (β-glucan and soluble arabinoxylan) in cereals are promptly fermented (Bach Knudsen *et al.,* 1997, Glitsø *et al.,* 1999). Insoluble dietary fibre is fermented in the large intestine, but at a slower rate than soluble polysaccharides. The complex cell-wall structure of insoluble dietary fibre restricts the accessibility of hydrolytic enzymes and bacteria (Glitsø *et al.,* 1999; Lebet *et al.,* 1998a). Bach Knudsen *et al.,* (1993a) and Hindrichsen *et al.,* (2006), studied the digestibility of beta glucan and arabinoxylan in pigs and found degradation of arabinoxylan was slower than beta glucan. Endosperm arabinoxylan (water-extractable) rapidly degraded in the caecum, whereas arabinoxylan of pericarp (more complex) was not degraded in the intestinal tract of pigs (Glitsø *et al.,* 1998, 1999).

The effects of weaning and gender (as well as heredity) on SCFA have yet to be studied in detail. Gender may be a significant factor for NSP and RS (resistant starch) fermentation. Digestibility of dietary fibre in wheat bran was 43% in women and 37% in men when they had 30 g of wheat bran fibre/day (Lampe *et al.,* 1993). Mouth to anus

transit was longer and fecal bulking was lower in women than in men (Lampe *et al.,* 1993).

Studies have found increased SCFA production by human faecal inocula with fibre-rich foods including bran fractions from wheat, oats, barley, corn and rice, soybean fibre, vegetable extracts, pea fibre and isolates of those substrates such as glucose, cellulose, guar gum, pectin, (Barry *et al.,* 1995; McBurney & Thompson, 1989; Mortensen & Nordgaard-Andersen *et al.,* 1993; Queenan *et al.,* 2007; Weaver *et al.,* 1992). Some fibres are fermented slowly and partly while glucose is fermented rapidly and completely. This shows that fermentability is dependent upon the characteristics of the substrate. Another example is that 97% of pectin and only 6-7% of cellulose in maize bran is fermentable (Bourquin *et al.,* 1992). Greater fermentability may be associated with more rapid digestion and higher SCFA formation (Bourquin *et al.,* 1992; Sayar *et al.,* 2007).

Substrates influence the formation of SCFA in animals and in humans. A diet with NSP (non starch polysaccharides), RS (resistant starch), and OS (oligosaccharides) produces high levels of SCFA. In addition fibre-enriched cereal fractions such as wheat bran (Cheng *et al.,* 1987; Folino *et al.,* 1995; McIntosh *et al.,* 1996; Roland *et al.,* 1995), oat bran (Zhang & Lupton, 1994), barley bran (McIntosh *et al.,* 1996), wheat aleurone (Cheng *et al.,* 1987), or rice bran (Folino *et al.,* 1995), have more potential to produce high levels of SCFA, leading to high concentrations of SCFA in the large bowel.

2.15 Concept of prebiotic, probiotic, and symbiotic

Probiotics are defined as the live microorganisms which are a dietary supplement that posses many beneficial effects to the host by their activities in the human gut (Guarner & Schaafsma 1998; Perdigon & Alvarez, 1992). They are able to

survive in the presence of the bile acids in the human gut and able to form colonies in the gastrointestinal tract without harming the host (Perdigon & Alvarez, 1992). Although many microorganisms exist in the human gut, only a few strains of microorganisms have probiotic characteristics (Gibson & Roberfroid, 1995). They are mainly *Lactobacillus* and *Bifidobacterium.*

Food that selectively stimulates the growth of these organisms in the human gut is known as prebiotics (FAO, 2007; Perdigon & Alvarez, 1992). Prebiotics either naturally occur in the food or can be specific supplements. Prebiotics are mainly dietary fibres (Gibson & Roberfroid, 1995). The intake of a prebiotic can increase a specific probiotic or a limited number of beneficial bacteria commensal in the colon (Gibson, 1998; Roberfroid, 1998) and can change the composition of the microorganisms in gut to a healthy flora (Kontula *et al.,* 1998a; Kontula *et al.,* 1998b). Prebiotics can induce luminal or systemic effects that are beneficial to the host's health (Gibson & Roberfroid, 1995). Any food that is a prebiotic should be neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract. A combination of probiotic microorganisms and prebiotic carbohydrates is called a symbiotic food (Gibson & Roberfroid, 1995).

2.16 Colonic food and prebiotics

Foods that are consumed by endogenous colonic bacteria in the colon are known as colonic food. These foods indirectly provide the host with energy, metabolic substrates and essential micronutrients (Gibson, 1998). Non digestible carbohydrates (oligo- and polysaccharides), some peptides, proteins, and certain lipids (both ethers and esters) are not absorbed in the upper part of the gastrointestinal tract or hydrolyzed by the human digestive enzymes. These foods can be considered as candidates for prebiotics (Gibson, 1998). Nondigestible carbohydrates (resistant starch, non-starch

poly saccharides, plant cell wall polysaccharides, hemicellulose, pectins, gums, and nondigestible oligosaccharides) (Delzenne & Roberfroid, 1994) can all be classified as colonic foods. Not all are prebiotics (Table 2.1) as many of these substances are unable to selectively stimulate the growth of beneficial bacteria such as *Lactobacillus* species and Bifidobacteria, or can stimulate growth of potentially harmful and beneficial bacteria (Maczulak *et al.,* 1993: Salyers *et al.,* 1982: Wang & Gibson, 1993).

2.17 Current probiotic position and state of the art

As noted in 2002 by Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are described as live microorganisms that most effective on health of human when consumed in adequate amounts. However, in this statement, "adequate amount" of probiotics or the number of live microbes required has not been defined yet. Metabolically active bacteria are useful in the human gut only if the number of microorganisms per gram of food is in excess of 10^6 at the time of consumption (Gilliland, 1989; Lee & Salminen, 1996).

Probiotics are important in,

- managing lactose intolerance (Sanders, 2000).
- prevention of colon cancer (Saikali *et al.,* 2004).
- lowering cholesterol (Ataie-Jafari *et al.,* 2009).
- lowering blood pressure (Sanders, 2000).
- improving immune function and preventing infections (Reid *et al.,* 2003).
- reducing antibiotic-associated diarrhea (Hickson *et al.,* 2007).
- reducing inflammation (Kirjavainen *et al.,* 2003).

Probiotics,

- should be alive when administered.
- should have a history of health benefits on the target host.

- should be a taxonomically defined microbe or combination of microbes.

- must be safe for its intended use (Canadian Nutrition Congress, 2007; FAO, 2002; FDA 2004; FDA 2007; ILSI, 2004).

However, to have the above features, a candidate probiotic should

- be of human origin (strains are isolated from either the intestinal wall or intestinal contents)
- be non-pathogenic;
- be resistant to gastric acid and bile;
- be adhere to gut epithelial tissue;
- be able to live in the gastrointestinal tract even for a short period (Brassart *et al.,* 1998; Guarner & Schaafsma, 1998; Huis in't Veld & Shortt, 1996; Marteau & Rambaud, 1993; Salminen *et al.,* 1996; Tannock, 1997).

Requirements for candidate probiotics are expanded to include both food components and non-food preparations as,

- each potential probiotic strain should be documented and assessed independently.
- extrapolation of data from closely related strains is not acceptable.
- only well defined strains, products and study populations should be used in experiments.
- all human studies should be randomised,
- results should be approved by independent research groups.
- a study should be published in a peer-reviewed journal (Berg, 1998; Salminen *et al.,* 1996, 1998a).

Probiotic-containing foods are popular in Japan and Europe (Sanders, 1999). Probiotic products are usually available in the market in the form of fermented milks

and yoghurts; (Heenan *et al.,* 2004). Some other probiotic products in the market are listed in Table 2.5. Several studies have reported inadequate populations of viable probiotic bacteria in products during their shelf life (Dave & Shah, 1997; Micanel *et al.,* 1997; Schillinger, 1999; Shah *et al.,* 2000; Shin *et al.,* 2000b). However, probiotic microorganisms incorporated into frozen yoghurt and ice cream products have good viability during the product shelf-life (Hekmat & McMahon, 1992; Laroia & Martin, 1991; Modler *et al.,* 1990).

Table 2.5 Different types of probiotic products

Strain	Commercial products	Source
L. acidophilus NCFM / *B. lactis* HN019 (DR10) / *L. rhamnosus* HN001 (DR20)	Sold as ingredient	Danisco (Madison WI)
Saccharomyces cerevisiae (boulardii)	Florastor	Biocodex (Creswell OR)
B. infantis 35264	Align	Procter & Gamble (Mason OH)
L. fermentum VRI003 (PCC)	Sold as ingredient	Probiomics (Eveleigh, Australia)
L. rhamnosus R0011 / *L. acidophilus* R0052	Sold as ingredient	Institut Rosell (Montreal, Canada)
L. acidophilus LA5 / *L. paracasei* CRL 431	Sold as ingredient	Chr. Hansen (Milwaukee WI)
B. lactis Bb-12	Good Start Natural Cultures infant formula	Nestlé (Glendale, CA) Chr. Hansen (Milwaukee WI)
L. casei Shirota / *B. breve* strain Yakult	Yakult	Yakult (Tokyo, Japan)
L.casei DN-114 001("L. casei Immunitas") *B. animalis* DN173 010 ("Bifidis regularis")	DanActive fermented milk Activia yogurt	DAnonymous (Paris,France) DAnonymous (Tarrytown, NY)
L. reuteri RC-14 / *L. rhamnosus* GR-1	Femdophilus	Chr. Hansens (Milwaukee WI) Urex Biotech (London, Ontario, Canada) Jarrow Formulas (Los Angeles, CA)
L. johnsonii Lj-1 (same as NCC533 and formerly *L. acidophilus* La-1)	LC1	Nestlé (Lausanne, Switzerland)

Table 2.5 Different types of probiotic products (continue)

L. plantarum 299V	Sold as ingredient Good Belly juice product	Probi AB (Lund, Sweden); NextFoods (Boulder, Colorado)
L. rhamnosus 271	Sold as ingredient	Probi AB (Lund, Sweden)
L. reuteri ATCC 55730 ("Protectis")	BioGaia Probiotic chewable tablets or drops	Biogaia (Stockholm, Sweden)
L. rhamnosus GG ("LGG")	Culturelle DAnonymous Danimals	Valio Dairy (Helsinki, Finland) The DAnonymous Company (Tarrytown, NY)
L. rhamnosus LB21 / *Lactococcus lactis* L1A	Sold as ingredient	Essum AB (Umeå, Sweden)
L. salivarius UCC118		University College (Cork, Ireland)
B. longum BB536	Sold as ingredient	Morinaga Milk Industry Co., Ltd. (Zama-City, Japan)
L. acidophilus LB	Sold as ingredient	Lacteol Laboratory (Houdan, France)
L. paracasei F19	Sold as ingredient	Medipharm (Des Moines, Iowa)
Lactobacillus paracasei 33/ *Lactobacillus rhamnosus* GM-020 / *Lactobacillus paracasei* GMNL-33	Sold as Ingredient	GenMont Biotech (Taiwan)
L. plantarum OM	Sold as Ingredient	Bio-Energy Systems, Inc. (Kalispell, MT)
Bacillus coagulans BC30	Sustenex, Digestive Advantage and sold as ingredient	Ganeden Biotech Inc., Cleveland, Ohio
Streptococcus oralis KJ3 / *Streptococcus uberis* KJ2 / *Streptococcus rattus* JH145	ProBiora3 EvoraPlus	Oragenics Inc. (Alachua FL)

Source : http://www.usprobiotics.org/products.asp. (Anonymous, 2010)

National and international authorities ensure that consumers are able to make well-informed decisions about the probiotic food choice. Products that claim to contain

"probiotics" may not contain adequate amounts of efficacious probiotic strains. The quality of probiotic products cannot be determined from just looking at the product. Research on specific probiotic products and their health benefits is required to ensure a particular product is effective.

The most common probiotics are *Lactobacillus* species and *Bifidobacterium* species, but microorganisms including *Enterococcus*, *Streptococcus*, *Escherichia coli,* and *Saccharomyces* species, have some characteristics of probiotics (Sullivan & Nord**,** 2002) (Table 2.6).

Table 2.6 Common probiotics

Lactobacillus **Species**	**Other Lactic Acid Bacteria**	**Non-Lactic Acid Bacteria**	***Bifidobacterium*** **Species**
L. acidophilus	*Enterococcus faecium*	*Bacillus subtilis*	*B. adolescentis*
L. bulgaricus	*Streptococcus thermophilus*	*Escherichia coli strain nisslle*	*B. adolescentis*
L. casei		*Saccharomyces boulardii*	*B. bifidum*
L. rhamnosus GG		*Saccharomyces cerevisiae*	*B. breve*
L. plantarum			*B. infantis*
			B. longum
			B. thermophilus

Sources : Gismondo et al., (1999)*; Holzapfel et al.,*(2001)

2.18 Lactobacillus Species

The *Lactobacillus* genus is Gram positive and varies in morphology from long, slender rods to short coccobacilli, frequently forming chains. These microorganisms produce lactic acid as the major end product during the fermentation of carbohydrates.

Some species are aerotolerant, while others are strictly anaerobic. The growth is optimum at pH 5.5-5.8 and temperatures of 35-45^0 C. The microorganisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Axelsson, 2004). Walter (2008), suggests that only a small number of *Lactobacillus* species are exact inhabitants of the mammalian intestinal tract and that most lactobacilli are allochthonous members. They are derived from fermented food, the oral cavity, or more proximal parts of the GIT.

The metabolic activity of lactic acid bacteria is species and strain specific, and depends upon adequate numbers of bacteria being available in the intestines. Benefits may be only applicable to particular strains. Some of the beneficial effects of *Lactobacillus* are as follows;

- Enhancement of nutritional quality of food and feed.
- Important to stimulate the vitamin synthesis and enzyme production.
- Compete with pathogens for nutrition and for space.
- Production of antimicrobial substances.
- Lower the serum cholesterol.
- Reduce the risk of colon cancer by detoxification of carcinogens (Naidu *et al.,* 1999).

The safety of probiotics has not been thoroughly investigated with particular concerns on safety for young children, elderly people, and people with compromised immune systems. Lactobacilli are generally safe and have been used for many years. However, prolonged use of lactobacilli has resulted in some reported health problems. For example systemic spread of *L. rhamnosus* GG (Rautio *et al.,* 1999) through the ingestion of raw milk or other dairy products (Pellizzer *et al.,* 1996) and dental

extraction because of caries caused by chewing capsules of lyophilized probiotic strains (*L. rhamnosus, L. acidophilus* and *S. faecalis*) (Mackay *et al.,* 1998).

2.19 Bifidobacterium species

Bifidobacteria are natural inhabitants of the human colon (Biavati & Mattarelli, 2001; Collado & Sanz, 2006). Bifidobacteria form the predominant species of the human colon microorganisms (Matsuki *et al.,* 2002) and the *Bifidobacterium* genus forms the third most numerous bacterial populations in the human intestine after the genera *Bacteroides* and *Eubacterium* (Charteris *et al.,* 1997). Bifidobacteria are Gram-positive, pleomorphic (rods and often appear as 'y' shape or bifid shape), non spore forming, non motile and anaerobic**.** These organisms produce lactic acid as the major end product during the fermentation of carbohydrates. Some species are aerotolerant and while others are anaerobic. The growth is optimum at pH 6.5-7 and temperature 20-46 ^{0}C (Arunachalam, 1999).

Research had found that Bifidobacteria appear a few days after birth and become dominant (10^{10}-10^{11}/g stool) in infants (Harmsen *et al.,* 2000) whereas the number of Bifidobacteria decrease significantly in the adult (Ballongue, 1998; He *et al.,* 2001b). Bifidobacteria have positive effects on human health. Some of the health benefits are as follows:

- Suppress harmful bacteria by producing strong acids (acetic acid, lactic acid) as end products of fermentation, thus control the pH of the large intestine (Gibson & Roberfroid, 1995).
- Promote immunological response against malignant cells (anticancerogenic) by acting as immunomodulators (Reddy & Rivenson, 1993; Mitsuoka, 1992).
- Reduce serum cholesterol (Pereira & Gibson, 2002).

- Mitigate lactose intolerance and vitamin B production (Fooks *et al.,* 1999; Gibson & Roberfroid, 1995).

2.20 Concept of symbiotic

To exploit the probiotic and prebiotic effects, the two can be combined as a symbiotic which is more active than either a probiotic or prebiotic alone. Synergy is another term for positive symbiotic activity (Saulnier *et al.,* 2007a*,* 2008). The obvious potential synergy between probiotic and prebiotic has been used to develop food products that include probiotics and prebiotics. However, symbiotic products should contain the prebiotic compound that selectively favors the probiotic (Schrezenmeir & DeVrese, 2001). For example, a product containing oligofructose and the probiotic *Bifidobacterium* would be a good symbiotic product, whereas a product that has oligofructose and the probiotic *Lactobacillus casei* was not, since oligofructose favours bifidobacteria rather than lactobacilli (Schrezenmeir & DeVrese, 2001). Another possible synergism could be obtained by the ingestion of lactobacilli on the one hand and the promotion of indigenous bifidobacterea on the other (Schrezenmeir & De Vrese, 2001).

Symbiotics can enhance the survival activity of the probiotic in the gastrointestinal tract (GIT), while promoting the growth of indigenous beneficial bacteria (Gibson & Roberfroid, 1995). Health effects of symbiotics have been observed (Perrin *et al.,* 2000; Schrezenmeir & DeVrese, 2001). The symbiotic activity of *Bifidobacterium* and *Lactobacillus* species with dietary fibre such as short chain fructo-oligosaccharides (Barrangou *et al.,* 2006; Goh *et al.,* 2006; Kaplan & Hutkins, 2003; Saulnier, *et al.,* 2007b, 2008) and with isomalto-oligosaccharide (Schell *et al.,* 2002) are believed to have health benefits. The combination of probiotics and dietary fibre may be a method of improving the stabilization of the probiotic activity.

2.21 Strategy for acetate, propionate and butyrate formation by Bifidobacteria and Lactobacillus species

The glycolytic pathway (Fig 2.7) rather than the Entner-Doudoroff pathway is used by most microorganisms of the human gut to metabolise carbohydrate (Macfarlane & Gibson, 1996). In the glycolytic pathway, carbohydrates are initially converted to pyruvate and acetyl-CoA.

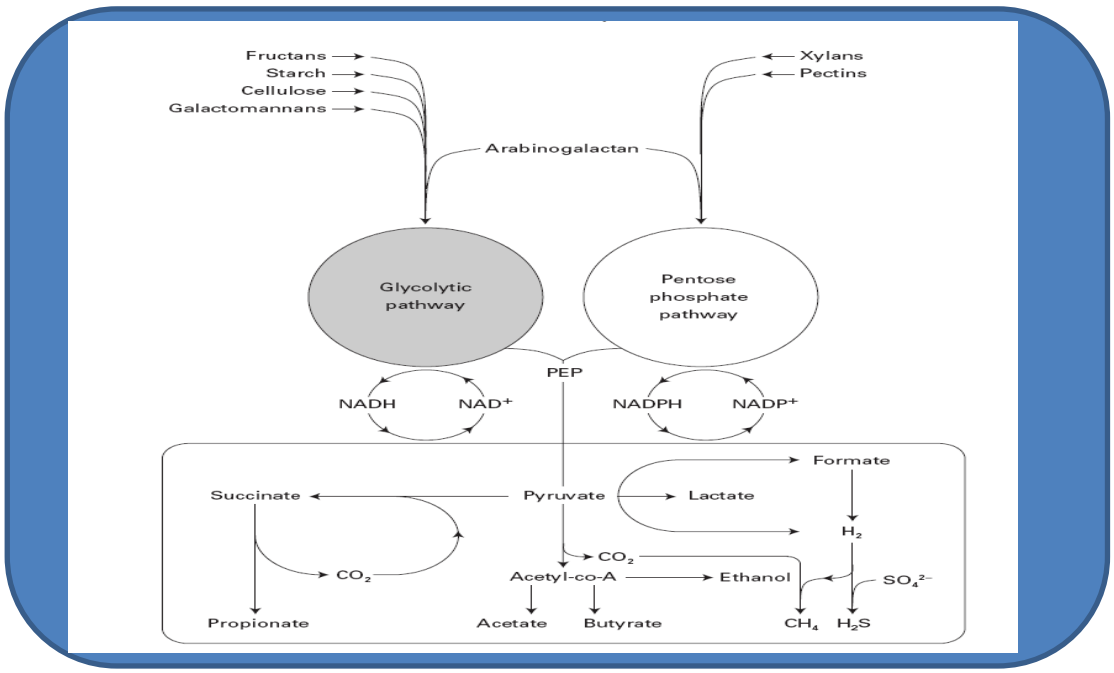


Figure 2.8 Glycolitic pathway

Source: Macfarlane and Gibson, (1996)

As in Fig 2.7, these metabolites are converted into a wide range of products including acetate, propionate, and butyrate (Macfarlane & Gibson, 1996).

Lactic acid bacterea (LAB) ferment sugars, especially hexoses and pentoses (glucose, fructose, mannose, and lactose) derived from dietary fibre by a heterofermentative pathway, known as the phosphoketolase (or oxidative pentose–phosphate) pathway, to form acetate (Fig 2.8). Homofermentative LAB use glycolysis through lactate dehydrogenase to ferment sugers to lactic acid. Homofermentative bacteria have the ability to shift to mixed acid fermentation with formate, acetate, ethanol, and lactate as the end products. This is regulated by lactate dehydrogenase and

pyruvate formate lyase enzymes. These enzymes activate as a result of catabolic and anabolic flux rates and changes in the NADH/NAD ratios (Garrigues *et al.,* 1997, 2001; Melchiorsen *et al.,* 2002). The pathway of formation of butyrate and propionate by lactic acid bacteria is still not clear (Stewart *et al.,* 2009).

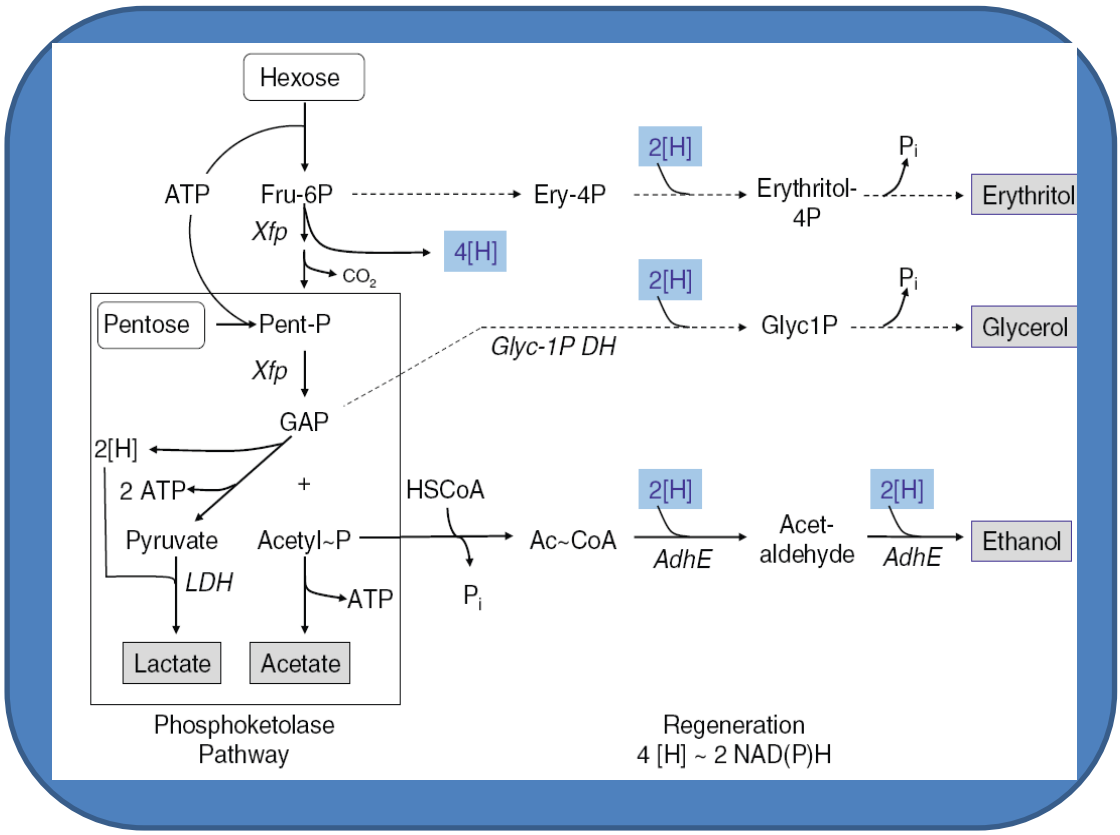


Figure 2.9 Fermentation of pentoses and hexoses by the phosphoketolase (oxidative pentose-P) pathway,

Source : Zaunmüller et al.,(2006)

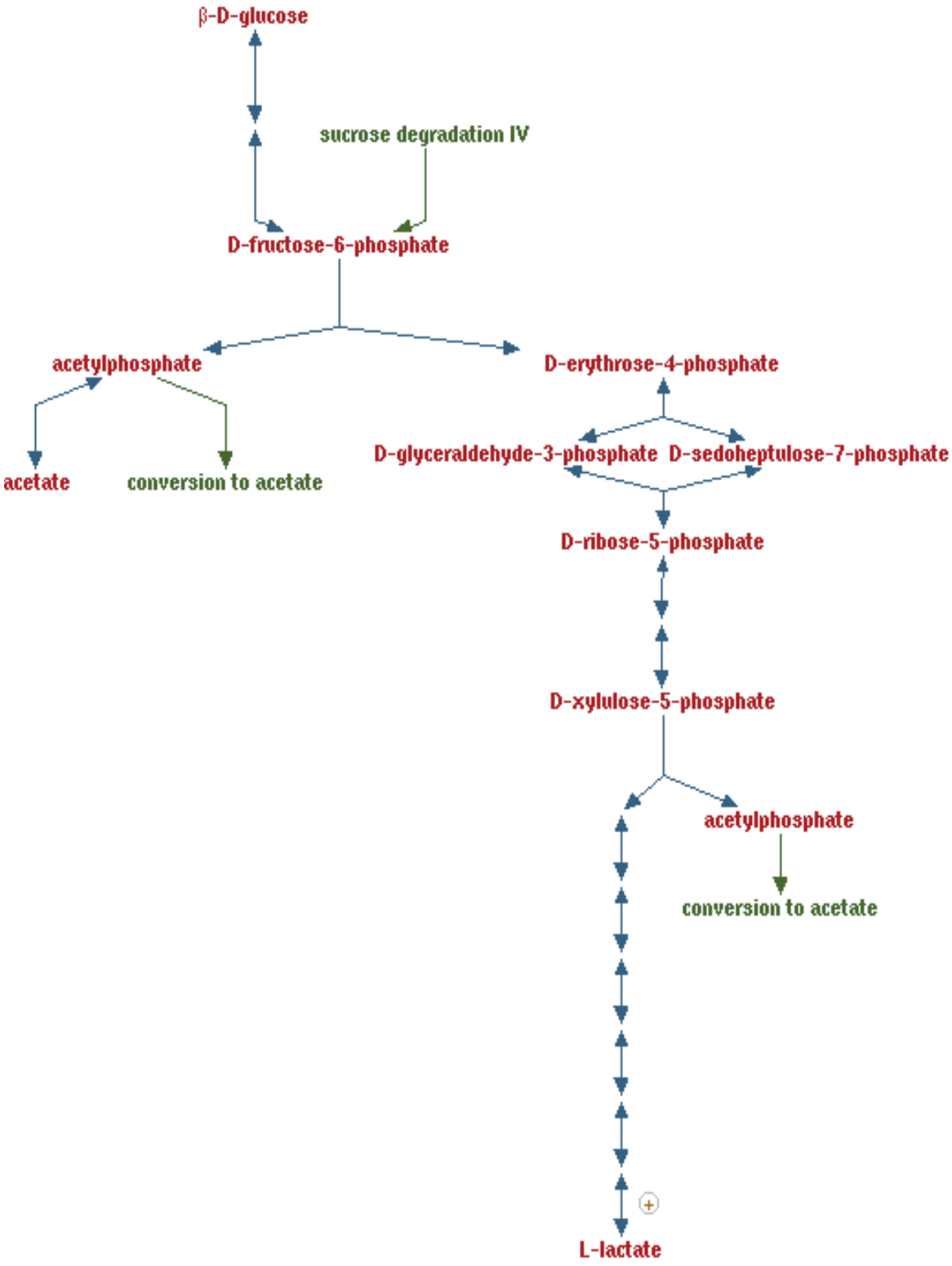


Figure 2.10 Bifidus pathway

Source: Scardovi *et al.,*(1971)

Bifidobacteria lack aldolase and glucose-6-phosphate NADP+ oxidoreductase (De Vries *et al.,* 1967), which is important for the usual glycolysis pathway or the hexose monophosphate shunt pathway. Therefore, to ferment carbohydrate, bifodbacteria use the special metabolic pathway called the bifidus pathway (Fig 2.9). In this pathway, Bifidobacteria use the enzyme fructose-6-phosphate phosphoketolase (F6PPK) (Fandi *et al.,* 2001) to form acetate and lactate in 3:2 ratios (Biavati *et al.,* 1992). However, formation of butyrate and propionate from Bifidobacteria is still not clear. Butyrate and propionate formation from frutooligosaccharides has been observed in the presence of bifidobacteria and lactobacilli (Nilsson *et al.,* 2006).

2.22 Microbial adhesion

Adhesion is a process where microorganisms adhere strongly to a surface by physicochemical interactions (Dankert *et al.,* 1986; Marshall, 1985; Oliveira *et al.,* 2003), including an initial phase of reversible physical contact followed by irreversible chemical and cellular interactions (Gristina, 1988; Marshall *et al.,*1971). Bacterial cell surface hydrophobicity (Busscher *et al.,* 1990; Oliveira *et al.,* 2001) and charge (vanLoosdrecht *et al.,* 1990) as well as the hydrophobicity, charge, roughness, and chemical composition of the biomaterial surface itself are important basic factors (An & Friedman, 1998) in microbial adhesion. In addition specific materials such as surface macromolecules, commonly lectins or lectinlike proteins or glycoproteins are important for adhesion and they are called adhesins (Tojo *et al.,* 1988; Timmerman *et al.,* 1991). According to Peumans and vanDamme (1995), adhesins carry at least one binding site which is particularly important for carbohydrate adhesion. Recent research had found that Lactobacilli produced more limited spectrum of lectins or lectin-like compounds with higher affinities to polysaccharides than Bifidobacteria having a more extended

polysaccharide-binding spectrum (Anokhina *et al.,* 2006; Kravtsov *et al.,* 2008; Lakhtin *et al.,* 2006).

The mechanisms of bacterial adhesion are very complex (Katsikogianni & Missirlis, 2004). Studies on the digestion of plant cell walls by cellulytic bacteria in the rumen suggest that adhesion is important for cellulose digestion and the breakdown of the fibre (Eryavuz & Dehority, 2009; Windham & Akin, 1984). However, there is some debate whether adhesion to a substrate is an essential prerequisite for digestion (Anderson & Salyers, 1989; Crittenden *et al.,* 2001; Morris & Cole, 1987).

Adhesion of bacteria to dietary fibre can occur in several steps, however, there is still much to learn about the process of fibre adhesion by probitics in the human digestive system. Adhesion of rumen cellulitic bacteria to fibre occurs in several steps (Miron *et al.,* 2001):

- Initial non-specific adhesion,
- specific adhesion,
- colonisation and the proliferation,

This is similar to the general process of adhesion to a variety of surfaces.

2.23 Conclusion

Although dietary fibre from many foods has been isolated and studied, there are still many sources of dietary fibre that have not. Most of the studies have involved cereals, especially wheat and maize. The physiological effect of fibre on digestion depends on the origin of the fibre.

Dietary substrates (fibre) that escape digestion by host enzymes in the human gut play an important role in bacterial fermentation which occurs later in the digestive system. SCFA, which is the result of the fermentation, varies depending upon the

dietary substrate, the transit time of the substrate through the colon and the type and number of microflora. Dietary fibre that promotes the formation of butyrate, propionate and acetate, is believed to lead to improved gut health. The genera *Bifidobacterium* and *Lactobacillus* are important in converting dietary fibre to SCFA. The rate and the extent of formation of SCFA depend upon the ability of bacteria to digest the substrate. Therefore, information on the microbiology of fibre metabolism is important in understanding gut health.

Metabolism of fibre by microorganisms in the human gut is still not totally understood. Factors that are believed to be important include the activity of microbial enzymes and bacterial adhesion to fibre. However, not all microbial species and fibre types are likely to be important in the microbial metabolism in the human gut have yet been studied.

CHAPTER 3

General Materials and methods

This Chapter explains the most common methods used in the following Chapters.

3.1 Sample collection

Four rice varieties were selected for the study based on their availability and their popularity in Sri Lanka: LD356 and AT353 (red pericarp), and BG352 and BG358 (white pericarp). Rice varieties were sourced from Labuduwa (LD356), Ambalantota (AT353) and Batalagoda (BG 352,358). In this thesis, LD 356 and AT353 are referred to as RR1 and RR2, and BG352 and BG358 are referred to as WR1 and WR2.

3.2 Sample preparation

The four rice varieties were ground using a coffee grinder and sieved with to a particle size of 300-400 μm (Asp, 1993).

3.3 Determination and extraction of soluble, insoluble and total dietary fibre

Rice samples were analyzed for soluble, insoluble and total dietary fibre according to AOAC method 991.43 (1995) using total dietary fibre assay kit from Megazyme International (Wicklow, Irland). This method consists of four main steps.

- Digesting the original sample with enzymes.
- Determination of insoluble dietary fibre.
- Determination of soluble dietary fibre.
- Determination of total dietary fibre.

3.3.1 Digesting the original sample with enzymes

Rice grains were powdered to 300-400 μm (Asp, 1993). With each assay, two blanks containing no rice were used along with the test samples to measure any contribution of the reagents to residue during enzymatic digestion. Samples were weighed in duplicate 1.000±0.005 g (Sartorius GD503 analatical balance) into 400 mL tall-form beakers. Forty millilitres (40 mL) of MES-TRIS (described below) buffer solution (pH 8.2) was added to each beaker with a magnetic stirring bar. The solution was stirred with magnetic stirrer until there were no lumps (lumps would make the sample inaccessible by enzymes). Fifty microliters (50 μL) of heat-stable α-amylase solution (E-BLAAM, 3,000 Ceralpha Units/mL) was added to the samples while stirring at low speed. Each beaker was covered with aluminium foil squares and placed in a shaking water bath at 95-100°C, and incubated for 35 min with continuous agitation. All sample beakers were removed from the water bath and cooled to 60 °C. The individual temperature of the samples was measured using a thermometer. The foil covers were removed and any deposits on the side of the beaker were scraped into the solution using a spatula. Any gels forming in the bottom of beaker were mixed into the solution with a spatula. The side walls of the beakers were rinsed with 10 mL distilled water using a pipette. Then 100 μL of protease solution (E-BSPRT, 50 mg/mL, 350 Tyrosine Units/mL) was added to each sample and covered with aluminium foil. Samples were incubated in a shaking water bath at 60±1 °C, with continuous agitation for 30 min. After incubation for 30 min, all sample beakers were removed from the water bath. Five millilitres (5 mL) of 0.561 M HCl solution was dispensed into the sample while stirring. The pH was measured and adjusted to 4.1-4.8 with 5 % NaOH solution or 5 % HCl solution.

Amyloglucosidase solution (200 μL) (E-AMGDF, 200 ρ-NP β-maltoside Units /mL) was added to the sample while stirring on a magnetic stirrer. After covering with aluminium cover, beakers were incubated in a shaking water bath at 60 °C for 30 min, with constant agitation.

3.3.2 Determination of insoluble dietary fibre

A wet sintered glass crucible containing celite (Megazyme G-CELL100) was weighed to the nearest 0.1 mg and redistributed as a bed of celite in a crucible using approximately 3 mL distilled water. Suction was applied to the crucible to draw celite onto fritted glass as an even mat. The enzyme mixture was filtered through a crucible into a filtration flask. The residue on the filter was washed twice with 10 mL distilled water preheated to 70 °C. A beaker was rinsed with hot water before washing the residue in the crucible. The filtrate was recoverd and filtrate was transferred into a separate beaker for the determination of SDF. The residue was then washed twice with 10 mL of:

- 95 % EtOH
- Acetone (reagent grade)

The crucibles containing the residues were dried overnight in an oven at 103 °C. Crucibles were cooled in desiccators for approximately 1 h. The crucible containing dietary fibre residues and celite were weighed.

Residue weight was calculated as follows

Residue weight (g) = weight (obtained) - weight of dried crucible and celite.

3.3.2.1. Protein and ash content of the sample

The protein content of the residue was analysed using the Kjeldahl method. A conversion factor is required to convert the nitrogen concentration to a protein concentration. Generally 6.25 (equivalent to 0.16 g nitrogen per gram of protein) is used

as the factor for many products; the conversion factor is dependant on its amino-acid composition (Anonymous, 2003b), however, the AOAC 984.13 method uses 5.7 for wheat grains. For ash analysis, the second residue was incinerated for 5 h at 525 °C. The container of ash was cooled in desiccators and weighed to the nearest 0.1 mg.

Calculation of the ash weight

Ash (g) = weight (obtained) - weight of crucible and Celite

3.3.3 Determination of soluble dietary fibre

The volume of the filtrate used to analyse soluble dietary fibre was measured. Ninty five percent (95 %) EtOH, preheated to 60 °C was added to the filtrate of each beaker. The volume of EtOH added to beakers was four times the volume of the filtarate. The solution was allowed to precipitate at room temperature for 60 min. The bed of celite was evenly distributed in the crucible, using 15 mL of 78 % EtOH. Suction was applied to the crucible to draw celite onto fritted glass as an even mat. Precipitated enzyme digest was filtered through the crucible, using 78 % EtOH and a rubber spatula, and all the remaining particles were transferred to the crucible. The residue was washed successively with two portions of 15 mL reagents of the following;

- 78 % EtOH
- 95 % EtOH
- Acetone (reagent grade)

The crucible containing residue was dried in an oven, overnight at 103 °C. Protein and ash analyses were determined as described in section 3.3.2

3.3.4 Determination of total dietary fibre

For the analysis of total dietary fibre, 225 mL of 95 % EtOH preheated to 60 °C was added to the enzyme digest in section 3.3.1. The volume of EtOH was measured after

heating. The ratio of volume of EtOH to sample was 4:1. All the samples were covered with large sheets of aluminium foil and allowed to precipitate at room temperature for 60 min. A wet crucible containing celite was weigh to the nearest 0.1 mg and redistributed in the crucible, using 15 mL of 78 % EtOH. Suction was applied to the crucible to draw celite onto fritted glass as an even mat. The precipitated enzyme digest was filtered through the crucible, by using a wash bottle with 78 % EtOH and a rubber spatula, and transferring all remaining particles to the crucible. The residue was washed successively with two portions of 15 mL of the following reagents;

- 78 % EtOH
- 95 % EtOH
- Acetone (reagent grade)

The crucible containing the residue was dried overnight in an oven at 103 °C. Protein and ash analyses were determined as described in section 3.3.2

MES/TRIS buffer preparation

2(N-morpholino) ethanesulfinic acid (19.52g) (MES) (Sigma, M 8250) and 14.2 g tris (hydroxymethyl) aminomethane (TRIS) (14.2 g) (Sigma, T1503) were dissolved in 1.7 L deionised water. The pH was adjusted to 8.3 (20 ºC) with 6.0 M NaOH.

Preparation of 0.561M hydrochloric acid solution

Ninety three point five millilitres (93.5 mL) of 6N HCLwas added to 700 mL distilled water and diluted up to 1000 mL.

Preparation of 78 % ethanol

Two hundred and seven millilitres (207 mL) of water was added to the 1-L volumetric flask and mixed with 95 % ethanol upto 1 L.

3.3.5 Calculation of percentage of dietary fibre in the samples

R1 = residue weight 1 from m1; R2 = residue weight 2 from m2;

M1= sample weight 1; M2 = sample weight 2;

A = ash weight from R1; P = protein weight from R2; and

B = blank.

Where:

BR = blank residue; BP = blank protein from BR1;

BA = blank ash from BR2;

Dietary fibre (%) = {((R1+R2)/2)-P-A-B}/ (M1+M2)/2*100;

Blank (B) = ((BR1+BR2)/2)* BP-BA.

3.3.5.1 Filtration

Filtration of dietary fibre fractions (residues) were conducted according to the AOAC method 991.43 that used celite to determine the percentage of fibre fractions present in the rice. However, filtration was modified by using filter paper 541 (Whatman International LTD, Maidstone, UK) twice, instead of celite as the substrate (Titgemeyer *et al.,* 1991). This step was carried out to purify the fibre fractions for the fermentation, since celite proved difficult to remove from the isolated fibre fractions.

3.3.6 Nitrogen Determination by the Kjeldahl method

(AOAC 984.13)

In this study block digester method was used and this method have three steps

- Digestion
- Distillation
- Titration

3.3.6.1 Digestion

- 1 g of sample was weighed into each digestion tube accurately. The following were added to the flasks – 2 Kjeldhal tablets (each containing 3.5 g K_2SO_4 and

0.0035 g Se-selenium), 15 mL concentrated H_2SO_4 (95-98 %). A blank was prepared by adding all the chemicals but without a sample. Tubes were digested in the block digest unit at 420 °C for 60-90 min.

- After cooling the tubes inside the fume hood with the airflow on for 10-15 min, 70 mL hot distilled water was added carefully by shaking gently to mix.

3.3.6.2 Distillation

- Twenty five milliliters (25 mL) of 4% boric acid was added to A titration flask (250 mL). Distillation was done using the distillation unit which added NaOH automatically to the sample tube (45% w/w, NaOH).

3.3.6.3 Titration

- Titration was done using 0.1 M HCL.

3.3.6.4 Calculation of crude protein in the sample

% of Nitrogen= ((A*B)*14*100)/ 1000*C

A= mLs HCL used, B=exact molarity HCL, C=weight (g) of original sample

3.4. Determination of purity of extracted fibre fractions

3.4.1 Determination of the presence of starch in extracted fibre

A few drops of Iodine-KI reagent were added to fibre samples and a colour reaction observed (a blue-black colour results if starch is present).

3.4.2 Determination of moisture content of extracted fibre

The two aluminum moisture content dishes fitted with lids were weighed accurately. Two grams of the fibre (IDF, TDF, and SDF) were placed in each dish and the dish was quickly reweighed. Each dish was placed in the oven, with the lid under the dish; at 130 ºC for one h. Dishes were covered with the lid before being removed from the oven and being placed in desiccators. Dishes were weighed accurately once

they had been cooled in the desiccator. Dishes were placed in the desiccators again for another 15-30 minutes before reweighing (if differences between weighing and reweighing are more than ± 1 mg, process has to be repeated).

3.4.2.1 Calculation of % Total Moisture (T.M) Content

% T.M= {(W3-W1)/ (W2-W1)}*100

Where, % T.M =% total moisture, W1 = weight in grams of crucible+lid

W2 = weight in grams of crucible +lid +sample (before drying),

W3 = weight in grams of crucible +lid +sample (after drying)

3.4.3 Determination of the presence of fat in the extracted fibre

Extracted fibre (5 mg) was transferred to separate test tubes (SDF, IDF, TDF) containing 5mL of water and boiled for one minute. Three drops of Sudan 111 stain was added to each test tube. The presence of fat was indicated by the development of a red stain.

3.5 In-vitro fermentation of fibre fraction with human inocula

3.5.1 Human subjects

Four human subjects studying at the University of Sri Jayawardenapura, Sri Lanka were selected on a random basis. Their ages were between 20-24 years (three males and one female). The volunteers were free of digestive diseases and had not received antibiotics for at least 6 months.

3.5.2 Diet

Volunteers were given the rice varieties for all three meals on a daily basis for four months. Each meal contained one rice variety as their main diet. Rice varieties were changed in each meal. The volunteers were not allowed to eat other sources of main food such as bread, roti, or cereals, which provide mainly carbohydrates. The volunteers were allowed to have their usual quantity of vegetables, fruits, meat, and

fish. However, the percentage of all the other foods (other than the rice) consumed was < 20% of the diet. This diet aimed to determine whether rice had an effect on the microflora of the human gut.

3.5.3 Preparation of inocula

Fresh faeces were collected from the four healthy human volunteers who had previously been assigned diets as described in section 3.5.2. Freshly passed faeces were immediately placed into an anaerobic chamber and mixed for one minute to prepare a homogeneous solution and to detach the cellulolytic microorganisms which might be associated with the faecal fibre matrix, using a 1:6 (w/v) phosphate buffered saline solution (Goering & VanSoest, 1970). This suspension was squeezed through a cheese cloth, and filtered through glass wool to remove faecal particles before transferring to a warm beaker (McBuney & Thomson, 1990) for immediate use as the fermentation inocula. The inoculm was prepared daily depending on the number of fermentations.

3.5.4 Analysis of faecal samples

To determine the effect of rice on the gut microflora, faecal samples from the volunteers were subjected to bacteriological analyses both before the consumption of rice varieties and four months after. Bacterological analysis was carried out within one h of defecation. Five grams of fresh faeces were homogenized under anaerobic conditions in 50 mL of 0.1 m/L sodium phosphate buffer (pH-7) to provide 10 % (wt/vol) faecal slurry (Gibson *et al.,* 1995). The slurry was centrifuged (to extract the bacteria) at 200*g for 10 min (Apajalahti *et al.,* 2003) and 1 g of the pellet was used to prepare a tenfold dilution series using anaerobic broth (Wilkins-Chalgren broth, Oxoid) in an anaerobic cabinet (10 % H2; 10 % CO2; 80 % N2) (Gibson *et al.,* 1995). Three sets of agar plates were inoculated using the appropriate media, for the enumeration of total anaerobes (under anaerobic conditions at 37 ºC for 3-5 days), total

aerobes (under aerobic conditions at 37 ºC for 24 h), *Bifidobacterium* (under anaerobic conditions 37 ºC for 3 days) and *Lactobacillus* (under anaerobic conditions 37 ºC for 3 days) following the procedures of Vinderola and Reinheimer, (1999). The media used were Wilkins-Chalgren agar (Oxoid), nutrient agar (Oxoid), Beerens agar (Oxoid) and Rogosa agar (Oxoid) for anaerobes, aerobes Bifidobacteria and Lactobacilli respectively. Media used in this study were chosen from previous work related to human faecal samples. (***Nutrient agar***- Greetham *et al.,* 2002, ***Wilkins-Chalgren agar***- Greetham *et al.,* 2002, ***Beerens agar***- Beerens, 1991; Silvi *et al.,* 1996; Hartemink & Rombouts, 1999; Greetham *et al.,* 2002; Apajalahti *et al.,* 2003; Awaisheh *et al.,* 2005; ***Rogosa agar*** - McCartney *et al.,* 1996; Walter *et al.,* 2001; Greetham *et al.,* 2002; Apajalahti *et al.,* 2003). The preparation of buffers and media are described in Appendix A. Enumeration of dead bacterial cells due to environmental stress and the depletion of nutrients was not done. The dead bacterial count in the faecal samples was assumed to be similer before and after the consumption of rice since the environmental stress was similar.

3.5.5 *In-vitro* fermentation with faecal inocula

Fermentations were conducted in sterile 100 mL bottles. Each bottle contained culture medium, substrate, faecal inoculum and a reducing solution (reducing solution contained (per litre): 6.25 g cysteine –HCl, 1.6 g NaOH, and 6.25 g $Na_2S.9H_2O$). The culture medium contained (per litre): 2.5 g trypticase peptone, 1.0 g NH_4HCO_3, 8.75 g $NaHCO_3$, 1.43 g Na_2HPO_4 anhydrous, 1.55 g KH_2PO_4 anhydrous, 0.15 g $MgSO_4$.$7H_2O$, 1.25 mg resazurin, 16.5 mg $CaCl_2$.$2H_2O$, 12.5 mg $MnCl_2.4H_2O$, 1.25 mg $CoCl_2$.$4H_2O$, and 10.0 mg $FeCl_3$.$6H_2O$. Culture medium (32 mL) and 400 mg substrate (extracted TDF, SDF or IDF from each of the separate rice varieties) was added to each bottle and sealed for 24 h for complete hydration of fibre before adding

the inoculum. One to two (1–2) h before inoculation, the bottles were placed in a 37 ºC shaking water bath to mix and bring the medium to the incubation temperature. Reducing solution (1.6 mL) was added and then the flasks were sealed with rubber stoppers. The faecal inoculum (8 mL) was added to each bottle and fermentation was conducted under strict anaerobic conditions (Goering & VanSoest, 1970) at 37 ºC. Strict anaerobic conditions were maintained using anaerobic jars (Anaerobic plus system, Oxoid, Dioxo, Prague) with palladium catalysts (Oxoid) and filled with CO_2/H_2 (10/90 %) by gas packs (Merck, Darmstad, Germany). One mL was used to prepare a 10-fold dilution series to analyse viable cell counts, a volume of 1.5 mL was taken to measure the optical density and 2-3 mL was taken for pH measurement. Aliquots of 2 mL were removed at 0, 2, 4, 6 and 24 h incubation, and the microbial growth was stopped by adding 1 mL of 10 g/L copper sulphate in preparation for SCFA analysis. Gas packs were replaced with new packs after each aliquot was removed. The samples were kept at -20 °C for further processing. Fermentation occurred in duplicates using a separate inoculum from the same subject taken on the same day. Two independent experiments were carried out for fermentation.

3.6 In-vitro fermentation of dietary fibre with probiotic cultures

3.6.1 Bactereial culture media

Microbiological media - de Mann Rogosa and Sharpe (MRS) (Sigma USA) and Reinforced Clostridial Medium (Sigma, USA) were used for the cultivation of pure cultures of *Lactobacillus* and *Bifidobacterium* respectively.

3.6.2 Bacterial strains

Bacterial strains *Lactobacillus rhamnosus* (ATCC 7469), *Lactobacillus acidophilus* (ATCC11975), *Bifidobacterium breve* (ATCC15700), *Bifidobacterium longum* (ATCC15707), were obtained from the culture collection at the Institute of

Environmental Science and Research Limited, New Zealand. These strains have been reported as probiotics in previous work (Matsumoto *et al.,* 2007; Kotikalapudi, 2009; Kwon *et al.,* 2004; Selcuk *et al.,* 2010).

3.6.3 Co-cultures

Pure cultures of bacterial strains were combined in a 1:1 (v/v) ratio to prepare nine combinations in equal proportions. The concentration of each micro-organism from a 24 h old culture was 10^7 CFU/mL, (OD 2.4 - 2.5, at 540 nm) at the time of mixing. The combinations were LA+LR, BB+BL, BB+LA, BB+LR, BL+LA, BL+LR, BB+BL+LA, BB+BL+LR, and BB+BL+LA+LR (these codes are explained in the abrevation list).

3.6.4 Preparation of cell suspensions

Freeze dried cultures were rehydrated by sub culturing *Lactobacillus* species in MRS medium and *Bifidobacterium* species in reinforced clostridial medium, under strict anaerobic conditions. Anaerobic jars were used to obtain anaerobic conditions. *Lactobacillus* species were incubated at 37 ºC for 24 h and *Bifidobacterium* species were incubated at 37 ºC for 72 h. Prior to the *in-vitro* fermentation, the bacteria were pre cultured twice in 10 mL of the appropriate medium containing 10 gL^{-1} glucose as the carbon source. After incubation, the bacteria cells were harvested, washed twice with physiological saline (0.85 % NaCl solution), and resuspended in the basal medium (PYF solution) to remove excess carbon. The suspension was then diluted to 1:10 with the basal medium (Jaskari *et al.,* 1998b; Kontula *et al.,* 1998a).

3.6.5 Preparation of growth medium

The basal medium, (Peptone/Yeast extract/Fildes (PYF) solution), was used as the carbohydrate-free medium. PYF medium consists of 10 g Trypticase Peptone, 5 g yeast extract, 0.5 g L-cysteine hydrochloride, 40 mL digested horse blood (Apendix A),

and 40 mL salt solution per 1 L. Salt solution contains 0.2 g $CaCl_2$, 0.2 g $MgSO_4 \cdot 7H_20$, 1.0 g $KH_2 PO_4$, 1.0 g K_2HPO_4 , 10 g $NaHCO_3$, and 2.0 g NaCl in 1 L deionized water (pH7.6) (Yoshimoto *et al.,* 2005) .

3.6.6 *In-vitro* fermentation with pure cultures or co-cultures

Fermentations were conducted in sterile 100 mL bottles. Each bottle contained culture medium, substrate, and pure cultures. Culture medium (50 mL) and 1% (v/v) substrate (extracted TDF, SDF or IDF from each of the rice varieties separately) were added to each bottle and sealed for 24 h for complete hydration of the fibre. The bottles were maintained at 37 °C for 2 h prior to inoculation (to boost the growth of bacteria when inoculated) and 10 % of the bacterial suspension of pure cultures or co-cultures (10^7 colony forming units [cfu]/mL) was used to inoculate the broth medium (pH 7.6). Fermentation was conducted under strict anaerobic conditions. All the bacteria were grown in duplicate fermentations in the appropriate medium and aliquots (2 mL) were removed at 0, 6, 12, 18, 24 and at 48 h. One mL was used to prepare a 10-fold dilution series to analyse viable count, 1.5 mL was taken to measure the optical density (540 nm) and 2-3 mL was taken for pH measurement.

Aliquots of 2 mL were removed at 0, 6, 12, 18, 24 and 48 h incubation, and the microbial growth was stopped by adding 1 mL of 10 g/L copper sulphate in preparation for SCFA analysis. Gas packs were replaced with new packs after each aliquot was removed. The samples were kept at -20 °C for further processing. Fermentation was carried out in duplicates using separate cultures and two independent experiments were done.

3.7 A study of in-vitro *fermentation with human inocula and pure probiotics cultures*

3.7.1 Determination of pH, optical density and viable cell counts

The pH of the aliquots was determined using a pH probe (Oakton pH 1100 series). Bacterial growth was determined by measuring the optical density of samples (1.5 mL) at 540 nm. Samples were subjected to optical density and viable count measurments immediately following the incubation periods.

The viable counts of the pure cultures were analysed using the pour plate technique, by inoculating 1 mL of ten-fold serial dilutions into PYF medium containing 10 g/L glucose as the carbon source and 1.5 % agar. The plates were incubated anaerobically at 37 ºC for 24 h for *Lactobacillus* species and 72 h for *Bifidobacterium* species (Crittenden *et al.,*2002; Perrin *et al.,* 2001). Viable cell counts of bifidobacteria and lactobacilli of BB+LA, BB+LR, BL+LA, BL+LR were measured using selective media as in 3.6.1. Viable counts of bifidobacteria and lactobacilli in BB+BL, LA+LR, BB+BL+LA, BB+BL+LR, and BB+BL+LA+LR were measured using selective media as in 3.6.1, but as total bifidobacteria and lactobacilli.

3.7.2 Specific growth rate

The specific growth rate was calculated according to the following equation.

$\mu = \ln Nt/N_0 \times 1/t$, N t = Viable cell count at time t,

N_0 = Viable cell count at time 0

μ = specific growth rate (h^{-1}), t = hours

3.7.3 Determination of the relative growth yield

The growth yield of organisms was calculated using the following formula.

Biomass yield on substrate compared with the yield on glucose = [(A-B)/(C-B)]*100%.

A= net change in OD 540 nm of culture grown on substrate X (IDF/SDF/TDF)

B= net change in OD 540 nm of culture grown without added substrate.

C= net change in OD 540 nm of culture grown on glucose. Biomass yield from growth on glucose was considered as 100 % (Crittenden *et al.,* 2002).

3.8 Determination of short chain fatty acids produced from fermentation by pure and mixed cultures

3.8.1 Determination of SCFA (short chain fatty acids)

In order to analyse the SFCA's produced by fermentation, a modified method of Pylkas et al., (2005) was used. This method has been used in previous work related to current study to anlyse the SCFA concentration (Stewart & Slavin, 2009). Samples (2 mL) that had been taken at the specific intervals and stored frozen were thawed and centrifuged for 30 minutes at 5000 x g at room temperature (In original method-3000 x g) . The supernatant (0.75 mL) was transferred to a sterile vial and vortexed with meta-phosphoric acid (20 %, 0.3 mL). Vials were incubated with meta-phosphoric acid at room temperature (25°C) for 30 min, after which samples were centrifuged for 20 min at 20,000 g for 10 min (In original method-5000 x g for 15 min) . The supernatant was analysed for SCFA by GLC. Samples were stored at -20 °C until required. Centrifugation conditions differed from the original method as these centrifugation comditions were found to produce more SCFA than the original centrifugation conditions.

3.8.2 Preparation of samples and standards for Gas Chromatography

Samples were thawed and the pH was adjusted to 6.5 with 4M KOH and 0.01 mL of 0.3M oxalic acid was added (Pylkas *et al.,* 2005).

3.8.2.1 Chemicals and reagents used to prepare standards

Acetic acid (100%) was obtained from Merck (Darmstadt, Germany). Propionic acid (100%)), *n*-butyric acid (99%), were purchased from Sigma (St Louis, MO, USA). The chemicals were used as standards for GLC. 2-Ethylbutyric acid purchased from

Sigma-Aldrich (Chemie GmbH, Steinheim, Germany) was used as the internal standard. The water used in the experiment was purified using a Milli-Q® reagent water system (Millipore, Molsheim, France).

3.8.2.2 Experimental conditions for gas chromatography

Analyses were performed using an Agilent 6890N GC (Hewlett Packard, Palo Alto, CA, USA) with a flame ionization detector (FID). A fused-silica capillary column with a free fatty acid phase (DB-FFAP 125-3237, J&W Scientific, Agilent Technologies Inc., USA) of 30 m × 0.53 mm and with 0.50 μm film thickness was used. Helium was the carrier gas at a flow rate of 14.4 mL/min. The initial oven temperature was 60 °C, maintained for 0.5 min, raised to 180 °C at 8 °C/min and held for 1 min, then increased to 250 °C at 20 °C/min, and finally held at 250 °C for 5 min. Glass wool (Supelco) was placed in the glass liner of the splitless injection port. These glass wool was prepared by immersing in H_3PO_4 (100 g/L) for 1 h and rinsed to remove the excess acid before placing at 100 °C for 1 h. The temperature of the flame ionization detector and the injection port was 200 and 240 °C, respectively. Flow rates of nitrogen, hydrogen and air were 20, 30 and 275 mL/min, respectively. The injected sample volume was 1 μL, and the run time for each analysis was 16.5 min. Data handling was carried out with HP ChemStation Plus software (A.09.xx, Agilent). Unidentofied peaks did not interfere with SCFA peaks and the appearance of ghost peaks was not significant. One μL of water was injected before starting the analysis and after 10 runs to clean the column.

3.8.2.3 Preparation of stock standard solution

An aqueous stock standard solution was prepared for each acid with a concentration of 500 mM for acetic acid and 200mM for propionic acid and n-butyric acid. 2-Ethylbutyric acid solution (4 mM) in methanol (99.9%) was prepared as an

internal standard stock solution. All the stock standard solutions were stored at −20 °C.

3.8.2.4 Preparation of standard mix solution

A standard mix solution of acetic, propionic and butyric acids was prepared in water using stock solutions at 50:10:10, 35:7.5:7.5, 20:10:10, 25:5:5 and 15:2.5:2.5 mM (Appendix D shows the calculations used to prepare the solutions). Five standard mix solutions were used to accurately identify the retention time of the SCFA.

3.8.2.5 Standard curve

Individual calibration curves were obtained for each compound (acetate, propionate and butyrate) using the standard SCFA mixtures. Each standard mixed solution was diluted to have following, concentrations,

Mix solution (mL)	Water (MiliQ, mL)
0	0.75
0.05	0.70
0.1	0.65
0.15	0.60
0.2	0.55
0.25	0.50

The different concentrations of each standard mix solution were treated as described in sample preparation for GLC (3.8.2). Forty micro liters (40 µL, 25mM) of internal standard was added to 960 µL of the prepared standard solutions to a final concentration of 1mM and to samples before injecting into the GLC. The linearity of response for each standard acid was tested five times by using the six concentrations described earlier in millimoles (mM). From the chromatograms, the peak area was plotted (response) against the concentration of each SCFA to find the calibration graph for each

SCFA. Calibration graphs were subjected to Excel regression analysis. Results of the graph were obtained as the slope *a*, intercept *b* and square correlation coefficients r^2 for each acid analysed. Correlation coefficients were approximately 0.9998 for all three acids.

To examine the precision of the method and to calculate the concentration of the SCFAs, one standard mixture of the SCFAs was used after determining the retention time for analysed SCFAs. This was 20:10:10 mM. For experiments, a standard mix solution (20: 10:10 mM) was prepared to plot the calibration graph using the described dilutions and also standards were checked after 10 runs of the GLC each day.

3.8.2.6 Retention time of standards

The acetic, propionic and butyric acid samples were identified on chromatograms by their specific retention times (min) under the above conditions (3.8.2.2) of GLC.

Acetic acid - 1.979± 0.003

Propionic acid - 2.471 ± 0.004

Butyric acid - 3.101 ± 0.003

2-ethyl butyric acid - 4.104 ± 0.003

3.8.2.7 Quantification

Quantification of the SCFA in the samples was done on the basis of peak area and relative response factor (Jouany, 1982; Zhao *et al*., 2006). Quantification was not based on the peak height, since peak height is less reliable. Peaks can be broadened, when the column is contaminated with compounds (non volatile) from previous injections. This broadening will cause less accuracy for peak heights but have no effect on the peak area.

Calculation of relative response factor (RRF)

RRF= Peak area of 1 mM of SCFA / Peak area of 1 mM internal standard

Concentration of SCFA mM in sample

= (Peak area of particular SCFA *Concentration of internal standard)/(RRF of particular SCFA * Peak area of internal standard)

3.9 Determination of indigestible fibre

3.9.1 Determination of the indigestible soluble dietary fibre following fermentation of SDF

The supernatant (5 mL) (used for SCFA analysis) was centrifuged at 24000*g for 15 min to remove cells of bacteria (Titgemeyer *et al.,* 1991). The resulting supernatant was mixed with four volumes of 95 % of ethanol to precipitate the soluble dietary fibre. The precipitate was isolated by filtration with Whatman No. 541 filter paper (Whatman Internationational Ltd, Maidstone, UK). The filter paper and residue were dried at 105 ºC and weighed. After correction for residue from the blank tubes, the residue was considered as the non-fermented soluble fibre. Blank tubes were prepared for each organism/combination without adding the substrate to make the appropriate corrections for the inocula.

3.9.2 Determination of the indigestible insoluble dietary fibre following fermentation of IDF

The pellet from the initial centrifugation of SCFA analysis (30 min at 5000 g at room temperature) was suspended in one mL acid pepsin solution and incubated for 48 h at 37 ºC. After incubation with acid and pepsin the suspension was filtered through Whatman No. 541 filter paper (Whatman Internationational Ltd Maidstone, UK). The filter paper and residue were dried at 105 ºC and weighed. After correction for the residue from the blank tubes, the residue was considered as non-fermented insoluble fibre (Tilly & Terry, 1963).

3.9.3 Preparation of acid pepsin solution

Pepsin (Pepsin a, 1:10000, Sigma Chemical Co) 47.6 mL was dissolved in 3 mL of 1.38 M HCL (11.5 mL of 12 M HCL was dissolved in 88.5 mL distilled water) and the total volume was adjusted up to 5 mL with 1.38 M HCL.

3.9.4 Determination of indigestible total dietary fibre following fermentation of TDF

The indigestible quantities of SDF and IDF were measured using 3.9.1and 3.9.2 from 5 mL of the aliquots of the fermentation broth at each time point. The sum of both SDF and IDF was considered as the indigestible amount of TDF.

3.9.5 Determination of the dry matter disappearance in fermented substrate

{[(Substrate dry matter – (residual dry matter (IDF/SDF/TDF)] - blank weight) / substrate dry matter]}*100

This calculation is a modification of previous formular by Meyer *et al.,*(1971); McBurney & Thompson, (1987). Modification is marked in red colour. In the original this is shown as: **(residual dry matter – 0 h inocula weight).**

3.10 Statistical analysis

Data were analysed as a randonmised complete design. The analyses were performed on duplicates and results were expressed as mean values and standard error of the mean. Duplicates (two independent experiments on different dates and 2 duplicates in each experiment), rather than triplicates were used in the experiments due to cost and time constraints. Other studies have used duplicate data (Crociani *et al.,* 1995; Khatoon & Prakash, 2006; Stewart *et al.,* 2009; Velázquez *et al.,* 2000; Wang *et al.,* 1999). Data were analysed using the statistical analysis package of Microsoft Excel 2003.

Differences between bacterial groups / human subjects and counts /OD /cell yield/ pH /SCFA /digestibility /growth rates / at interval of fermentation for each fibre fraction were tested for significance using following tests and differences were considered significant if $p \leq 0{\cdot}05$.

Compare two paired groups	Paired *t* test for equal veriance
Compare three or more unmatched groups	One-way ANOVA
Compare three or more matched groups	Repeated-measures ANOVA

CHAPTER 4

IDF, SDF and TDF Dietary Fibre Content of Rice

4.1 Abstract

Total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) content of four rice varieties were analysed. The official AOAC method 991.43 was used to analyse IDF, SDF and TDF from Sri Lankan rice varieties LD 356 (RR1), AT 353 (RR2), BG 352 (WR1) and BG 358 (WR2). Total dietary fibre varied significantly among the rice varieties. The rice variety RR1 (LD 356) gave a highest yield of fibre while the variety WR2 (BG 358) gave the least. The relative proportions of IDF in the different rice varieties were the same as for TDF (RR1< WR1<RR2<WR2). However, the relative proportions of SDF were different (RR1<RR2<WR1<WR2).

4.2 Introduction

There are thousands of rice cultivars all over the world. Most of the product is consumed in the form of cooked grain (Khatoon & Prakash, 2006). The method of cooking (boiled, fried, ground, etc.) and the cooking type (conventional rice cooker, oven or microwave) may have an effect on the dietary fibre composition as seen in vegetables (Kahlon & Chow, 2000; Rehman, *et al.,* 2003; Varo *et al.,* 2007).

In the present study, the rice grain was selected before cooking (to avoid variations caused by cooking) to identify the content of IDF, SDF and TDF in rice. Many studies have concentrated on the nutritional quality of rice bran (Carroll, 1990; Hammond, 1994; Kahlon & Chow, 2000; Khatoon & Prakash, 2006; Prakash & Jyothilakshmi, 1995; Sekhon *et al.,* 1997) since it has a higher amount of fibre and

nutritional components than the other layers of the rice. However, the current investigation concentrated on the “whole grain”.

Total dietary fibre is the sum of soluble and insoluble dietary fibre. Insoluble and soluble dietary fibres have different characteristics and these influence the physiological functions of the rice once consumed (Chapter 2). Dietary fibre information contained in this Chapter aims to help consumers improve their diet by selecting fibre-rich rice, and by informing food processors and nutritionists explore rice varieties for local and foreign markets. The aim of this study was to assess the quantity of IDF, SDF and TDF of four rice varieties to serve as a basis for selecting varieties most suitable for the daily diet.

4.3 Materials and Methods

Samples were taken as described in Section 3.1 and 3.2. The TDF, SDF and IDF of three different rice fibres used in this trial were determined using the method detailed in 3.3. Confirmation of purity of extracted fibre is described in 3.4.

4.4 Results

LD 356 (RR1) **AT 353(RR2)**

BG 358 (WR1) **BG352 (WR2)**

Figure 4.1 Physiological appearances of selected rice varieties.

Source: Anonymous (2007), Department of Agriculture Sri Lanka, www.agridept.gov.lk.

(Images show the appearance of rice grain before and after processing)

According to Haard *et al.,* (1999), the milling grades, are categorized as brown rice (dehulled: LD356, BG352), unpolished rice (hull, bran and most of germ removed: AT353) and polished rice (aleurone layer removed from unpolished rice: BG358) in selected rice varieties. The pericarp of the rice grian contains pigments and forms the unique colour for particular rice varieties. Varities RR1 and RR2 have a red pericarp, therefore the grain appears red and WR1 and WR2 have a white pericarp and the grain appears white.

Table 4.1 shows the average quantities of TDF, SDF and IDF in each rice variety. The selected rice varieties had different quantities of fibre..

Table 4.1 Content (%) of dietary fibre in rice varieties as TDF, SDF and IDF

Rice variety	Total Dietary Fibre % (TDF)	Soluble Dietary Fibre % (SDF)	Insoluble Dietary Fibre % (IDF)
LD356 (RR1)	16.73±0.01	3.57±0.01	11.90±0.01
AT353 (RR2)	10.80±0.02	2.07±0.03	8.73±0.02
BG352 (WR1)	11.15±0.01	1.18±0.01	9.97±0.02
BG358 (WR2)	2.01±0.01	0.58±0.01	1.27±0.01

Results are expressed as the mean value of ten trials ± standard error.

Protein, starch, fat and moisture of extracted fibre were analysed to determine the purity (Table 4.2).

Table 4.2 Analysis of purity of extracted fibre

Rice type	Fibre type	Starch (%)	Protein (%)	Ash (%)	Moisture (%)	Fat	Fibre %
RR1	IDF	-	0.01	0.07	-	-	99.92
	SDF	-	0.01	0.03	-	-	99.96
	TDF	-	0.01	0.08	-	-	99.91
RR2	IDF	-	0.01	0.06	-	-	99.93
	SDF	-	0.01	0.05	-	-	99.94
	TDF	-	0.01	0.08	-	-	99.91
WR1	IDF	-	0.01	0.06	-	-	99.93
	SDF	-	0.01	0.05	-	-	99.94
	TDF	-	0.01	0.07	-	-	99.92
WR2	IDF	-	0.01	0.05	-	-	99.94
	SDF	-	0.01	0.03	-	-	99.96
	TDF	-	0.01	0.07	-	-	99.92

Results are expressed as the mean value of three trials. In all cases the standard error was less than 0.001.

4.5 Discussion

The dietary fibre of rice varied between 1-17 g/100g, which is lower than rye, maize or wheat (Juliano, 1985a; Souci *et al.,* 1986). The current investigation used the "whole grain" to separate fibre fractions from rice as SDF, IDF and TDF. Selected rice varieties were different in terms of fibre content (Table 4.1). A key factor in the different compositions is the milling grade of these rice varieties. However, physiological factors and environmental factors such as climate conditions, soil and storage period would also have contributed to the different nutritional compositions, as discussed in the introduction of this Chapter.

The highest amount of total dietary fibre was found in RR1 (LD356, brown rice) and the least in WR2 (BG358, polished rice). Previous studies have shown that

whole grain (contain bran, germ and endosperm) had higher fibre contents compared to white rice (Callegaro & Tirapegui, 1996; Slavin, 2003, 2004). In this study, the fibre content (TDF and IDF) of each rice variety in decreasing order was RR1< WR1 <RR2<WR2 (Table 4.1). The varieties RR1 and WR1 were unrefined where only the hull of the grain is removed. These varieties included the bran and germ layers which contain the highest amount of the dietary fibre in rice (Eggum *et al.,* 1982; Juliano, 1985a). Therefore, RR1 and WR1 had more TDF and IDF than other rice types. However, sample RR1 had significantly higher amounts of dietary fibre in terms of TDF ($p<0.05$), IDF ($p<0.05$), and SDF ($p<0.05$), than WR1. This may be due to the different varieties. Red rice (RR2), which is milled and unpolished to remove the bran and most of the germ, had relatively low levels of total dietary fibre (TDF+SDF+IDF) compared with RR1. However, the level of soluble dietary fibre followed a different pattern: RR1<RR2<WR1<WR2, though the study expected the same pattern as TDF and IDF. This suggests that RR2 may contain significantly more soluble dietary fibre ($p<0.05$) in the germ and the endosperm than the unrefined WR1. WR1 has bran, germ and the endosperm while RR2 is predominantly germ and the endosperm. The results indicated that the percentage of dietary fibre in different layers of rice may be variety specific and the distribution of IDF, SDF and TDF among the layers of rice may be different from one another. Therefore, the milling of rice may have less effect on some rice varities compared with others based on the distribution of different fibre fractions. However, this needs to be confirmed with many varities. Polished rice (WR2) has a lower content of dietary fibre than the rest of the rice varieties. This is due to the absence of layers up to the endosperm of WR2. TDF, SDF and IDF in the WR2 rice was significantly ($p<0.05$) lower than in RR1, RR2 and WR1.

Previous studies by Alais & Linden, (1991); Dendy, (2001); and FAO, (2004) determined the quantity of fibre in both brown rice and white rice. The current study indicates that this broad generalisation of fibre content based on milling grade may not explain the quantity of fibre in brown rice and white rice, because even among the brown rice varieties, the quantity of fibre varies as seen in RR1 and WR1 (Table 4.1). This needs to be confirmed with more rice varieties.

The amount of TDF extracted was more or less similar to the sum of the SDF and IDF quantity of all rice varieties. However, the percentage of SDF was very low compared with IDF in the rice analysed. The highest amount of SDF was found in WR2 (28.85%), because this contained the least amount of total dietary fibre (IDF+SDF+TDF). Rice varieties RR1, RR2, and WR2 contained SDF at 21.33%, 19.16% and 10.58% of the sum of SDF, IDF and TDF respectively.

As shown in Table 4.1, measured TDF values agreed with the TDF estimated by summing SDF and IDF; that is, both measured and calculated values agreed with a low standard deviation for all the rice varieties analysed.

The isolated fibre fractions corresponding to total, soluble and insoluble compounds were analysed for starch, protein, fat, moisture and ash content (Table 4.2). Results indicated the isolated fibre fractions were pure (99%). This shows that the methods used for isolating fibre fractions for fermentation studies were highly effective.

4.6 Conclusion

The milling grade has a great impact on the fibre content of rice. The rice varieties selected for this study varied in their fibre content. LD356 had highest amount of total dietary fibre and BG358 had the least. In this trial, soluble fibre was less than the amounts of total and insoluble dietary fibre in analysed rice. Total dietary fibre is expected to be the sum of the soluble and insoluble fibre fractions in rice.

CHAPTER 5

Growth of Fecal Organisms on Rice Fibre during *In-vitro* Fermentation

5.1 Abstract

The effects of dietary rice fibre on fermentation by human faecal microflora were determined. Dietary fibre was extracted from four commercially available Sri Lankan rice varieties (LD 356, AT 353 (red in colour); BG 352, and BG 358 (white in colour)) and separated into total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). In order to enhance the growth of microflora that ferment rice fibre, four healthy human subjects (3 males / 1 female) were given a diet containing the four rice varieties for four months prior to the study. The faecal microflora from these individuals was incubated anaerobically with individual rice fibre fractions. The viable count of total aerobes, total anaerobes, total *Bifidobacterium* species and total *Lactobacillus* species, and the optical density and pH of the fermentation broth were measured at 0, 2, 4, 6 and 24 h.

Rice fibre fractions at a concentration of 2.5 % (v/v) were sufficient to enhance the growth of *Lactobacillus* species and *Bifidobacterium* species after 24 h of fermentation. Fermentation with TDF resulted in slight increase in the number of total *Bifidobacterium* species and total *Lactobacillus* species compared with SDF and IDF but not significant. The rice variety WR2 appeared to enhance the growth of microflora more than the other rice varieties although this was not significant. However, there were no significant differences in optical density (OD_{540}) and viable cell counts although there was a significant change in pH ($p<0.05$) among the human inocula.

5.2 Introduction

Major groups of bacteria involved in dietary fibre fermentation in the intestinal tract are species from the genera *Bacteroides, Bifidobacterium, Clostridium* and *Lactobacillus* (Macfarlane & Cummings, 1999). There are reports on the fermentation of non-digestible oligosaccharides by the consortia of intestinal bacteria (Hopkins *et al.,* 1998; Kontula *et al.,* 1998a; Mortensen & Nordgaard-Andersen, 1993; Wang & Gibson, 1993). However, the fermentation of specific dietary fibre fractions of rice has not been reported. Fermentation of fibre by colonic flora has been shown to be affected by the chemical composition and physiological composition of fibre (Lebet *et al.,* 1998a), as well as the microbial composition. Many factors also affect the composition of the microorganisms of the large-intestine in humans. These include the age, susceptibility to infections, nutritional requirements, and immunologic status of the host and the pH, transit time, interactions between flora components, and presence and availability of fermentable material in the gut (Collins & Gibson, 1999). The type of dietary fibre potentially affects the size and activity of specific bacterial populations within the intestinal microorganisms. Therefore, fermentation of dietary fibre by different bacterial populations may produce distinct functional effects in the host (Flamm *et al.,* 2001; Stevens & Selvendran, 1988).

The growth of the microbial population on fibre can be measured by studying the increase in the cell numbers. Cell count, OD (Optical density), and the dry weight are commonly used to assess microbial growth. The growth rate can also be used as a measure of the relative ecological success of gut organisms in adapting to different environments. The growth rates of microbes in fermentation processes are influenced by a variety of factors including pH, temperature and aeration (Gupthar *et al.,* 2000).

The objective of this study was to determine the ability of rice to enhance the growth of *Bifidobacterium* and *Lactobacillus* species in the human gut. The study was also designed to investigate the effect of individual fractions of rice fibre as IDF, SDF and TDF on the growth of *Bifidobacterium, Lactobacillus* species and the total anaerobic count in faeces.

5.3 Materials and Methods

In order to obtain faecal inocula, samples were taken as described in Section 3.1. The TDF, SDF and IDF of three different rice fibres used in this trial were determined using the method detailed in 3.3. Human subjects, diet of humans, analysing faecal samples, inocula preparation, *in-vitro* cell count are described in 3.5.1, 3.5.2, 3.5.4, 3.5.5 and 3.7.1

5.4 Results

Figure 1 shows the effect of rice rich diet on the bacterial counts in human faeces.

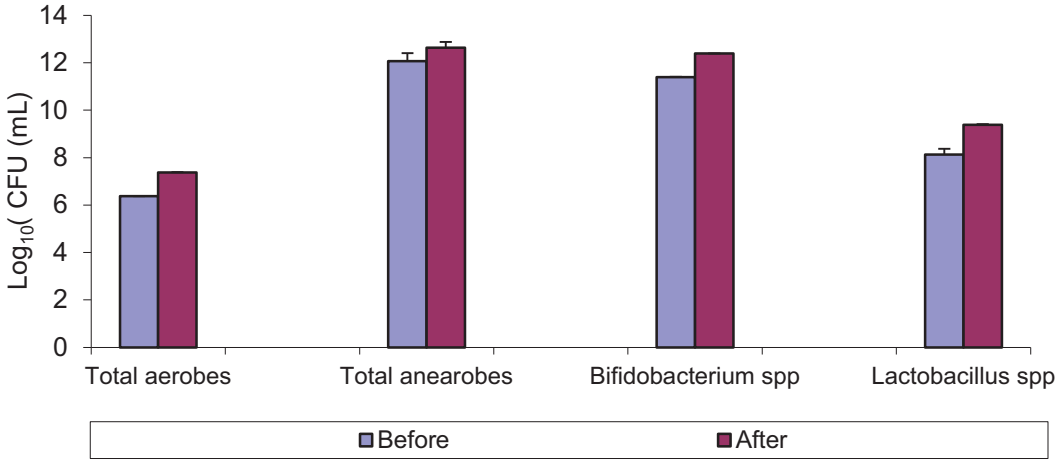


Figure 5.1 Comparison between the faecal bacteria cell counts before and after consuming rice for 4 months.

Results are expressed as the average of human samples H1, H2, H3 and H4 (mean value ± standard error from three replicates. Note the standard error < 0.03).

There was no significant difference (p>05) in bacterial cell counts before and after the subjects were given rice rich diets.

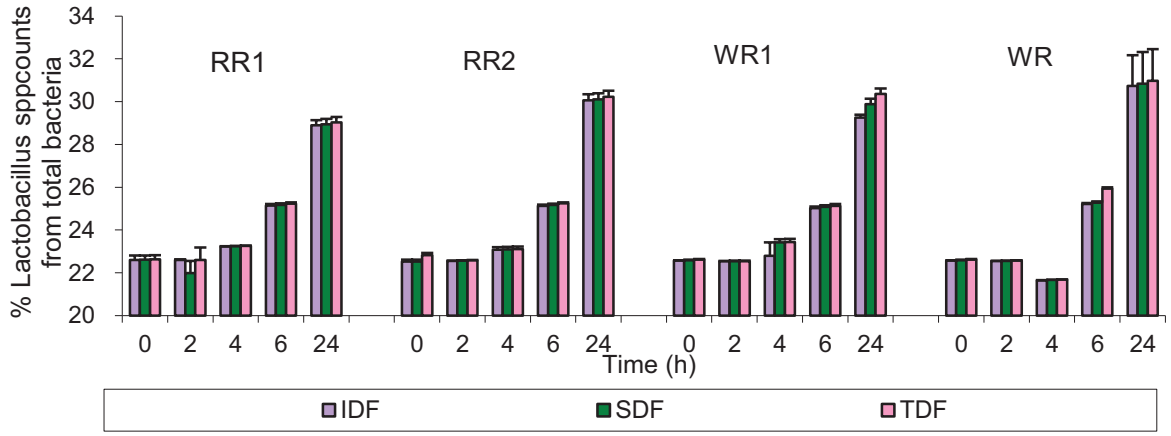


Fig 5.2 A) *Lactobacillus*

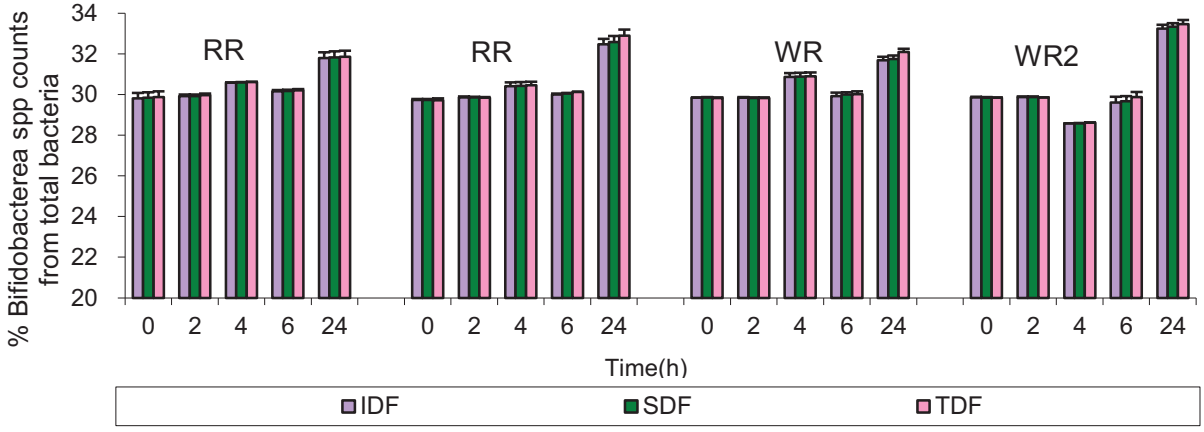


Fig 5.2 B) *Bifidobacterium*

Figure 5.2 (A-B) Proportion of *Lactobacillus* (A) and *Bifidobacterium* (B) species from the total bacterial count at each sampling time.

Results are expressed as the average value of viable cell counts of faeces on each rice variety. Results are expressed as the mean value ± standard error of duplicate samples.

Micro-organisms had the ability to utilise IDF, SDF and TDF equally as seen in Fig 5.2. The percentage of viable count of lactobacilli that increased with the incubation time was not observed with the *Bifidobacterium* species. This may represent a difference in the ability of the two groups of microorganisms to adapt to their environment.

Figure 5.3 iIllustrates the growth of total anaerobes, total aerobes, genus *Bifidobacterium* and genus *Lactobacillus* species on the rice tested.

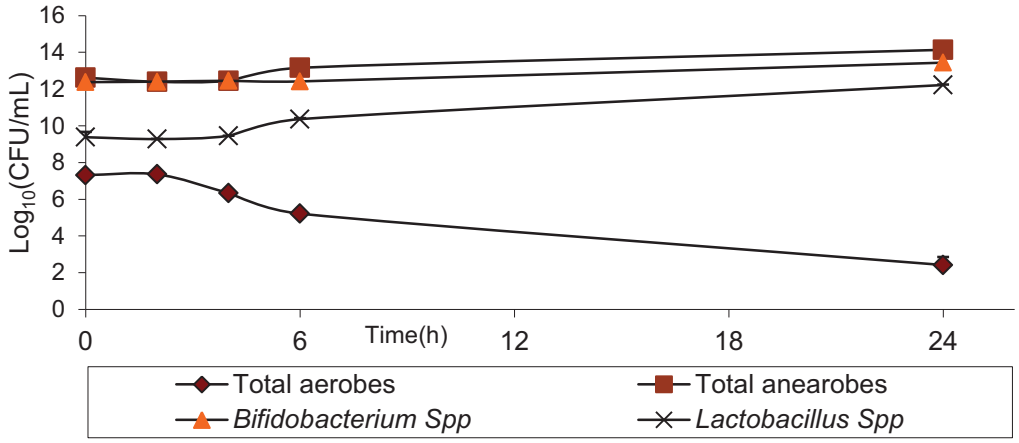


Fig 5.3 A) Growth of faecal microorganisms on RR1

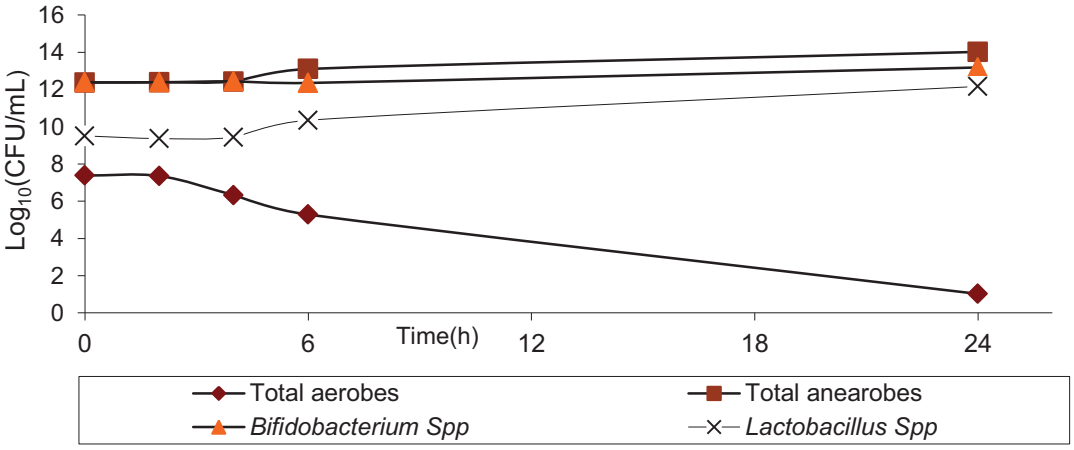


Fig 5.3 B) Growth of faecal micro organisms on RR2

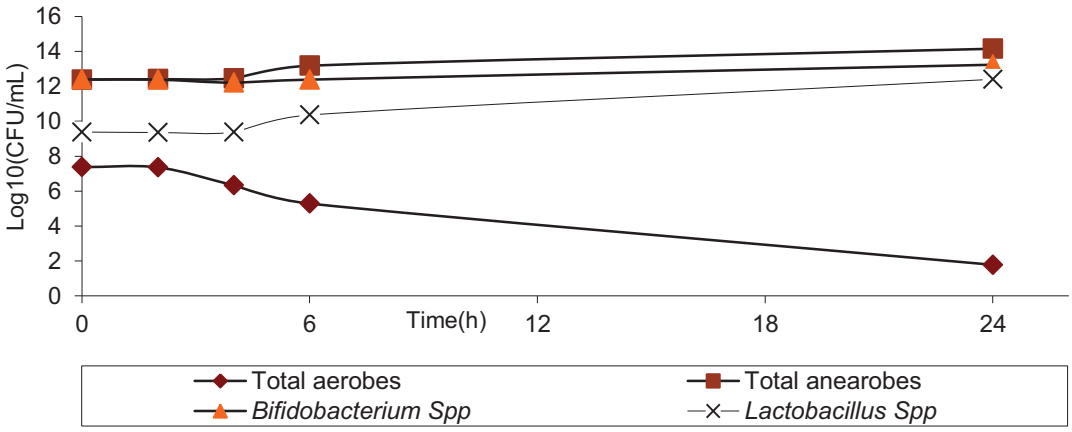


Fig 5.3 C) Growth of faecal micro organisms on WR1

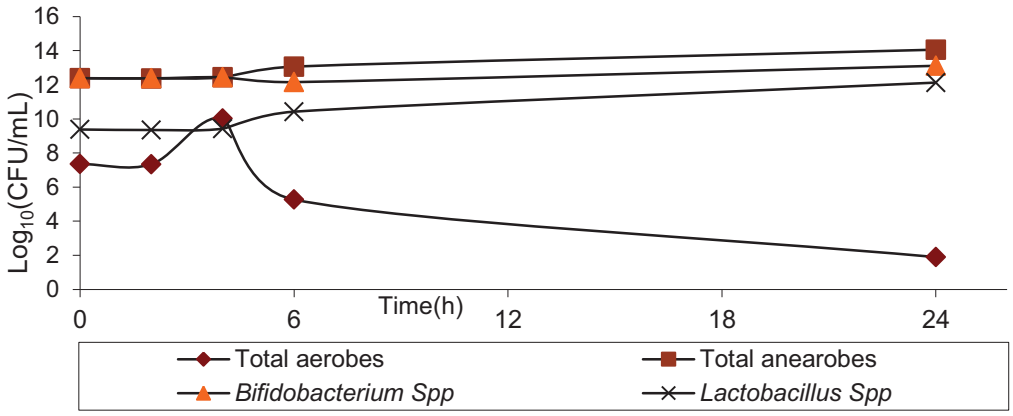


Fig 5.3 D) Growth of faecal micro organisms on WR2

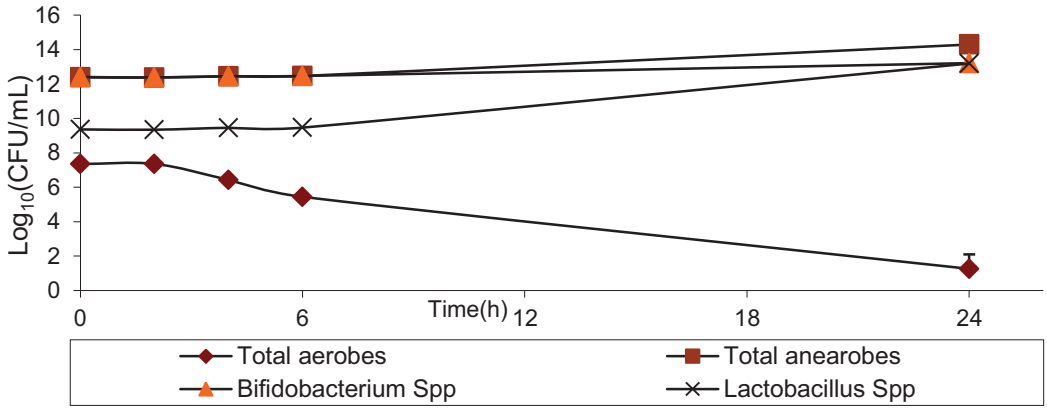


Fig 5.3 E) Growth of faecal micro organisms on Glucose

Figure 5.3 (A-E) Growth of Total aerobes, Total anaerobes, Genus *Bifidobacterium* and Genus *L*actobacillus on rice varieties

Results are expressed as the average viable cell counts on IDF, SDF and TDF, at each time point. Results are expressed as the mean value ± standard error of duplicate samples.

The growth of *Bifidobacterium* species, *Lactobacillus* species and the anaerobes were similar for all rice varieties (Fig 5. 3 A, B, C, D, E).

Members of the genus *Bifidobacterium* increased from 29 – 32% and lactobacilli from 23 – 29% (Fig 5.2) of the total anerobic microflora over the 24 h incubation.

There was no significant difference in the results from the inocula from the different individuals ($p > .05$) in terms of their ability to use the rice dietary fibre fractions and the increase in anaerobes, total *Bifidobacterium* and total lactobacilli. The viable cell counts during the 24 h incubation for each group of microorganisms (except total aerobes) in all the inocula increased on all fractions of rice fibre (Fig 5.3).

Growth and the metabolite formation was further analysed by measuring the OD at 540 nm and the pH of the anaerobic fermentation broth at different sampling times over 24 h (Fig 5.4). There was no correlation between pH and the OD though, as expected, the pH drop followed a corrosponding rise in OD at 540 nm (Fig 5.4). There was no significant difference ($p>.05$) in the results from the fermentation of IDF, SDF, and TDF based on the optical density readings, but there was a significant difference in pH values between IDF/SDF ($P<.05$, $P= 4.40E-11$), SDF/TDF ($P<.05$, $P=7.29E-06$) and TDF/IDF ($P<.5$, $P=7.63E-14$).

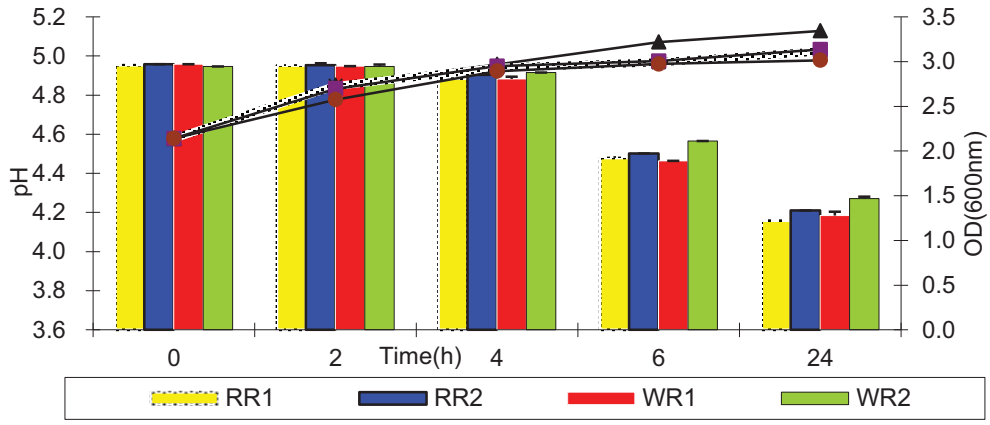


Fig 5.4 A) TDF

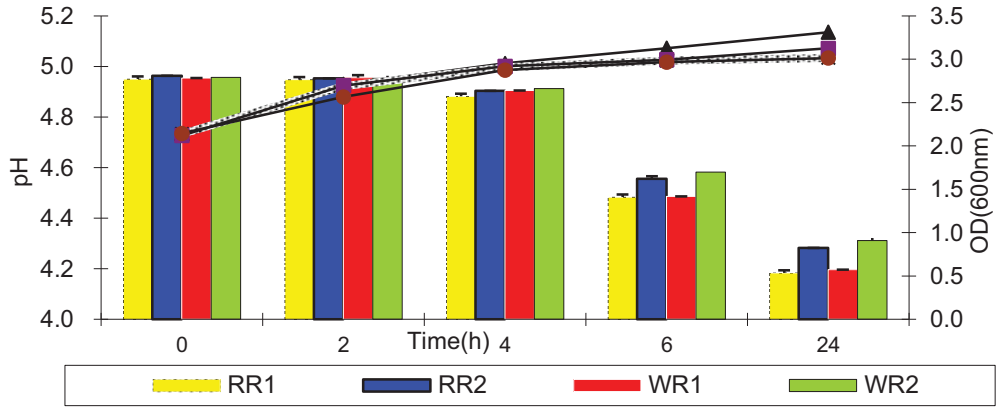


Fig 5.4 B) SDF

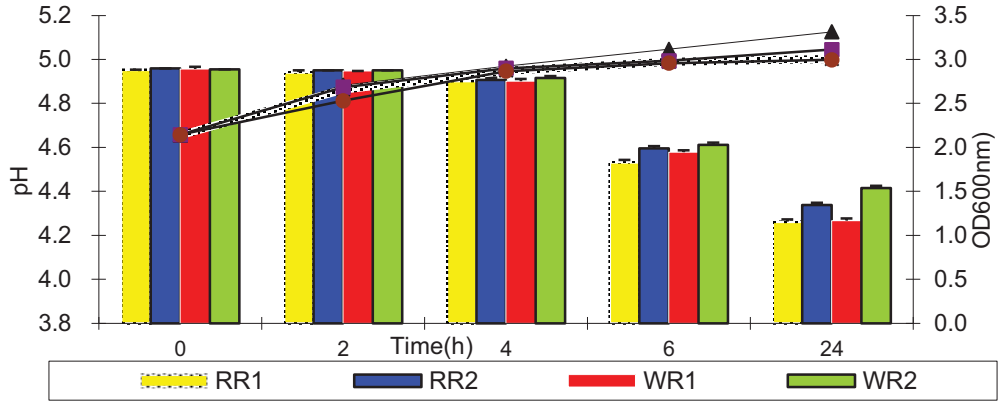


Fig 5.4 C) IDF

Figure 5.4 (A-C) pH values (bars) and the optical density (lines) during rice fibre fermentation

Results are expressed as the mean value ± standard error of duplicates.

The inoculum was added when the pH was around 4.96 and decreased to 4.19 - 4.27 after 24 h incubation. The highest decrease in pH was during the fermentation of TDF from RR1 and WR1. There was no significant difference (p>05) between the different types of inocula.

5.5 Discussion

There is little published information on the relationship between rice dietary fibre fractions and the ability of human faecal *Lactobacillus* species and *Bifidobacterium* species to ferment rice. In this study, the fermentation of fibre from different rice varieties was compared under *in-vitro* conditions. This study compared the growth of faecal microflora on IDF, SDF, and TDF from four rice varieties. The predominant groups of bacteria in faeces were cultivated using selective growth media to understand the effect of rice fibre on the specific gut flora.

Bacteria in the human gut mainly depend on dietary substrates for energy and growth (Gibson *et al.,* 1995). In this research the total anaerobe count, total *Bifidobacterium* count and total *Lactobacillus* count in faeces did not change significantly after consuming the rice for four months (p>.05 at 95% CL) (Total *Lactobacillus* counts increased by 10-12%, the total *Bifidobacterium* count by 2.5 % - 4.5 %, and the total anaerobes by 5-6.5%) (Fig 5.1). Both a positive (Gibson *et al.,* 1995; McBurney & Thomson, 1987) and negative (Woods & Gorbach, 1993) impact on bacterial numbers after manipulating the diet have been reported. However, the results from the current study do not show any changes in the gut microflora following the consumption of rice. One explanation for this may be that few *Bifidobacterium* and *Lactobacillus* are capable of digesting rice fibre. Therefore, the presence of rice fibre as whole grain has little effect on the numbers of these microorganisms. The actual species that digest rice fibre needs to be determined.

The fermentation media used in these trials was free of any other carbon sources other than the TDF, SDF, and IDF from the rice. TDF increased the microbial count by 1-2% compared with SDF and IDF with the largest increase for the TDF of WR1 (Fig 5.2). Although the quantity of fibre was the same for all the fermentations, the fibre fraction might have different sugar components depending on the rice variety producing a different response for the different rice varieties. *In-vitro* results confirm the growth of faecal microorganisms on rice fibre. The proportion of *Bifidobacterium* from the total bacteria (anearobe+ aerobe+ *Bifidobacterium* + *Lactobacillus*) population increased by 2 or 3% within the 24 h of fermentation, whereas the *Lactobacillus* proportion increased by 7or 8% of total counts. This was achieved with 2.5 % (v/v) of dietary fibre of rice. The greatest increase in microbial numbers was seen after 24 h incubation using rice variety WR2. This shows differences in the fermentation of different rice fibres. Previous research reports high growth of *Bifidobacterium* and *Lactobacillus* with oligofructose and inulin (Gibson *et al.,* 1995). This indicates that growth of *Bifidobacterium* and *Lactobacillus* can be enhanced by the composition of dietary fibre and this is known to vary with different fibre sources. .

In general, *Bifidobacterium* and *Lactobacillus* growth followed a similar pattern for all rice varieties (Fig 5.3). However, one difference was that *Lactobacillus* species rapidly had a heigher growth) reaching maximum growth after 24 h. This was apparent even with the limited number of data points in this experiment. In contrast *Bifidobacterium* species exhibited slower growth for first 6 h, reaching maximum growth at 24 h. *Lactobacillus* species had a slow growth initially for four hours for all the rice varieties indicating a rapid adjustment to the new environment and faster utilization of fibre than the *Bifidobacterium* species. Previous studies (Kaplan & Hutkins, 2000) with the fructooligosaccharides showed a short lag phase for

Lactobacillus species *(Lactobacillus acidophilus)* compared with *Bifidobacterium* species and Perez-Conesa *et al.,* (2005) observed a long lag phase for *Bifidobacterium* for glucose, oligosaccharides, and inulin fermentation.

Since the microorganisms had not reached the stationary phase (Fig 5.3) after 24 h fermentation, the carbon source (dietary fibre) may not have become the limiting factor. Differences in the pH from the fermentation of IDF, SDF, and TDF (Fig 5.4) indicates that the amount of metabolite produced (short chain fatty acids) may be different for the different substrates. This result suggests that the growth of potential probiotics in the inoculum increased slightly due to dietary fibre digestion in the fermentation broth, despite the fact that total microbial growth showed no significant difference ($p>0.05$) for the different fibre fractions. The most effective fibre fractions fermented by faecal inocula based on the pH drop were TDF (Fig 5.4A) and SDF (Fig 5.4B) from the rice varieties RR1 and WR1 with the fermentation being similar to that observed for glucose fermentation. . Insoluble dietary fibre (IDF) (Fig 5.3C) from all rice varieties had the least effect on pH at the end of the 24 h fermentation, suggesting that the IDF is less accessible than SDF and TDF to the human gut microorganisms.

This study assumes that the natural micro-flora used was typical of the human gut. Variations in the proportion of different microorganisms may influence the results. As in other studies, (Bielecka *et al.,* 2002: Crittenden *et al.,* 2002; Kneifel *et al.,* 2000; Poolman, 1993) the present study observed changes in the growth of probiotics in terms of cell numbers, pH and OD in response to a fibre substrate. Interactions between prebiotics (substrate) and the different probiotics depend on diet and location in the human GIT, so the actual fermentation in the gut may differ from the observations in this study.

5.6 Conclusion

This study found that diet with rice did not increase the total number of human gut microorganisms measured in faecal samples. However, the proportion of *Bifidobacterium* and *Lactobacillus* species in the faecal microflora increased by 2 or 3% and 7 or 8% respectively following 4 months of a rice rich diet. However, *in-vitro* fermentation of dietary rice fibre fractions (IDF, SDF, and TDF) by gut microflora enhanced the growth of *Lactobacillus* species by 10 or 12 % and *Bifidobacterium* species by 2.5 % or 4.5 %. This enhancement occurred with a 2.5 % (v/v) rice dietary fibre fraction.

The pH and OD for different individual faecal material was similar, with most of the growth occurring between 6 – 24 h. The most promising substrate was TDF.

More specific information on the fermentation of rice fibre may be produced by using pure cultures of bifidobacteria and lactobacilli rather than using the natural undefined faecal microflora as the inocula. Future studies should also look at more frequent sampling over a longer fermentation period.

CHAPTER 6

Short Chain Fatty Acid Formation from the *In-vitro* Fermentation of Rice Fibre with Human Inocula

6.1 Abstract

Short chain fatty acid production (SCFA) from the fermentation of rice fibre by human faecal microflora was examined. The substrates for the study, were prepared from total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) were separated from four rice varieties (LD 356, AT 353 (red colour pericarp); BG 352, and BG 358 (white colour pericarp). The inocula for the study were prepared from the faeces of four healthy human subjects (3 males / 1 female) given a diet containing above four rice varieties for more than four months prior to the study. Individual fibre fractions were incubated anaerobically with the faecal microflora obtained from human volunteers, and short chain fatty acid (SCFA) production was analysed by gas liquid chromatography. Analysis was done at 0 and 24 h for fermentations, using faecal inocula taken from individuals before starting the rice diet and at 0, 2, 4, 6, and 24 h for fermentations using faecal inocula from individuals after being fed the rice diet.

SCFA produced by rice fibre fermentiations using faecal microflora taken from subjects fed a 4 month rice diet, showed a significant increase compared with fermentations using faecal inocula taken before the subjects started the rice diet. Acetic acid was the most abundant acid formed in the fermentation of all rice varieties. The fibre fractions from rice variety RR1 gave the highest yield of SCFA while the variety

WR2 gave the least. Total dietary fibre (TDF) of all rice varieties contributed to produce more SCFA than soluble dietary fibre and insoluble dietary fibre.

The highest SCFA was obtained between 6 and 24 h fermentation. The molar ratio and concentration of SCFA was different, depending on the fibre substrate, faecal inocula and fermentation time. The molar ratio of SCFA was highest after 24 h fermentation for all the faecal inocula and substrates.

6.2 Introduction

Fermentation of fibre results in the formation of short chain fatty acid (SCFA), (acetate, propionate and butyrate) and the level of the production of SCFA can be influenced by the properties of the substrate and the composition of the predominant intestinal microbial population responsible for fibre digestion in the human gut (Eastwood, 1992). The degradation of fibre in the human gut is mainly associated with the structure and physiological and chemical properties of the dietary fibre (Kabel *et al.,* 2002). According to Bouhnik *et al.,* (1996), dietary fibre may be fermented by bacteria in the human gut either partiality or completely, depending on its structural and physicochemical properties. Additionally, the rate and the extent of the fermentation can depend on the particle size, time, solubility and lignification of fibre (Edwards, 1995). Salvador *et al.,* (1993) stressed that these factors are also governed by the type of substrate. The rate and the extent of the fermentation are important to predict the effects of the dietary fibre in the large intestine. The slower the fermentation rates the higher the retention time of the fibre in the human gut.

The composition of microflora changes in response to the variation of food consumption patterns. Due to these differences in microbial populations in different individuals, the molar ratio of different SCFA and the quantity of the SCFA formation can vary (Eastwood, 1992; Kabel *et al.,* 2002). However, *in-vitro* fermentation can

provide valuable information about the fibre fermentation in humans by minimizing the donor variability. The current research used *in-vitro* fermentation of fibre, using faecal inocula to study the effect of different fibre components (extracted from commercially available rice varieties) on the amount of SCFA production. There is no standard method for *in-vitro* fermentation. Usually, faeces are mixed thoroughly with the medium used in the experiment and filtered to remove debris under anaerobic conditions. The filtrate is used as the inoculum for fermentation. However, the quantity of inoculum and the substrate used for such experiments vary from study to study. The amount of the substrate varies in general from 100 mg to 500 mg (Casterline *et al.,* 1997, Lebet *et al.,* 1998a, Wisker *et al.,* 1998). Fermentation times also vary from 24 to 48 h. For most studies, the fermentation is up to 24 h. Sampling times vary as follows: 1, 2, 3, 3.5, 5, 6,7, 7.5, 9.5, 12, 16, 22 and 24 h (Jeracp & Horvath, 1989); 3, 6, 12, 24 and 48 h (Titgemeyer *et al.,* 1991); 6, 12 and 24 h (Fardet *et al.,* 1997;Salvador *et al.,* 1993); 0, 6, 24 and 48 h (Casterline *et al.,* 1997); 0, 24 and 48 h (Wisker *et al.,* 1998); and 0, 2, 4, 6, 8 and 24 h (Lebet *et al.,* 1998a); 0, 5, 10, 24 h (Kedia *et al.,* 2009) and 0,2, 4, 8, 12, 24 h (Kim & White, 2010). Samples are often collected over narrow time intervals in the early stages of the fermentation, and over longer periods during the later stages of fermentation (12 -18 h). Based on these time intervals, the current work selected 0, 2, 4, 6 and 24 h sampling times.

SCFA measurements to determine faecal microflora activity have been largely limited to faecal samples. This is inaccurate, because a high percentage of bacterially derived SCFA, which appear in faeces are subject to colonic absorption (McNail *et al.,* 1978, Topping & Clifton, 2001), and these methods provide the information of SCFA formation from a mixture of substrates. Faecal cultures are an *in-vitro* model of the human colon that avoids the absorption and utilization of SCFA by colon. Thus, the use

of faecal cultures in this study allowed the direct comparison of the contributions of fibre to SCFA production. The present study is focused on the SCFA formation from dietary rice fibre under anaerobic conditions in *in-vitro* fermentation. In this study, dietary fibres from four rice varieties were fermented as insoluble dietary fibre (IDF), soluble dietary fibre (SDF) and total dietary fibre (TDF).

6.3 Materials and methods

In order to obtain faecal inocula, samples were taken as described in Section 3.1. The TDF, SDF and IDF of three different rice fibres used in this trial was determined using the method detailed in 3.3. Human subjects, diet of humans, inocula preparation, *in-vitro* fermentation of fibre with human inocula, determination of SCFA formation and statistical analysis of data are all described in 3.5.1, 3.5.2, 3.5.4, 3.5.5 and 3.10, respectively.

6.4 Results

The following two tables summarise the results from these experiments, examining the SCFA produced by all fermentations using faecal inocula from each human subject (Table 6.1) and by all fermentations using specific rice fibre substrates (Table 6. 2) after the consumption of rice diets.

Table 6.1 SCFA molar ratios (%) measured after 24 h fermentation with rice fibre samples using specific human faecal inocula.

Inoculum (Human)	Acetate	Propionate	Butyrate
H1	68.93±1.37	29.08±1.26	1.98±0.11
H2	70.84±.25	22.35±0.19	6.81±0.05
H3	80.63±0.05	18.18±0.05	1.19
H4	76.06±1.06	21.84±1.09	2.10±0.03

The molar ratio is calculated as an average total SCFA as acetate, propionate, butyrate (Table 6. 1) of RR1+RR2+WR1+WR2 of each individual at 24 h. Results are expressed as the mean value of two trials ± standard error.

Table 6.2 SCFA molar ratios (%) measured after 24 h fermentation with human faecal inocula for specific rice varieties

Substrate	Acetate	Propionate	Butyrate
RR1	66.78±0.15	28.21±0.12	5.02±0.04
RR2	73.78±1.27	23.94±1.34	2.28±0.07
WR1	80.20±0.97	18.29±0.89	1.52±0.08
WR2	83.21±0.56	15.30±0.52	1.48±0.04

The molar ratio for each rice variety was calculated as the average total SCFA formed by all subjects on particular rice type at 24 h. Results are expressed as the mean value of two trials ± standard error.

The study observed the variation of SCFA formation before and after the consumption of rice for four months.

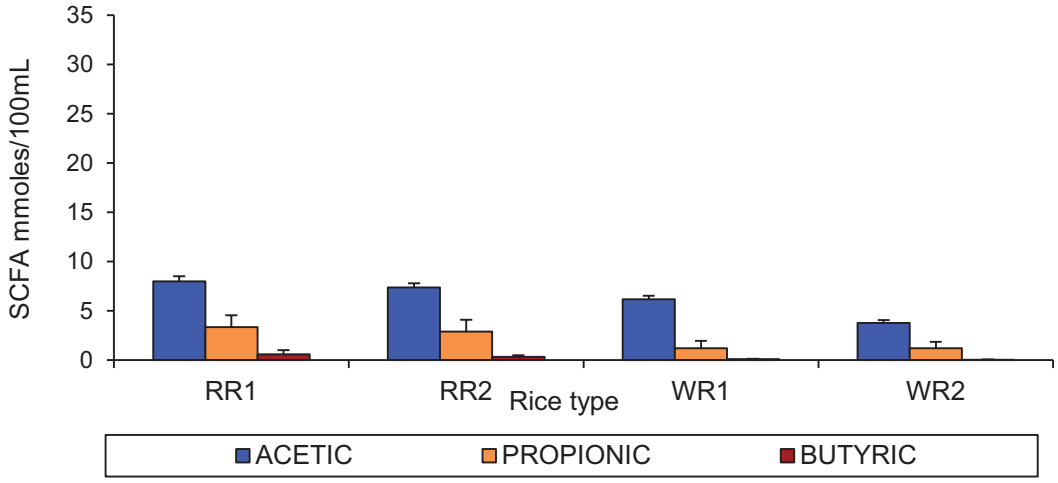


Figure 6.1 SCFA formation from rice fibre fermentation using human faecal microflora before the rice diet

Results are expressed as the average value of SCFA formed from the subjects (H1-H4) ± standard error. SCFA results of Fig 6.1 were calculated as:

SCFA (24h) mmoles/100 mL – SCFA (0 h) mmoles/100 mL. *In-vitro* fermentation was carried out (Fig 6.1) prior to the study using human faecal inocula with the fibre isolates.

The SCFA produced from fermentations of different rice fibre fractions using inocula from subjects, after the rice diets are shown in Figures 6.2 – 6.4.

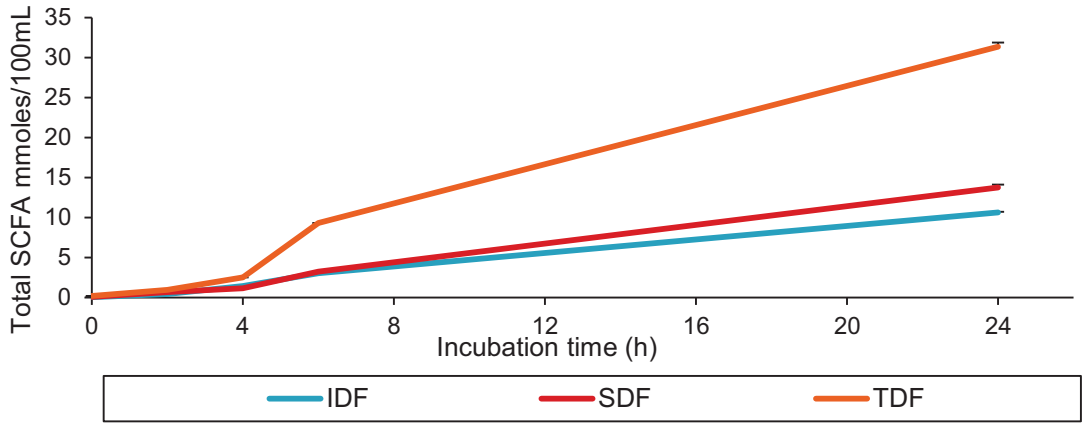


Fig 6.2 A) Total SCFA formation using the H1 inoculum

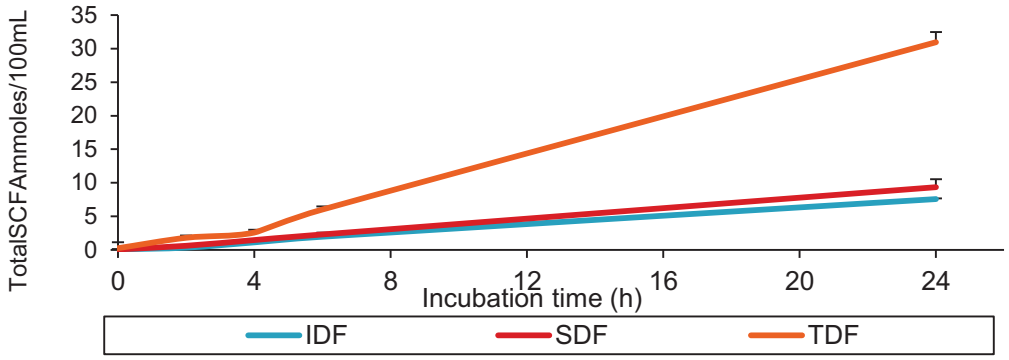


Fig 6.2 B) Total SCFA formation using the H2 inoculum

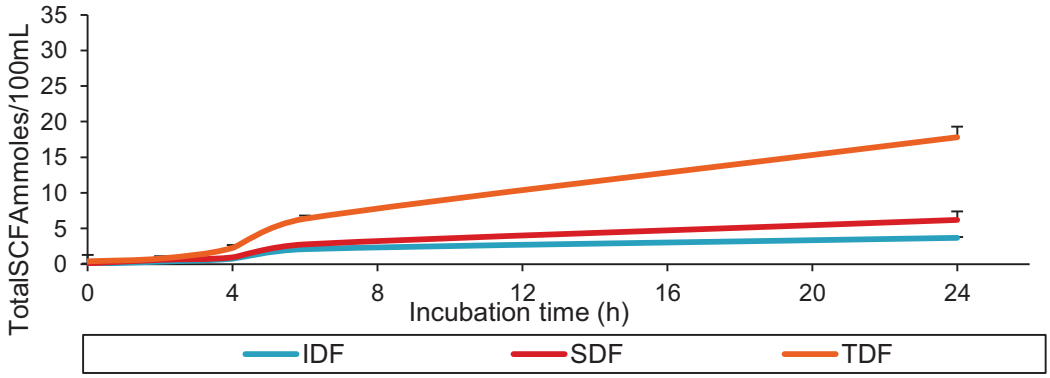


Fig 6.2 C) Total SCFA formation using the H3 inoculum

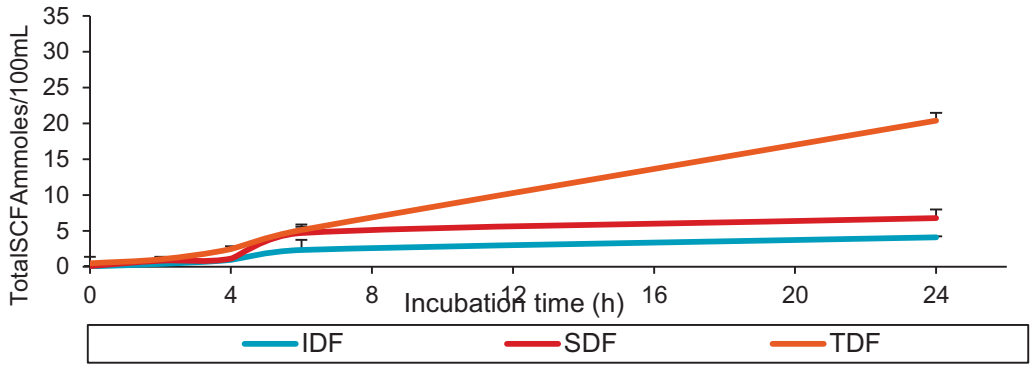


Fig 6.2 D) Total SCFA formation using the H4 inoclulum

Figure 6.2 (A-D) Total SCFA (acetate + propionate + butyrate) formation by individual human faecal inocula (H1 – H4) after the consumption of rice

Results of two independent experiments were calculated from the average of total SCFA formed by IDF, SDF and TDF of all the rice types ± standard error.

Following figures illustrates the effect of rice varieties on SCFA formation.

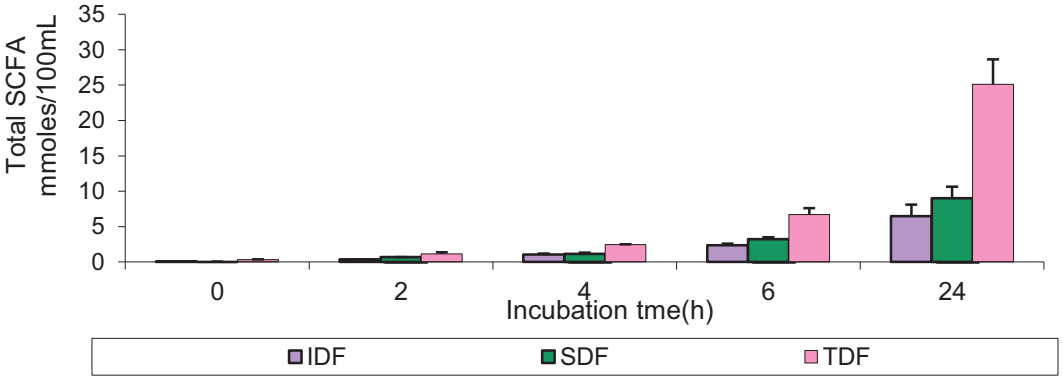


Fig 6.3 A) Total SCFA formation during the fermentation of RR1

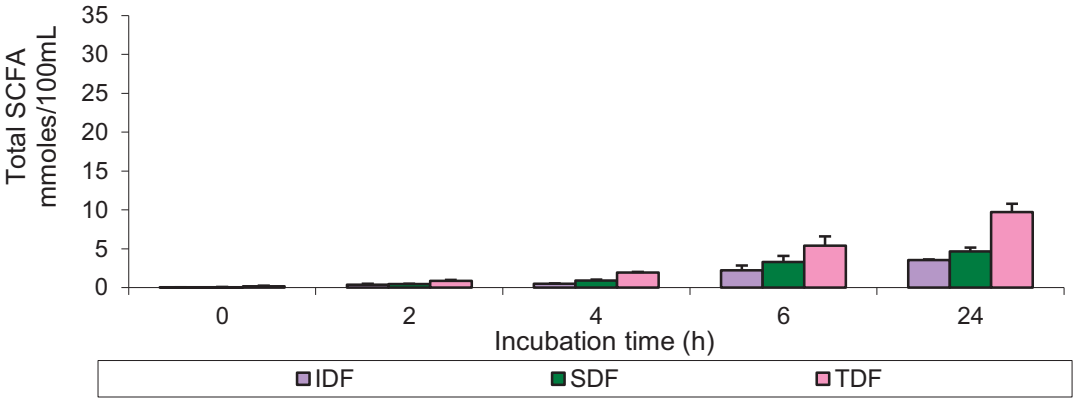


Fig 6.3 B) Total SCFA formation during the fermentation of RR2

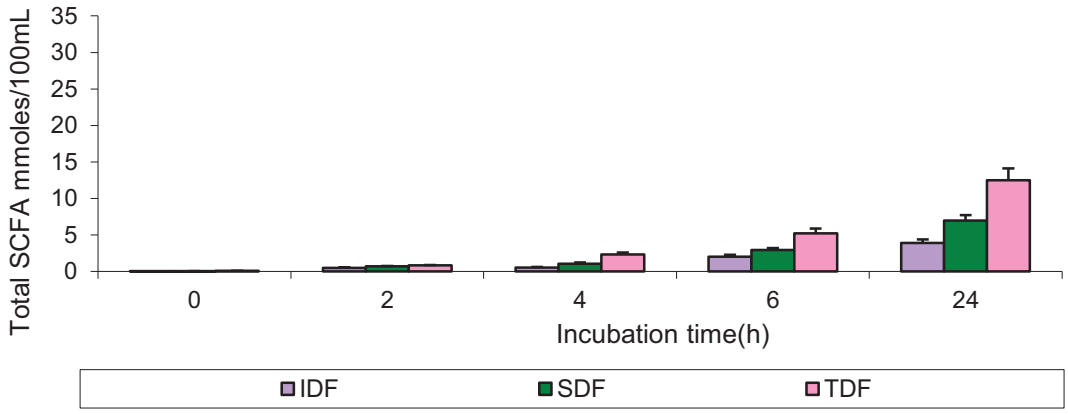


Fig 6.3 C) Total SCFA formation during the fermentation of WR1

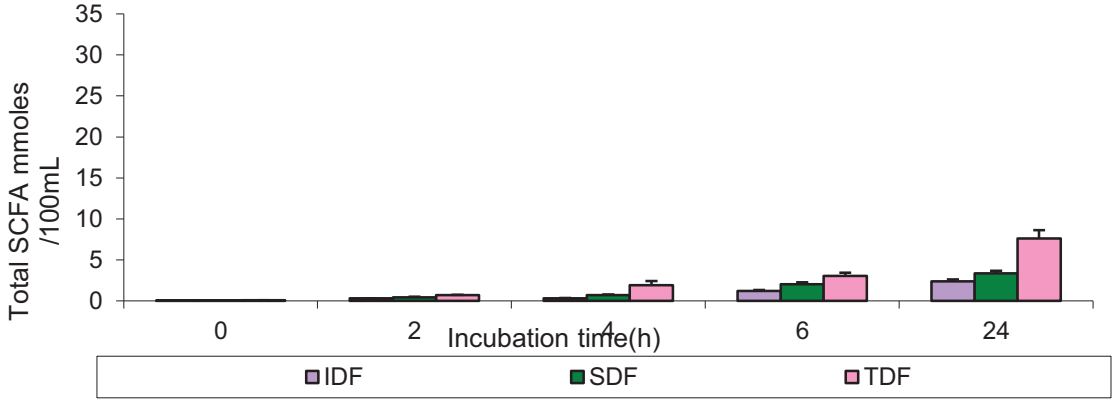


Fig 6.3 D) Total SCFA formation during the fermentation of WR2

Figure 6.3 (A-D) Total SCFA (acetate + propionate + butyrate) formation by rice types

Results of two independent experiments were calculated from the average value of total SCFA formed by the average of the inocula (H1 – H4) for IDF, SDF and TDF for each rice variety ± standard error.

The following figures show the variation in SCFA formation following fermentation of selected rice varieties using microflora from human subjects.

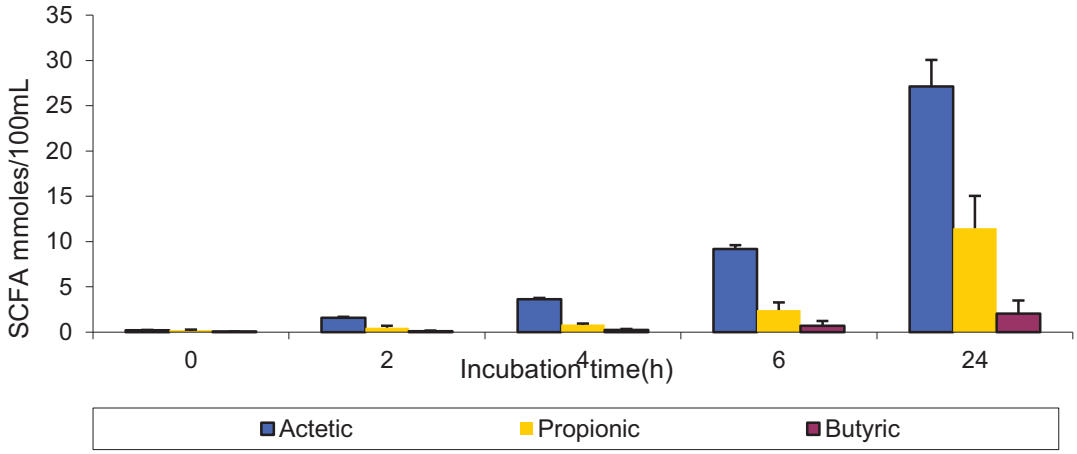


Fig 6.4 A) SCFA formation during the fermentation of RR1

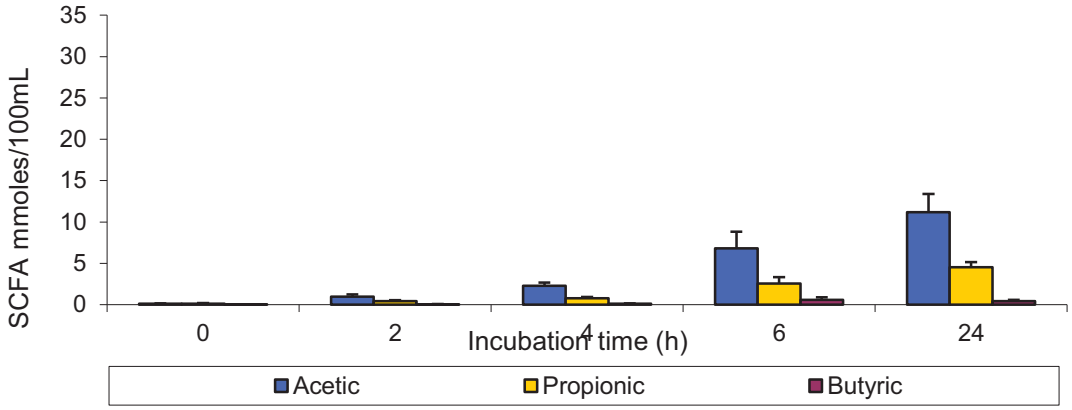


Fig 6.4 B) SCFA formation during the fermentation of RR2

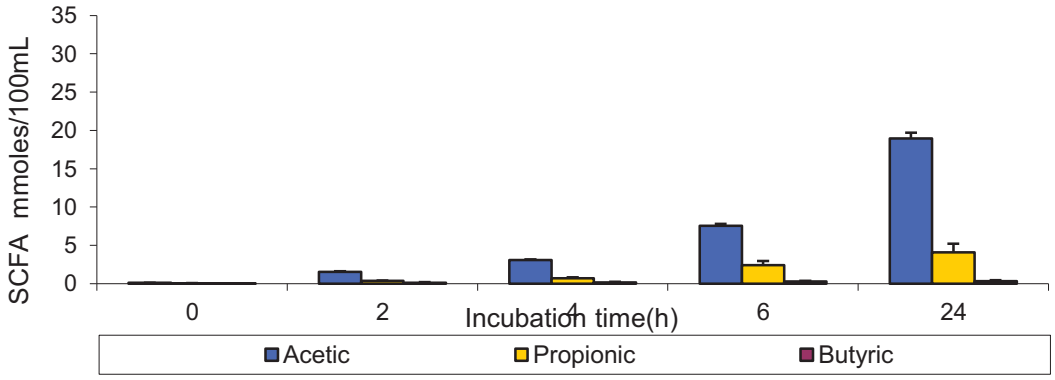


Fig 6.4 C) SCFA formation during the fermentation of WR1

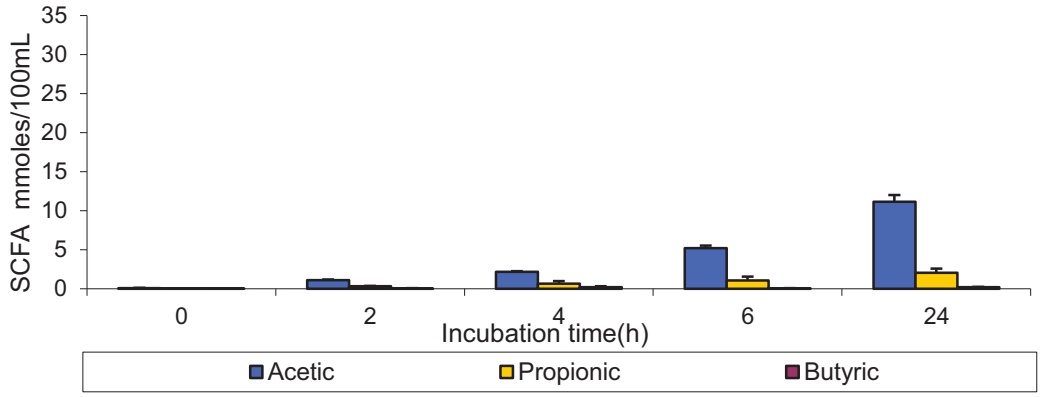


Fig 6.4 D) SCFA formation during the fermentation of WR2

Figure 6.4 (A-D) Individual SCFA formation (Acetic, Propionate and Butyrate) by rice varieties during 24 h fermentation using human faecal microflora

Results of two independent experiments were calculated from the average value of SCFA formed by the average of inocula (H1-H4) ± standard error.

6.5. Discussion

The microbial growth on rice dietary fibre (Chapter 5) is believed to promote colonic health, partly due to the formation of SCFA. This study examined SCFA produced from the fermentation of fractions of rice fibre (IDF, SDF and TDF) from four different rice varieties using human faecal inocula.

Previous Chapters of this study observed that the percentage of dietary fibre varied depending upon the rice cultivar and processing conditions used (Chapter 4). Moreover, the profile of the fibre within the total dietary fibre fractions (IDF /SDF /TDF) varied. These differences lead to variations in total SCFA production and the composition of SCFA production during 24 h *in-vitro* fermentation. Such differences in the SCFA production may be associated with the composition, chemical structure and the physiological nature of the fibre extracts obtained from the different rice varieties.

In the present study, natural faecal microflora was used as inocula for *in-vitro* fermentation of rice fibre fractions an experiment that would more closely mimic the human digestive system with a mixed microflora. Purified microbial enzymes also

have a potential to simulate the digestion in the hind gut (Boisen & Fernanndez, 1991, 1997), but only live microbes are able to ferment a substrate (Coles *et al.,* 2005). This is due to the complex systems that are needed to break down the substrate into simple molecules, which is not possible with individual enzymes or mixtures (Coles *et al.,* 2005).

In this study, dietary fibre fractions were used instead of whole rice as the substrate because substrate that is available for fermentation in human gut is already digested from the food which is consumed (Velazquez *et al.,* 2000). Dietary fibre is not fully digested in the upper part of the digestive tract and may reach the colon as intact molecules (Hunt & Groff, 1990), although subject to physical changes that have occurred in the upper tract (Åman *et al.,* 1994; Salvador *et al.,* 1993). Fibre fractions provide more information about the physiological and metabolic changers in the human gut than the rice.

The study observed an increase in SCFA formation by the faecal microflora after the consumption of rice by the human subjects (Fig 6.1 and Fig 6.2). This indicates the ability of rice fibre to stimulate the SCFA formation in the human body and justifies using faecal microflora from rice-fed subjects for this study. This supports the slight increase in microbial viable count observed in faecal samples in Chapter 5, following the consumption of rice for four months. This confirms that rice fibre can produce SCFA in the human colon that may enhance colon health.

The results showed that the fibre fractions were not used equally by the mixed faecal microflora throughout the fermentation period, demonstrated by the rate of SCFA production during the first 6 h. A lag phase was observed in first few hours of SCFA formation and with a log phase of SCFA formation starting within 4-6 h fermentation for all inocula and substrates (Fig 6.2). Although there was some variation in the initial

phase of the growth between the different inocula, this was not significant ($p>.05$), (inocula from H1 and H3 reached the heigher value of cell nubmer quicker than H 2 and H 4). Such variations are expected with the natural biological variability in microflora that can be expected in the faeces from different individuals. However, the production of SCFA for all treatments continuously increased as fermentation time proceeded. A homogenate of pooled human faeces of individuals has been used as the inocula for batch fermentations in other reports, and was reported to produce reproducible fermentation profiles (Barry *et al.,* 1995; McBurney & Thompson, 1987; Michel *et al.,* 1998; Mortensen *et al.,* 1991). However, the profile of microorganisms is different in each individual based on the sex, age, etc (Delgado *et al.,* 2006). Therefore, the current study used the inocula of each individual without pooling.

Total SCFA (acetate+propionate+ butyrate) formed from the different rice varieties during 24 h fermentation (Fig 6.3 and Fig 6.4), varied from the most to the least with RR1> WR1>RR2>WR2. The total SCFA formation (acetate+propionate +butyrate) also differed between the dietary fibre fractions (IDF, SDF, TDF) for each of the rice fibres. Although the quantity of dietary fibre used in fermentation was standardised, the differences in SCFA production between varieties may be further explained by the presence of different amounts of sugars such as galactose, arabinolactose, pectin, etc in the different fibre fractions and, accordingly rice varieties.

Soluble fibre appeared to contribute to SCFA more rapidly than IDF. The solubility and the structure of soluble fibre are likely reasons for the rapid fermentation. The low final pH (Fig 5.5) during the fermentation of SDF might induce high acid tolerant species to aid digestion. It is important to note that the proportion of soluble fibre in the rice varieties studied was small. The highest rate and the quantity of total SCFA formation were obtained from TDF. Functional foods have a mix of

soluble and insoluble fibre. If those foods include fibre from rice then TDF, containing both SDF and IDF, will provide the most representative result.

The total SCFA concentration (mmoles/100 mL) varied significantly ($p<0.05$) from 6 to 24 h for all rice types (Fig 6.2 and Fig 6.3). The peak total SCFA formation was observed at 24 h suggesting that the anaerobic fermentation of fibre cannot be completed within 24 h. These results are in agreement with the research of Stewart and Slavin (2006) who examined the fermentation dynamics of guar gum fibre. The possible reason for selecting the 24 h fermentation period by the majority of the researchers, as explaned in the introduction of this Chapter, may be to prevent microorganisms in the human inocula using the SCFA as a carbon source for their continued growth. This would make, difficult to evaluate the direct effect of fibre on the formation of SCFA. However,the total transit time for food in human gut has been estimated as 24 to72 h (Wrick *et al.,* 1983).

The quantity of acetate, propionate, and butyrate increased, but by different amounts during fermentation. Acetate formed in the greatest proportions, followed by propionate and butyrate, and the rate of acetate formation (slope of the graph 6.2) was always higher than that of the propionate and butyrate. This result suggests that rice fibre is more prone to produce acetate than the propionate and butyrate. This was common for all fractions for all rice varieties studied. Acetate is the main compound from which the human body obtains energy out of dietary fibre and is important for lipogenisis and for glucogenisis as explained on page 37. Due to the large number of health benefits of acetate propionate and butyrate, it may be possible for food manufactures to provide the information about the SCFA formation when rice fibre is included in food as an additive. Butyrate also is important to human health (Roediger & Moor, 1981; Spina *et al.,* 2007). Some studies have revealed that the molar ratio of

butyrate may be higher due to the conversion of acetate into butyrate by Embden-Meyerof-Parnas metabolic pathways (Romano & Conway, 1996). However, this study could not evaluate the exact rate of SCFA formation during the 6-24 h fermentation due to the sampling times that would not allow an accurate determination of the rate of SCFA formation. An approximate rate can be calculated based on the slope of the graph.

Wong *et al.,* (2006) reported that a substrate reduces the ratio between acetate and propionate is beneficial to human health by reducing cholesterol synthesis. In the present study, microflora of human subject one (H1) produced a lower ratio of acetate: propionate compared with the other subjects, and the fermentation of substrate RR1 resulted in the least difference between acetate to propionate (Table 6.1 and 6.2). These results suggest that both the substrate and inoculum influence the acetate to propionate ratio produced during fermentation. In a mixed diet, the increase in acetate: propionate produced by WR2 (Table 6.1 and 6.2) may be offset by additional formation of propionate by RR1. Therefore, a combination of fibre fractions from different rice varieties may have a synergistic effect to benefit human health. Compared with previous studies on different cereals (Marsono *et al.,* 1993; McBurney & Thomson, 1990; Stewart & Slavin, 2006), the present study obtained a higher molar ratio of acetate, similar or less molar ratio of propionate, and a significantly less molar ratio of butyrate (Table 6.1 and 6.2). In all these previous studies, the fermentation process was similar to the one adopted in this experiment. However, microbial composition is likely to be different with human faecal inocula from different subjects. This makes it difficult to compare results without standardising the inocula even if the substrates are same.

The results indicated that rice varieties are different, not only through physical appearance, but also from the metabolic products they produce during fermentation.

This shows the source and type of rice fibre determines the physiological effect they will exert on the body by formation of SCFA. Other studies have revealed that there is a difference in fibre fermentation from different food varieties (McBurney *et al.,* 1985; McBurney & Thomson, 1990; Van soest *et al.,* 1983).

6.6 Conclusion

Differences in SCFA formation after consumption of rice indicate the ability of rice fibre to increase the SCFA formation in human gut.

Among the fibre fractions, TDF produced the most SCFA irrespective of the substrate. Acetate was the most abundant SCFA formed during 24 h of fermentation with the order of SCFA produced being acetate > propionate > butyrate for all the substrates. The formation of SCFA from the different rice varieties was in the order, RR1 > WR1 > RR2 > WR2. The molar ratio between acetate to propionate was the least in rice variety RR1, Therefore, this rice variety may be preferable as a dietary fibre source when compared with the other rice varieties tested.

CHAPTER 7

Role of Rice Fibre in Stimulating *in-vitro* Growth of *Lactobacillus* and *Bifidobacterium* species

7.1 Abstract

In this Chapter, the effects of dietary fibre from rice on fermentation by *Lactobacillus* and *Bifidobacterium* species were investigated. Dietary fibre was extracted from two commercially available Sri Lankan rice varieties; LD 356 (brown rice, red pericarp), AT 353 (unpolished rice, red in pericarp) and separated into total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). Four probiotic species (*Lactobacillus rhamnosus* (ATCC 7469), *Lactobacillus acidophilus* (ATCC11975), *Bifidobacterium breve* (ATCC15700) and *Bifidobacterium longum* (ATCC15707) were grown in medium containing rice fibre as IDF, SDF, TDF or glucose as the sole source of carbon for fermentation. Growth of the probiotics was studied at 0, 6, 12, 18, 24, and 48 h of incubation.

The ability of the strains to ferment the fibre differed. *Lactobacillus* species showed the highest growth irrespective of the substrate up to 24 h whereas *Bifidobacterium* species demonstrated the highest growth at both 24 and 48 h.

The microorganisms used in these trials had different preferences for the different rice varieties. Rice fibre at 1% concentration increased the growth of all probiotics tested. It may be possible to select fibre sources most suited to supporting the growth of probiotics in the human colon.

7.1 Introduction

The importance of *Bifidobacterium* species and *Lactobacillus* species to human health is well known (Degnan & Macfarlane 1993; Ding *et al.,* 2005; Fooks *et al.,* 1999; Kurmann, 1993; Macfarlane & Cummings, 1999, 2002; Roller *et al.,* 2004; Rinne *et al.,* 2005; Wang & Gibson 1993). High numbers of *Bifidobacterium* species and *Lactobacillus* species in the human gut are important and can be influenced by diet supplements. Therefore, identifying the foods that increase numbers of these bacteria is imperative. Most of the supplements consist of oligosaccharides and fructooligoscharides (Gibson & Roberfroid, 1995; Rada & Koc, 2000). However, there are no published data on the use of rice fibre to promote the growth of these probiotics in the GIT.

Feeding studies with human volunteers have confirmed that *Bifidobacterium* species and *Lactbacillus* species populations can be stimulated by fructooligosaccharides (Gibson *et al.,* I995; Roberfroid, 1998). In earlier Chapters in this thesis, the effect of dietary rice fibre on faecal cultures was reported (Fernando *et al.,* 2008). Although there have been a number of publications on the fermentation of non-digestible oligosaccharides by probiotic bacteria, (Crittenden,1999) and the fermentation of fibre by the consortia of human and animal intestinal bacteria (Hopkins *et al.,* 1998; Kontula *et al.,* 1998a; Mortensen & Nordgaard-Andersen, 1993; Saarela *et al.,*2002; Wang & Gibson, 1993), there is little information on the fermentation of specific dietary fibre polysaccharides by individual probiotic and intestinal bacteria (Crittenden,1999; Shin & Ustunol, 2005).

Growth is the most important measure of bacterial physiology (Wang & Levin, 2009). Therefore, the growth of specific bacterial species, such as *Bifidobacterium* and

Lactobacillus species on fibre will help determine the ability of these species to ferment specific dietary fibre as in Nithyaja *et al.,* (2009).

Agar plate counts were used to measure microbial growth in this study**.** The success of probiotic bacteria in fermenting fibre can also be determined by the growth rate and amount of substrate used. The growth rate of *Bifidobacterium* species and *Lactobacillus* species on dietary fibre will influence the ability to compete with other bacteria in the colon. The amount of substrate converted into bacterial mass or cell number can be used as an indicator to assess the efficiency of prebiotic or probiotic activity using rice fibre.

The current study used different fibre fractions from two rice varieties to understand the effect on growth of different probiotic strains on fibre fermentation and determine the ability of rice fibre to stimulate the growth of selected *Bifidobacterium* species and *Lactobacillus* species.

This research aimed to provide useful information about the human gut bacteria, with the ability to digest non-starch polysaccharides of rice fibre, leading to the health-promoting properties of *Bifidobacterium* and *Lactobacillus* species.

7.3 Materials and methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, bacterial strains, preparation of cell suspensions, preparation of growth medium, *in-vitro* fermentation, determination of pH, optical density and viable count, specific growth rate, determination of the growth yield and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.2, 3.6.4, 3.6.5, 3.5.6, 3.7 and 3.12 respectively.

7.4 Results

This study was done using two rice varieties (RR1 and RR2). RR1 and RR2 are fibre rich sources of food and had a higher quantity of fibre than white rice (WR1 and

WR2). Therefore, it was decided to use these two rice varieties in this experiment to understand the growth. Fibre was extracted from these rice varieties as SDF, IDF, and TDF and fibre fractions from rice variety RR1 were labelled as IDF1, SDF1 and TDF1 and fibre fractions from rice variety RR2 were labelled as IDF2, SDF2, and TDF2. These non starch polysaccharide fractions were subject to fermentation with pure cultures of probiotics. This study focussed on the growth kinetics of organisms on different fibre fractions.

Bifidobacterium and *Lactobacillus* species demonstrated different growth patterns on rice fibre (Fig7.1). The major difference between the two species was that *Lactobacillus* species rapidly gained a heigher growth, and did not have a constant growth in selected time points. In contrast, *Bifidobacterium* species exhibited slower growth for first 6 h and constant growth from 24 - 48 h. Interestingly, both species reached the maximum growth at 24 h. A similar growth pattern for *Bifidobacterium* species was observed by Perez-Conesa *et al.,* (2005) for substrates glucose, and the oligosaccarides inulin and oligofructose.

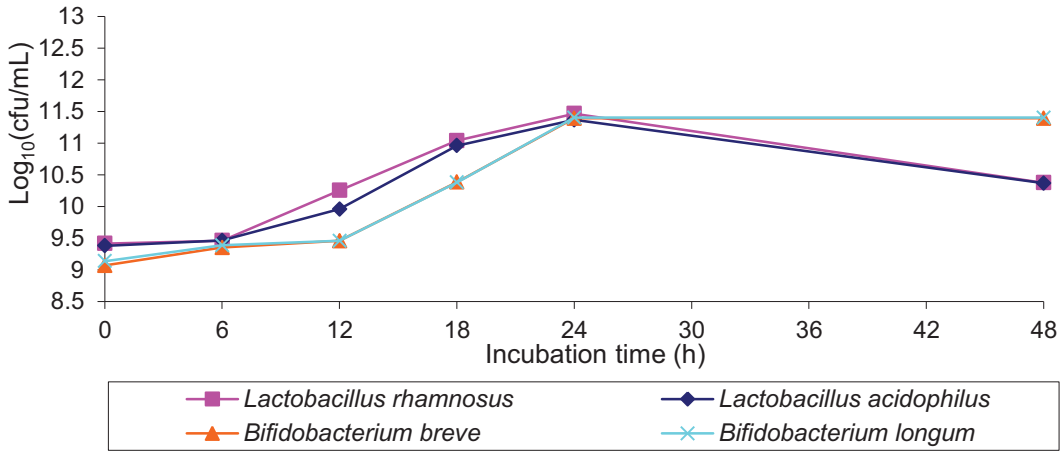


Fig 7.1 A) Growth on fermentation of IDF 1

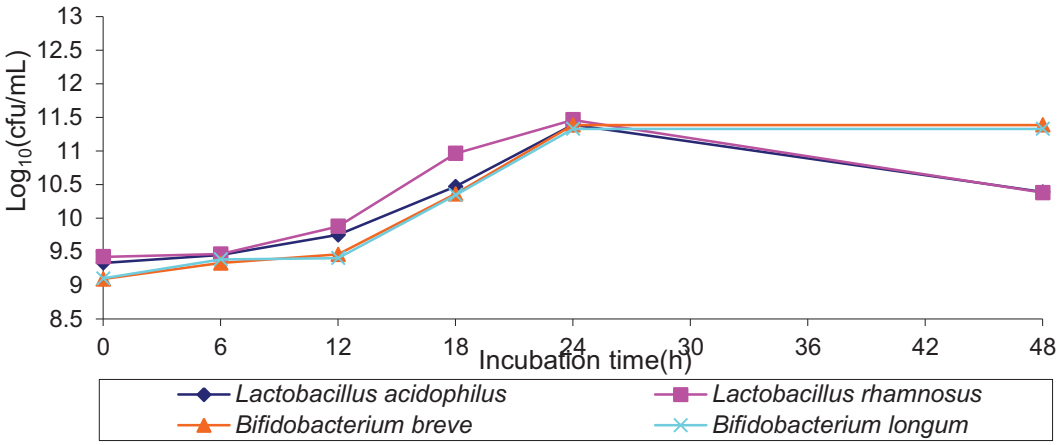


Fig 7.1 B) Growth on fermentation of SDF1

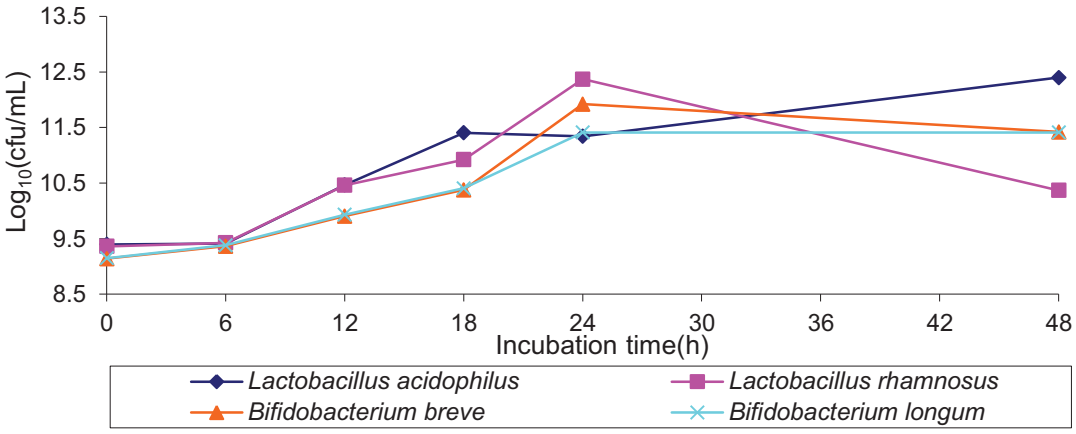


Fig 7.1 C) Growth on fermentation of TDF1

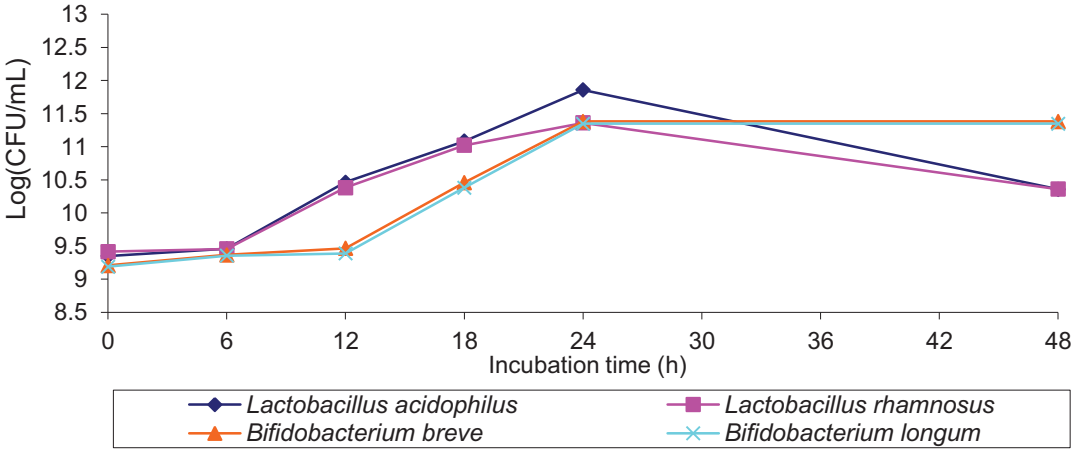


Fig 7.1 D) Growth on fermentation of IDF2

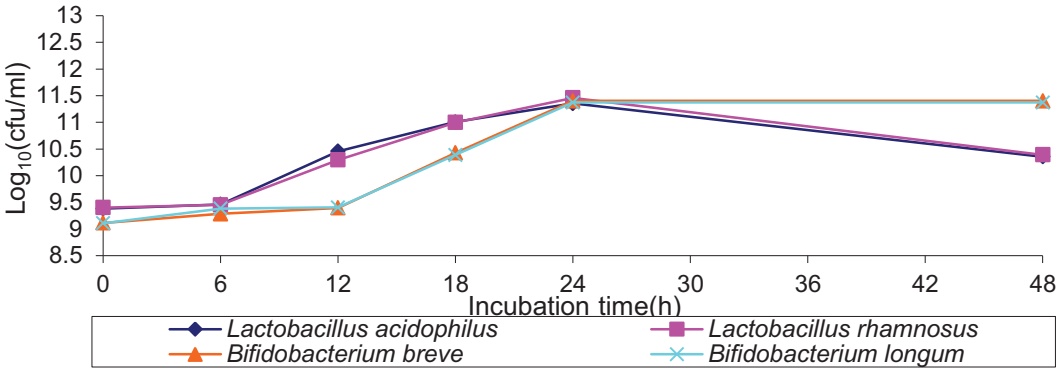


Fig 7.1 E) Growth on fermentation of SDF2

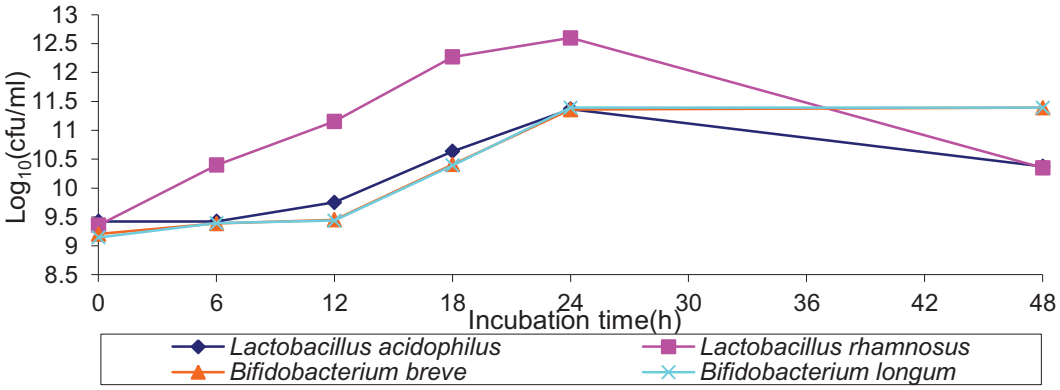


Fig 7.1 F) Growth on fermentation of TDF2

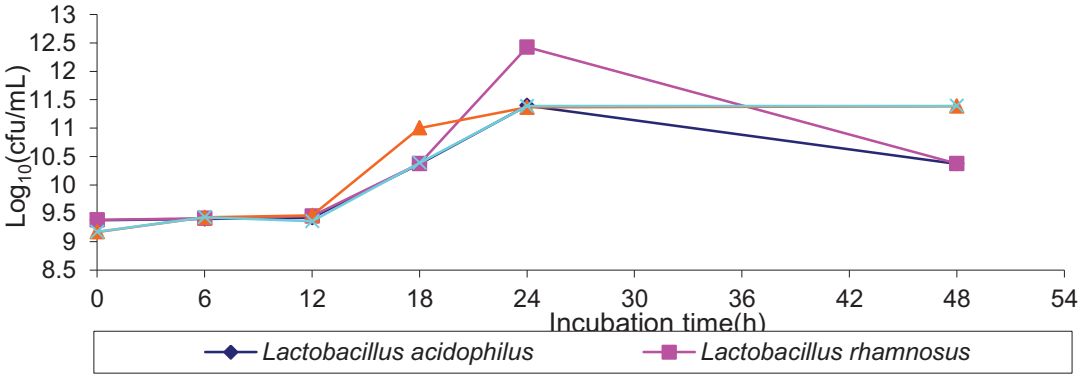


Fig 7.1 G) Growth on fermentation of Glucose

Figure 7.1 (A - G) Bacterial growth in media containing different dietary fibre isolates and in glucose

Results are expressed as the mean value of two separate fermentations ± standard error.

The standard error of the mean was smaller than 0.01, therefore, it is not visible.

The growth measured using optical density readings had a higher slope compared with the viable count (Fig 7. 2). This may be due to the effect of dead cells and undigested substrate in the broth.

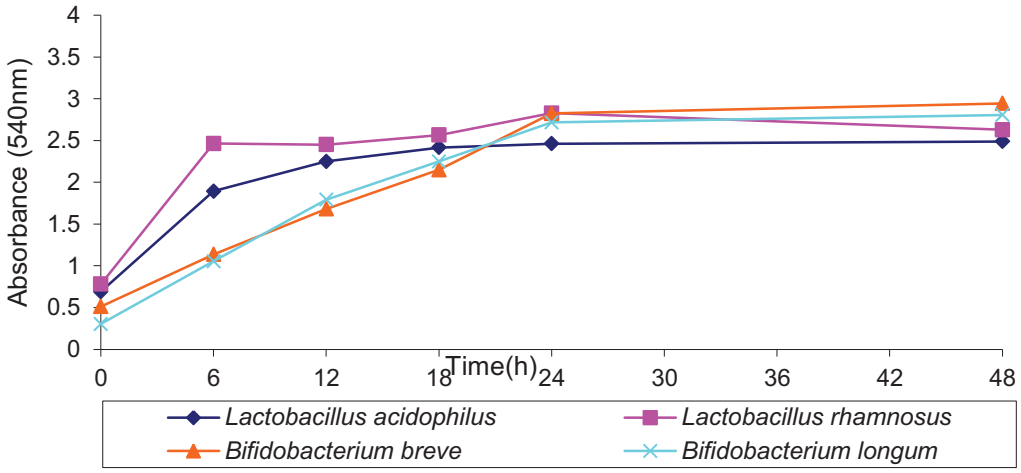


Fig 7.2 A) Absorbance on the fermentation of IDF 1

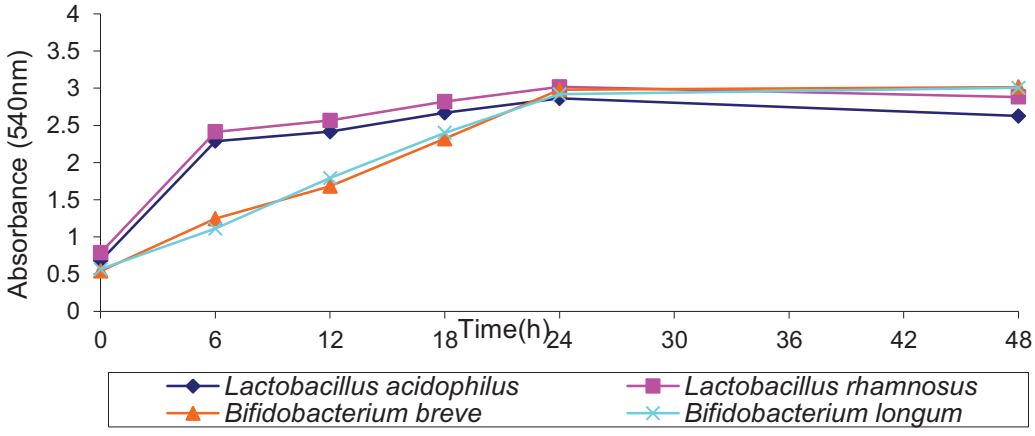


Fig 7.2 B) Absorbance on the fermentation of SDF 1

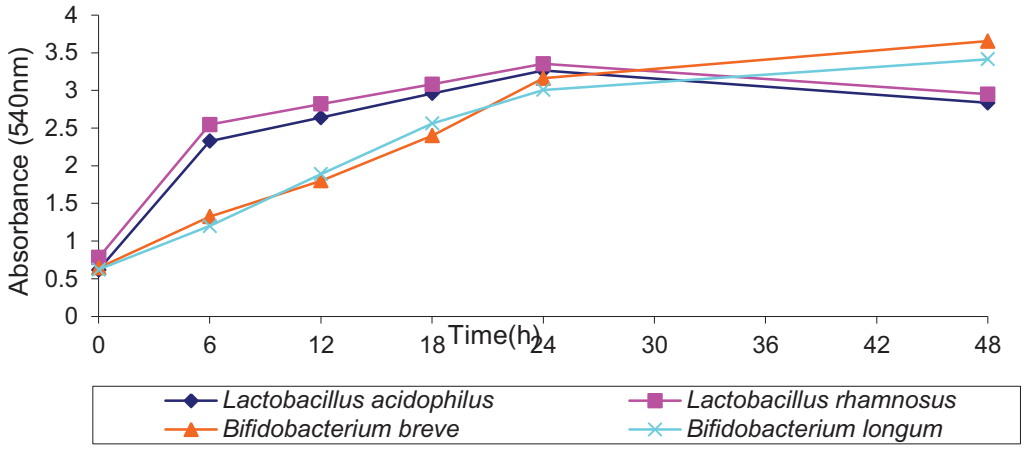


Fig 7.2 C) Absorbance on the fermentation of TDF1

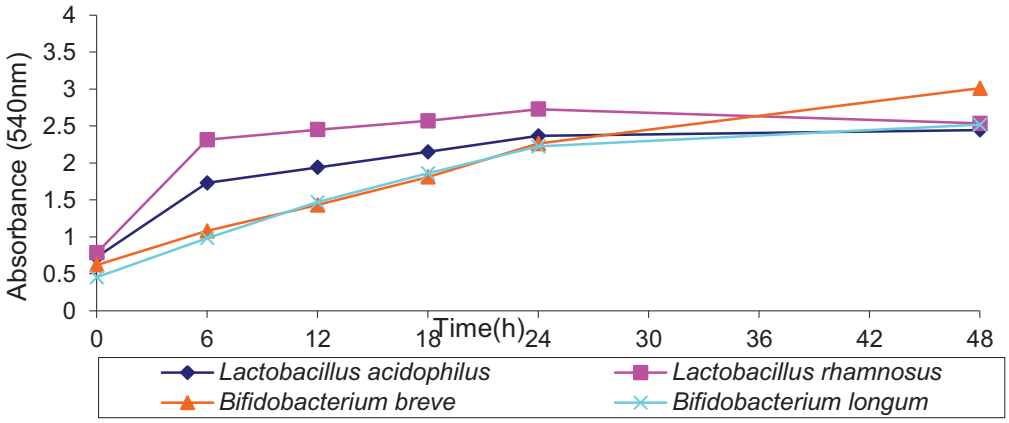


Fig 7.2 D) Absorbance on the fermentation of IDF 2

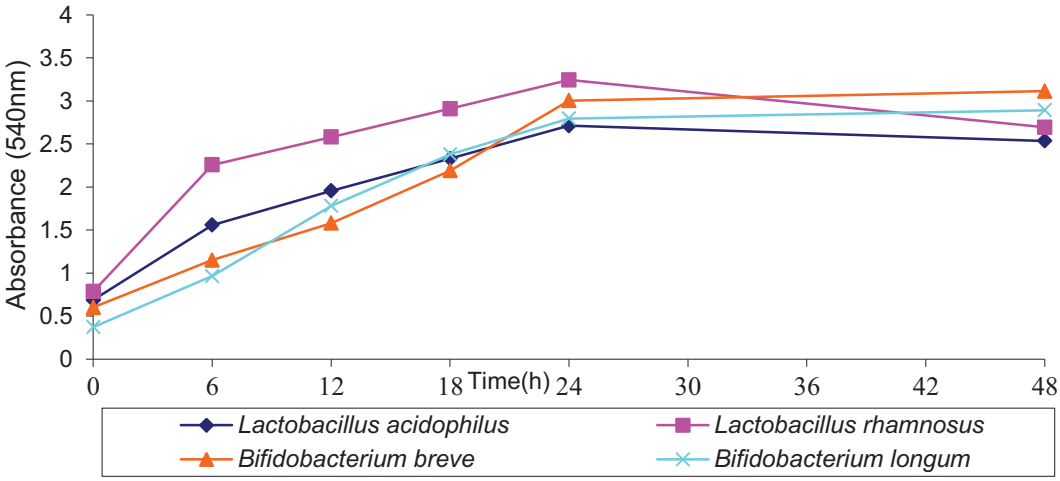


Fig 7.2 E) Absorbance on the fermentation of SDF2

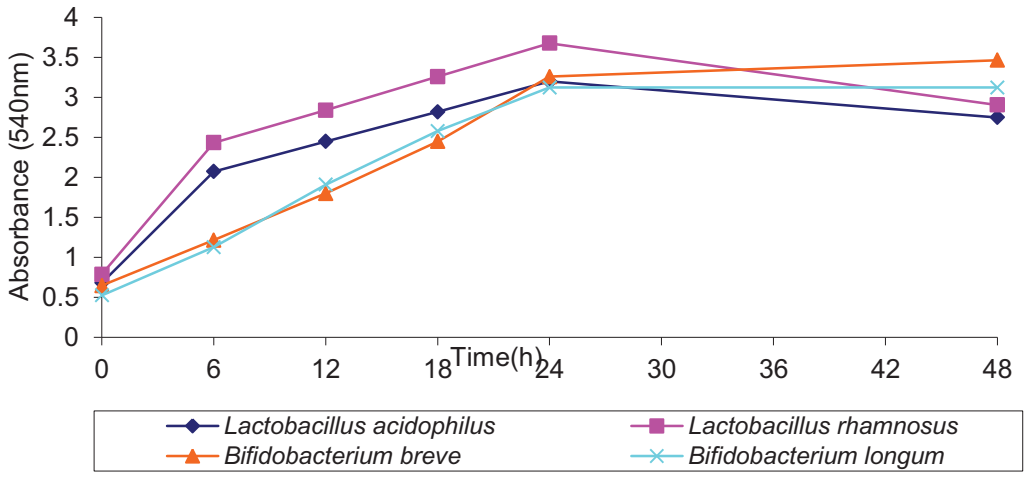


Fig 7.2 F) Absorbance on the fermentation of TDF2

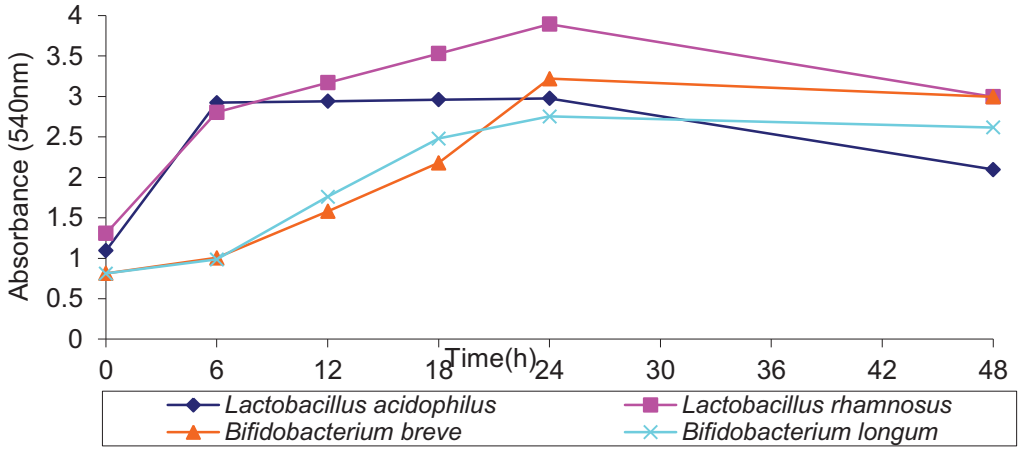


Fig 7.2 G) Absorbance on the fermentation of Glucose

Figure 7.2 (A-G) Optical density (OD_{540}) readings during fermentation, of three different fractions of dietary fibre and glucose

Results are expressed as the mean value of two separate fermentation ± standard error. Standard errors was smaller than 0.01. Therefore, it is not visible.

The pH of the four species of probiotics with 7 substrates is shown in Table 7.1. Initially the pH values were around 5.5-5.75 in all the fermentation flasks. A decrease in pH values was observed with the increase in the length of fermentation for all substrates. pH values was significantly different ($p<0.05$) between the *Bifidobacterium*

and *Lactobacillus* species after 6 h incubation on all substrates and for glucose, after 24 h incubation. Generally the pH decreased by 0.5-0.9 points after 6 h of incubation except for the substrates SDF1, IDF1, TDF1 and glucose with *Bifidobacterium* species (0.03-0.1).

Table 7.1 pH values at different incubation times

0 H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**	**Control**
Lactobacillus acidophilus	5.65±.02	5.64±.02	5.645±.03	5.53±.03	5.51±.05	5.48±.05	5.49±.1	5.5±.05
Lactobacillus rhamnosus	5.565±.03	5.56±.04	5.555	5.55±.05	5.57±.04	5.51±.04	5.58±.04	5.72±.01
Bifidobacterium breve	5.56±.04	5.58±.02	5.55±.03	5.51±.05	5.52±.08	5.38±.04	5.57±.04	5.7±.03
Bifidobacterium longum	5.63±.02	5.79±.03	5.5±.01	5.55±.01	5.56±.04	5.55±.02	5.49±.10	5.67±.03
6H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**	**Control**
Lactobacillus acidophilus	4.75±.02	4.78±.05	4.675±.01	4.59±.05	5.1±.3	5.05±.30	4.33±.08	5.54±.09
Lactobacillus rhamnosus	4.74±.03	4.64±.03	4.615	4.53±.08	4.53±.07	4.57±.03	3.83±.05	5.6±.06
Bifidobacterium breve	5.54±.02	5.48±.04	5.51±.01	5.43±.08	5.47±.03	5.36±.03	5.35±.05	5.55±.09
Bifidobacterium longum	5.56±.04	5.57±.03	5.51±.01	5.5±.06	5.51±.04	5.41±.06	5.62±.02	5.56±.10
24 H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**	**Control**
Lactobacillus acidophilus	4.46±.02	4.3±.04	4.33±0	4.33±.06	4.23±.08	4.26±.05	3.85±.07	5.69±.02
Lactobacillus rhamnosus	4.3±.06	4.36±.02	4.26±.03	4.28±.07	4.26±.04	4.19±.08	3.58±.02	5.67±.03
Bifidobacterium breve	4.57±.04	4.48±.04	4.46±.03	4.54±.07	4.5±.06	4.38±.06	5.53±.05	5.67±.05
Bifidobacterium longum	4.61±.06	4.63±.01	4.53±.01	4.59±.05	4.56±.04	4.38±.06	5.45±.05	5.61±.10
48 H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**	**Control**
Lactobacillus acidophilus	4.75±.03	4.67±.03	4.58±.04	4.59±.07	4.57±.04	4.51±.09	4.69±.02	5.54±.09
Lactobacillus rhamnosus	4.67±.03	4.65±.03	4.56±.03	4.56±.07	4.46±.10	4.46±.04	4.1±.05	5.65±.03
Bifidobacterium breve	4.57±.03	4.47±.03	4.49±.05	4.51±.07	4.43±.06	4.33±.07	4.25±.06	5.6±.06
Bifidobacterium longum	4.93±.12	4.94±.05	4.845±.08	4.89±.11	4.88±.08	4.69±.27	5.52±.06	5.61±.06

Results are expressed as mean values of two separate fermentations ± standard error.

The decline in pH for *L. rhamnosus* grown in glucose was high after 6 and 24 h incubation (pH 3.83, 3.58, respectively). *Bifidobacterium breve* showed the highest acidification for IDF, and TDF2 (4.38 and 4.27 respectively, 24 h), *L. acidophilus* showed the lowest pH value for SDF1 (4.36, 24 h), and *L. rhamnosus* showed the lowest pH values for TDF1, SDF2, and for Glucose (4.3, 4.31 and 3.61 respectively, 24 h). *Bifidobacterium* species showed a higher pH value for growth on glucose (pH 5.5-

5.6) than the *Lactobacillus* species. At 24 h, the pH values for all strains grown on all dietary fibre substrates had dropped to pH4.3-4.6. After 48 h incubation, pH values for all strains on all substrates had increased by 0.35-0.42 units from the 24 h readings. The pH variation is an indication of the growth of the strains on the different substrates and production of short chain fatty acids.

To obtain further information on the growth of the microorganisms on rice fibre, biomass yield was calculated. However, an absolute biomass yield for each microorganism could not be evaluated due to the presence of undigested fibre in the fermentation broth. Therefore, the relative biomass yield was calculated.

Table 7.2 Relative biomass yields on different fibre extracts compared with growth on glucose

6th H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**
Lactobacillus acidophilus	++	++	++	+	+	++
Lactobacillus rhamnosus	++	++	++	++	++	++
Bifidobacterium breve	++++	++++	++++	++++	++++	++++
Bifidobacterium longum	++++	++++	++++	+++	+++	++++
24th h	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**
Lactobacillus acidophilus	++	+++	+++	++	++	+++
Lactobacillus rhamnosus	++	++	++	++	++	+++
Bifidobacterium breve	++	++	+++	++	++	+++
Bifidobacterium longum	+++	+++	+++	++	+++	++++
48th H	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**
Lactobacillus acidophilus	++++	++++	++++	++++	++++	++++
Lactobacillus rhamnosus	++	+++	+++	++	++	+++
Bifidobacterium breve	+++	+++	++++	+++	+++	++++
Bifidobacterium longum	+++	++++	++++	+++	++++	++++

The relative growth yield compared with growth on glucose '-'= no growth, '+' =0-40% of the OD on glucose, '++ '=40-90% of the OD on glucose, '+++ '=90-120% of the OD on glucose, '++++'=120% of the growth on glucose.

B. longum generally demonstrated a higher biomass on fibre fractions compared with glucose. *L. acidophilus* too had a higher biomas on fibre compared with glucose after 48 h incubation (Table 7. 2).

In this study, the specific growth rates were calculated for pure cultures grown on rice fibre fractions. The use of the equation μ_{max}= (1/ average X) dx_t/dt normally used to calculate the specific growth rate is invalid in this study as growth trends were non-linear and the viable count was measured every 6 h which is considered a relatively large time interval.

Table 7.3 Specific growth rates of microorganisms at different time points

microorganisms	Time -(h)	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2	Glucose
	6-12							
Lactobacillus acidophilus		0.19±0.007	0.23±0.008	1.53±0.006	0.77±0.008	0.76	0.25±.194	0.02±.018
Lactobacillus rhamnosus		0.30±0.16	0.32±0.002	0.80	0.71±.002	0.65±.013	0.03±.046	0.03±.003
Bifidobacterium breve		0.04±0.001	0.10±0.05	0.42±0.18	0.07±.005	0.09±.006	0.05±.005	0.03±.002
Bifidobacterium longum		0.03±0.002	0.01±0.006	0.42±0.19	0.03±.006	0.01±.022	0.03	0.05±.003
	12-18							
Lactobacillus acidophilus		0.38±0.019	0.74±0.001	0.73±0.002	0.42±.001	0.42±.005	0.69±.183	0.73±.211
Lactobacillus rhamnosus		0.08±0.017	0.60±0.113	0.34±0.198	0.28±.113	0.32±.097	0.39±.394	0.95±.078
Bifidobacterium breve		0.36±0.001	0.68±0.011	0.37±0.002	0.73±.011	0.78±.019	0.71±.015	0.95±.12
Bifidobacterium longum		0.35±0.003	0.72±0.006	0.36±.198	0.76±.006	0.76±.019	0.72	0.79±.004
	18-24							
Lactobacillus acidophilus		0.19±0.013	1.09±0.218	0.67±0.409	0.59±.218	0.27	0.20±.143	0.40±.189
Lactobacillus rhamnosus		0.35	0.38±.389	1.11±0.187	0.26±.389	0.35±.004	0.85±.014	0.81
Bifidobacterium breve		0.39±0.001	0.79±.002	1.18±0.193	0.71±.002	0.75±.001	0.75±.019	0.29
Bifidobacterium longum		0.39±0.001	0.76±.002	0.77	0.74±.002	0.76±.001	0.77	0.77±.003

Specific growth rates (approximate) results are expressed as the mean value of two separate fermentations ± standard error.

Therefore, the specific growth rate was determined assuming linearity between the closest two time points in the growth using the formula, $\mu=\ln(x_2/x_1)/ (t_2-t_1)$ (Gupthar *et al.,* 2000). However, the regression of values considered to plot the graph (using average values) ln x Vs Time was generally more than 90% indicating a reasonable correlation between the data points.

Generally, there were no significant variations in the specific growth rates between ***B. longum*** and *B. breve* for all the substrates ($p>0.05$). *L.rhamnosus* and *L.acidophillus* showed similar results except for TDF and for glucose.

The low specific growth rate of *Bifidobacterium* species at the early stages of growth may be linked to the long lag phase. Results from previous studies with xylo-oligosaccharides showed a long lag phase and low specific growth rate for the *Bifidobacterium* species (Crittenden *et al.,* 2002). *Lactobacillus* species showed higher specific growth rates for fibre during the first 6 h of growth after the slow initial growth than the *Bifidobacterium* species and this may be linked to the shorter lag phase compared with the *Bifidobacterium* species. Specific growth rates varied with the dietary fibre substrate (Table 7.3).

7.5 Discussion

An evaluation of the amount of fermentation of individual probiotic species on different dietary fibre may help to understand polysaccharide fermentation in the human intestine. This study used dominant saccharolytic intestinal bacterial species to determine the ability of these microorganisms to ferment rice dietary fibre as the sole carbon source. Pure culture experiments aimed to reveal differences in the efficiency of the growth of *Bifidobacterium* species and *Lactobacillus* species on different fractions of dietary rice fibre.

The condition of the inocula has a strong impact on the duration of the lag phase of the organisms fermenting fibre. To minimize the differences in the lag phase of the microorganisms, inocula were obtained from healthy exponentially growing cultures. However, *Bifidobacterium* species showed a longer slow growth at early stages than the *Lactobacillus* species irrespective of the substrate (Fig 7.1 and Fig 7.2). A short initial slow growth phase suggests rapid adjustment of *Lactobacillus* species to the new environment and indicates that these bacteria did not require an initiation period to utilize fibre as a sole carbon source. Previous studies (Kaplan & Hutkins, 2000) with the fructooligosaccharides also showed a shorter lag phase for *L.acidophilus*, compared with *Bifidobacterium* species. Research with xylooligosacharides showed a 4 to 6 h lag

phase for *Bifidobacterium* species (Crittenden *et al.,* 2002).*Bifidobacterium* species showed a long slow growth phase and a long constant growth phase. In this study, the increase of number of cells of *Lactobacillus* species and *Bifidobacterium* species continued for up to 24 h indicating that organisms need 24 h to digest fibre (Fig7.2). Previous studies support the present results for *Lactobacillus* species, showing a similar duration for the log phase with oat bran as the substrate (Kontula *et al.,* 1998a). The decline in cell numbers of *Lactobacillus* species after 24 h may be due to the intolerance of the metabolites formed during growth, the low pH (Table 7.1) in the fermentation medium, or substrate limitation.

Bacterial strains, as individuals produced similar growth for IDF1/IDF2, SDF1/SDF2, and for TDF1/TDF2 for two rice types. This indicates a similar capability to use different fibre fractions of the two rice varieties (Fig 7.1 and 7.2), although these rice varieties do differ as explained in Chapter 4. This indicates that the probiotics tested have the ability to use rice fibre from different rice varieties.

From the biomas (relative to glucose) results, there were differences in the preference or ability of the different microorganisms to ferment glucose or rice fibre as a carbohydrate source. *B. longum* showed a preference for fibre rather than glucose, whereas *L. rhamnosus* grew better on glucose rather than rice fibre (Table 7.2). *L. rhamnosus* grew better on glucose than β-glucooligomers xylooligomers, raffinose and fructooligomers (Jaskari *et al.,* 1998b).

Some explanation is required for the differences in the relative biomas yield and viable cell counts at certain stages in the growth of the different microorganisms. The, biomass yield after 6 and 24 h incubation for *Bifidobacterium* species growing on fibre was higher than the *Lactobacillus* species, yet the viable cell count for the *Bifidobacterium* species was less than the *Lactobacillus* species. The high biomas

reading for the *Bifidobacterium* species may represent undigested fibre resulting in a higher optical density. *L. acidophilus* produced the maximum biomass at 48 h, whereas this species showed a reduction in viable cell count at 48 h compared with a 24 h sample. This may be due to the limitation in the availability of fermentable substrate and the production of toxic metabolites in the later stages of fermentation, resulting in a surplus of dead cells later in the fermentation (Table 7.2 and Fig. 7.1).

The specific growth rate (Table 7.3) of microorganisms was used to determine the fermentation capacity of the microorganisms for a substrate. The growth rate is depends on the rate of substrate hydrolysis, as this affects the availability of substrate to the bacteria (Macfarlane & Macfarlane, 1993). *Bifidobacterium* species had a higher specific growth rate than the *Lactobacillus* species at later stages of the growth, whereas *Lactobacillus* species had a higher specific growth at the initial stages growth. The higher specific growth rate of *Bifidobacterium* species at later stages may mean that the bacterium reached its fermentation capacity for certain substrates later, resulting in the formation of more metabolites than the *Lactobacillus* species at later stages of fermentation. This suggests that *Bifidobacterium* species are more capable of fermenting fibre over a longer period than the *Lactobacillus* species.

The degree of depolymerisation of the substrate affects the rate of fermentation. Cereal contains arabinoxylans, beta-D- Glucose (soluble), cellulose and lignin (insoluble), (Brennan & Cleary, 2005). Fermentation and the growth of organisms on fibre substrates depend on the linkages of the cell wall. The structure of fibre governs the availability of the substrate to the organisms for the fermentation and therefore, affects the metabolite formation. Quantitative data on this subject is limited.

pH measurement is an indicator of the metabolite (short chain fatty acid) formation by microorganisms and a good indication of the predilection for the substarte

during the fermentation (Table 7.1). The reduction in pH by *Bifidobacterium* species growing on glucose was less than the pH reduction by *Lactobacillus* species growing on the same substrate. This suggests that the *Lactobacillus* species are able to ferment glucose better than the *Bifidobacterium* species, producing SCFA. However, this does not tell us about the amount or type of SCFA produced.

This study indicates that the presence of a 1% (v/v) concentration of fibre in the medium enhanced the growth of probiotics. Similar results were observed by Yoshimoto *et al.,* (2005) for *Bifidobacterium* species with dietary fibre prepared from sweet potato. More research is needed to find out the optimum concentration of fibre to facilitate the growth of probiotics.

Probiotics showed differences in their preference for substrates. Generally *B. breve* utilized the substrate TDF1 most efficiently and L. *acidophilus* utilized TDF1 and IDF2 (LD356, AT353) and glucose, *L. rhamnosus* utilized the TDF1, IDF2, SDF2 and glucose and *B.longum* utilised SDF1 and TDF2 more than the other substrates. Among the fibre fractions, TDF was the most accessible fibre fraction for probiotics. This was shown by the highest viable count, pH decrease, and biomass for the fermentation of TDF by both species.

Differences in the preferences of *Lactobacillus* species and *Bifidobacterium s*pecies towards the different fibres has been reported (Crittenden *et al.,* 2002; Kontula *et al.,* 1998a; Kaplan & Hutkins, 2000). However, this is the first study on the fermentation of rice fibre, using probiotics.

7.6 Conclusion

The aim of this study was to determine the dietary factors that stimulate the growth of *Bifidobacterium* species and *Lactobacillus* species using rice fibre extracts to enable the maintenance of a healthy intestinal flora. Rice fibre promoted the growth of the *Bifidobacterium* species and *Lactobacillus* species. The two species behaved differently in the fermentation experiments, with *Bifidobacterium* species showing longer slow and constant growth compared with *Lactobacillus* species. *Bifidobacterium* species had a higher specific growth rate, than *Lactobacillus* species possibly due to enzyme-based specificity of the microorganisms on the different fractions of fibre. *Lactobacillus* species and *Bifidobacterium* species needed a minimum of 24 h to metabolise fibre. The presence of a 1 % (v/v) concentration of fibre in the medium enhanced the growth of probiotics. It was difficult to conclude the most preferred rice type based on the pH, OD, and the viable count for *Lactobacillus* and *Bifidobacterium* species tested in this work. Glucose was utilised poorly by *Bifidobacterium longum* and most efficiently by *Lactobacillus rhamnosus.* However, further studies are needed to determine what specific sugars constituents are in the extracted fibre stimulate the growth of the microorganisms. *In-vitro* studies with pure cultures are not ideal as a model of the human colon, to determine the fermentation of polysaccharides in the colon involves combination of different probiotic species (Topping & Clifton, 2001), thus this work needs to be followed up by studies on the fermentation of cereal fibres using co-cultures of known probiotics.

CHAPTER 8

Enhancement of Short Chain Fatty Acid (SCFA) Formation by Pure Cultures of Probiotics on Rice Fibre

8.1 Abstract

The influence of rice fibre fermentation on the formation of short chain fatty acids (SCFA) using four probiotics was investgated. Dietary fibre was extracted from two commercially available Sri Lankan rice varieties; LD 356 (brown rice, red in colour), AT353 (unpolished rice, red in colour) and separated into fibre fractions of total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). Four probiotic species (*Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium breve)* were grown on the medium containing rice fibre fractions; IDF, SDF, TDF or glucose as the sole source of carbon for fermentation. SCFA production of the probiotics was measured at 0, 6, 12, 18, 24, and 48 h using gas liquid chromatography (GLC).

Incubation with total dietary fibre (TDF) resulted in a steady rate of SCFA production over 24 h of fermentation with the genus *Lacobacillus*, and 48 h with the genus *Bifidobacterium*. SCFA formation among the fibre fractions followed the pattern of TDF>SDF>IDF irrespective of rice variety, indicating that TDF is the best possible dietary fibre for SCFA production.

All four probiotics utilized the rice fibre with the main end products being acetate, propionate, and butyrate. Acetate was the most abundant SCFA formed comprising 50-85% of total SCFA at different time points by all probiotics. The

quantity of different SCFA's produced was acetate>propionate >butyrate. The ratio of acetate: propionate: butyrate was lower for the genus *Bifidobacterium* with a higher proportion of butyrate produced by *Bifidobacterium* than genus *Lactobacillus*. The ratio of acetate to propionate was lowest with *Bifidobacterium breve* and highest with *Lactobacillus acidophilus*.

Lactobacillus species digested only 60%-80% of the rice fibre whereas *Bifidobaceria* digested 75%-85%. None of the organisms were able to digest the fibre completely during the incubation period.

The results from this study will help to prepare new food products through an understanding of the effect of individual organisms on digesting dietary fibre.

8.2 Introduction

The impact of dietary fibre as carbon sources for fermentation has been studied using faecal cultures **(**McNail *et al.,* 1978; Topping, 1991; Topping & Illman 1986; Topping & Clifton 2001; Wang & Gibson, 1993)**.** In these experiments SCFA measurements were largely confined to faecal samples and to faecal cultures **(**McNail *et al.,* 1978; Rossi *et al.,* 2005; Topping & Clifton 2001; Wang & Gibson, 1993). Faecal cultures comprise of a large variety of different organisms, but give no information on the role of individual organisms on the digestion of dietary fibre. Pure cultures provide more specific information than mixed cultures on the contributions of dietary fibre or sugars to SCFA formation (Belenguer *et al.,* 2006; Duncan *et al.,* 2004) in the human gut.

Previous research used *in-vivo* methods to investigate SCFA formation using pure cultures of *Lactobacillus* species and *Bifidobacterium* species (Kaplan & Hutkins, 2000; McKellar & Modler, 1989; Gibson & Wang, 1994a; Hopkins *et al.,* 1998; Perrin *et al.,* 2001**;** Palframan *et al.,* 2003; Stewart, *et al.,* 2009; Yamazaki & Dilawri, 1990)

with dietary fibre, or components of fibre. Some studies have used *in-vitro* methods to understand the impact of dietary fibre on selected probiotics (Goderska *et al.,* 2008

Different types of dietary fibre isolates have been used as prebiotics to understand how these fibres can influence the formation of SCFA by probiotics (Crittenden & Playne, 1996). Studies have identified a large number of dietary fibre fractions (inulin, oligofructose, lactulose, galacto-oligosaccha-rides (GOS), transgalacto-oligosaccharides (TOS), and iso-maltooligosaccharides (IMO), that act as prebiotics (Blaut, 2002; Fooks *et al.,* 1999; Rastall & Maitin, 2002). However, few studies have examined the prebiotic activity of insoluble dietary fibre, soluble dietary fibre and total dietary fibre, from the same food source (Titgemeyer *et al.,* 1991) rather than the isolates.

The synergy between probiotics and prebiotics has led to the development of food which is known as symbiotic (Roberfroid, 1998; Ziemer & Gibson, 1998). It is important that probiotics are viable in the presence of the prebiotic so that they produce expected metabolites such as SCFA. The main objective of this study was to understand the relationship between the growth of microorganisms on rice fibre and SCFA formation.

8.3 Materials and Methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, bacterial strains, preparation of cell suspensions, preparation of growth medium, *In-vitro* fermentation, determination SCFA formation, determination of percentage of indigestible fibre quantity and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.2, 3.6.4, 3.6.5, 3.5.6, 3.10, 3.11 and 3.12, respectively.

8.4 Results

For this experiment, only two rice varieties were used (RR1 and RR2). RR1 and RR2 are fibre rich sources of food and had a higher quantity of fibre than white rice (WR1 and WR2). Therefore, it was decided to use these two rice varieties in this experiment to understand the SCFA formation. Fibre was extracted from these rice varieties as SDF, IDF and TDF. Extracted fibre fractions from rice variety RR1 is known as IDF1, SDF1 and TDF1 and extracted fibre fractions from rice variety RR2 is known as IDF2, SDF2 and TDF2. These non starch polysaccharide fractions were subject to fermentation with pure cultures of probiotics.

Table 8.1 indicates the individual ability of microorganisms to produce SCFA and their preferred substrate at 24 h. SCFA formation by pure cultures at 0, 6, 12, 18 and 48 h is described in Appendix B. The microorganisms produced more acetate than propionate and butyrate.

Table 8.2 indicates the relative percentage of SCFA formation at 24 h. Relative percentage of SCFA formation by pure cultures at 0, 6, 12, 18 and 48 h is described in Appendix B. The relative percentage of acetate (Table 8.2) was higher in fermentations using *Lactobacillus* species than the *Bifidobacterium* species after 6 h (Appendix B). However, the ratio between acetate, propionate and butyrate was lower with the *Bifidobacterium* species than with the *Lactobacillus* species (Appendix B).

Table 8.1 Fermentation of rice dietary fibre with probiotics leading to the formation of short chain fatty acids (SCFA, m moles/100mL)

A) At 24h

RICE	Fibre	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.53±0.01	0.16	0.06	0.45±0.02	0.21	0.07	0.50±0.01	0.20	0.15	0.58	0.18	0.11
	SDF1	0.63±0.01	0.16	0.06	0.79±0.02	0.23	0.08	0.63±0.04	0.21	0.15	0.68	0.19	0.12
	TDF1	0.73±0.06	0.17	0.07	0.89 ±0.2	0.29±0.01	0.09±0.01	0.77±0.02	0.24	0.25	0.73±0.02	0.22±0.01	0.11
RR2	IDF2	0.51±0.02	0.14	0.06	0.72±0.01	0.18	0.07	0.45±0.01	0.14	0.14	0.55±0.01	0.17±0.01	0.10
	SDF2	0.54±0.02	0.15	0.06	0.86±0.02	0.20	0.07	0.57±0.02	0.17	0.16	0.60±0.03	0.18	0.11
	TDF2	0.63±0.01	0.16	0.06	0.98±0.02	0.23±0.01	0.08	0.72	0.23	0.18	0.66	0.20	0.13
	GLUCOSE	0.76±0.03	0.24	0.10	1.52±0.04	0.35	0.14	1.57±0.1	0.21	0.22	0.54±0.05	0.22	0.09
	CONTROL	0.31	0.04	0.03	0.32±0.01	0.04	0.03	0.30	0.04	0.03	0.32±0.01	0.01	0.03

B) At 48 h

RICE	Fibre	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.56±0.01	0.15	0.06	0.40±0.01	0.15	0.06	0.64±0.05	0.22	0.23	0.60±0.01	0.21	0.16
	SDF1	0.61	0.16	0.07	0.51±0.02	0.16	0.06	0.85±0.01	0.23	0.24	0.65±0.03	0.24	0.17
	TDF1	0.72±0.03	0.17	0.08	0.58	0.19	0.08	1.07±0.02	0.24	0.34	0.93±0.05	0.28	0.18
RR2	IDF2	0.55	0.14	0.06	0.52±0.04	0.14	0.05	1.08±0.04	0.21	0.24	0.49	0.19	0.14
	SDF2	0.59	0.14	0.06	0.60	0.15	0.06	0.91±0.01	0.22	0.25	0.51	0.20	0.15
	TDF2	0.67	0.15	0.07	0.67	0.17	0.06	1.06	0.22	0.26	0.59±0.06	0.21	0.15
	GLUCOSE	0.72±0.01	0.19	0.09	0.48±0.02	0.21	0.09	1.68±0.09	0.31	0.33	0.56	0.18±	0.16
	CONTROL	0.32±0.01	0.04	0.03	0.29	0.04	0.03	0.33	0.04	0.03	0.39	0.00	0.03

Results are expressed as the mean values of two trials± standard error.

Table 8. 2 Relative percentage of SCFA formation by probiotics at 24h

Rice	Fibre	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF 1	84.22±0.07	9.28	6.50±0.07	61.48±0.85	28.28±0.63	10.24±0.21	59.10±0.44	23.53±0.26	17.37±0.19	67.11±0.02	20.74	12.16±0.02
	SDF 1	85.69±0.2	8.36±0.14	5.95±0.05	71.26±0.19	21.08±0.07	7.66±0.12	63.38±0.77	21.22±0.32	15.40±0.45	69.33±0.2	19.24±0.12	11.43±0.07
	TDF 1	87.72±1.02	7.48±0.72	4.81±0.3	70.06±0.16	22.66±0.3	7.28±0.46	60.73±0.44	19.22±0.2	20.05±0.24	68.29±0.09	20.63±0.14	11.09±0.23
RR2	IDF 2	83.72±0.58	9.71±0.35	6.57±0.23	74.39±0.19	18.87±0.14	6.74±0.05	61.85±0.54	18.77±0.38	19.38±0.17	67.04±0.92	20.85±0.81	12.10±0.12
	SDF 2	84.87±0.46	8.67±0.34	6.46±0.12	76.26±0.80	17.64±0.55	6.11±0.24	63.55±0.62	18.78±0.06	17.67±0.56	67.80±0.86	20.32±0.54	11.88±0.32
	TDF 2	87.60±0.15	7.10±0.11	5.30±0.04	75.79±0.07	18.08	6.13±0.07	63.97±0.17	20.34±0.21	15.69±0.05	67.35±0.19	20.15±0.04	12.50±0.23
	GLUCOSE	81.78±0.61	11.13±0.43	7.09±0.18	75.73±0.27	17.32±0.05	6.95±0.21	78.61±0.86	10.39±0.44	11.00±0.42	59.95±1.82	24.48±1.11	15.57±0.71

Results are expressed as the mean value of two trials± standard error.

Percentage of acetate, propionate, and butyrate are calculated from the total SCFA formation at particular time point

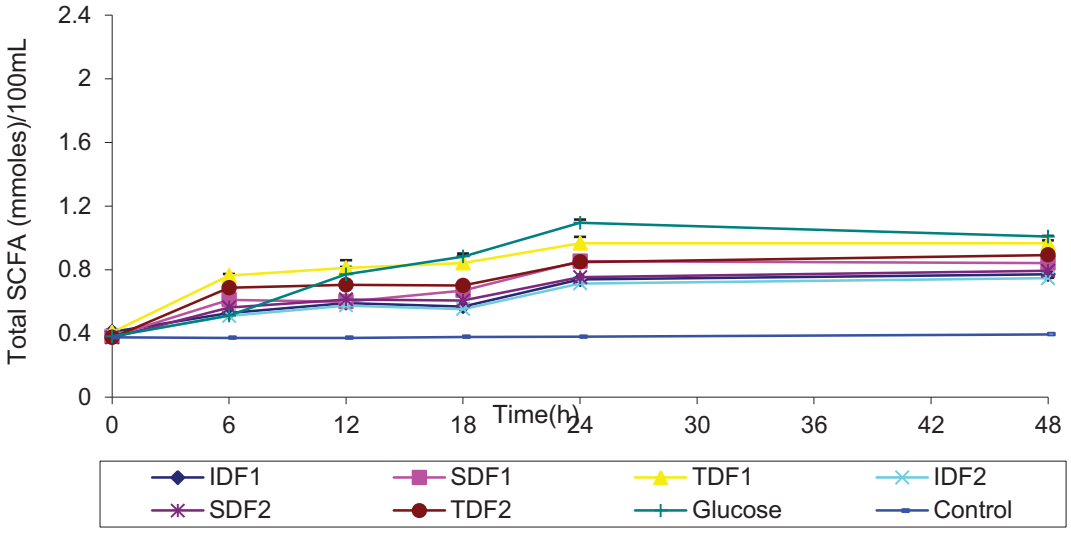
The ability to metabolise dietary fibre depends on the time and genera. As seen in Table 8.3, the *Lactobacillus* species in most cases metabolise more than 50% of the fibre within the first 24 h of 48 h fermentation. However, the *Bifidobacteriium* species fermented less than 50 % of the fibre within the first 24 h.

Table 8. 3 Percentage of substrate remaining after each time point

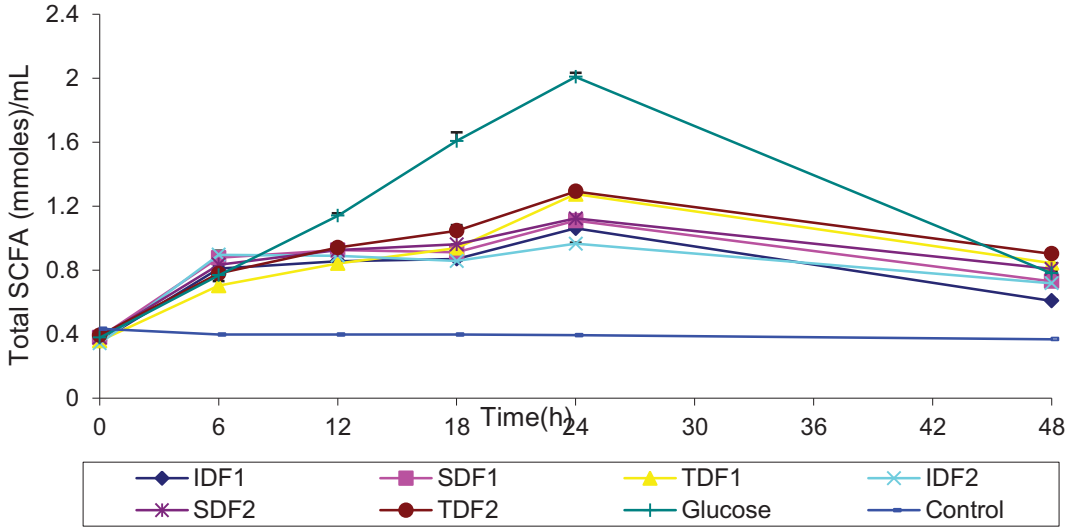
Probiotics	H	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2
Lactobacillus acidophilus	6h	83.8±6	79.7±2.1	72.5±0.9	86.8±1.1	76.6±1	84.6±4.4
	12h	69.3±5.5	70.4±2.2	68.4±1.4	79.2±1.1	69.3±3.3	78.1±2.5
	18h	64.9±1.1	62.7±3.3	68.2±1.6	70.4±2.2	63.80	74.37±1.1
	24h	41.8±4	44.8±3	58.8±1	44.7±1	45.8±2	60.4±0.71
	48h	31.2±0.6	42.1±0.3	20.3±0.1	32.2±2.1	38.7±0.9	39.4±0.22
Lactobacillus rhamnosus	6h	67.7±5.9	70.7±3.1	86.1±1.1	78.7±3.1	78.7±5.1	84.53±2.35
	12h	57.2±2.2	66.00	79.9±1.3	67.1±3.3	72.60	79.12±.46
	18h	46.2±2.2	58.3±3.3	76.1±1.3	57.2±4.4	66±2.2	68.24±7.4
	24h	29.8±4	34.7±3.1	50.8±2.4	40.8±4	38.7±0.9	46.05±1.66
	48h	16.9±1.06	29.8±2	25.32±0.16	31.7±1	27.8±2	26.67±2.05
Bifidobacterium breve	6h	83.58±2.22	78.7±0.9	97.4±2.4	84.7±3.1	78.7±3.1	81.52±3.5
	12h	71.28±5.72	69.3±3.3	89.7±3.1	73.7±3.3	72.60	80.53±1.45
	18h	69.08±5.72	66±2.2	79.6±2.2	66±4.4	67.1±1.1	68.24±7.4
	24h	47.58±5.78	55.7±4.1	65.2±3.4	63.6±4	60.7±3.1	46.05±1.65
	48h	19.6±2	14.8±3	27.94±0.16	22.8±1	19.7±2.1	26.67±2.05
Bifidobacterium longum	6h	79.7±6.1	82.7±3.1	91.38±2.4	88.7±5.1	81.52±3.5	81.52±3.5
	12h	72.6±4.4	72.60	85.71±3.1	82.5±1.1	75.9±1.1	78.53±3.45
	18h	67.1±1.1	69.3±1.1	82.589±2.2	75.9±3.3	70.4±2.2	73.26±2.36
	24h	66.8±1	58.7±0.9	71.4±3.4	65.7±2.1	59.6±2	62.11±2.09
	48h	29.7±4.1	23.8±2	22.6±0.92	28.7±5.1	24.7±5.1	14.43±0.47

Results are expressed as the average of two trials±standard error.

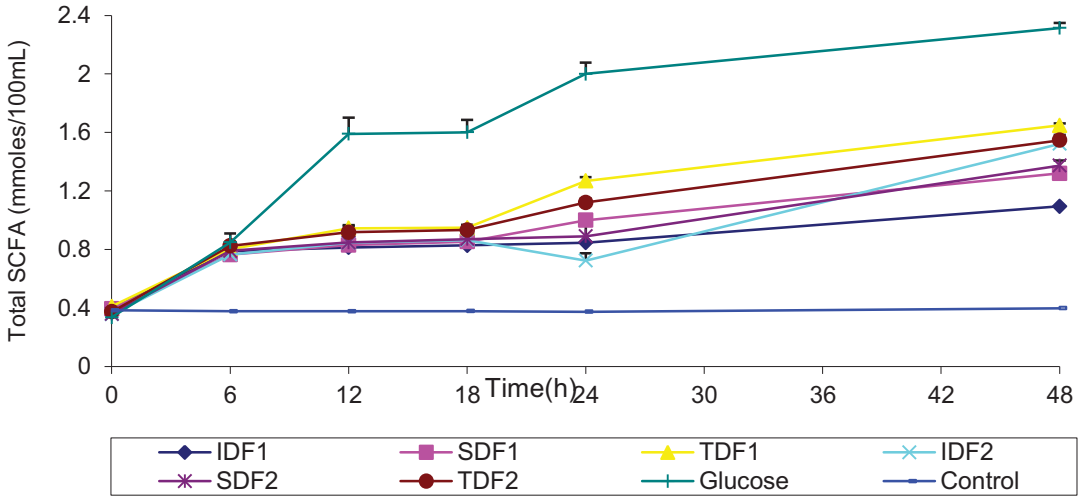
The pattern of SCFA formation differed between different microorganisms and rice varieties (Fig 8.1). The *Lactobacillus* species produced less total SCFA than the *Bifidobacterium* species after 24 h.



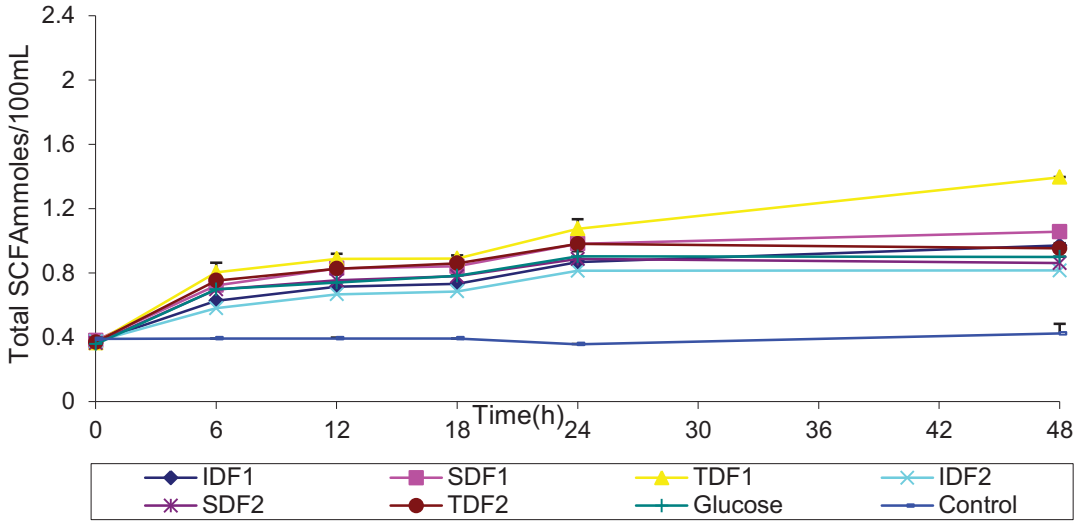
A) *Lactobacillus acidophilus*



B) *Lactobacillus rhamnosus*



C) *Bifidobacterium breve*



D) *Bifidobacterium longum*

Figure 8.1(A-D) Total SCFA formation by probiotics on RR1 and RR2 rice varieties

Results are expressed as the averages of two trials±standard error.

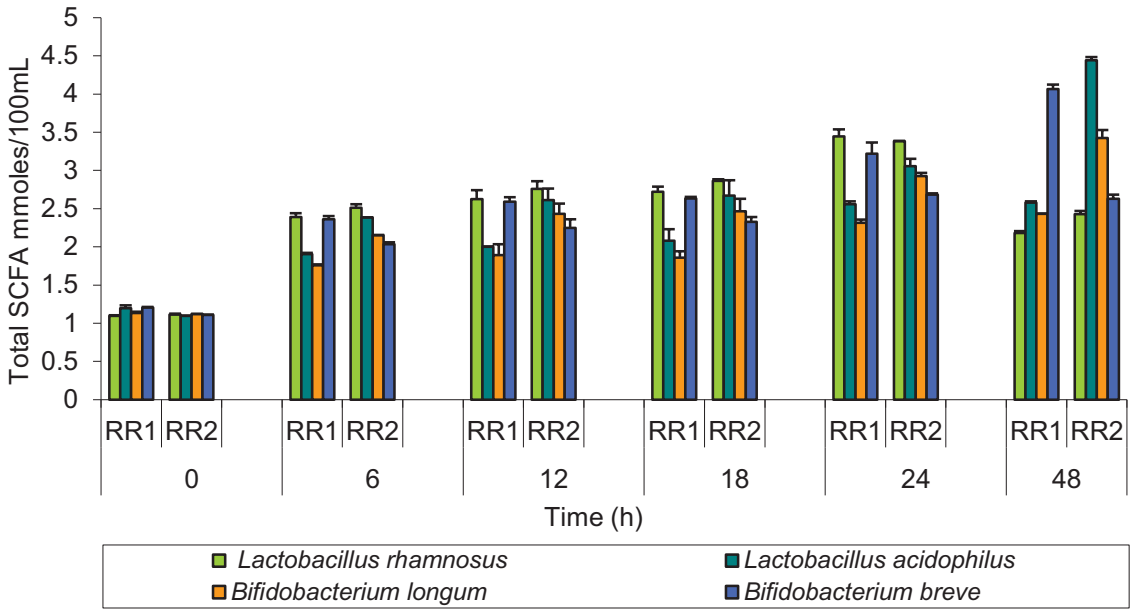
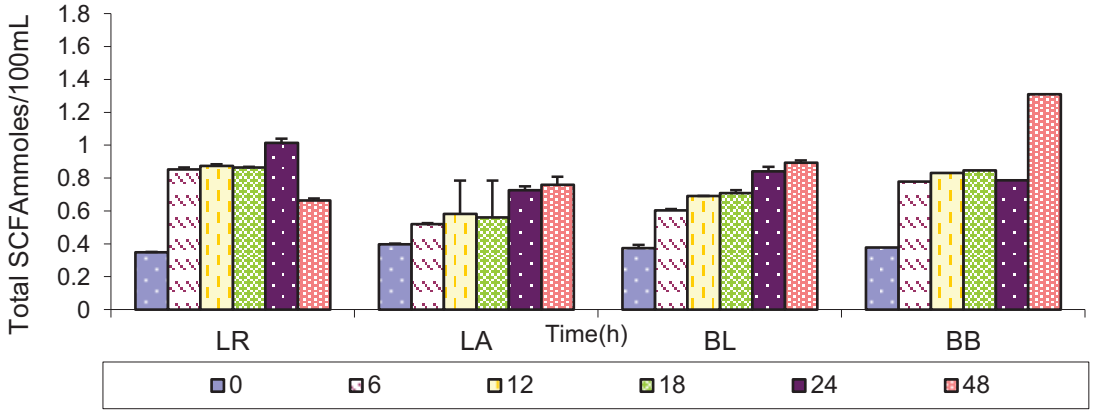


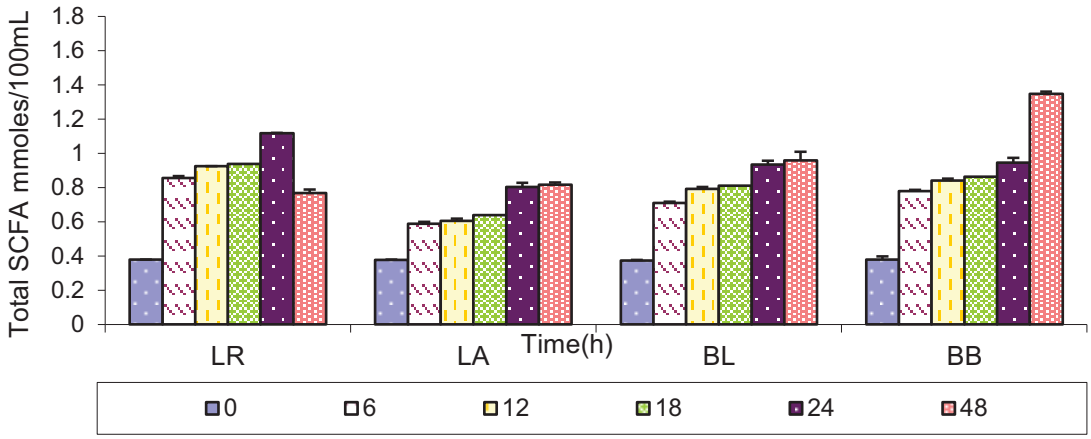
Figure 8.2 Total SCFA formation on rice varieties

Results are expressed as the averages of two trials± standard error.

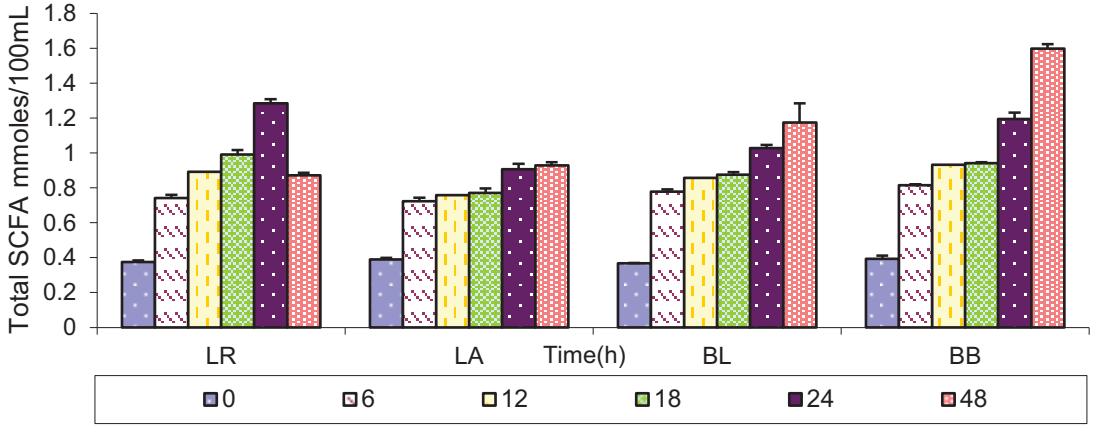
All microorganisms showed no significant differences in the ability to utilise rice varieties ($p>0.5$) (Fig 8.2). However, the microorganisms differed in the time required to ferment the substrates with *Lactobacillus rhamnosus* completing most of the fermentation within 24 h, while the *Bifidobacterium* species required 48 h.



A) IDF



B) SDF



C) TDF

Figure 8.3 Total SCFA formation from IDF, SDF and TDF

Results are expressed as the averages of two trials±standard error.

The microorganisms produced different amounts of SCFAs at different sampling times during the fermentation of IDF, SDF and TDF. However, the difference in SCFA formation was not clear for *Bifidobacterium longum* after 6 h fermentation for IDF and SDF. Although there was more SCFA produced for TDF than IDF or SDF, this difference was not significant ($p>0.5$).

8.5 Discussion

In this study, the fermentation of insoluble dietary fibre, soluble dietary fibre, and total dietary fibre of rice, by pure cultures of probiotics led to a significant increase in SCFA ($p<0.05$) (Table 8.1). All the probiotic strains on rice fibre formed SCFA, especially acetate, at almost twice the level compared with the negative control which contained only the growth media without fibre and probiotics (Table 8.1). The low SCFA profile from the control fermentation flask indicates the components of the medium and the substrate did not contribute to SCFA. This observation was contrary to the previous work on the fermentation of dietary fibre with faecal inocula (Stewart *et al.,* 2009) where there was more SCFA in the control fermentation than for the fermentation on fibre and it is in agreement with other research on different types of fibre using pure cultures and mixed faecal cultures (Beatrice & Michael, 2006; Sembries *et al.,* 2003).

The higher concentration of SCFA produced from the fermentation of TDF when compared with other fibre fractions of both rice varieties shows that this substrate contains more fermentable material than the individual SDF and IDF fractions. Most of the SCFA from the fermentation of TDF using *Lactobacillus* species was produced in 24 h, while 48 h was required for the genus *Bifidobacterium*. A comparison between the SDF and IDF as fermentation substrates showed SDF had more fermentable fibre and produced more SCFA than the IDF. This result was in agreement with previous work on different fibre as cellulose and dietary fibre in common clinical use, such as Inolaxol, Fibreform, Vi-Siblin, Lunelax, pectin (Mortensen & Nnordgaard-Aandersen, 1993). Generally SCFA formation from the fermentation of rice fibre fractions followed the pattern: TDF>SDF>IDF, irrespective of the rice variety (Table 8.1).

The growth of microorganisms on glucose used to prepare inocula for these experiments ensured the bacteria were metabolically active during this study. In this study glucose was used as the positive control, since it is readily available for bacterial digestion.

Total SCFA produced from the microbial fermentation of complex dietary fibre have been identified by several investigators to be linearly substrate dependant (Cummings & Englyst, 1987; Casterline *et al.,* 1997; Mortensen *et al.,* 1988, 1991) when combinations of microorganisms are used. In those experiments increasing concentrations of glucose, wheat bran, pectin, cellulose, or albumin inoculated with human faecal homogenates and incubated for 24 h resulted in a linear increase in the production of short-chain fatty acids. Quantities and ratios of short-chain fatty acids produced were dependent on the type of substrate fermented. However, the current study did not find a significant difference ($p>0.05$) in SCFA formation for the rice varieties used (RR1 and RR2), which may have been due to the fact that mixtures of bacteria were not used in the fermentation (Table 8.1 and Table 8.2) compared with the published experiments. However, studies on pure cultures are important so as to understand the contribution of specific bacteria to the fermentation of dietary fibre to assess the fermentation capacity in the human gut.

The rice varieties, RR1 and RR2 produced similar amounts of SCFA when fermented with the individual probiotics (Fig 8.1 and Fig 8.2). This current study showed that *Lactobacillus acidophilus* (Fig 8.1 and Fig 8.3) has the least ability to utilize RR1 and RR2, and that *Lactobacillus acidophilus* is more active during the first 24 h than the second 24 h. Previous studies have indicated that variations in SCFA formation may be due to the chemical and structural differences between fibres (Stewart *et al.,* 2009). This study indicates that the effect of chemical and structural differences

on SCFA formation was minimal for the individual organisms grown on these rice fibres. However, this should be confirmed using other sources of fibre.

The percentage of indigestible material (Table 8.3) indicates that none of the microorganisms was able to digest the fibre completely during the incubation period, *Lactobacillus* species digested 60%-80% and *Bifidobacterium* digested 75%-85% of the rice fibre. It is important to note that recovered dry matter may be contaminated with the microbial cell mass even though methods were used to remove bacteria. Therefore, the digestion percentage may be higher than it appears. The substrate used was related to the SCFA production, with an increase in SCFA corresponding to a lower level of dry matter remaining after fermentation. The amount of substrate remaining after 48 h fermentation suggests that rice fibre will require a long retention time in the human colon in order to complete the digestion. However, 48 h incubation may be sufficient to yield the optimal concentration of SCFA from the *in-vitro* fermentation from the microorganisms studied (Fig 8.2). Among the *Lactobacillus* species, *Lactobacillus rhmanosus* was the most efficient ($p<0.05$) in the digestion of rice fibre and *Bifidobacterium breve* was more efficient ($p<0.05$) than *Bifidobactrium longum* (Fig 8.3). *Bifidobacterium breve* was the most efficient ($p<0.05$) at digesting rice fibre fractions at 48 h and *Lactobacillus rhmanosus* was the most efficient ($p<0.05$) at digesting rice fibre fractions at 24 h. This result confirms that *Bifidobacterium breve* was the most efficient tested organism in digesting fibre fractions within 48 h of incubation.

The formation of acetate was most favoured by the probiotics while butyrate and propionate were comparatively ($p<0.05$) less favoured in this experiment. Acetate comprised of 50-85 % total SCFA at different time points by all strains (Table 8.2,). Previous work on different fibre fractions resulted in higher acetate and lactate

formation than other SCFA by *Bifidobacterium* and *Lactobacillus* species (Garrigues, *et al.,* 1997, 2001; Melchiorse, *et al.,* 2002; Vlkova, *et al.,* 2002).

Both rice varieties (RR1 and RR2) contributed to acetate production and the difference between them was not significant ($p>0.05$). This indicates that the milling grade of these two selected rice varieties had little effect on the formation of SCFA with selected probiotics. It also suggests that the composition of fibre of these rice varieties contains similar amounts of fermentable substrates such as pectin or arabinoxylan. However, these two rice varieties vary in the percentages of IDF, SDF and TDF (Chapter 4).

A low ratio of acetate to propionate has been suggested to inhibit cholesterol synthesis from acetic acid in the liver (Wright *et al.,* 1990; Wolever *et al.,* 1991; 1996). Among the organisms tested, *Bifidobacterium* species generally showed a lower ratio for acetate to propionate than the *Lactobacillus* species (Table 8.1 and 8.2) at the 24 and 48 h. This may be the most effective choice of probiotics for a functional food incorporating probiotics with rice fibre. This is an important finding as the formation of propionate and butyrate from the *Bifidobacterium* species has been rarely reported.

Previous studies have shown that *Lactobacillus rhamnosus* has the ability to increase the serum propionate level (Vogt *et al.,* 2004) in the human body. The current investigation observed low propionate compared to acetate by *Lactobacillus rhamnosus* and a lower ratio of acetate to propionate than *Lactobacillus acidophilus*, more or less similar to the *Bifidobacterium* species tested. An increased faecal *Bifidobacterium* species count has been observed with the increase of propionic acid in human clinical studies (Mårtensson *et al.,* 2005). The observation of a higher viable count of the *Bifidobacterium* and *Lactobacillus* species during the later stages (Chapter 7) of fermentation of the current work may indicate the use of propionic acid for the growth

of the probiotics tested. Therefore, the resultant amount of propionic acids in this study may be lower**.**

The ratio of acetate: propionate: butyrate was lowest with the *Bifidobacterium* species, and a higher proportion of butyrate was formed by the *Bifidobacterium* species than by the *Lactobacillus* species (Table 8.2). Therefore, *Bidfidobacterium* species had a lower ratio of acetate to propionate and a higher proportion of butyrate than the *Lactobacillus* species and this is important for human health. For example, the colonic mucosa obtains energy by oxidizing mainly SCFAs in the order of butyric> propionic> acetic acid (Clausen & Mortensen, 1994). This is described in detail in Chapter 2.l.

Changes in the biochemical characteristics of the dietary fibre after *in-vitro* fermentation could also contribute to the different concentrations of SCFAs. For example, Lambo *et al.* (2005) observed that the physico-chemical properties, (viscosity and the quantity of fibre) lowered after incubation with a mixture of lactic acid bacteria. *Bifidobacterium breve* produced more acetate after 24 h, and *Lactobacillus rhamanosus* formed more acetate during the first 24 h of incubation. The *Bifdobacterium* species produced a greater proportion of propionate and butyrate than the *Lactobacillus* species; However, *Lactobacillus rhamnosusus* produced more propionate at 24 h compared with *Lactobacillus acidophilus*. These results suggest that the *Bifidobacterium* species have the ability to form individual SCFA from rice fibre more efficiently for a longer period than the *Lactobacillus* species.

Previous studies found that the relative proportions of acetate, propionate, and butyrate production are related to the monosaccharide composition of the fibre (Mortensen *et al.,* 1988). However, results from this study propose that the profile of SCFA is governed mainly by the microorganisms. Individual SCFA formation can be maximized by selecting the organisms on the basis of their ability to form particular

SCFA. This factor is important when microorganisms are combined with food to form food supplements for specific health purposes.

In the human digestive system, the formation of SCFA from fibre depends on the retention time of the substrate in the colon (Titgemeyer *et al.,* 1991**).** However, in batch fermentation, SCFA formation is dependent on the availability of the substrate for the digestion by microorganisms. The availability of substrate is determined by the hydration of substrate prior to the fermentation and the rate of substrate disappearance. As seen in this study, the pure cultures used fibre at different rates (Table 8.3). Fibre was extensively fermented by the *Lactobacillus* species during the first 24 h and for *Bifidobacterium* after

48 h.

The formation of SCFA from rice dietary fibre by selected probiotics in the current investigation could also be interpreted as a probable indication of the survival of these microorganisms in the gastrointestinal tract. Acetic acid formation by these microorganisms might help to convert propionic and butyric acid via the microorganisms in the human gut. The production of butyric acid is formed by the conversion of acetic acid and lactic acid by acid microorganisms such as *Megaspaera elsdenii* (Tsukahara *et al.,* 2002). These organisms will play a major role in the balance of the microbial gastrointestinal flora by fermenting rice fibre and increasing the number and activity of endogenous bacteria of benefit to human health.

This study supports the use of rice fibre as a prebiotic for the micro-oranisms tested. However, in the GI tract, microorganisms do not ferment the fibre as individual organisms. To extrapolate the *in-vitro* studies to *in-vivo* studies, digestion has to be studied when organisms work synergistically. There is a need to identify the optimal gut microbial communities to ferment fibre in the human gut.

8.6 Conclusion

Rice fibre facilitates the formation of acetate, propionate, and butyrate by fermentation with *L. acidophilus*, *L. rhamnosus*, *B. breve*, and *B. longum*. The formation of SCFA followed the pattern acetate>propionate>butyrate for all the strains tested. The acetate to propionate ratio was lower for the *Bifidobacterium* species than the *Lactobacillus* species. The *Bifidobacterium* species produced most SCFAs after 48 h fermentation while the *Lactobacillus* species required less than 24 h. Among the dietary fibre fractions, TDF produced more SCFA than the SDF and IDF. However, glucose did not produce more SCFA than the rice dietary fibre for most of the strains. An examination of the indigestible percentage of substrate indicated that none of the organisms were able to digest the fibre completely during the incubation period. However, the *Lactobacillus* species digested 60%-80% of rice fibre and Bifidobacteria digested 75%-85%. The *Bifidobacterium* species were the most efficientt microorganisms tested for the digestion of rice fibre and the formation of SCFA. In summary, *L. acidophilus* had the least ability to utilize RR1 and RR2 and *B. breve* was the most efficient in using rice dietary fibre.

The results from this study are important to understand the behavior of different dietary fibres capable of producing different levels of SCFA in the human colon. Knowing the characteristics of microbial fermentation in the colon can help food technologists when preparing foods containing probiotics. The results from this experiment suggest that dietary fibre can be used by lactic acid bacteria. This is supported by the work on the fermentation of fructooligosaccharides with probiotics (Kalplan & Hutkins, 2000).

CHAPTER 9

The Effect of Rice Fibre Fractions on the Growth of Co-Cultures of Probiotics

9.1 Abstract

The fermentation of rice dietary fibre using nine co-cultures of four probiotics was measured by the viable counts, pH, optical density, specific growth rate and biomass over 48 h incubation. The results from the fermentation of the soluble, insoluble and total dietary fibre (SDF, IDF, and TDF) of two rice varieties RR1 and RR2 were compared.

Overall, there was no significant difference ($p>0.05$) in the fermentation of the six different rice fibre fractions. However, the co-cultures showed a preference for glucose as a fermentation substrate rather than the fibre fractions.

Bifidobacteria produced a higher cell counts than the *Lactobacillus* species after 24 h and 48 h of fermentation ($p<0.05$). There was evidence of synergistic activity with increased growth when *Lactobacillus* and *Bifidobacterium* species were grown together. Growth was limited by the pH reaching 4.2- 4.5. Specific growth rates of the co-cultures varied for different culture combinations. Combinations of the same species produced less biomass than combinations of mixed species. *Bifidobacterium breve* + *Bifidobacterium longum* + *Lactobacillus rhamnosuss* produced most growth in TDF, SDF and IDF from both rice varieties and glucose as substrates.

These findings showed that synergism between microorganisms within the co-cultures affected the degree of fermentation of dietary fibre.

9.2 Introduction

Microorganisms in the human gut have the potential to hydrolyse and ferment dietary fibre (Gibson & Roberfroid, 1995). To study this fermentation, an *in-vitro* fermentation system is more practical than expensive human and animal trials (Stevenson *et al.,* 1997). Among the 400 species of bacteria in the human gut (Tannock, 1995), *Bifidobacterium* and *Lactobacillus* genus are the most important (Gibson, 1998; Mitsuoka, 1990) in terms of digestion, absorption of nutrients, prevention of colonisation by pathogens (Lankaputhra *et al.,* 1996; Shin *et al.,* 2000a), and stimulation of immune responses (Yaeshima, 1996).

Oligosaccharides are selectively used by all *Bifidobacterium,* and some *Lactobacillus* and *Bacteroides* species (Bouhnik *et al.,* 1997; Smiricky-Tjardes *et al.,* 2003; Tzortzis *et al.,* 2005). These microorganisms in the human gut work as a consortium (Tannock, 1995). To understand the fermentation of dietary fibre by microorganisms, the following study used combinations of probiotics.

The aim of the current work was to study the fermentation of the rice dietary fibre by mixed cultures in order to understand the growth kinetics associated with this fermentation.

Measuring culture viability in a fermentation medium is believed to be a good model to understand the probiotic activity in the human gut. The emphasis of this work was to determine the ability of co-cultures to achieve high viable cell populations using rice fibre fermentation. A concentration of approximately 10^7 cells mL^{-1} as the inoculum, is considered suitable (Gomes & Malcata, 1999; Shortt, 1999) to initiate the utilization of dietary fibre.

High cell growth and acidification rates helped to reduce the fermentation time and boost the viability of the specific strain in the fermentation medium (Marklinder & Lönner, 1992) and presumably in the human gut.

Physiological and taxonomical characteristics of the *Lactcobacillus* and *Bifidobacterium* genus are important to select the most effective combinations for fermentation. Lactobacilli are fastidious microorganisms, and require fermentable carbohydrates, amino acids, vitamins of the B-complex, nucleic acids and minerals to achieve a high cell yield. Nutrient requirements are dependent on the strains of lactobacilli (Gomes & Malcata, 1999). In contrast, the genus *Bifidobacterium* has the ability to utilise a wider variety of nutrients, thus they have the potential to adjust and compete for nutrients with other organisms in the environment (Crittenden, 1999). Therefore, substrate composition and nutritional requirements of the strain affect the overall performance of the microbial combination. Microbial growth also depends on extrinsic factors such as the pH, temperature and accumulation of metabolic end products in the fermentation medium (Ganzle *et al.,* 1998; Mercier *et al.,* 1992).

Fermentation, following the inoculation of rice fibre with combinations of microorganisms, was measured using viable cell counts, biomass formation, acidification and the specific growth rate.

This is the first study to report the growth kinetics of co-cultures of probiotics on rice fibre.

9.3 Materials and methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, co-cultures, preparation of cell suspensions, preparation of growth medium, *in-vitro* fermentation, determination of pH, optical density and viable count, specific

growth rate, determination of the growth yield and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.3, 3.6.4, 3.6.5, 3.5.6, 3.7 and 3.12, respectively

9.4 Results

This study used two rice varieties (RR1 and RR2). Fibre was extracted from these rice varieties as SDF, IDF, and TDF and fibre fractions from rice variety RR1 were labelled IDF1, SDF1 and TDF1 and fibre fractions from rice variety RR2 were labelled as IDF2, SDF2, and TDF2. These non starch polysaccharide fractions were fermented with co-cultures of probiotics. The current study focused on the growth kinetics of co-cultures on different fibre fractions.

In this study, nine combinations of probiotics were selected from the following microorganisms (*L. acidophilus, L. rhamnosus, B. breve, B.longum*) with most of the combinations incorporating the *Bifidobacterium* species. Previous work (Chapters 7 and 8), showed that *Bifidobacterium* species are more efficient than *Lactobacillus* species in fermenting rice fibre, therefore, most of the combinations used in Chapter 9 and Chapter 10 contained *Bifidobacterium.*

The viable cell count differed from one co-culture to another for different rice varieties (Fig 9.1 and Fig 9.2). The differences were significant ($p<0.05$) when comparing co-cultures formed from members of the same genus with co-cultures formed from members of different genus. However, all co-cultures were able to use both rice varieties as substrates. Co-cultures formed from more than two strains produced higher viable counts than the other combinations. Co-cultures produced maximum numbers of viable cells after 24 h incubation, suggesting that this is the optimal time for the fermentation of fibre.

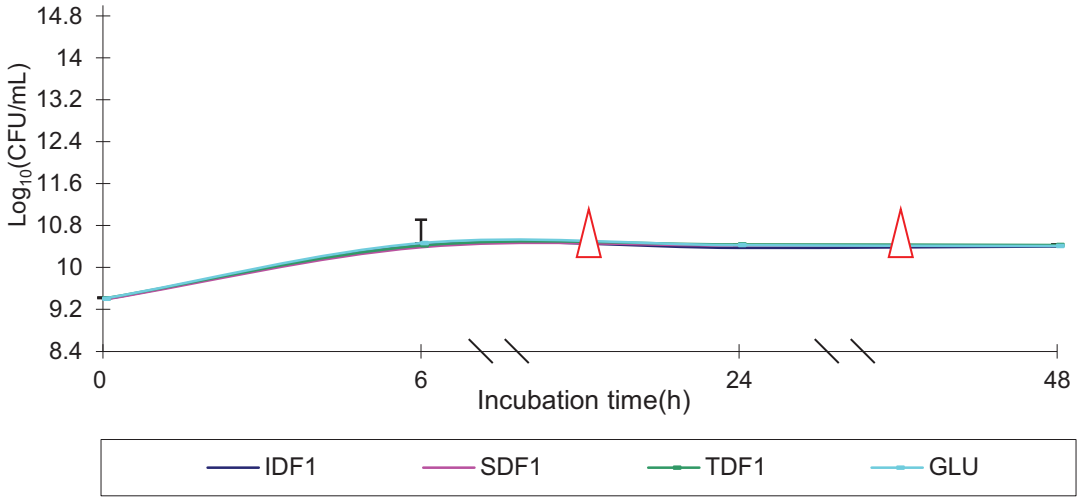


Fig 9.1 A) LA+LR

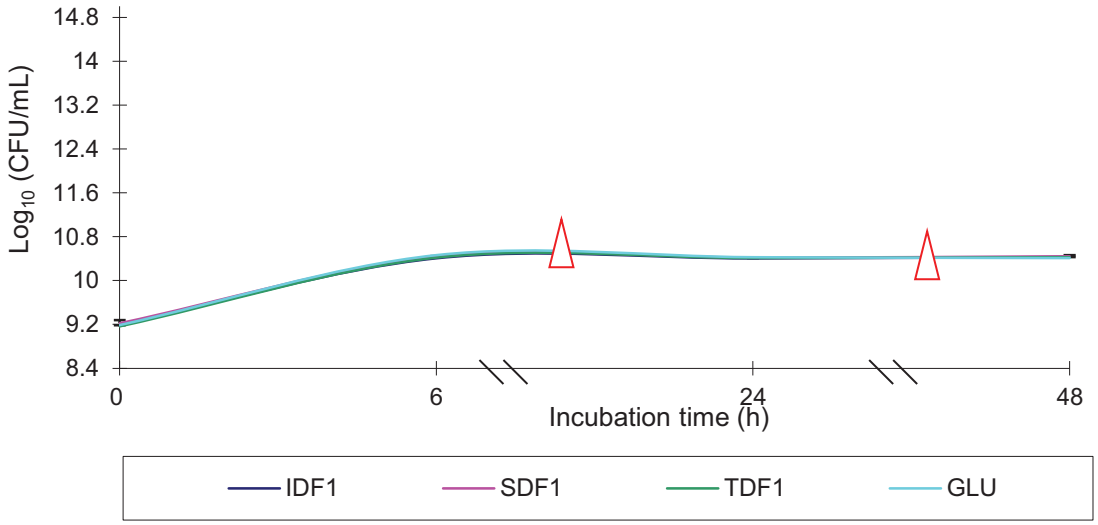


Fig 9.1 B) BB+BL

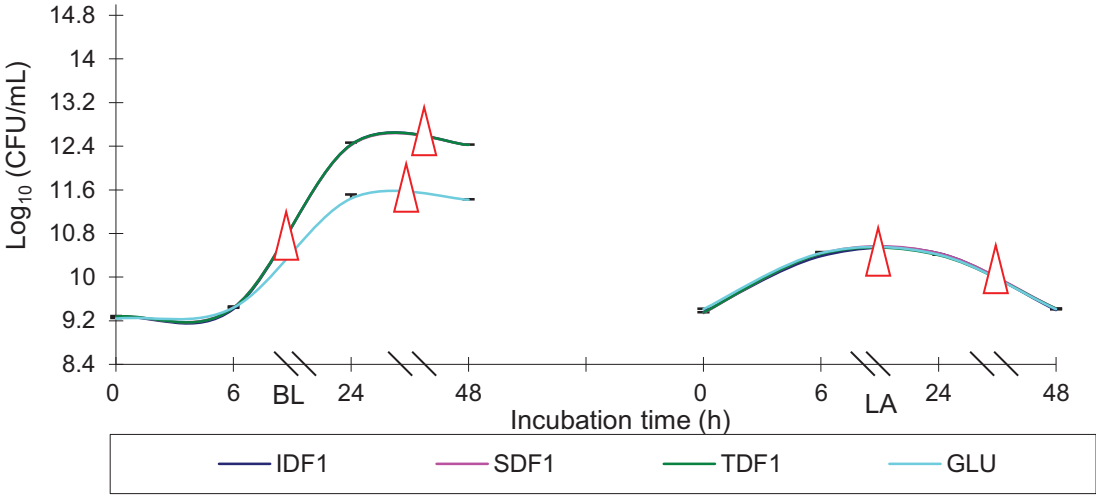
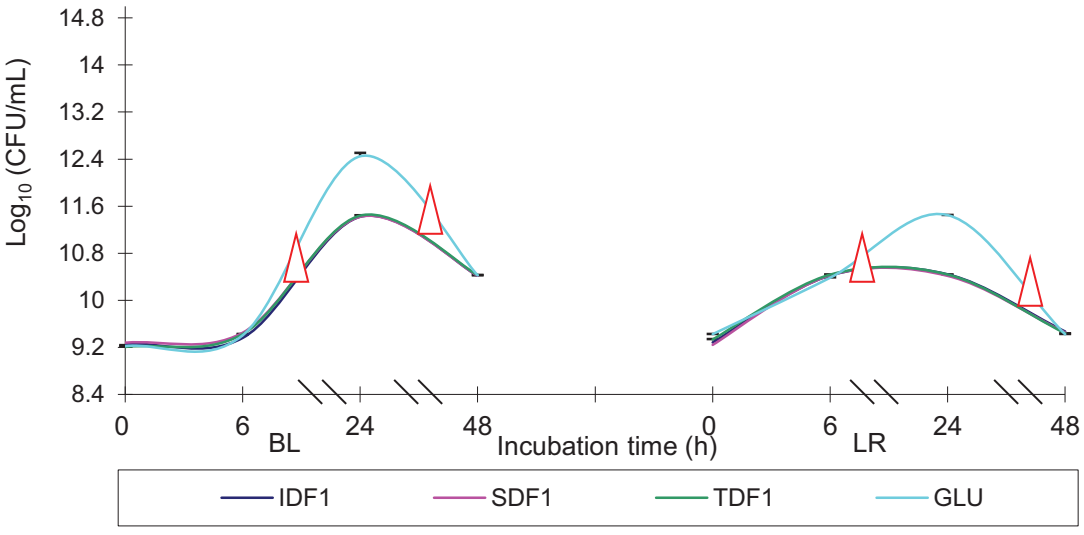


Fig 9.1 C) BL+LA



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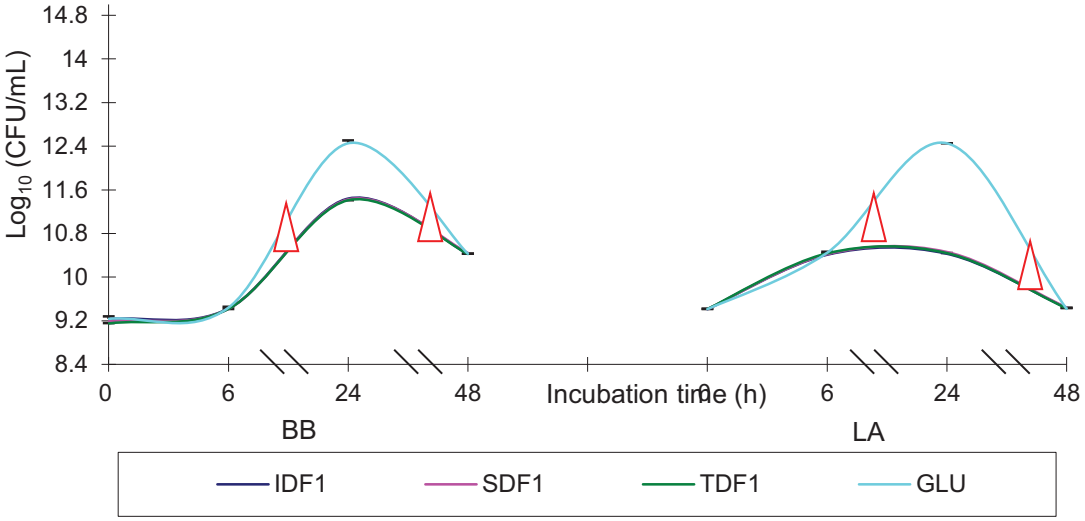

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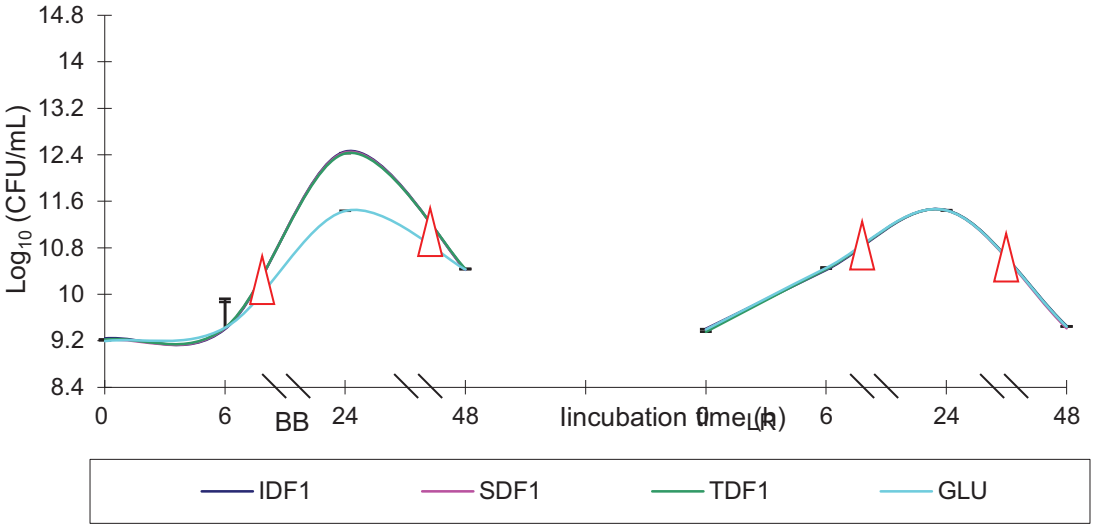


Fig 9.1 F) BB+LR

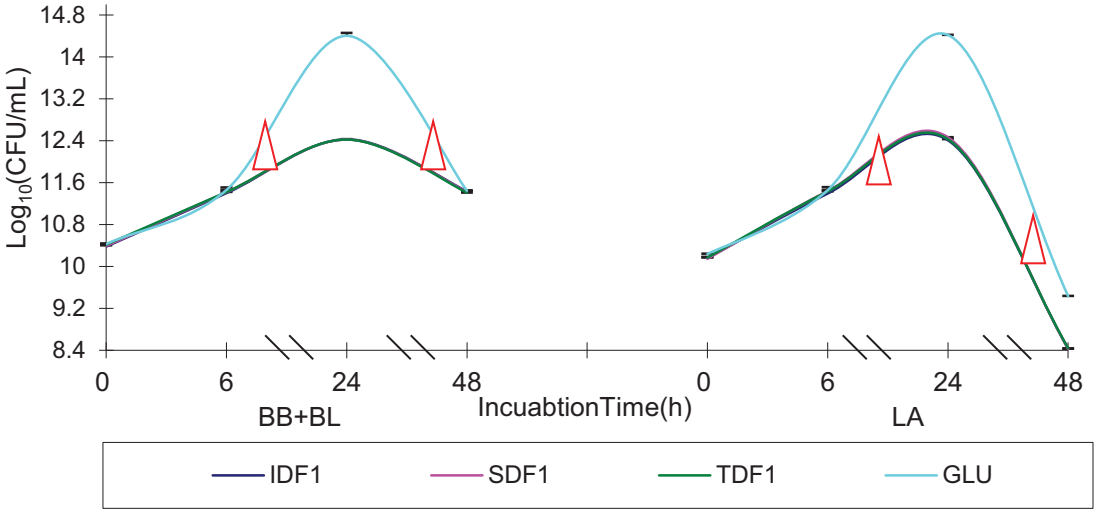


Fig 9.1 G) BB+BL+LA

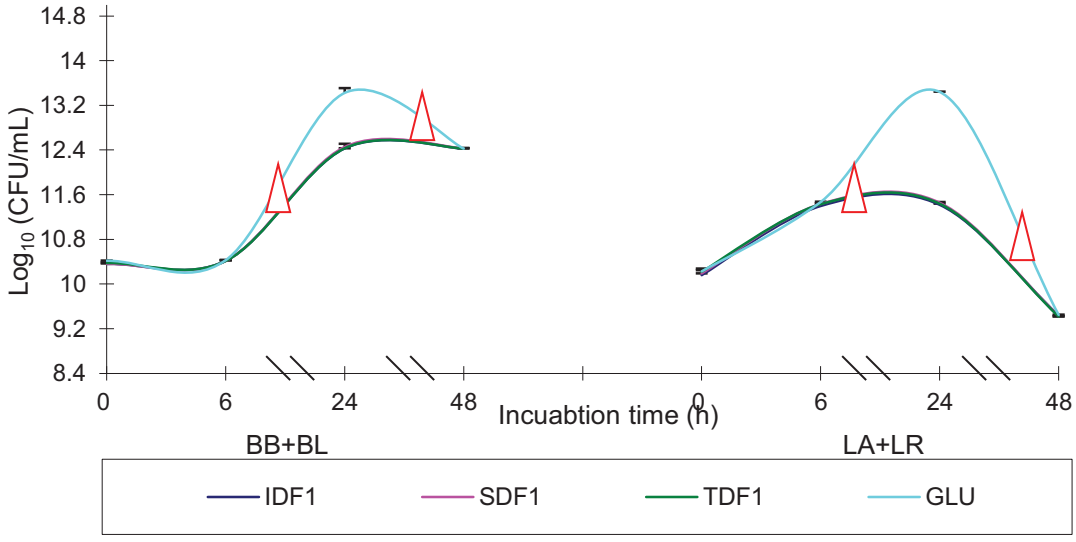


Fig 9.1 H) BB+BL+LA+LR

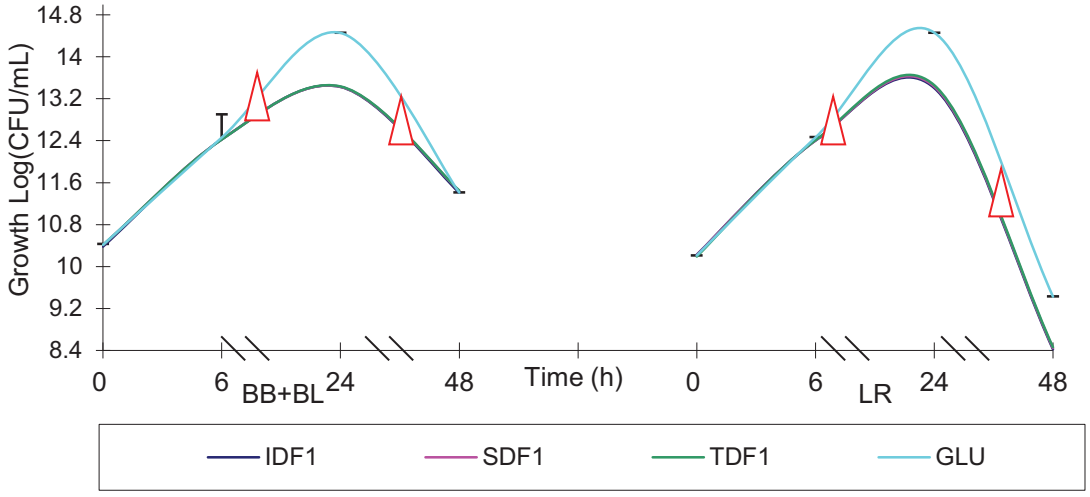
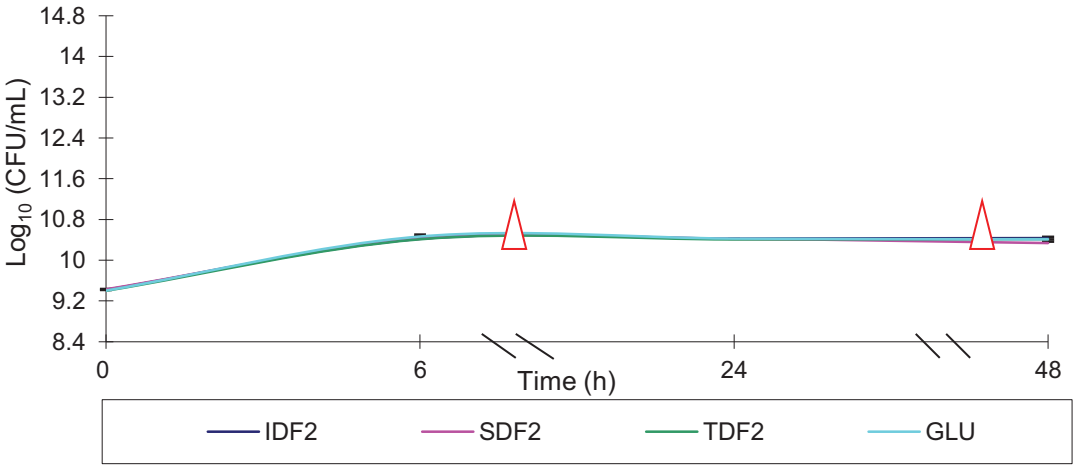


Fig 9.1 I) BB+BL+LR

Figure 9.1(A-I) Growth of individual organisms in combinations on rice variety RR1

Results are expressed as the mean value of two trials ± standard error. Standard error was smaller than 0.1. Break down of the axis and the chart is represented by



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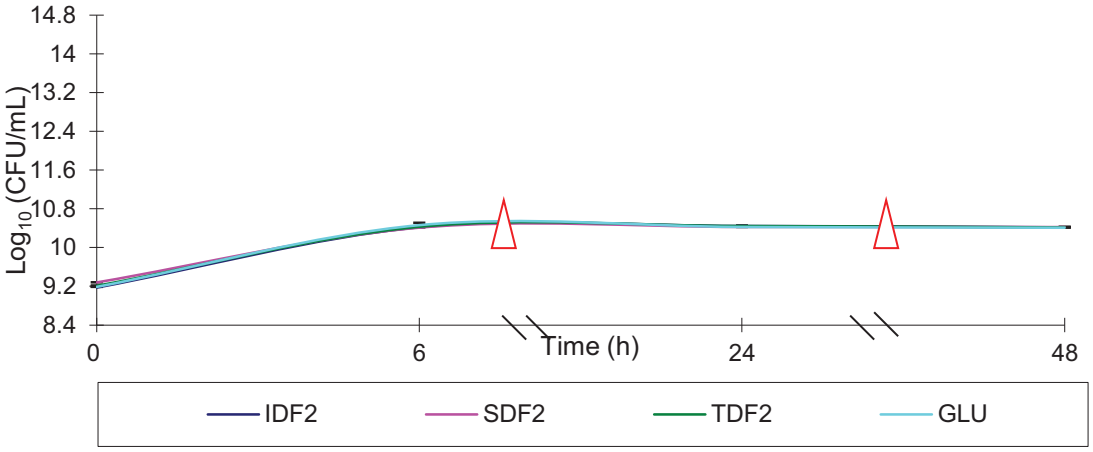

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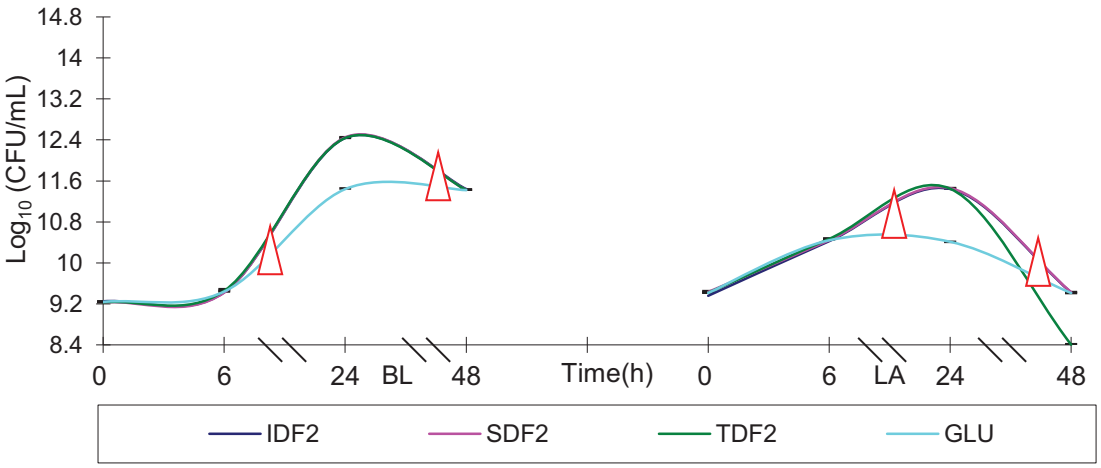

Fig 9.2 C) BL+LA

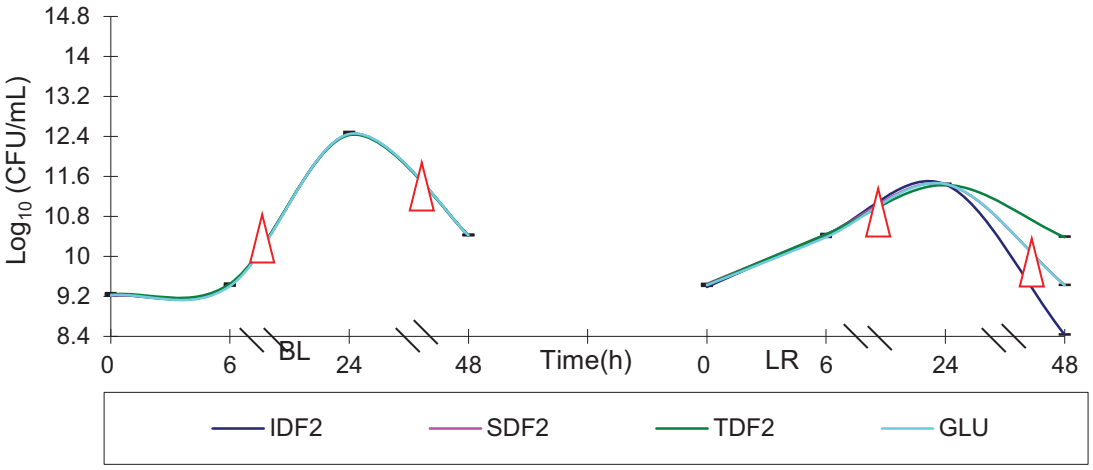

Fig 9.2 D) BL+LR

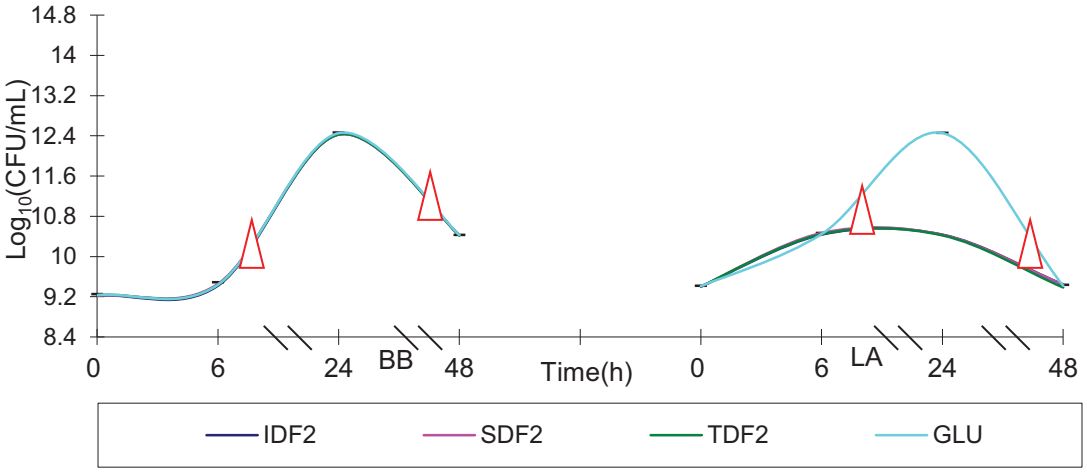

Fig 9.2 E) BB+LA

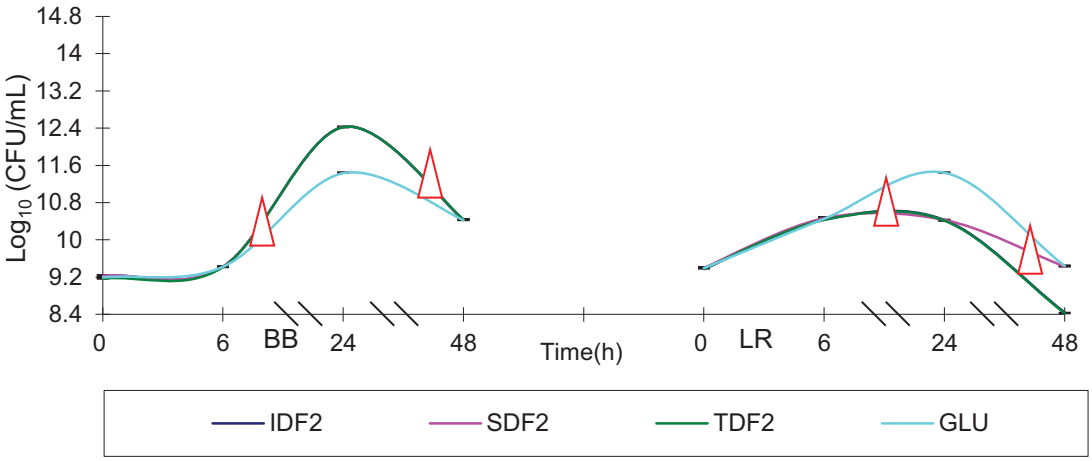

Fig 9.2 F) BB+LR

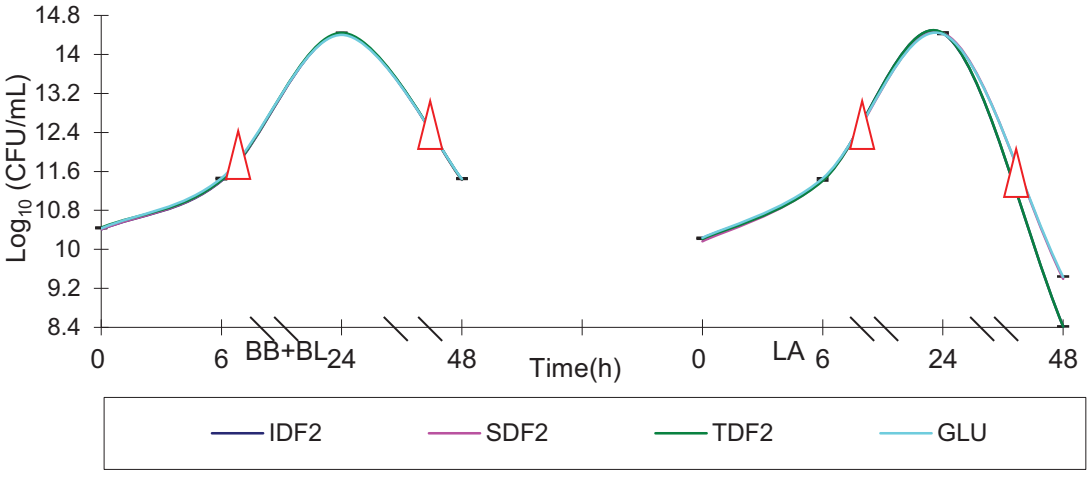

Fig 9.2 G) BB+BL+LA

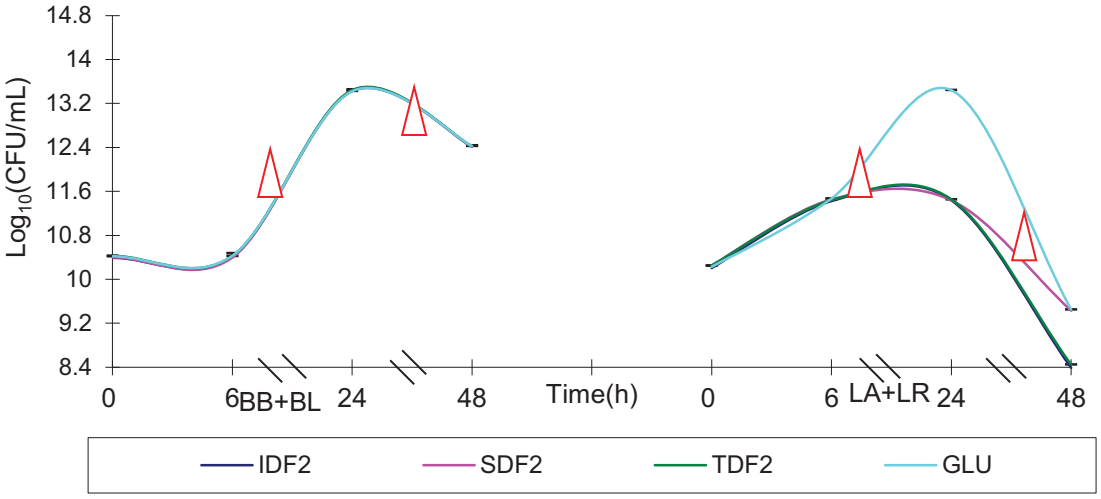

Fig 9.2 H) BB+BL+LA+LR

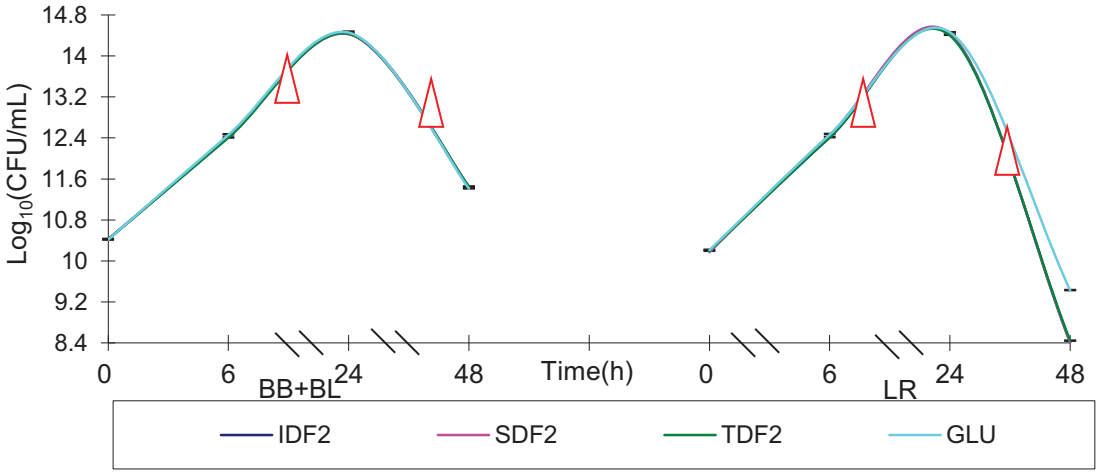

Fig 9.2 I) BB+BL+LR

Figure 9.2 (A-I) Growth of individual microorganisms in combinations on rice variety RR2

Results are expressed as the mean value of two trials ± standard error. Standard error was smaller than 0.1. Break down of the axis and the chart is represented by

Measuring the optical density is a convenient method to measure the growth of co-cultures on rice fibre. Viable counts of counterpart organisms demonstrated a rapid decline after the 24 hours. However, this was not observed in the results from the

optical density readings for the mixed species co-cultures. This represents (Fig 9.1 and 9.2) a change in the viable cell population but, not in the total cell content. The optical density readings varied with different substrates (Fig 9. 3).

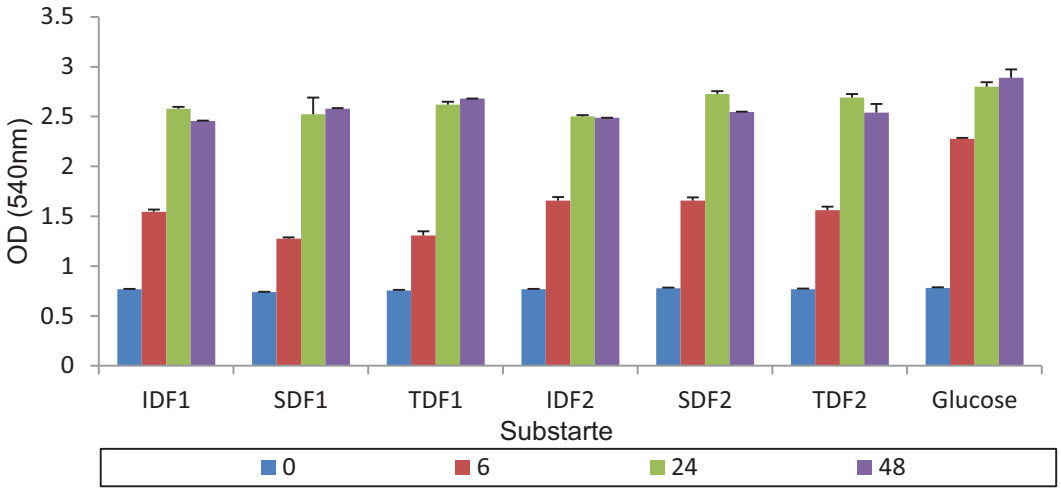

Fig 9.3A) LA+LR

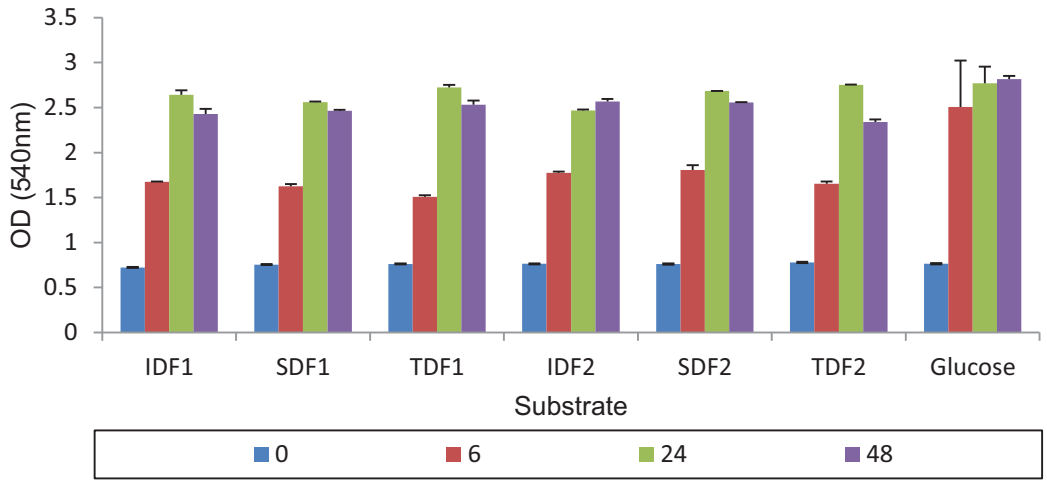

Fig 9.3B) BB+BL

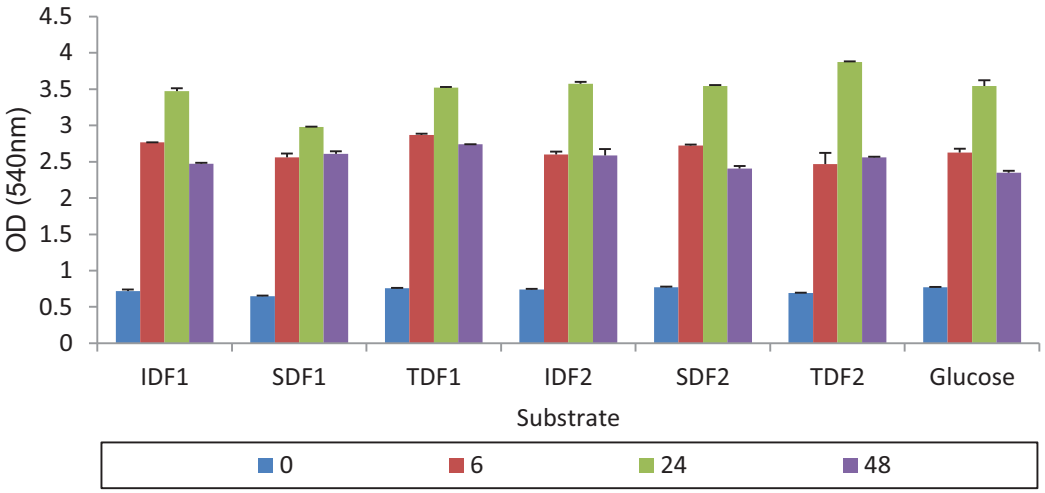

Fig 9.3C) BL+LR

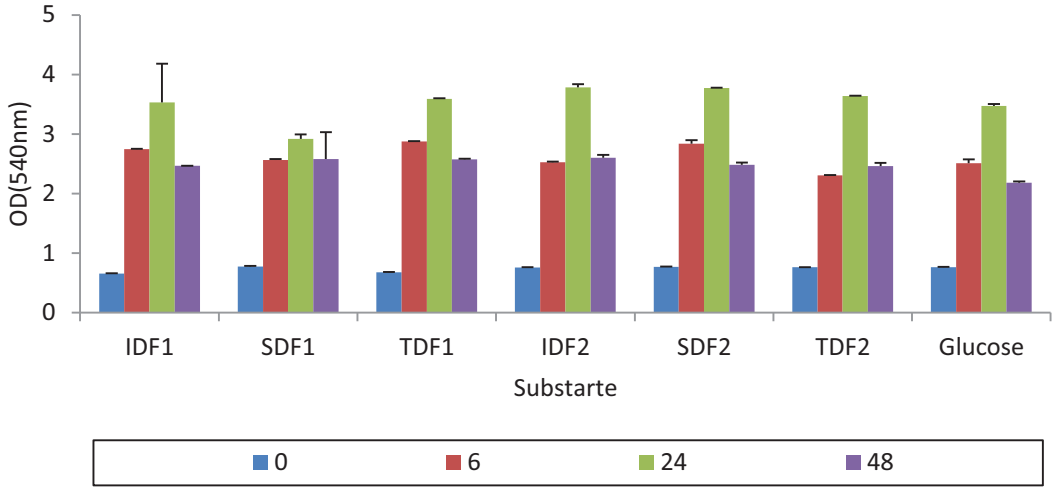

Fig 9.3D) BB+LR

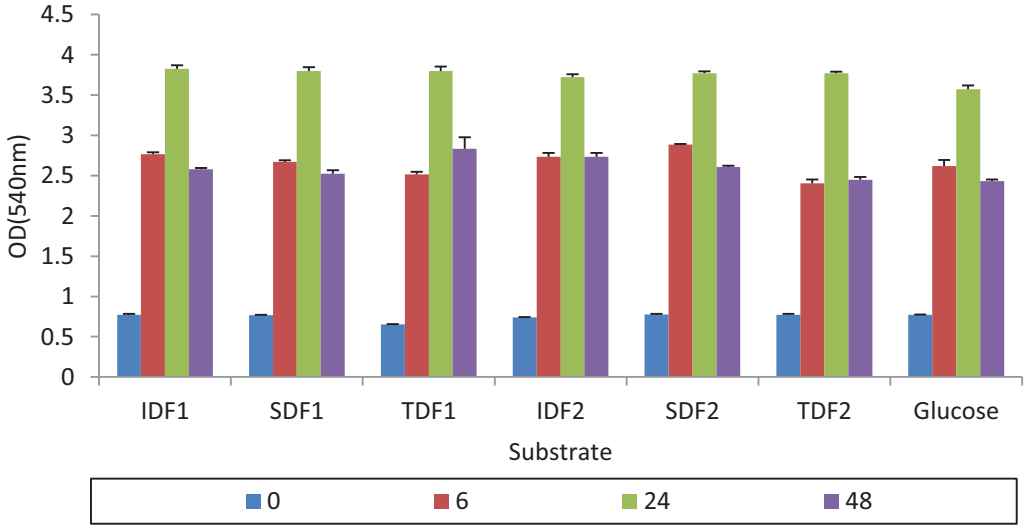

Fig 9.3E) BB+LA

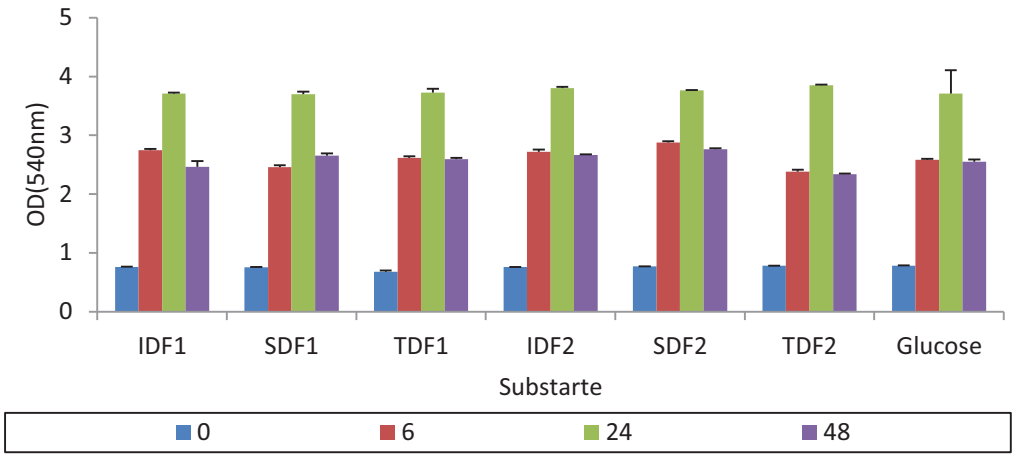

Fig 9.3F) BL+LA

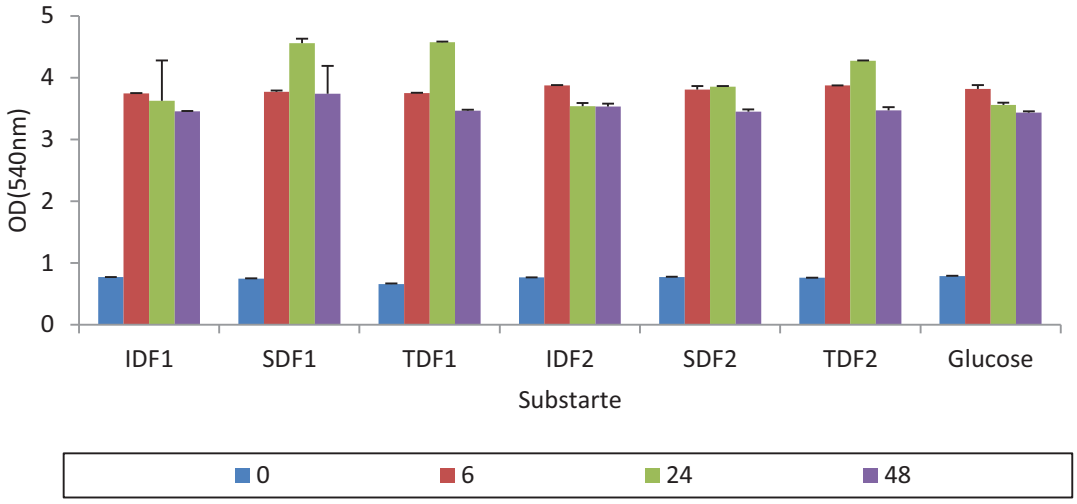

Fig 9.3G) BB+BL+LA+LR

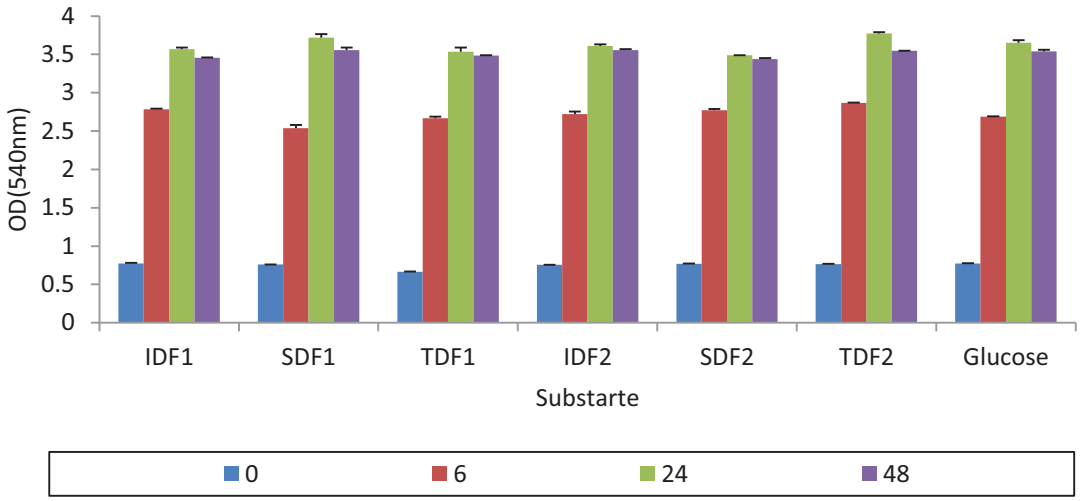

Fig 9.3H) BB+BL+LA

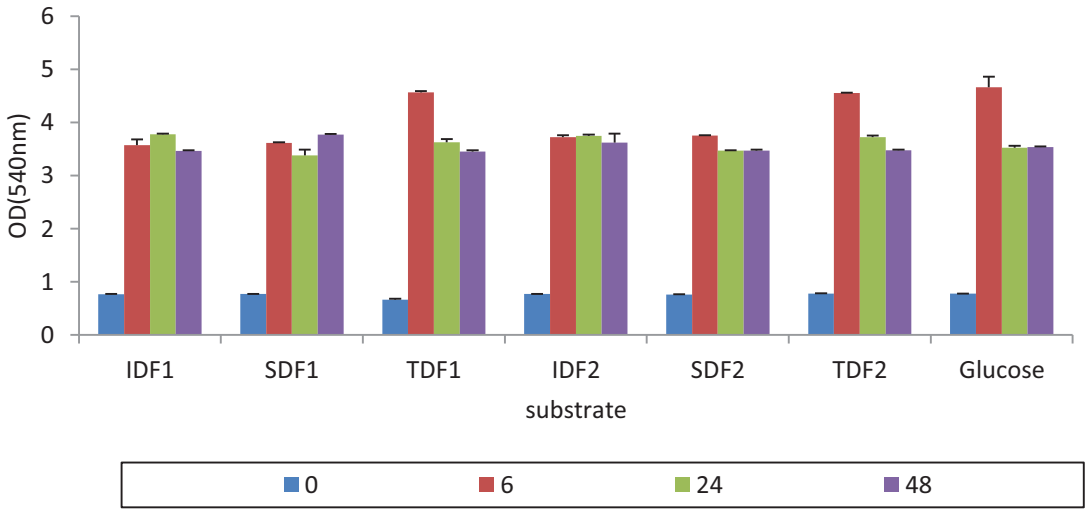

Fig 9.3 I) BB+BL+LR

Figure 9.3 (A-I) Optical densities of combinations of microorganisms at each time point

Results are expressed as mean values of two trials± standard error.

pH was one of the measurements used to evaluate the growth of co-cultures

Table 9. 1 pH value of culture combinations at each time point

0 H	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2	Glucose	Control
LA+LR	5.62±0.01	5.66±0.02	5.6±0.06	5.66±0.2	5.66±0.02	5.55±0.01	5.65±0.03	5.73±0.02
BB+BL	5.68±0.01	5.57±0.03	5.6±0.04	5.65±0.4	5.63±0.01	5.58±0.01	5.59±0.01	5.65±0.03
BL+LR	5.58±0.15	5.65±0.03	5.64±0.01	5.55±0.03	5.68±0.02	5.66±0.02	5.63±0.01	5.75±0.02
BB+LR	5.67±0.01	5.64±0.03	5.6±0.02	5.65±0.02	5.52	5.65±0.03	5.69±0.01	5.77±0.01
BB+LA	5.61±0.05	5.63±0.01	5.64±0.03	5.63±0.01	5.58±0.01	5.65±0.03	5.66±0.03	5.78±0.01
BL+LA	5.64±0.03	5.63±0.05	5.57±0.03	5.64±0.03	5.53±0.01	5.65±0.02	5.63±0.02	5.62±0.03
BB+BL+LR+LA	5.63±0.01	5.56±0.03	5.65±0.03	5.66±0.03	5.58±0.04	5.63±0.02	5.6±0.04	5.7±0.03
BB+BL+LA	5.61±0.07	5.64±0.03	5.68±0.01	5.66±0.02	5.65±0.03	5.64±0.04	5.56±0.02	5.72±0.01
BB+BL+LR	5.65±0.04	5.63±0.03	5.53±0.01	5.67±0.03	5.55±0.04	5.56±0.02	5.66±0.04	5.69±0.05
6^{th} H	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2	Glucose	Control
LA+LR	4.65±0.03	4.65±0.04	4.63±0.01	4.64±0.02	4.64±0.02	4.65±0.02	4.62±0.01	5.67±0.03
BB+BL	4.68±0.01	4.65±0.01	4.62±0.01	4.64±0.01	4.66±0.02	4.62±0.01	4.66±0.01	5.62±0.01
BL+LR	4.52±0.01	4.55±0.03	4.55±0.04	4.56±0.01	4.52±0.01	4.52±0.02	4.57±0.01	5.66±0.02
BB+LR	4.465±0.02	4.435±0.03	4.42±0.01	4.46±0.03	4.45±0.02	4.43±0.01	4.4±0.01	5.66±0.02
BB+LA	4.52±0.01	4.57±0.01	4.53±0.01	4.54±0.03	4.52±0.01	4.56±0.025	4.47±0.02	5.63±0.01
BL+LA	4.52	4.535±.03	4.54±0.03	4.55±0.02	4.55±0.03	4.53±0.01	4.42±0.01	5.76±0.02
BB+BL+LR+LA	4.44±0.03	4.435±0.03	4.46±0.02	4.42±0.01	4.44±0.01	4.47±0.01	4.47±0.01	5.68±0.04
BB+BL+LA	4.36	4.305±0.04	4.34±0.02	4.34±0.01	4.32±0.01	4.37±0.01	4.34±0.01	5.66±0.02
BB+BL+LR	4.24±0.01	4.25±0.01	4.27±0.01	4.26±0.01	4.26±0.03	4.26±0.01	4.385±0.01	5.73±0.01
24^{th} H	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2	Glucose	Control
LA+LR	4.54±0.02	4.54±0.02	4.51	4.55±0.02	4.54±0.02	4.55±0.02	4.52±0.01	5.72±0.01
BB+BL	4.53±0.03	4.52±0.01	4.54±0.03	4.51±0.05	4.52±0.01	4.52±0.01	4.44±0.02	5.65±0.04
BL+LR	4.41	4.46±0.01	4.43±0.01	4.43±0.02	4.45±0.02	4.47±0.01	4.39±0.01	435±0.01
BB+LR	4.39	4.4±0.01	4.35±0.04	4.36±0.01	4.38±0.01	4.44±0.03	4.42±0.01	5.73±0.01
BB+LA	4.44±0.02	4.41±0.02	4.45±0.04	4.48±0.01	4.44±0.015	4.47±0.02	4.38±0.01	5.77±0.01
BL+LA	4.44±0.01	4.45±0.02	4.47±0.01	4.45±0.03	4.47±0.01	4.43±0.02	4.36±0.02	5.75±0.01
BB+BL+LR+LA	4.23±0.01	4.28±0.03	4.3±0.02	4.27±0.01	4.29±0.04	4.33±0.02	4.34±0.03	5.76±0.03
BB+BL+LA	4.24±0.01	4.3±0.03	4.32±0.06	4.28±0.03	4.28±0.02	4.28	4.27±	5.73±0.01
BB+BL+LR	4.26±0.01	4.26±0.03	4.23±0.02	4.27±0.01	4.26±0.01	4.27±0.02	4.27±0.01	5.74±0.03
48^{th} H	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2	Glucose	Control
LA+LR	4.67±0.02	4.65±0.04	4.62±0.01	4.65±0.03	4.55±0.2	4.65±0.03	4.54±0.02	5.65±0.03
BB+BL	4.67±0.02	4.64±0.01	4.65±0.02	4.66±0.03	4.61	4.61±0.03	4.52±0.01	5.63±0.02
BL+LR	4.57±0.01	4.55±0.01	4.53±0.01	4.57±0.01	4.5±0.02	4.49±0.01	4.44±0.02	5.73±0.02
BB+LR	4.47±0.01	4.52±0.02	4.44±0.02	4.48±0.01	4.45±0.04	4.42±0.01	4.4±0.01	5.73
BB+LA	4.47±0.01	4.52±0.01	4.48±0.01	4.49±0.01	4.42±0.01	4.43±0.03	4.41±0.03	5.74±0.02
BL+LA	4.55±0.02	4.44±0.02	4.52±0.01	4.57±0.01	4.49±0.01	4.45±0.03	4.37±0.01	5.73±0.01
BB+BL+LR+LA	4.46±0.01	4.42±0.02	4.41±0.02	4.47±0.02	4.43±0.02	4.38±0.01	4.37±0.02	5.75±0.03
BB+BL+LA	4.44±0.02	4.44±0.03	4.35±0.04	4.47±0.01	4.42±0.01	4.42±0.02	4.42	5.72±0.01
BB+BL+LR	4.41±0.02	4.35±0.02	4.38±0.01	4.46±0.01	4.45±0.02	4.39±0.01	4.42±0.01	5.65±0.04

Results are expressed as the mean value of two trials ± standard error.

The pH of the fermentation media varied with time (Table.9. 1). The lowest pH was reported after 24 h incubation, indicating maximum metabolite formation. In this trial, it was not possible to measure the acidification of each of the microorganisms

making up the co-culture separately. The acidification will result from the combined metabolic pathways of each microorganism (Chapter 2).

Table 9.2 Specific growth rates of combinations as individuals/ species

A) Between 0-6 h of fermentation

Time points	**0-6h**	**0-6h**	**0-6h**	**0-6h**	**0-6h**	**0-6h**	**0-6h**
LA+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Lactobacillus species	0.40±0.01	0.38±0.01	0.39±0.01	0.38	0.38±0.01	-	0.41
BB+BL	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.43±0.01	0.48±0.02	0.50±0.01	0.50±0.01	0.50±0.01	-	0.47
BL+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium longum	0.04	0.07±0.01	0.08±0.01	0.08	0.08±0.01	0.38±0.01	0.07
Lactobacillus rhamnosus	0.44	0.45	0.42	0.40±0.01	0.38±0.01	0.09	0.37
BB+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium breve	0.06	0.08	0.08	0.08±0.01	0.08±0.01	0.38±0.01	0.09
Lactobacillus rhamnosus	0.39	0.40±0.01	0.41	0.41±0.02	0.41	-	0.41
BB+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium breve	0.07	0.09±0.01	0.10	0.08±0.01	0.09±0.01	0.38±0.01	0.08
Lactobacillus acidophilus	0.38	0.39	0.39	0.40±0.02	0.41	-	0.40
BL+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium longum	0.05±0.01	0.07	0.06	0.07±0.01	0.07±0.03	0.38±0.01	0.08
Lactobacillus acidophilus	0.39	0.42	0.41	0.41	0.39	0.09	0.39±0.01
BB+BL+LR+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.02±0.02	0.02	0.01	0.01	-	0.39±0.01	0.00
Lactobacillus species	0.48	0.48	0.46	0.47±0.01	0.46	0.02	0.48
BB+BL+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.39±0.02	0.39	0.39	0.38	0.38±0.021	0.01	0.40
Lactobacillus species	0.47	0.50	0.48±0.01	0.46±0.01	0.48±0.01	0.39	0.47
BB+BL+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.79	0.77	0.77	0.77	0.77	0.19	0.79
Lactobacillus species	0.84	0.84±0.01	0.85±0.01	0.85±0.02	0.88	0.19	0.87

Results are expressed as the mean value of two trials ± standard error. Standared error less than 0.01is not indicated.

B) Between 6-24 h of fermentation

Time points	6-24h	6-24h	6-24h	6-24h	6-24h	6-24h	6-24h
	IDF1	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Lactobacillus species	-	-	-	-	-	-	-
BB+BL	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	-	-	-	-	-	-	-
BL+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium longum	0.26	0.25	0.26	0.39	0.39	0.38	0.39
Lactobacillus rhamnosus	-	-	-	0.13	0.13	0.13	0.14
BB+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium breve	0.39	0.39	0.38	0.38	0.38	0.38	0.26
Lactobacillus rhamnosus	0.13	0.13	0.13	-	-	-	0.13
BB+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium breve	0.26	0.26	0.26	0.38	0.38	0.38	0.39
Lactobacillus acidophilus	0.00	0.01	-	-	-	-	0.26
BL+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium longum	0.39	0.38	0.38	0.39	0.39	0.38	0.26
Lactobacillus acidophilus	0.01	-	-	0.13	0.13	0.13	-
BB+BL+LR+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.26	0.26	0.26	0.39	0.39	0.39	0.38
Lactobacillus species	-	-	-	0.02	0.02	0.02	0.25
BB+BL+LA	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.13	0.13	0.13	0.39	0.38	0.39	0.38
Lactobacillus species	0.13	0.13	0.13	0.38	0.39	0.39	0.38
BB+BL+LR	**IDF1**	**SDF1**	**TDF1**	**IDF2**	**SDF2**	**TDF2**	**Glucose**
Bifidobacterium species	0.13	0.13	0.13	0.26	0.26	0.26	0.26
Lactobacillus species	0.13	0.13	0.13	0.26	0.25	0.25	0.26

Results are expressed as the mean value of two trials ± standard error and the standarded error was smaller than 0.01.

As detailed in Chapter 7, the specific growth rate (CFU/mL/h) was established using the closest two points in the growth using the formula, $\mu=\ln(x_2/x_1)/(t_2-t_1)$ (Gupthar *et al.,* 2000). However, in this Chapter, the time intervals for most of the combinations were 6 and 18 h. Therefore, the values of the specific growth rate can be considered only as approximate values. The specific growth rates indicate the ability of the co-cultures to utilise the rice fibre substrates (Table 9.2). Genera *Lactobacillus* and *Bifidobacterium* had different specific growth rates in different co-cultures. Even in the same co-culture, organisms demonstrated different specific growth rates indicating variation in the fermentation capacity of *Bifidobacterium* and the *Lactobacillus* species.

Some of the co-cultures produced maximum biomass at 24 h and some at 48 h incubation (Fig 9.3). Since the co-cultures obtained the maximum viable count at 24 h, those producing the highest biomass yield at 24 h suggest that the maximum metabolite formation is also occurring at this time. The combinations with the highest biomass at 48 h, suggest metabolite formation occurs for longer and there are a number of non-viable cells at this later stage of fermentation, since organisms are in the death phase based on the viable counts.

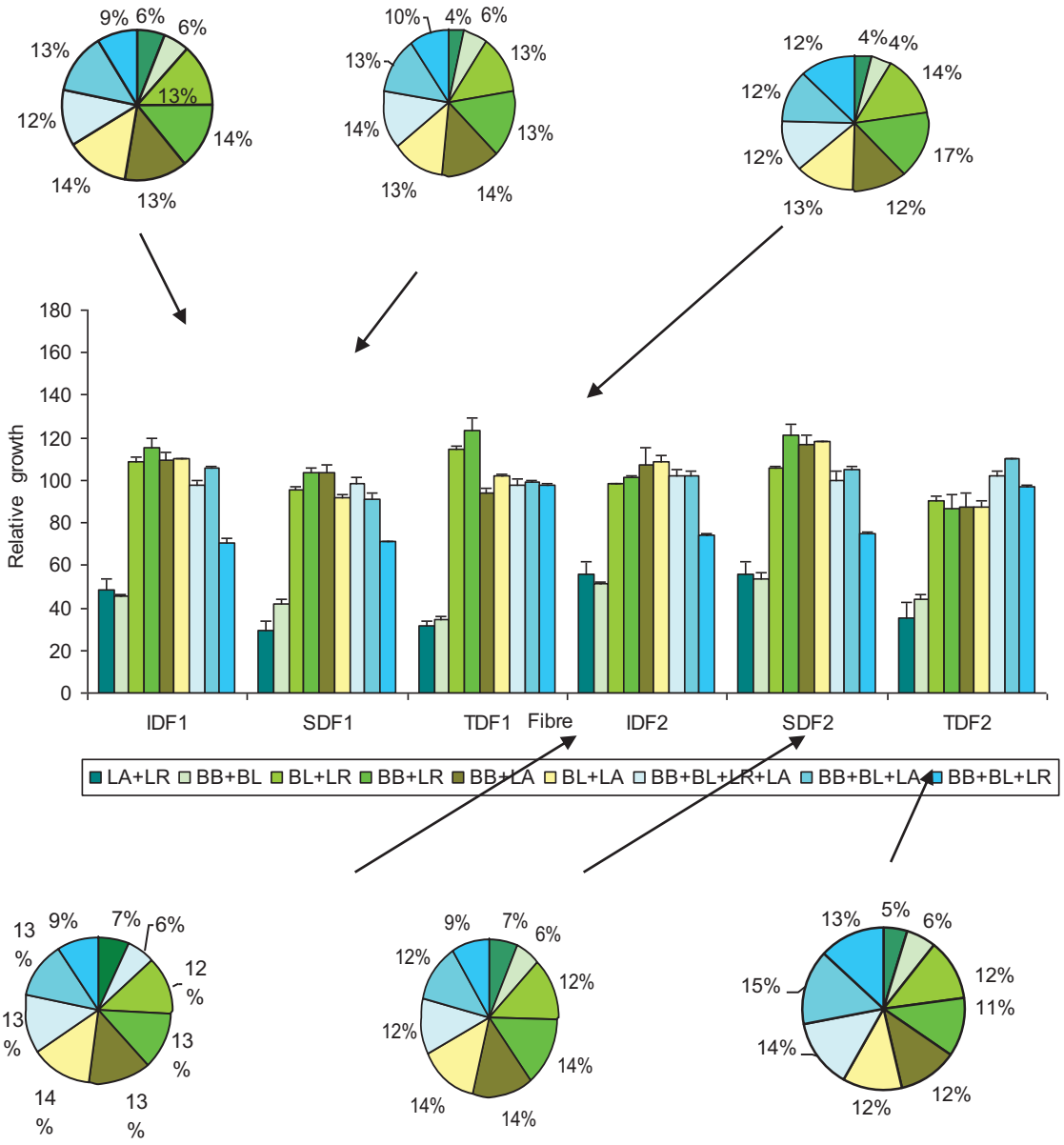

Fig 9.4 A) Relative growth at 6th h

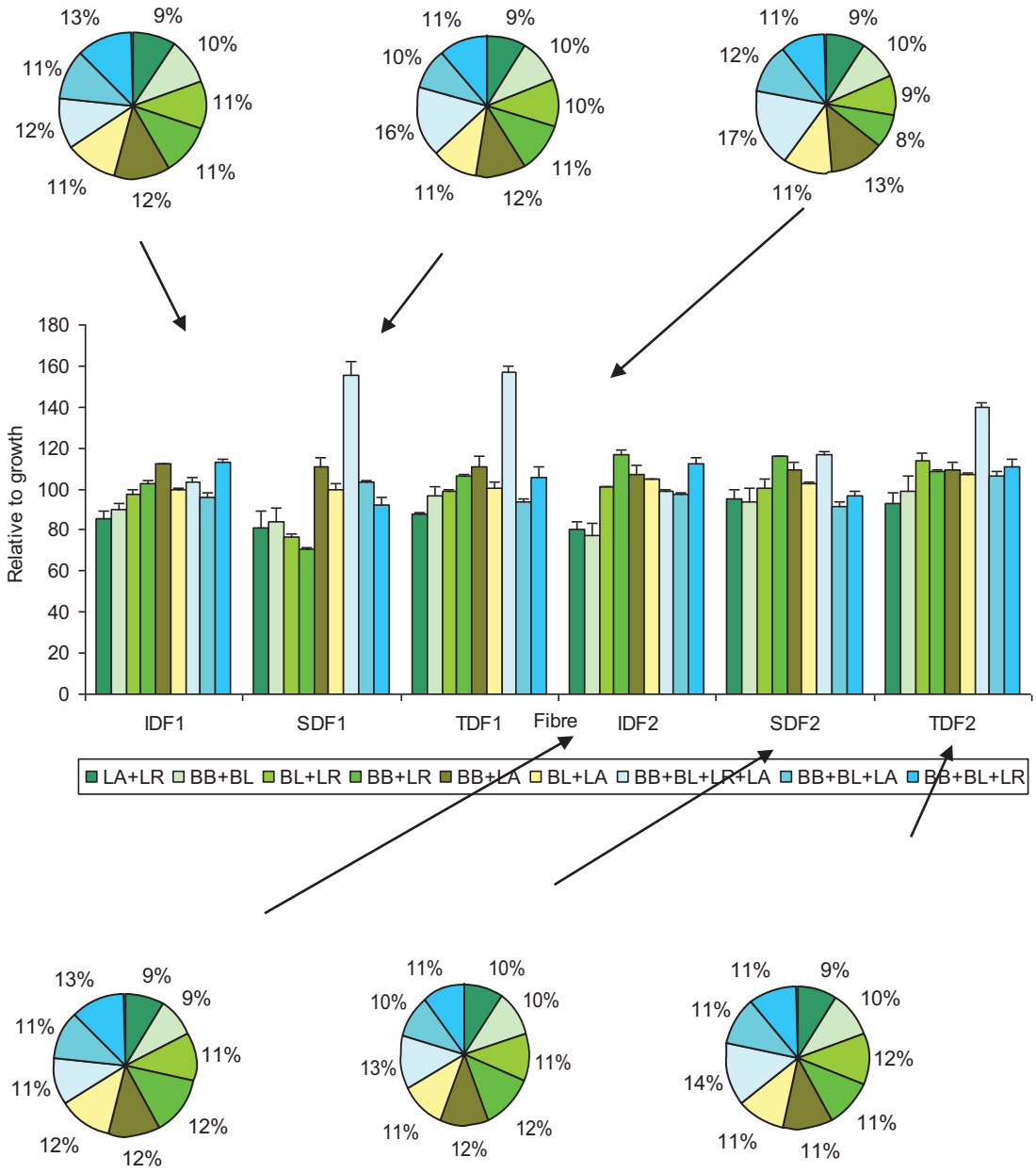

Fig 9.4 B) Relative growth at 24th h

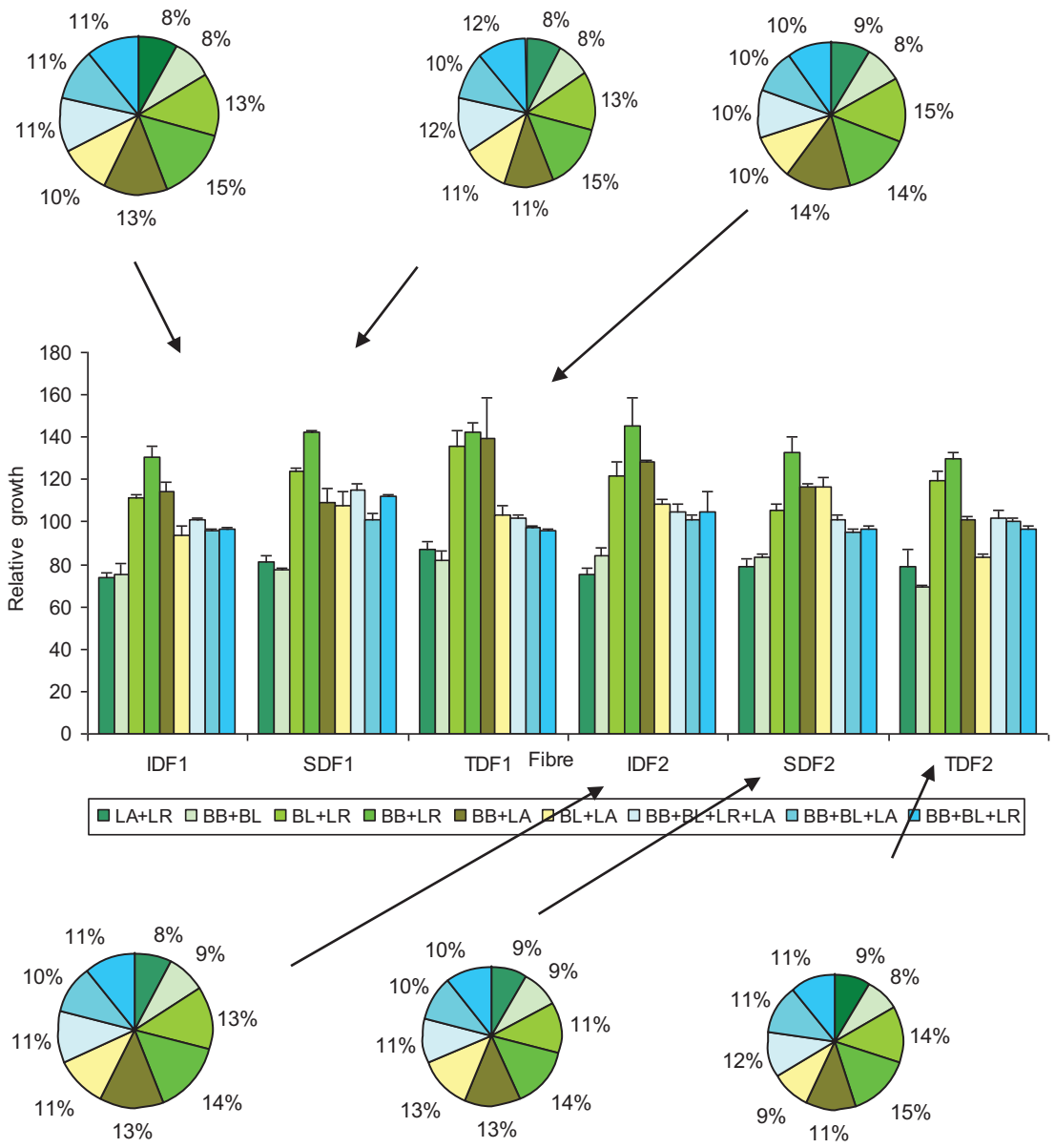

Fig 9.4 C) Relative growth at 48th h

Figure 9.4 Comparative growth of different microbial combinations on fibre fractions (A-C).

The bar charts represent the percentage of relative growth of each combination and pie charts represent the ratio of relative growth between each combination. Results are expressed as mean values of two trials ± standard error.

9.5 Discussion

The aim of this research was to investigate and compare the growth characteristics of combinations of pure cultures of *L. acidophilus*, *L. rhamnasus*, *B. breve* and *B. longum* on unformulated dietary rice fibre. The composition of raw material, specific growth rate of the combinations, final cell count, biomass and acidification rates were the parameters used to evaluate the fermentation of dietary fibre.

This study is the first to compare the *in-vitro* fermentation of rice fibre by co-cultures of probiotics.

In this study, high cell counts of the starter cultures ensured the high initial levels of cell counts in the fermentation medium: 10^8-10^9 cfu/mL. The addition of fibre substrate to the fermentation medium in general had an observable ($p<0.05$) effect on the culture growth and the viability, for all the microbial combinations, especially at 24 h incubation, (Fig 9.1 and 9.2). This indicates the ability of the co-cultures to use rice fibre as a fermentable substrate. Previous work with oat fibre using some species of the genus *Lactobacillus* produced a 2.8 log increase in the cell count (Angelov *et al.,* 2005), less than the increase observed in the current research. This may be due to the different substrate and/or different fermentation capabilities of the strains used in each experiment.

Cell counts during fermentation in the current study were mainly species and time dependant. The *Bifidobacterium* species produced a higher cell count ($p<0.05$) than the *Lactobacillus* species, especially at 48 h incubation, for all the substrates (Fig 9.1 and 9.2). Fibre had a greater effect on the cell numbers of *Bifidobacterium* than the *Lactobacillus* species. Previous work with species of the genus *Bifidobacterium* has shown that Bifidobacteria can ferment a variety of carbohydrates (Bezkorovainy, 1999)

and that Bifidobacteria vary in their fermentation profiles on different monosaccharides (Beverly & George, 1991). This particular ability of *Bifidobacterium* will help these organisms to out-compete other organisms in the human gut when dietary fibre is provided in the diet.

Most of the combinations of microorganisms showed significantly ($p<0.05$) higher growth on glucose compared with the fibre (Fig 9.3). Therefore, the potential prebiotic supplement that accelerated the viable count of most of the co-cultures was glucose, and to some extent, TDF. This indicates the preference of the co-cultures used in this experiment is for glucose rather than the fibre fractions. However, microorganisms of the same genus in co-cultures showed an equal preference for glucose and for fibre, while co-cultures of more than two species showed a higher preference for glucose than fibre. This result disagrees with previous work showing higher growth by *Bifidobacterium* on dietary fibre (fructo- oligosaccharides) than on glucose (Gibson & Wang, 1994; Wang & Gibson, 1993). Co-cultures of different species showed a preference for glucose and for TDF in terms of the viable count and optical density measurements. It was interesting to note that all potential prebiotics (IDF, SDF and TDF) produced similar fermentations with the co-cultures used, irrespective of the rice variety. Generally the difference in cell count for co-culture fermentations of fibre substrates (IDF, SDF, and TDF) at each time point was not significant ($p>0.05$). The combined cultures utilized the fibre fractions equally well. This study used the same quantity of fibre as IDF, SDF and TDF from two different rice varieties. These fractions were 99 % pure. It is factual that the initial fibre content of these rice varieties varied (Fernando *et al.,* 2008).

However, there were differences in the growth for the fermentation of the different fibre fractions - IDF, SDF, and for TDF (Fig 9.1 and 9.2). The growth for co-

cultures of species from same genus were different from the mixed co-cultures (different genus) and among the mixed cultures the binary combinations had a different pattern than those consisting of more than two species, demonstrating synergism between the *Lactobacillus* and *Bifidobacterium* species. This indicates different synergistic relationships between the different microbial combinations during fermentation. This relationship is considered as synergistic since higher numbers of viable cells were produced during the growth using co-cultures, when compared with the individual organisms. The variations in cell numbers between the different co-cultures were statistically significant ($p<0.05$). Previous work has found evidence of symbiosis between *L. acidophilus* and *B. lactis* when grown in milk (Gomes *et al.,* 1998). Lactobacilli have a higher proteolytic activity and can supply *Bifidobacterium* with peptides and amino acids (Gomes *et al.,* 1998), which they need to produce a high yield. *Bifidobacterium* species can also be inhibited by a fast growing *Lactobacillus* strain (Gomes & Malcata, 1999). However, this study did not observe any higher growth of the *Lactobacillus* species, compared with the *Bifidobacterium* species (Fig 9.1, 9.2 and 9.3). This indicates the *Lactobacillus* strains used in this trial are not fast growing organisms when fibre is used as the fermentation substrate. However, among the *Lactobacillus* species, *L. acidophilus* showed slower growth than the *L. rhamnosus* (Fig 9.1 and 9.2). The slower increase in cell numbers of *L. acidophilus* could be explained by the absence of the nutrients in the medium (Gomes & Malcata, 1999). Previous research has demonstrated that this organism has a high nutrient requirement and has poor growth in media lacking supplements, such as yeast extract and peptone (Gomes & Malcata, 1999).

The combinations of species from each different genus showed increase of the cell number for a long time and very short period without increasing the number of cells

at leater stages of the growth except for the LA+LR and BB+BL combinations (Fig 9.1and 9.2). A rapid growth indicates greater utilization of fibre by the microorganisms. Fast depletion (Chapter 10) of the fibre after the accelerated growth, the pH drop and accumulation of toxic metabolites, might have caused the short stationary phase before the populations started to decline. Maximal cell counts of 10^{10}-10^{12} /mL were obtained in 24 h and positive factor for fibre digesion in the human gut since total transit time of fibre in human gut has been estimated as 24 to72 h (Wrick *et al.,* 1983). Co-cultures of BB+BL and LA+LR did not enter the decline phase like the other combinations. Their population was small compared with other combinations, thus nutrition limitation might not have affected them as rapidly as other combinations.

Microorganisms made up of combinations belonging to a different genus, showed slow growth when the pH was 4.2- 4.45 after 24 h incubation (Table 9.1). These experiments were conducted in a synthetic medium without pH control. These uncontrolled conditions in the fermentation medium would have resulted in the accumulation of metabolic products such as short chain fatty acids, reducing the pH after 24 h incubation. These organic acids can inhibit microbial growth (Passos *et al.,* 1993). However, previous studies have found that the contribution of metabolites (organic acids) in limiting the growth of microorganisms is less than the effect of pH (Giraud *et al.,* 1991). Thus the higher cell population of *Bifidobacterium* species compared with the *Lactobacillus* species in combinations can be attributed to the higher growth of *Bifidobacterium species* in the fermentation medium before the pH value decreased to 4.2-4.45. Previous studies have found that the pH value that limits the growth of *Lactobacillus* species (*L. acidophilus*) is dependent on the cereal used in the fermentation (Lönner & Preve-Åkesson, 1988). Less acidification in the medium may allow an increase of the cells of the microbial combinations, and allow the formation of

more metabolites in the fermentation medium. It is still not clear whether the limit of microbial growth with these microbial combinations is due to the acidification of the medium or deficiency in the nutrients. However, since these organisms are sensitive to low pH, in this study it is assumed that the limitation of microbial growth may be due to pH.

In this study, the specific growth rates were expected to be similar for the same organisms in binary and more than binary combinations, for fermentations of the same fibre fraction. Interestingly, the same organism in different co-cultures exhibited different specific growth rates for the same substrates and the specific growth rate was also different between the different co-cultures. The variations in chemical structure and the quantity of sugars of fibre fractions may contribute to variations in the specific growth rates. Specific growth rates may change when the substrate concentration decreases with the incubation time. In this study, substrates used by the microorganisms as the energy source, would have been used for more than just growth (reproduction, metabolite formation). Therefore, the growth rate of the microorganisms may be slower than expected. On the other hand, the energy requirement of the microorganisms living as co-cultures may differ from combination to combination. Therefore, organisms may exhibit different specific growth rates for the same substrate when organisms are present in different combinations. Metabolic waste that accumulates in the medium may inhibit the cells and may affect the specific growth rate. The capacity of microorganisms to compete for nutrients with the other microorganisms in the combination might also affect the specific growth rate.

This combination of factors may explain the different specific growth rates for the fermentation of the same substrate when microorganisms are in different co-cultures (Table 9.2). Therefore, it is impossible to know whether *Lactobacillus* or

Bifidobacterium species had a higher specific growth rate on rice fibre when they are in combinations (BB+BL, LA+LR, BB+BL+LA,BB+BL+LR) rather than as individual species.

The combinations were evaluated, and those with species from same genus demonstrated less biomass formation than the other combinations during the fermentation ($p<0.05$). The study expected to show significantly higher biomass at 24 h with the peak viable count at 24 h incubation for combinations of species from different genera. However, the study did not observe any significant increased biomass at 24 h incubation. Highly metabolically active cultures at 24 h might have utilised the fibre and might have reduced the optical density to give a lower value for biomass. There was no significant difference in the biomass yield from the different fibre substrates ($p>0.05$). The highest pH drop after 24 h incubation indicates that the physiological state of cells at 24 h favours the formation of acids rather than biomass.

The combination of BB+BL+LR showed the greatest stimulation of growth in the presence of the prebiotics TDF, SDF, IDF and Glucose in terms of viable count, optical density and pH. With an average count of 13.6 log CFU/mL for this combination, this indicates a good synergistic relationship between these probiotic microorganisms, capable of using the rice fibre fractions. LR or LR in co-culture with BB or BL or BB+BL, produced high counts (12-14 log CFU/mL on average), but only in the presence of glucose.

9.6 Conclusion

Co-cultures grew on fibre from both rice varieties, reaching high cell yields, higher than those obtained by individual cultures. This demonstrates synergistic relationships between these bacterial combinations. This study found that there was no preference for any particular dietary fibre fraction. However, most co-cultures showed a preference for glucose rather than fibre as a substrate.

This study observed an increase in the cell count of *Bifidobacterium* and *Lactobacillus* species, with combinations from a different genus rather than combinations from the same genus. This suggests a certain degree of symbiosis between the members of each genus.

The microorganisms making up the combinations reached the death phase after a very short stationary phase, between 24 and 48 h, except in fermentations consisting of organisms from the same genus. The pH drop in all fermentations occurred rapidly, reaching the lowest level after 6 h fermentation. Co-cultures made up of more than one genus reached a lower pH than those made up of organisms from the same genus, adding further evidence for the synergistic association in these co-cultures.

The specific growth rate differed among the co-cultures on all the fibre substrates indicating the differences in the ability to utilize fibre. BB+BL+LR were the combination showing the most growth on all substrates tested (TDF, SDF, IDF and Glucose). The results from this work suggest that fermentation of dietary fibre from rice could be enhanced by the addition of probiotics bacteria in specific combinations.

CHAPTER 10

SCFA Formation by Combinations of Probiotics on Rice Fibre

10.1 Abstract

Sri Lankan rice varieties, LD 356 and AT 353 were separated into the following fibre fractions: total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). Nine combinations of *Bifidobacterium longum* (BL), *Bifidobacterium breve* (BB) and the *Lactobacillus* species *Lactobacillus rhamnosus* (LR) and *Lactobacillus acidophilus* (LA) were given the rice fibre (IDF, SDF, and TDF) and glucose as sole sources of carbon for the fermentation processes. Short Chain Fatty Acid (SCFA) production and the digestion of rice fibre were analyzed after 0, 6, 24 and 48 h incubation. The metabolite formation (SCFA) was low with combinations of microorganisms belonging to the same genus. The amount of SCFA produced by different substrates favoured the substrates in the following order, TDF>SDF>IDF. Acetate was the most abundant SCFA formed from all the combinations comprising more than 90% of the total SCFA formation at each time period. Combinations of the same genus resulted in the lowest ratio of acetate to propionate. Most microbial combinations digested 60 % - 80 % of the fibre; however, the combination of BB+ BL+ LR digested the fibre (SDF and IDF) completely.

The slowest co-cultures for SCFA formation were LA+LR and BB+BL and the most efficient combination was BB+ BL+LR.

10.2 Introduction

This study aimed to explore the formation of SCFA from rice fibre with co-cultures of probiotics. This study is the first to report the effect of the interaction of co-cultures of probiotics on SCFA formation from the fermentation of dietary rice fibre.

Dietary fibre and its digestion and the subsequent fermentation by colonic microorganisms provide energy via the formation of short chain fatty acids, mainly acetate, propionate, and butyrate (Cummings & MacFarlane, 1991; Hoverstad & Bjorneklett, 1984; Salyers & Leedle, 1983; Wolever *et al.,* 1991). The fermentation of fibre results in different amounts and types of SCFA in the colon depending on the solubility and degree of polymerization, type of linkages, branching, and monomeric composition of the fibre substrate (Berggren *et al.,* 1993; Nilsson & Nyman, 2005). The rate and the extent of fibre digestion depend on the number of microorganisms in the colon and their metabolic interactions (Berggren *et al.,* 1993; Nilsson & Nyman, 2005). The form of interactions in the human colon is difficult to determine *in-vivo* due to the complexity of the colon environment. Previous studies have found that probiotics may influence SCFA formation by producing SCFAs by themselves or by stimulating or suppressing the activity of other SCFA producing bacteria in the colon (Johansson *et al.,* 1998).

Interactions can result in metabolic consequences that cannot be identified simply from the substrate preferences of pure cultures. Therefore, studies with combinations of microorganisms under *in-vitro* conditions are more useful.

The following series of experiments aimed to compare the fibre utilization and the relative SCFA formation by co-cultures of probiotics in a substrate-limited environment. The results from this study and the mechanisms underlying this will help

to understand the fibre utilization and the interactions among the probiotics in the human gut.

The main objective of the present study was to study the effects of the different combinations of *Lactobacillus rhamnosus* (LR), *Lactobacillus acidophilus* (LA), *Bifidobacterium longum* (BL) and *Bifidobacterium breve* (BB) on the rate of SCFA development in the media containing rice fibre. Another objective was to investigate the synergistic effects between these microorganisms on the fermentation of fibre and to investigate the extent of fermentation of dietary fibre by combinations of probiotics. This study aimed to understand the relationship between digestion and the SCFA formation by co-cultures.

10.3 Materials and Methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, co-cultures, preparation of cell suspensions, preparation of growth medium, *in-vitro* fermentation, determination SCFA formation, determination of percentage of indigestible fibre quantity and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.3, 3.6.4, 3.6.5, 3.5.6, 3.10, 3.11 and 3.12, respectively.

10.4 Results

This study used two rice varieties (RR1 and RR2). Fibre was extracted from the rice varieties as SDF, IDF, and TDF and fibre fractions from rice variety RR1 were labelled as IDF1, SDF1 and TDF1 and fibre fractions from rice variety RR2 were labelled as IDF2, SDF2 and TDF2. These fractions were fermented with co-cultures of probiotics. This study focused mainly on SCFA formation of co-cultures on different fibre fractions.

All the combinations produced more acetate ($p<0.05$) than propionate and butyrate (Table 10.1). However, the amount of the acetate formed by combinations of

the same species was less than combinations of different species. There was no significant difference ($p<0.5$) in propionate and butyrate produced by different combinations. This indicates that when these microorganisms work as co-cultures, they produce more acetate from fibre fermentation than propionate and butyrate. The molar fraction of butyrate remained constant or slightly increased (non significant, ($p>0.05$) in batch cultures derived from most of the combinations of probiotics (Table 10.2). This implies that the quantity of propionate and butyrate did not change significantly with or without fibre using the co-cultures in this trial. Tables 10.1 and 10.2 show the results of combinations of BB+BL, BB+LR and BB+BL+LR. Results from the balance of the combinations are shown in Appendix C. Results are expressed as the mean values of two trials±standard error.

Table 10.1 Fermentation of rice dietary fibre with culture combinations leading to formation of short chain fatty acids (SCFA, m moles/100mL)

A) BB+BL

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.27±0.02	1.36±0.01	1.64±0.01	1.81±0.03	0.04	0.05	0.07	0.06	0.03	0.03	0.05	0.04
SDF-1	0.30±0.01	1.44±0.01	1.75±0.04	1.99±0.02	0.04	0.06	0.07	0.07	0.03	0.03	0.05	0.05
TDF-1	0.28±0.03	1.49±0.01	1.82±0.01	2.01±0.02	0.04	0.06	0.09	0.09	0.03	0.03	0.05	0.05
IDF-2	0.33±0.01	1.31±0.01	1.62±0.01	1.81±0.02	0.04	0.06	0.07	0.08	0.03	0.03	0.04	0.05
SDF-2	0.31±0.02	1.42±0.01	1.83±0.01	1.95±0.01	0.04	0.06	0.08	0.09	0.03	0.03	0.05	0.06
TDF-2	0.28±0.01	1.49±0.01	1.86±0.04	1.97±0.03	0.04	0.06	0.09	0.10	0.03	0.03	0.05	0.05
GLU	0.25	1.58±0.03	1.90±0.02	2±0.01	0.04	0.06	0.10	0.11	0.03	0.03	0.05	0.04
CON	0.25±0.02	0.20±0.03	1.20±0.02	1.22±0.02	0.04	0.05	0.05	0.05	0.03	0.03	0.03	0.03

B) BB+LR

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.28±0.01	1.40±0.03	3.74±0.03	3.20±0.02	0.04	0.05	0.05	0.06	0.03	0.03	0.04	0.05
SDF-1	0.30±0.01	1.54±0.02	4.28±0.01	3.24±0.03	0.04	0.05	0.06±0.01	0.05	0.03	0.03	0.04	0.03
TDF-1	0.28±0.03	2.15±0.05	6.44±0.02	4.11±0.02	0.04	0.06	0.08	0.06	0.03	0.03	0.05	0.04
IDF-2	0.30±0.01	1.45±0.04	2.94±0.03	3.01±0.03	0.04	0.05	0.05	0.05	0.03	0.03	0.04	0.04
SDF-2	0.25	1.75±0.04	3.10±0.03	3.04±0.04	0.04	0.04	0.06	0.06	0.03	0.03	0.04	0.04
TDF-2	0.30±0.01	1.92±0.03	3.77±0.02	3.79±0.04	0.04	0.05	0.06	0.12	0.03	0.03	0.04	0.03
GLU	0.31±0.02	1.59±0.06	2.97±0.04	3.55±0.06	0.04	0.05	0.05	0.05	0.03	0.03	0.04	0.03
CON	0.23±0.01	1.27±0.03	1.20±0.04	1.25±0.06	0.02	0.05	0.05	0.04	0.03	0.03	0.04	0.03

C) BB+BL+LR

H	**0**	**6**	**24**	**48**	**0**	**6**	**24**	**48**	**0**	**6**	**24**	**48**
SCFA	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF1	0.28±0.01	4.71±0.05	5.09±0.03	2.72±0.03	0.04	0.05	0.06	0.05	0.03	0.04	0.04	0.03
SDF-1	0.32±0.01	5.13±0.09	5.45±0.03	4.52±0.04	0.04	0.05	0.07	0.06±0.01	0.03	0.04	0.04	0.03
TDF-1	0.35±0.01	5.41±0.01	5.77±0.04	5.68±0.03	0.04	0.09±0.01	0.07	0.07	0.03	0.04	0.04	0.04
IDF-2	0.30±0.01	5.25±0.04	4.49±0.02	3.70±0.07	0.04	0.05	0.06	0.05	0.03	0.04	0.04	0.04
SDF-2	0.33±0.01	5.69±0.02	6.22±0.04	5.45±0.04	0.04	0.06	0.06	0.05	0.03	0.04	0.03	0.03
TDF-2	0.32±0.01	5.93±0.07	8.18±0.03	5.73±0.06	0.04	0.07	0.07±0.01	0.07	0.03	0.04	0.04	0.03
GLU	0.30±0.01	5.36±0.06	5.48±0.12	5.13±0.03	0.04	0.05	0.06	0.05	0.03	0.04	0.04	0.03
CON	0.23±0.01	1.24±0.01	1.21±0.04	1.24±0.02	0.04	0.05	0.05	0.05	0.03	0.04	0.03	0.03

Results are expressed as the mean value of two trials±standard error. Standard error which is less than 0.01 is not included

Table10.2 Molar fraction of acetate: propionate: butyrate

A) BB+BL

H	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate %	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate %	Butyrate%
IDF-1	78.03±1.15	12.94±0.68	9.02±0.47	94.42±0.03	3.30±0.02	2.28±0.01	93.58±0.03	3.75±0.02	2.67±0.01	94.59±0.08	3.29±0.05	2.12±0.03
SDF-1	79.68±0.49	11.98±0.29	8.35±0.2	94.16±0.12	3.68±0.08	2.16±0.05	93.55±0.12	3.95±01.08	2.50±0.05	94.39±0.05	3.37±0.03	2.24±0.02
TDF-1	78.53±1.64	12.65±0.97	8.82±0.68	94.36±0.17	3.56±0.19	2.09±0.02	93.15±0.17	4.46±0.19	2.40±0.02	93.40±0.06	4.09±0.03	2.51±0.02
IDF-2	81.48±0.41	10.91±0.24	7.61±0.17	93.63±0.03	4.02±0.02	2.36±0.01	93.41±0.03	4.27±0.02	2.31±0.01	93.46±0.06	4.10±0.04	2.44±0.02
SDF-2	80.13±0.94	11.71±0.56	8.16±0.39	93.82±0.03	4.00±0.02	2.18±0.01	93.41±0.03	4.21±0.02	2.38±0.01	92.90±0.03	4.20±0.02	2.90±0.01
TDF-2	78.64±0.55	12.59±0.32	8.77±0.22	94.36±0.12	3.56±0.08	2.09±0.04	93.10±0.12	4.56±0.08	2.34±0.04	92.88±0.09	4.56±0.06	2.56±0.03
GLU	76.88	13.62	9.50	94.66±0.06	3.37±0.04	1.97±0.02	92.83±0.06	4.88±0.04	2.29±0.02	93.22±0.03	4.89±0.02	1.89±0.01
CON	75.44±1.44	14.47±0.85	10.09±0.59	93.71±0.09	3.71±0.05	2.57±0.04	93.61±0.09	3.78±0.05	2.61±0.04	94.38±0.08	3.54±0.05	2.08±0.03

B) BB+LR

H	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	80.51±0.51	11.55±0.3	7.94±0.21	94.80±0.08	3.21±0.05	1.99±0.03	97.66±0.01	1.26±0.01	1.08±0.02	96.71±0.22	1.79±0.09	1.50±0.13
SDF-1	81.48±0.46	10.97±0.27	7.55±0.19	94.79±0.031	3.21±0.28	2.00±0.02	97.59±0.05	1.42±0.13	0.99±0.07	97.62±0.24	1.51±0.1	0.87±0.13
TDF-1	80.40±1.54	11.61±0.91	7.99±0.63	96.00±0.1	2.51±0.06	1.49±0.04	98.03±0.03	1.15±0.01	0.83±0.01	97.68±0.07	1.38±0.1	0.93±0.03
IDF-2	81.48±0.46	10.97±0.27	7.55±0.19	94.71±0.44	3.36±0.4	1.94±0.04	97.03±0.19	1.73±0.13	1.24±0.06	97.03±0.26	1.61±0.12	1.36±0.14
SDF-2	78.87	12.52	8.61	95.90±0.08	2.37±0.05	1.73±0.03	97	1.78±0.02	1.22±0.02	96.92±0.24	1.92±0.1	1.16±0.15
TDF-2	81.48±0.46	10.97±0.27	7.55±0.19	96.15±0.05	2.28±0.03	1.58±0.02	97.38±0.09	1.65±0.03	0.96±0.07	96.25±0.23	2.97±0.15	0.78±0.08
GLU	81.90±0.88	10.72±0.52	7.38±0.36	95.38±0.16	2.73±0.09	1.89±0.07	97.29±0.03	1.55±0.02	1.15±0.01	97.89±0.09	1.39±0.07	0.72±0.01
CON	76.88±0.72	13.70±0.42	9.42±0.29	94.28±0.11	3.38±0.06	2.34±0.04	93.54±0.18	3.71±0.1	2.75±0.08	94.54±0.32	3.42±0.22	2.04±0. 1

C) BB+BL+LR

H	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	80.51±0.51	11.55±0.30	7.94±0.21	98.26±0.02	0.99±0.01	0.74±0.01	98.16±0.01	1.12±0.01	0.72	97.27±0.14	1.79±0.12	0.94±0.01
SDF-1	82.36±0.42	10.45±0.25	7.19±0.17	98.27±0.06	1.04±0.05	0.69±0.01	98.14±0.02	1.19±0.01	0.67	98.07±0.18	1.24±0.13	0.69±0.05
TDF-1	83.89±0.35	9.5±0.21	6.57±0.14	97.77±0.1	1.54±0.01	0.69	98.09±0.03	1.28±0.03	0.64	98.17±0.08	1.20±0.02	0.63±0.07
IDF-2	81.48±0.46	10.97±0.27	7.55±0.19	98.30±0.07	1.00±0.06	0.70±0.01	97.98±0.03	1.21±0.03	0.82	97.65±0.07	1.37±0.04	0.98±0.11
SDF-2	83.16±0.38	9.98±0.23	6.87±0.16	98.26±0.04	1.09±0.03	0.65	98.49±0.05	0.97±0.06	0.54±0.01	98.63±0.04	0.85±0.02	0.51±0.02
TDF-2	82.36±0.42	10.45±0.25	7.19±0.17	98.12±0.02	1.21±0.01	0.66±0.01	98.66±0.07	0.86±0.07	0.48	98.27±0.05	1.15±0.06	0.58±0.01
GLU	81.48±0.46	10.97±0.27	7.55±0.19	98.36±0.02	0.99±0.03	0.65±0.01	98.15±0.01	1.14±0.01	0.71±0.02	98.35±0.02	1.01±0.03	0.65
CON	76.88±0.72	13.70±0.42	9.92±0.29	93.70±0.04	3.60±0.02	2.69±0.02	93.65±0.22	3.76±0.13	2.60±0.09	93.90±0.13	4.06±0.11	2.04±0.02

Results are expressed as the mean value of two trials±standard error.

One measure of the fermentation for each culture combination was the percentage of substrate remaining at each sample time during fermentation (Table 10.3).

Table 10.3 Percentage of substrate remaining after each time point

Co-cultures		RR1	RR1	RR1	RR2	RR2	RR2
	H	IDF	SDF	TDF	IDF	SDF	TDF
LA+LR	6 h	58.3	41.8	80.3	56.1	51.7	94.8
	24h	28.6	15.4	47.3	22.0	26.4	36.5
	48h	14.3	9.9	11.0	13.2	12.1	16.5
BB+BL	6 h	64.9	35.1	88.0	62.7	58.3	90.2
	24h	25.3	26.2	49.5	26.4	29.7	49.5
	48h	11.0	11.0	15.4	14.3	15.4	9.9
BL+LR	6 h	61.6	49.5	89.1	57.2	50.6	88.0
	24h	18.7	20.9	41.8	22.0	22.0	45.1
	48h	6.6	7.7	18.7	9.9	7.7	19.8
BB+LR	6 h	42.9	63.8	88.0	58.3	52.8	88.0
	24h	24.2	20.9	40.7	27.5	22.0	37.4
	48h	13.2	11.0	9.9	14.3	7.7	9.9
BB+LA	6 h	70.4	69.3	89.1	48.0	61.6	86.9
	24h	17.6	12.1	56.1	23.1	26.4	51.7
	48h	12.1	6.6	17.6	8.8	11	17.6
BL+LA	6 h	50.6	50.6	79.2	48.4	44.0	91.3
	24h	22.0	12.1	46.2	23.1	28.6	38.5
	48h	14.3	8.8	19.8	7.7	12.1	12.1
BB+BL+LA+LR	6 h	48.4	60.5	85.8	51.7	49.5	95.7
	24h	23.1	19.8	50.6	25.3	23.1	33.0
	48h	6.6	11.0	15.4	12.1	5.5	9.9
BB+BL+LA	6 h	50.6	49.5	90.2	53.9	45.1	80.3
	24h	20.9	27.5	23.1	24.2	18.7	19.8
	48h	5.5	7.7	11.0	5.5	4.4	6.6
BB+BL+LR	6 h	38.5	38.5	72.6	44.0	0.2	73.7
	24h	11.0	8.8	35.2	22.0	0.0	17.6
	48h	4.4	-	2.2	4.4	-	-

Results are expressed as the mean values of two trials±standard error.

The total SFCA produced (acetate+propionate +butyrate) in each fermentation setup (fibre fraction) is shown in Fig 10.1.

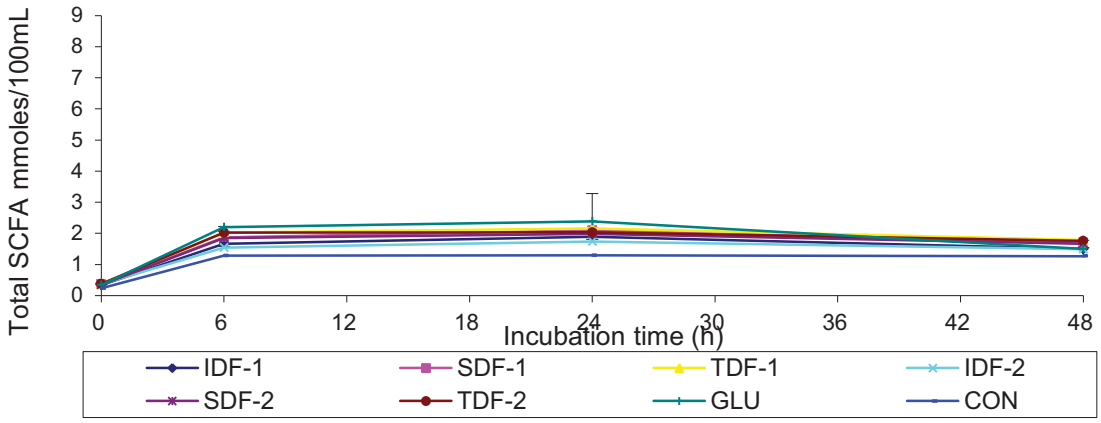

Fig 10.1 A) LA+LR

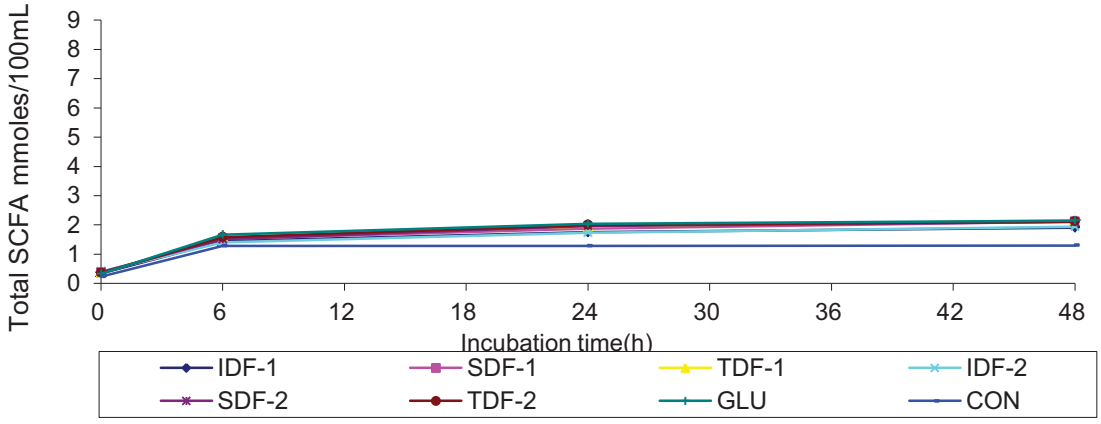

Fig 10.1 B) BL+BB

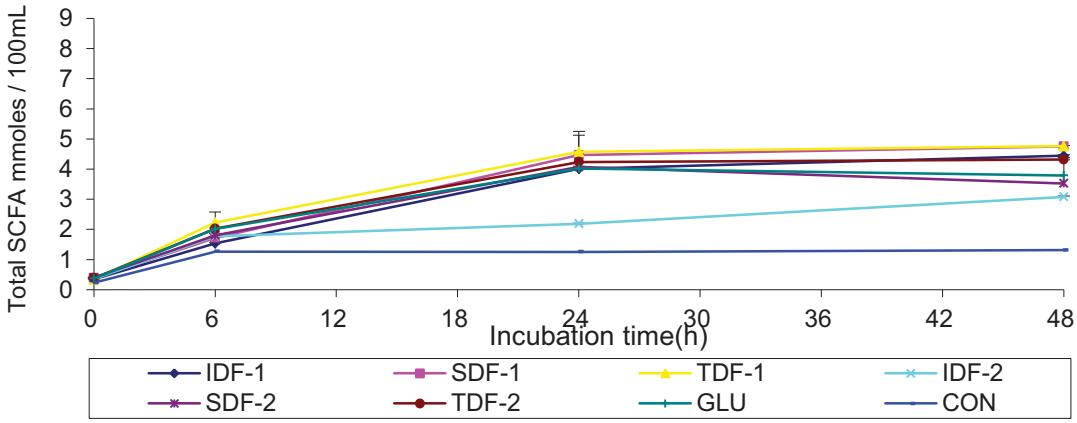

Fig 10.1 C) BL+LR

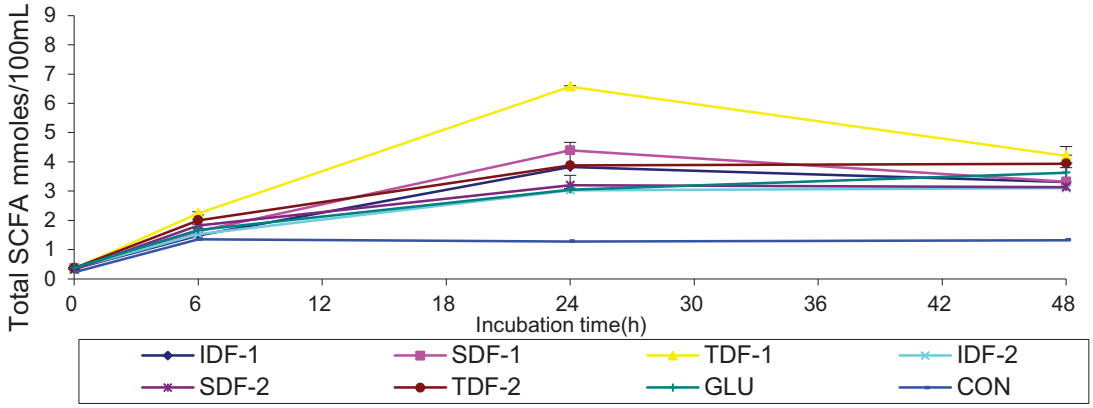

Fig 10.1 D) BB+LR

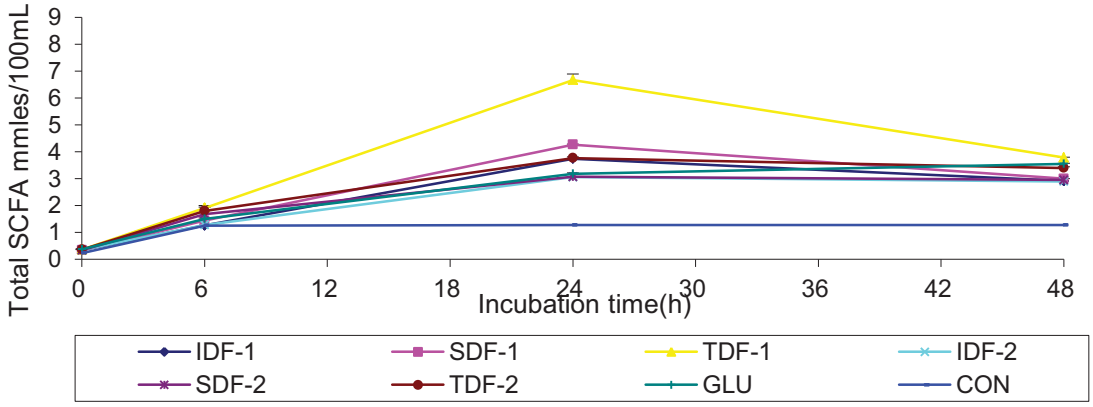

Fig 10.1 E) BB+LA

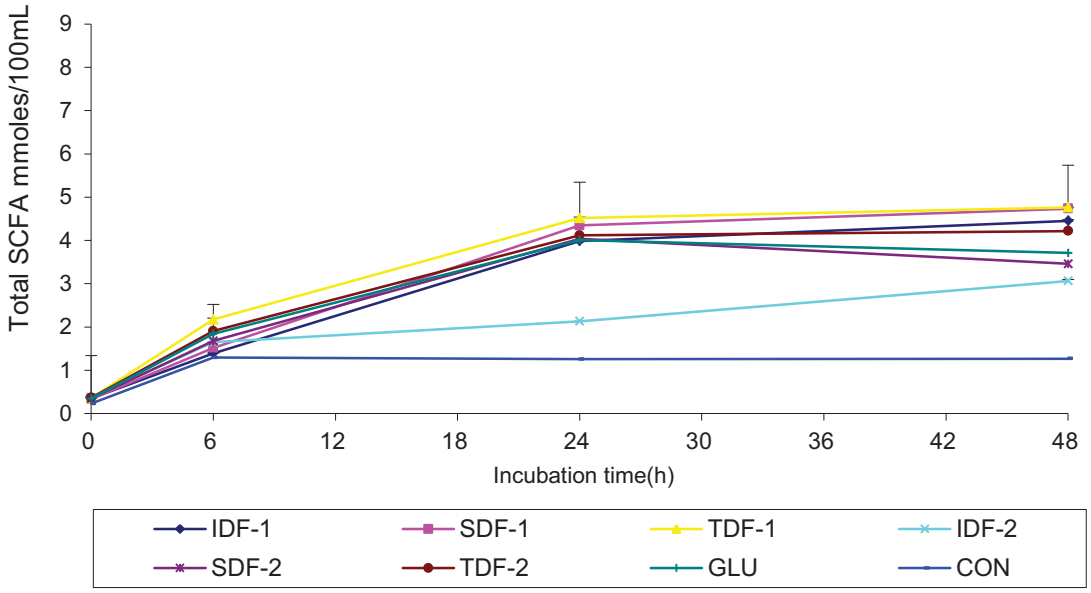

Fig 10.1 F) BL+LA

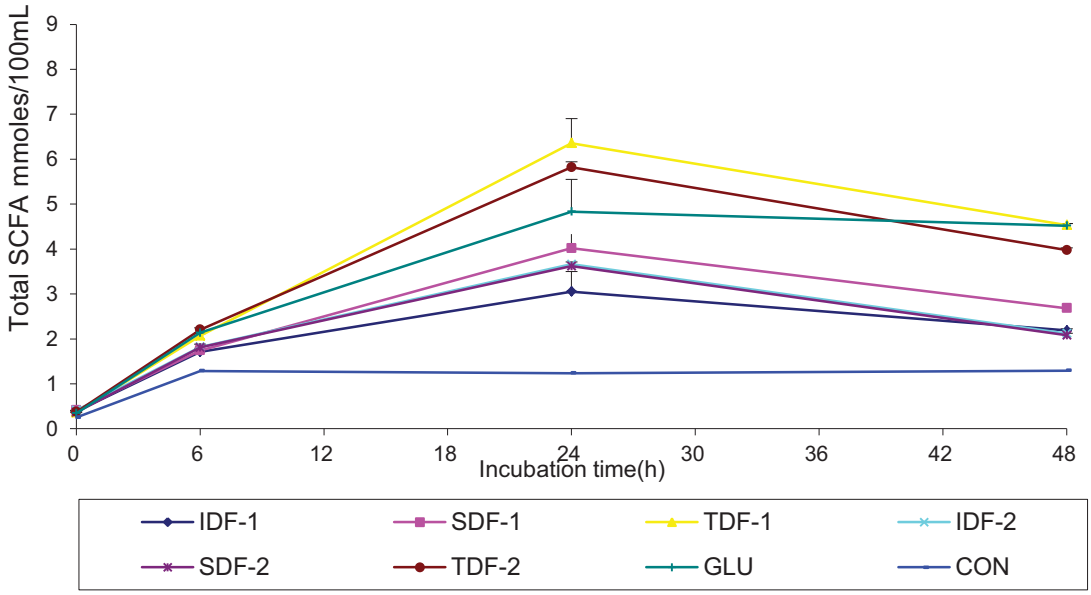

Fig 10.1 G) BB+BL+LA +LR

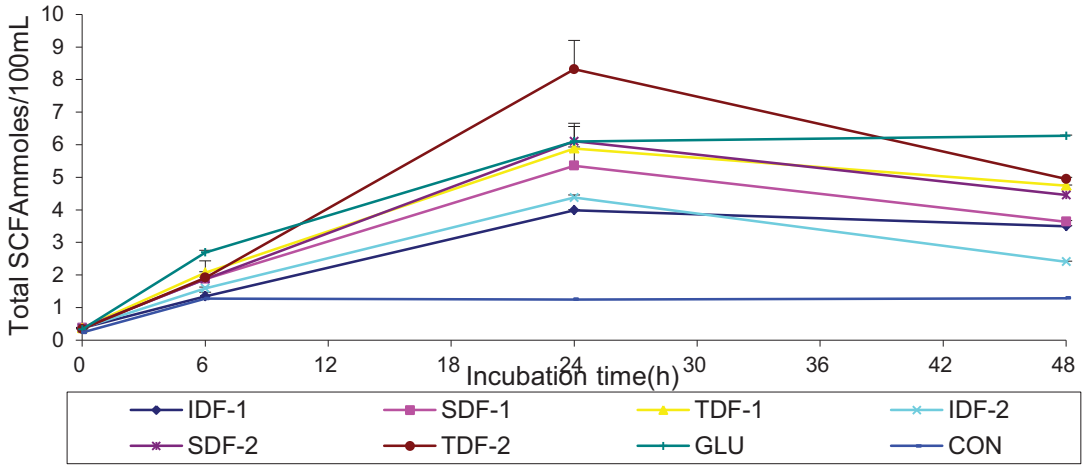

Fig 10.1 H) BB+BL+LA

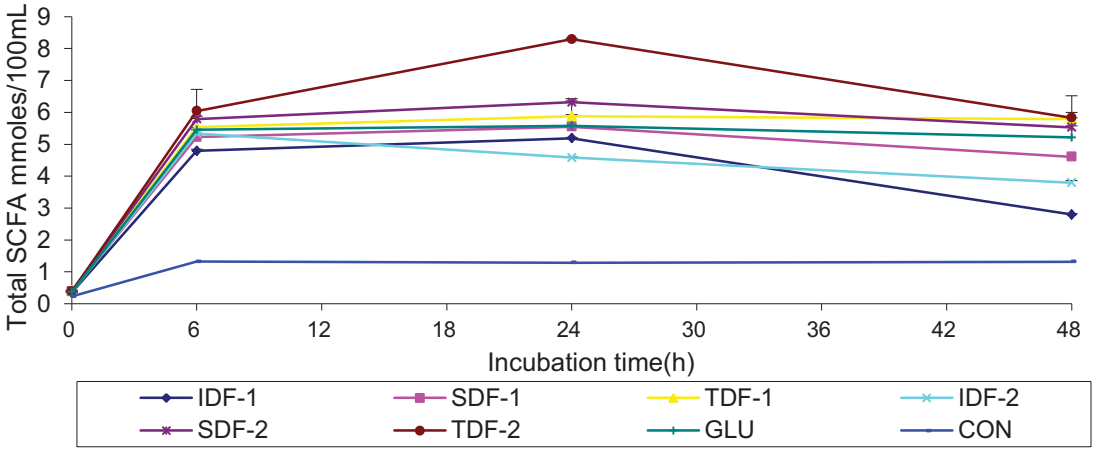

Fig 10.1 I) BB+BL+LR

Figure 10.1 Total SCFA produced on different substrates (A-I)

Results are expressed as the mean value of two trials±standard error. Units are mmoles /100mL. GLU-glucose,Con-Control. Total SCFA is addition of acetate+ propionate+butyrate of each substrate.

The total SFCA produced by nine different probiotic combinations on the two different rice varieties is shown in figure 10.2.

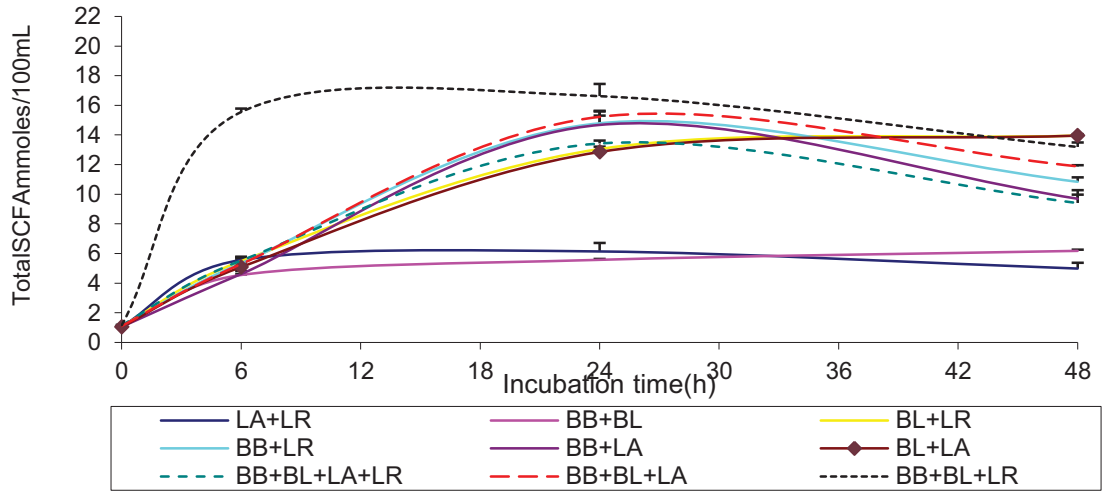

Fig 10.2 A) Total SCFA on RR1

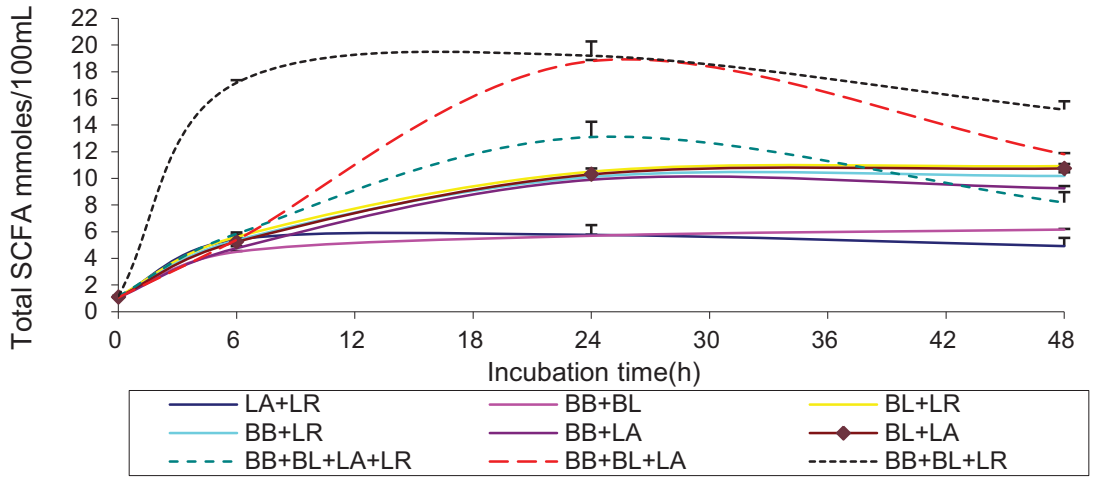

Fig 10.2 B) Total SCFA on RR2

Figure 10.2 (A-B) Total SCFA on different rice varieties using nine probiotic combinations

Results are expressed as the mean values of two trials±standard error. Units are mmoles/100mL. Total SCFA is the sum of acetate + propionate + butyrate of IDF+SDF+TDF.

Fig 10 .3 shows the proportion of the total SFCA produced by different probiotic combinations on the different rice fibre fractions (IDF, SDF and TDF) at each sampling time.

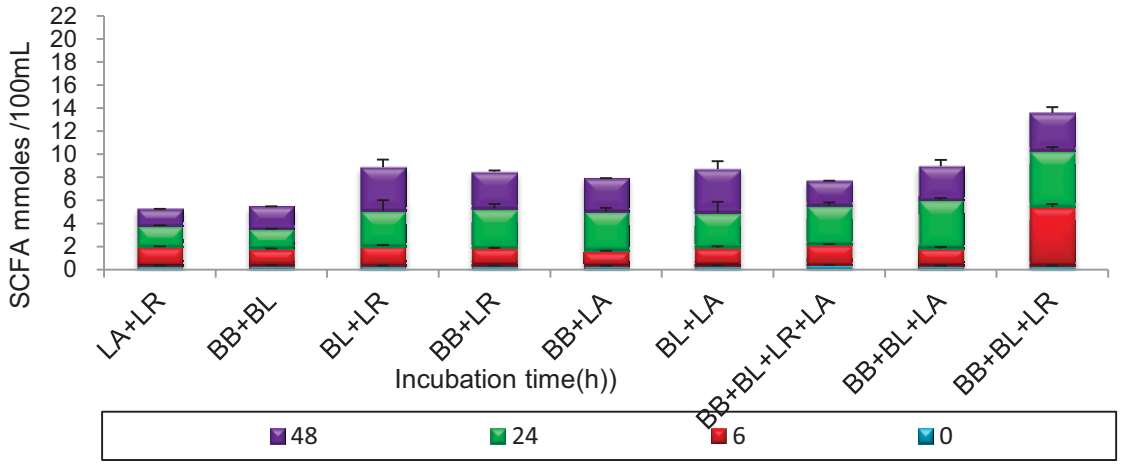

Fig 10.3A) IDF

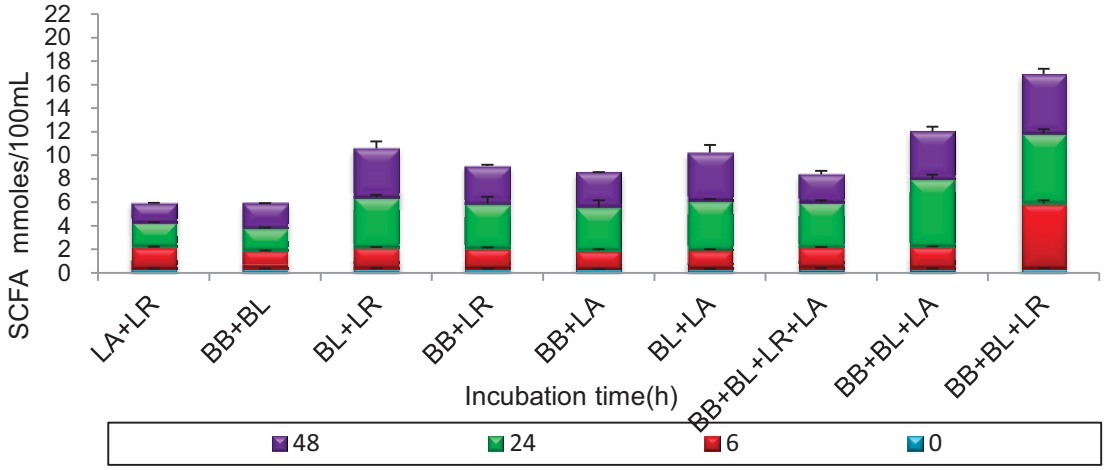

Fig 10.3 B) SDF

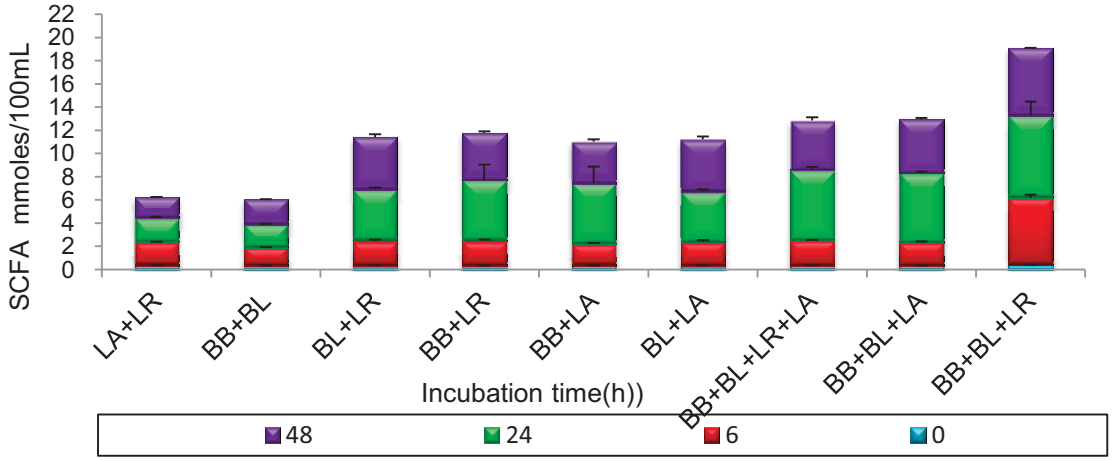

Fig 10.3 C) TDF

Figure 10.3 (A-C)Total SCFA on fibre fractions

Results are expressed as the mean value of two trials±standard error. SCFA is calculated as the sum of the average of acetate + propionate+butyrate of RR1.

10.5 Discussion

In-vitro fermentation studies using co-cultures and monocultures have limitations in interpreting the relevance of these results to the human gut, due to the complexity of human colon. However, *in-vitro* studies using batch culture experiments are effective in understanding the metabolism of pure cultures and inter species interactions (Belenguer *et al.,* 2006; Meulen *et al.,* 2006).

Previous studies found that a high proportion (90–95%) of SCFAs produced in the human colon are absorbed, leaving 5 –10 % in the faeces (Berggren *et al.,* 1993). This makes it difficult to study changes in SCFA production in the gut by studying faecal composition. However, SCFA of faecal excretion will give some indication of the SCFA production in the proximal colon (Scheppach *et al.,* 1988). Microorganisms in the human gut behave as a consortium, fermenting food as a combination of organisms and as a result of inter relationships between the species (Freter, 1992). Therefore, studies with combinations of organisms on food using *in-vitro* conditions can help to understand these interactions, the way they affect the fermentation of different substrates and metabolite formation.

The present study of co-cultures fermentation led to the identification of two clearly distinct types of SCFA formation. Combinations of the same genus formed less SCFA than those containing members of a different genus (Fig 10.1, 10.2 and 10.3). For instance, the combinations of BB+BL and LA+LR produced significantly ($p<0.05$) less SCFA than those containing more than one genus. This indicates that the fermentation of rice fibre can be done most efficiently by microbial combinations from more than one genus.

Previous studies with cellulytic bacteria in a batch system found two different microbial interactions - competition and synergism (Dehority, 1993; Odenyo *et al.,* 1994a). Competition has a negative impact on microbial growth. Microorganisms

belonging to the same genus are likely to compete for the same substrate. Similar nutrient requirements of organisms belonging to the same genus (Fredrickson, 1977) may result in competition for adherence to fibre, fibre hydrolysis, and utilization of hydrolytic products, all leading to a reduction in the potential amount of metabolite formation. Microorganisms, each belonging to a different genus, are more likely than those of the same genus to interact synergistically (Shi *et al.,* 1997).

Metabolite formation with mixed cultures may be strain specific. This has been observed previously with short chain fructooligosaccharides (Bouhnik *et al.,* 1997; Saulnier *et al.,* 2007b). However, this was not observed clearly in the present trials with combinations of different genera.

This study found different amounts of metabolite formed from rice fibre by different combinations of more than two microorganisms. The total amount of SCFA produced by combinations of more than two microorganisms varied with BB+BL+LR> BB+BL+LA> BB+BL+LA+LR. These three combinations produced more SCFA than the other combinations and much more than the BB+BL and LA+LR combinations. This suggests that BB+BL metabolised rice fibre fractions via different pathways when LA or LR is added, as indicated by the different profiles of SCFA. For example, the proportion of acetate increased, whereas that of propionic and butyric decreased. As indicated in the Table 10.1, 10.2 and Appendix C, rice fibre stimulates the formation of SCFA from both Bifidobacteria and *Lactobacillus* species. The combination of BB+BL with LA or LR would have created a more complex environment in the fermentation medium with more metabolic interactions among the species than the binary combinations of same species. These interactions between the microorganisms may result in competition initially, leading to commensalism and the production of more SCFA. This type of situation was observed with gut organisms of the rat with the

addition of *Bifidobacterium* with inulin and pectin (Nilsson *et al.,* 2006). The results from the present trial indicated that BB+BL have the ability to support the growth of *Lactobacillus* species. Cross feeding between bacteria in this group (combination of BB+BL with LR) may be another reason for the high metabolite formation of this group. Some of the metabolites produced by one bacterium may provide the substrate to enhance the growth of other, resulting in higher overall metabolite formation (Belenguer *et al.,* 2006).

This study expected to show the highest amount of SCFA produced from the combination of all microorganisms (Table 10.1, Appendix C, Fig 10.1and 10.3). The lower amount of SCFA produced by the combination of all species, compared with the combination of more than two species, implies that synergism and competition between the organisms is quite specific. In this combination, the effects that noted in the other combinations, competition and synergism would be occurring together producing a similar amount of SCFA to the binary combinations. The results from the current and previous work confirm that the combination with *Bifidobacterium* has the ability to form more SCFA with rice dietary fibre than the combinations lacking the *Bifidobacterium* species.

The slowest co-cultures in terms of SCFA formation were LA+LR and BB+BL and these also produced the least amount of SCFA. Combinations with members of a different genus produced more SCFA after 24 h irrespective of the fibre fraction used as the substrate. As shown in earlier work, the growth of the individual organisms used in this trial reached a maximum after 24 h fermentation. This may explain the maximum metabolite formation seen after 24 h in these combinations. However, the combination of BB+BL+LR produced a significantly higher amount of SCFA than the other combinations at 6 h and 24 h (Fig 10.1 and 10.3). This result suggests that fermentation

of dietary fibre can occur faster if there is a compatible combination of microorganisms, and the most effective co-cultures in terms of SCFA formation was BB+BL +LR in this trial.

Acetate was the most abundant SCFA formed from all the combinations comprising more than 90% of the total SCFA formation at each hour (Table 10.2 and Appendix C). The microbial combinations produced a higher amount of acetate than other SCFA from both rice varieties. Combinations of the same genus produced significantly less acetate than the other microbial combinations with more than one genus. Combinations of members of the same genus may have a similar uptake of dietary fibre which makes them prone to competition. Previous research has found that Bifidobacteria have a greater ability to form more SCFA from dietary fibre than the *Lactobacillus* species (Kolida *et al.,* 2002). However, the current research found no significant difference in SCFA formation for fermentation using different combinations of *Bifidobacterium* or *Lactobacillus* species. Acetate formation in all the combinations increased within the time of incubation 0 to 24 h. This was not observed with propionate and butyrate. Less propionate and butyrate, and almost unchanged quantity of propionate and butyrate from the start of the experiment to end, indicate that microorganisms might have utilized propionate and butyrate as an energy source for their survival in the mixed culture environment. The short chain fatty acid that was less well used after 48 h of incubation by the co-cultures was acetate.

The ratio of propionate and butyrate was significantly less ($p<0.05$) than acetate (Table 10.2 and Appendix C). Interestingly, combinations of the same species produced a higher percentage of propionate and butyrate than the other culture combinations. The pattern of SCFA formation was acetate >propionate ≥ butyrate for all the combinations. A result from previous work on dietary fibre (oligofructose) reported increased butyrate

formation (Berggren *et al.,* 1993; Khan & Edwards, 2005) by organisms in the rat caecum. However, other work with *Bifidobacterium* and *Lactobacillus* species found a higher formation of acetate than propionate or butyrate with oligofructose, supporting the results of the current research (Kolida *et al.,* 2002).

Differences in the individual SCFA formation between different combinations may be due to the physiological properties of the strains, ability to ferment the substrate, and the composition of the medium (Angelov *et al.,* 2005). SCFA is the end product of microbial fermentation of dietary fibre as decribed in Chapter 2. Previous work found that butyrate production from the fermentation of dietary fibre is largely produced from an interconversion reaction from acetate (Morrison *et al.,* 2006). A certain percentage of butyrate reported during this study may have resulted from acetate. It is important to understand how butyrate is formed from these culture combinations since butyrate is the most important SCFA for human health (Cummings & Macfarlane, 1991). Butyrate can be stimulated by increasing the acetate formation in the fermentation medium.

Research on the fermentation of inulin in rats suggested that a higher amount of propionate can be produced when a surplus of substrate is available in the system (Nilsson *et al.,* 2006). However, a higher percentage of propionate was not observed in this study even when there was a surplus of fibre at the end of the fermentation in all the combinations except BB+BL+LR. There was no significant difference in propionate from the combination of BB+BL+LR and other combinations. This shows that more research is required to understand the quantity of individual SCFA formed.

A low ratio between acetate to propionate is important to reduce the human serum cholesterol level (Wolever *et al.,* 1996). Combinations of microorganisms belonging to the same genus produced the lowest ratio for acetate to propionate, since acetate formation was less in these combinations.

The amount of metabolites formed varied with the different substrates. All the co-cultures formed a greater amount of SCFA with TDF than with the IDF and SDF (Fig 10.3). This indicates that most effective fermented fibre fraction of rice was TDF for selected microbial combinations. Total dietary fibre (TDF) resulted in SCFA reaching a maximum after 24 h of fermentation with co-cultures of different species. Generally, SCFA formation among the fibre fractions followed the pattern TDF>SDF>IDF irrespective of the rice variety. There was less difference between IDF and SDF in terms of metabolite formation indicating that co-cultures have an equal ability to ferment IDF and SDF. Interestingly, the formation of SCFA with glucose was not higher than the TDF. Glucose is a pure monosaccharide whereas rice fibre fractions are likely to contain unknown components which might be less easily fermented by microorganisms than glucose. Therefore, more research is required to understand the difference in the fermentation of complex and simple structures by microorganisms. The results from this study indicate that both rice varieties form SCFA from all the microbial combinations, thus rice can be considered as a potential prebiotic for these microbial combinations. The milling grade of rice had a minimal effect on the fermentation of fibre by co-cultures.

Some of the combinations had the ability to digest more than 90% of the fibre while others used 80-85% (Table 10.3). Binary combinations digested rice fibre less than the other combinations. Culture combinations of members of the same genus had the least ability to digest the fibre. The combination of BB+BL+LR digested the SDF of both rice varieties completely and 100% TDF of RR2. The higher percentage (100%) of digestion of dietary fibre by this combination suggests that the co-operative growth between these bacterial strains is stronger, in terms of digesion of rice fibre, than

the other combinations. This result shows that the incubation period for this experiment was long enough to enable complete digestion of the fibre.

These results in total not only confirm that BB+BL+LR is the most effective co-cultures for the rice fibre fermentation out of selected combinations, but also other combinations of more than two employed in this study would be able to ensure satisfactory results in terms of metabolite formation and fermentation time.

The results from the fermentation of fibre by combinations of microorganisms are assumed to relate to the microbial activity that is likely to occur in the human gut. The findings of the present study indicate that supplementing the diet with rice fibre will stimulate the formation of acetate in human gut. All the microbial combinations produced the SCFA under low oxygen and highly reducing experimental conditions, similar to conditions in the distal colon. However, the place of SCFA formation may vary in the human gut depending on the length of the fibre fraction. For instance, short chain fibre fractions may ferment most rapidly and therefore, the site of fermentation is likely to be more proximal towards the caecum (Morrison *et al.,* 2006). Hence, it is important to understand the structure of rice fibres and how this may affect fermentation. This, in turn, may result in variable SCFA production depending on the amount of oxygen present at different sites in the gut. The site of fermentation in human gut may also influence the quantity of SCFA.

10.6 Conclusion

The present study showed that the fermentation of rice fibre by most of the mixed cultures significantly increased ($p<0.05$) the concentration of SCFA when compared with pure cultures. It is improbable that the SCFA increase was due to the fermentation of rice fibre per se, but rather due to the ability of compatible combinations of organisms

Combinations of microorganisms belonging to more than one genus produced more SCFA than the other combinations.

Acetate was the most abundant SCFA formed from all the combinations comprising more than 90% of the total SCFA formation at each hour. The pattern of SCFA formation was acetate > propionate >butyrate and the TDF produced more SCFA than from SDF or IDF.

Some of the combinations have an ability to digest more than 90% of the fibre, while others digested only 80-85%. BB+BL+LR was the most effective co-culture to facilitate the fermentation of rice fibre.

CHAPTER 11

The Influence of Environmental Factors on the Adhesion of Probiotics to Insoluble, Soluble and Total Dietary Fibre of Rice

11.1 Abstract

The adhesion of pure cultures of probiotics to insoluble dietary fibre (IDF1, IDF2), soluble dietary fibre (SDF1, SDF2), and total dietary fibre (TDF1, TDF2) of two rice varieties (RR1 and RR2) was investigated. *Bifidobacteria* and *Lactobacilli* adhered to dietary fibre fractions within 15 to 30 minutes. A higher percentage of *Bifidobacteria* (40-70%) adhered to rice fibre than *lactobacilli* (<40%). Adhesion was not affected by NaCl or Tween 80 but, was reduced by various sugars and polysaccharides (maltose, moltodextrin, amylopectin and amylose). The optimal temperature for adhesion was 37 ^{0}C and live cells are important for adhesion. Adhesion was inhibited by protease and by low pH (pH 3-4). Adhesion was not influenced by proteins or peptides linked with the fibre, but involved bacterial cell surface proteins or glycoproteins.

11.2 Introduction

Bacterial adhesion is dependent on many factors including their morphological and physiological properties, the nature of substrate and the environment (pH, other molecules, etc) (Gordon *et al.,* 1981). In addition, specific materials such as surface macromolecules, commonly lectins or lectin like proteins or glycoproteins may also influence adhesion (Tojo *et al.,* 1988).

Adhesion may be specific or nonspecific (Timmerman *et al.,* 1991; Tojo *et al.,* 1988). Specific adhesion involves selective binding by specific molecular components on the surface of bacteria and is less influenced by electrolytes, pH or temperature than non-specific binding (Timmerman *et al.,* 1991; Tojo *et al.,* 1988). Most of the attachment between bacteria and proteins are specific ligand- and receptor like interactions (McDowell *et al.,* 1995; Muller *et al.,* 1991). Non-specific adhesion results from Van der Waals forces acting between the bacteria and the solid substrate and does not involve specific adhesins or ligands (Busscher &Weerkamp 1987). Colonisation of bacteria on cellulose is partially due to nonspecific interactions (Miron *et al.,* 2001).

Proteins (albumin, fibronectin, fibrinogen, laminin, denatured collagen etc) may increase or decrease the bacterial adhesion to the substrate. They may bind to the bacterial surface, or be present in liquid medium during the adhesion period (Miron *et al.,* 2001).

Adhesion of bacteria to dietary fibre may occur in several steps, (initial non-specific adhesion, specific adhesion and growth) as for example with rumen cellulitic bacteria (Miron *et al.,* 2001). However, the process by which probiotics adhere to dietary fibre has not been investigated. Dietary fibre fractions (inulin, oligofructose, lactulose, galacto-oligosaccha-rides (GOS), transgalacto-oligosaccharides (TOS), and iso-maltooligosaccharides (IMO), can act as prebiotics (Blaut, 2002; Fooks *et al.,* 1999; Rastall & Maitin, 2002) for probiotics belonging to the *Bifidobacterium* and *Lactobacillus* genera which are important to human health (Degnan & Macfarlane, 1993; Ding *et al.,* 2005).

The adherence of *Lactobacillus spp* or *Bifidobacterium spp* to rice fibre has not been investigated. Moreover, it is not known whether adhesion to rice dietary fibre is a general characteristic of *Bifidobacterium spp* and *Lactobacillus spp* or whether adhesion

only occurs when the fibre is being digested by these species. The present work studied the ability of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium breve* and *Bifidobacterium longum* to adhere to several rice fibers in an attempt to understand the mechanism of adhesion of probiotics to rice fibers.

11.3 Materials and methods

11.3.1 General methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, bacterial strains, and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.2, and 3.12, respectively.

11.3.2 Preparation of cell suspensions

Freeze dried cultures were rehydrated by culturing *Lactobacillus spp* in MRS (de Man, Rogosa, and Sharpe) medium and *Bifidobacterium spp* in Reinforced Clostridial Medium under strict anaerobic conditions. *Lactobacillus* spp were incubated at 37ºC for 24 hours and *Bifidobacterium spp* were incubated at 37ºC for 72 hours. In preparation for *in-vitro* fermentation, the bacteria were pre cultured twice in 10 mL of the appropriate medium containing $10gL^{-1}$ glucose as the carbon source. After incubation the bacterial cells were centrifuged (12000xg, 10 min) (Imam &Harry-O'Kuru, 1991), washed twice with physiological saline (0.85% NaCl solution), and resuspended in the basal medium (Fildes PYF solution) to remove excess carbon before testing (Kontula *et al*., 1998).

11.3.3 Adhesion to fibre

Bacterial adhesion was determined using a co-sedimentation method (Crittenden *et al*., 2001). This was chosen as a simple, rapid, reproducible method that allowed the study of many factors on microbial adhesion to fibre and more accurate than methods

involving centrifugation (Minato & Suto, 1978), which cause the non-adherent bacterial cells to be trapped within the fibre (Rasmussen *et al*., 1989).

The bacterial cells were first washed twice with 10 mL of 0.1 M phosphate buffer (pH 7.0) to remove components of medium and cell products such as bacteriocins that may interfere with the assay. The washed cells were then resuspended in the same buffer to a concentration of approximately 10^7 cells ml^{-1}, determined using a Petroff_Hauusser counting chamber.

Two milliliters of the bacterial suspension were thoroughly mixed for 1 min in a 1-cm-diameter test tube with an equal volume of a suspension of the fibre (10 gL^{-1}) in 0.1 M phosphate buffer (pH 7.0). In this experiment each fibre was added to the 4 test tubes before adding the inoculum. These test tubes were labeled as 15 min, 30 min 45 min and 1 h. The bacterium-fibre suspension was then allowed to stand at room temperature for 15, 30, 45 min or 1 h to sediment. This step was taken to minimize any disturbance of adhesion onto the fibre. Two 1.5 mL samples were then taken from 0.5 cm below the surface of the liquid of the sample and the optical density was measured at 540 nm (OD_{540}) with a spectrophotometer using phosphate buffer as a blank. In order to calculate the percentage of cells that adhered to the fibre and subsequently co-sedimented to bottom of the test tube, the OD_{540} of these samples were compared with the OD_{540} of similar samples taken from two control tubes containing (i) bacteria without fibre and (ii) fibre without bacteria. The percentage of bacteria that adhered to the fibre was calculated as follows:

$$\textbf{Percentage of cells adhering to fibre} = 100\% - \{[(a - b)/c] \cdot 100\%\},$$

where "a" is the OD_{540} of a sample from the tube containing fibre plus bacteria, "b" is the OD_{540} of a sample from a control tube containing fibre but no bacteria, and "c" is the OD_{540} of a sample from a control tube containing bacteria but no fiber.

Bacterial strains with more than 70% of the cells adhered to the fibre were named as highly adherent. Bacterial strains with 40 to 70% adhesion were named as moderate, while those with less than 40% adhesion were named as poor adherent strains.

11.3.4 Influence of growth of bacteria on adhesion

The influence of the growth phase on adhesion was examined. Adhesion of the bacteria to fibre was determined with cells that were at 6h, 24 h, and at 36 h (Crittenden *et al*., 2001).

11.3.5 Study of adhesion mechanisms

Studies of the mechanisms of adhesion were aimed to determine whether adhesion was due to the growth medium components, or specific cellular or extracellular proteins produced by the bacteria. The nature of the receptor sites in the adhesion process was evaluated by using number of potential inhibitors of adhesion, including glucose, maltose, maltodextrin, amylose, and amylopectin. The treatments used in the adhesion assay are described in Crittenden *et al*. (2001) (Table 1). Each assay was performed twice. The bacteria were in the stationary phase when they were used in the adhesion assay. For each assay 2 mL of bacterial suspension were mixed with the same volume of fibre (10 g L^{-1}) suspended in the appropriate treatment medium (Phosphate buffer, fresh medium etc).

Table 11. 1 Treatments used in the investigation of mechanisms of adhesion

Treatment	Description
Phosphate buffer	No cell pretreatment; the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
Fresh medium	No cell pretreatment; the adhesion experiment was performed in fresh growth medium (pH 6.8). MRS and Reinforced Clostridial for *Lactobacillus* and for *Bifidobacterium* respectively
Spent medium	No cell pretreatment; the adhesion experiment was performed in PYF supernatant from a 24-h

	culture of the bacterial strain (pH 4.5). Bacteria are grown in this medium and supernatant was collected aftre removing the cells by centrifuging. Tubes were gently spun for 10s (two bursts of 5s each 15, 600 g) (Imam and Harry-O'Kuru , 1991). Non adhering cells will be in the supernatant.
Pepsin-treated spent medium	No cell pretreatment; the adhesion experiment was performed in spent medium(PYF) treated with 30 U of pepsin A (P 7000 Sigma USA pepsin from hog stomach, 800-2,500 units/mg protein) mL^{1} for 6 h at 37°C; for pepsin treatment, the pH of the medium was reduced to 2.0 by using 0.1 M HCl and then readjusted prior to the adhesion experiment to 4.5 by using 0.1 M NaOH
Pepsin-treated fibre	No cell pretreatment; the adhesion experiment was performed by using fibre that had been treated with 30 units of pepsin (P 7000 Sigma USA pepsin from hog stomach, 800-2,500 units/mg protein) mL^{-1} (at pH 2.0) for 6 h at 37°C; the fibre was washed and resuspended in phosphate buffer (pH 7.0) for the adhesion experiment
Proteinase K-treated cells	Cells (10^{8} CFU mL^{-1}) were pretreated with 10 units of proteinase K (P6556 sigma , USA lyophilized powder, ≥30 units/mg protein, 3.0-15.0 unit/mg solid) mL^{-1} at pH 7.0 for 6 h at 37°C; the cells were then washed twice, and the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
NaCl	No cell pretreatment; the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl (0.03mg/mL)
Tween 80	No cell pretreatment; the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 3.0 g of Tween 80/ L (Polyoxyethylenesorbitan monooleate, Polysorbate 80, P4780 Sigma)
Pepsin-treated cells	Cells (10^{8} CFU/ mL) were pretreated with 30 U of pepsin mL^{-1} at pH 2.0 for 6 h at 30°C; the cells were then washed twice (12000*g, 10 min) with phosphate buffer, and the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
Heat-treated cells, cells treated at room temperature (22-25°C), human body temperature (37°C)	Cells (10^{8} CFU/ mL) were heat treated at 65°C,22-25°C and 37°C for 30 min at pH 7.0; the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)

Effect of potential inhibitors	No cell pretreatment; the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 5 g of one of the following carbohydrates per liter: glucose, Sigma-Aldrich, G6152 maltose (M5885 Sigma-Aldrich),sucrose, (S0389 Sigma),lactose (L8773 Sigma), trehalose,(T0299, Sigma), cellobiose(C7252 Sigma), maltodextrin (419672 Aldrich) amylose (A 6211 , Sigma), and amylopectin.

Source: Crittenden et al.,(2001)

11.3.6 Adhesion under conditions to simulate to the environment of the human stomach and small intestine

To determine the effect of some of the conditions of the stomach and small intestine on bacterial adhesion to fibre, bacterial adhesion to fibre was determined under the conditions listed in Table 2 (Crittenden *et al*., 2001).

Table 11. 2. Treatments used to simulate conditions for adhesion during passage through the upper gastrointestinal tract

Treatment	Description
Effect of pH	No cell pretreatment; the adhesion experiments were performed in 0.1 M citrate-phosphate buffers at pH, 3.0, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, and 5.
Effect of acid plus pepsin	No cell pretreatment; the adhesion experiments were performed in 0.2 M HCl-KCl buffer (pH 3.0) containing 30 U of pepsin A (P 7000 Sigma USA pepsin from hog stomach, 800-2,500 units/mg protein) mL^{-1}
Effect of bile	No cell pretreatment; the adhesion experiments were performed in 0.1 M phosphate buffer (pH 7.0) containing 3.0 g of porcine bile (Sigma Chemical Co.) L^{-1}
Effect of pancreatin	No cell pretreatment; fibre was pretreated for 6 h at 37°C with 0.01 g of porcine pancreatin (Sigma Chemical Co.) g^{-1}; the fibre was washed twice, and the adhesion experiments were performed in 0.1 M phosphate buffer (pH 7.0)

Source: Crittenden et al., 2001

11.4 Results

This study used two rice varieties (RR1 and RR2). Fibre was extracted from these rice varieties as SDF, IDF, and TDF and fibre fractions from rice variety RR1 were named as IDF1, SDF1 and TDF1, and fibre fractions from rice variety RR2 were named as IDF2,

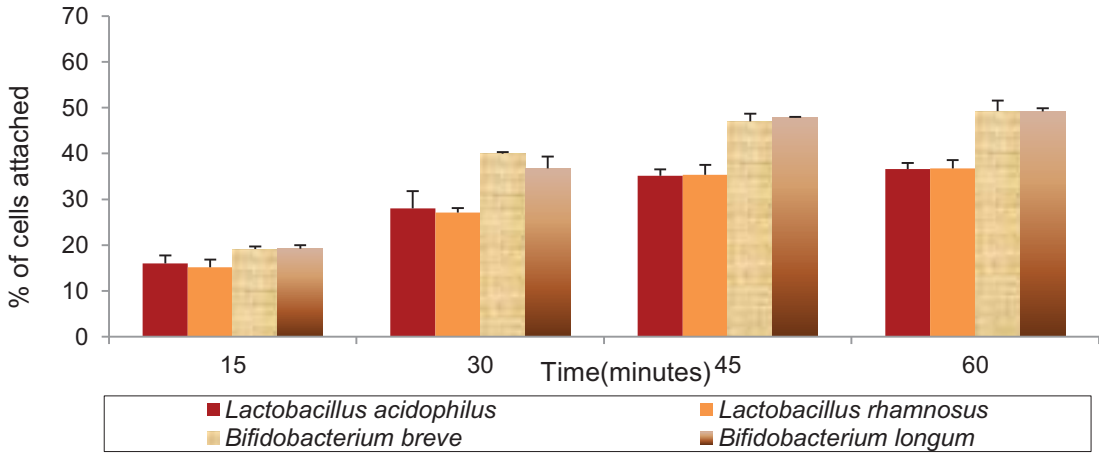

Figure 11.1 Effect of time on adhesion (have considered average of fibre fractions of RR1 and RR2) on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error.

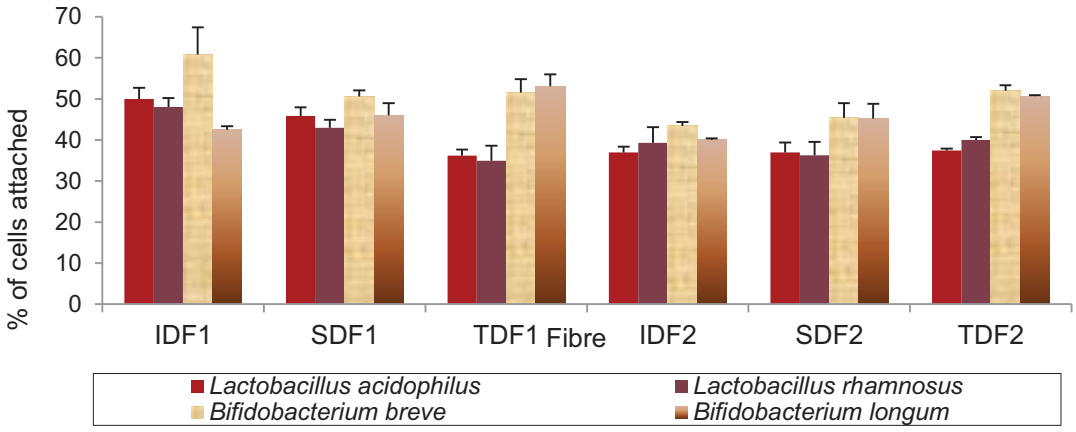

Figure 11.2 Adhesion of bacteria to fibre fractions.

Results are expressed as the mean value of two trials±standard error.

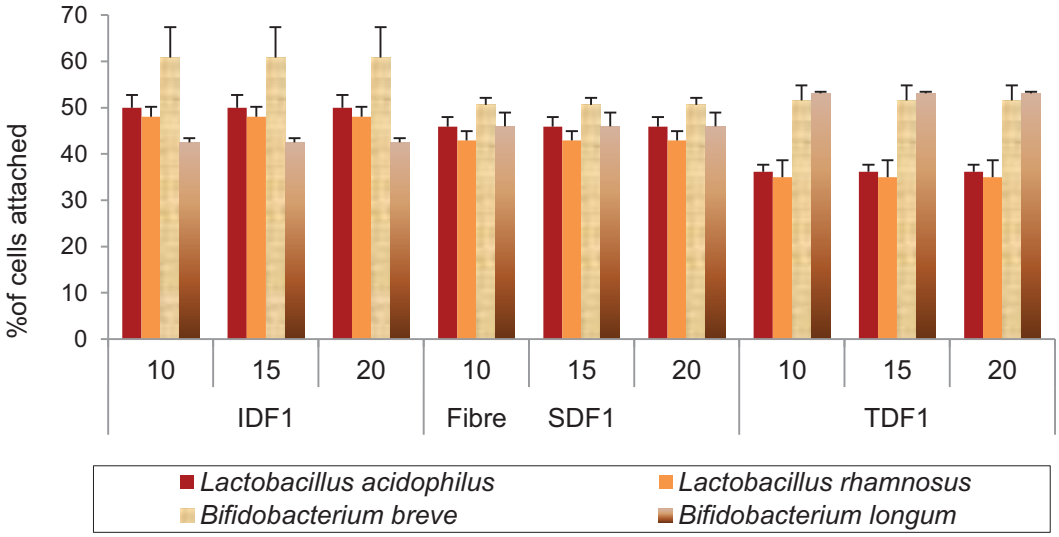

Figure 11.3.A. Effect of concentration of substrate from RR1 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error**.** 10g/l-10, 15g/l-15, and 20g/l-20

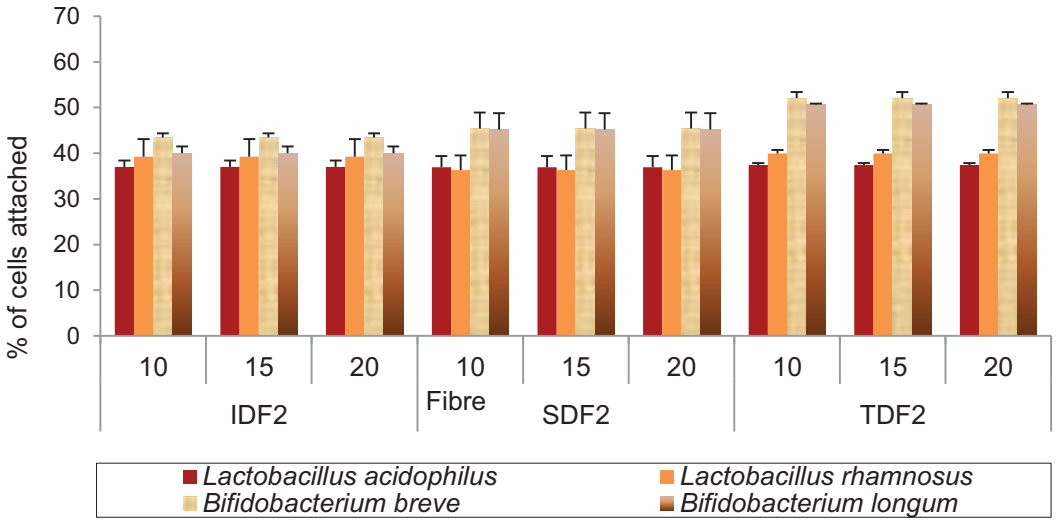

Figure 11.3.B. Effect of concentration of substrate from RR2 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. 10g/l-10,15g/l-15, and 20g/l-20

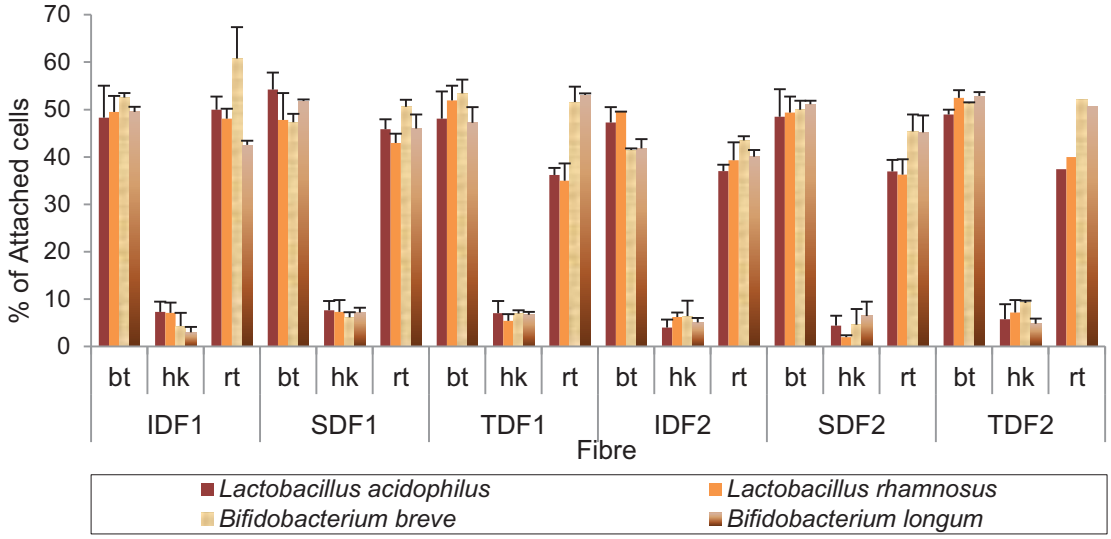

Figure 11.4 Effect of body temperature (37^0C), heat killed cells and, room temperature on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Body temperature (37^0C)- bt, Heat killed cells- hk, Room temperature- rt

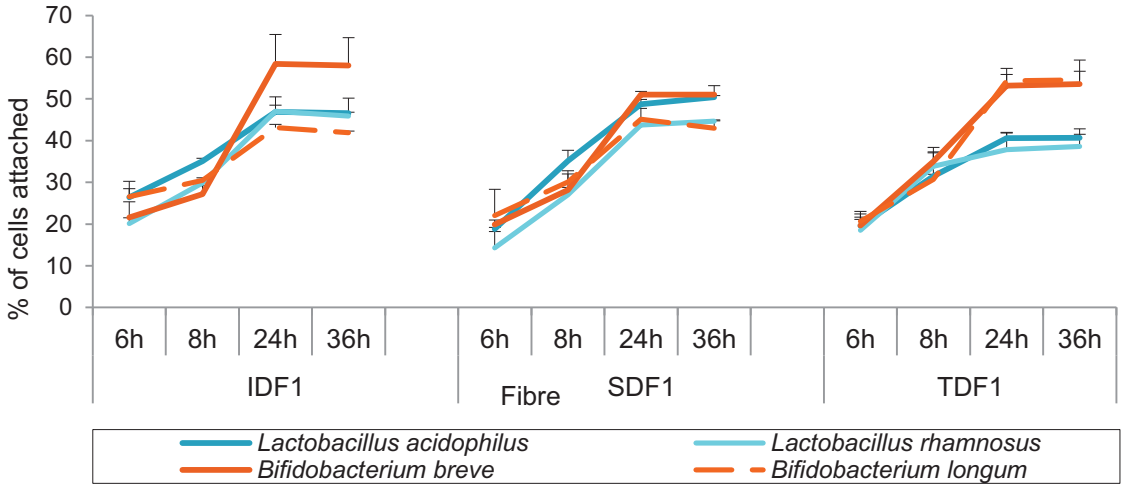

Figure 11.5.A. Effect of growth on adhesion of RR1

Results are expressed as the mean value of two trials±standard error.

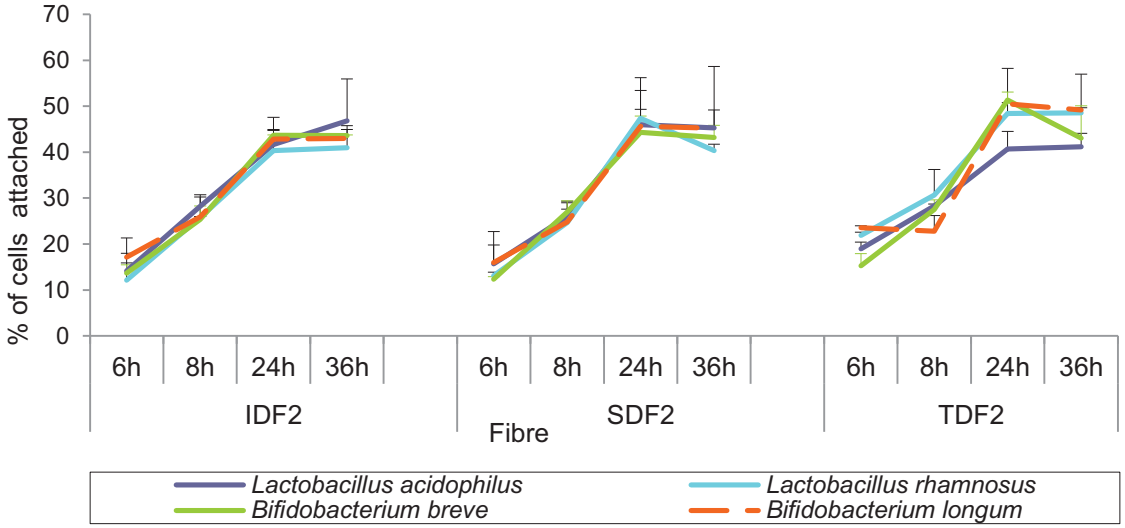

Figure 11.5.B. Effect of growth phase on adhesion of RR2.

Results are expressed as the mean value of two trials±standard error.

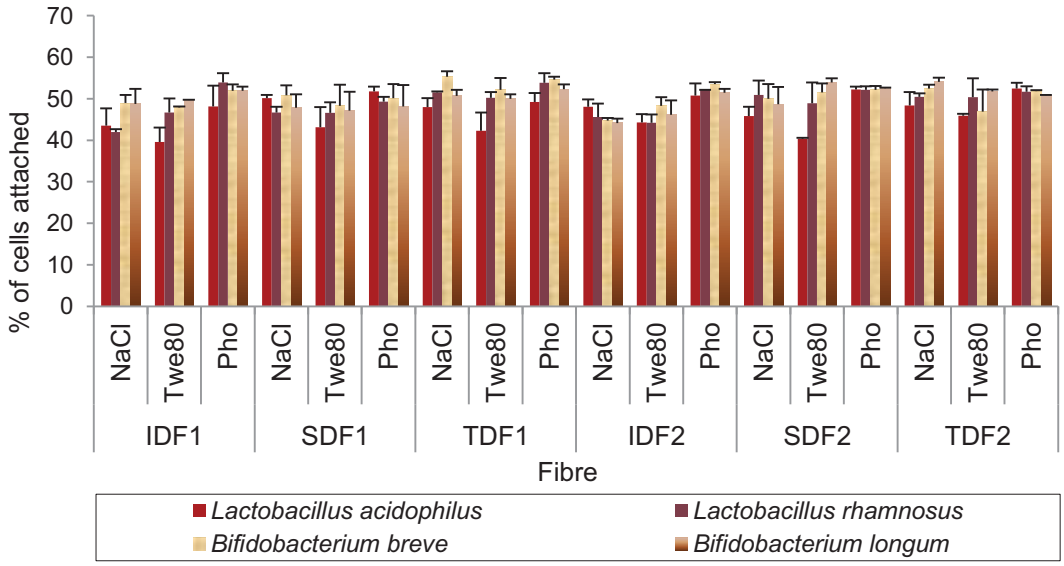

Figure 11.6 Effect of Chemicals NaCl, Tween 80 and Phosphate buffer on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Tween 80-Twe80, Phosphate buffer-Pho

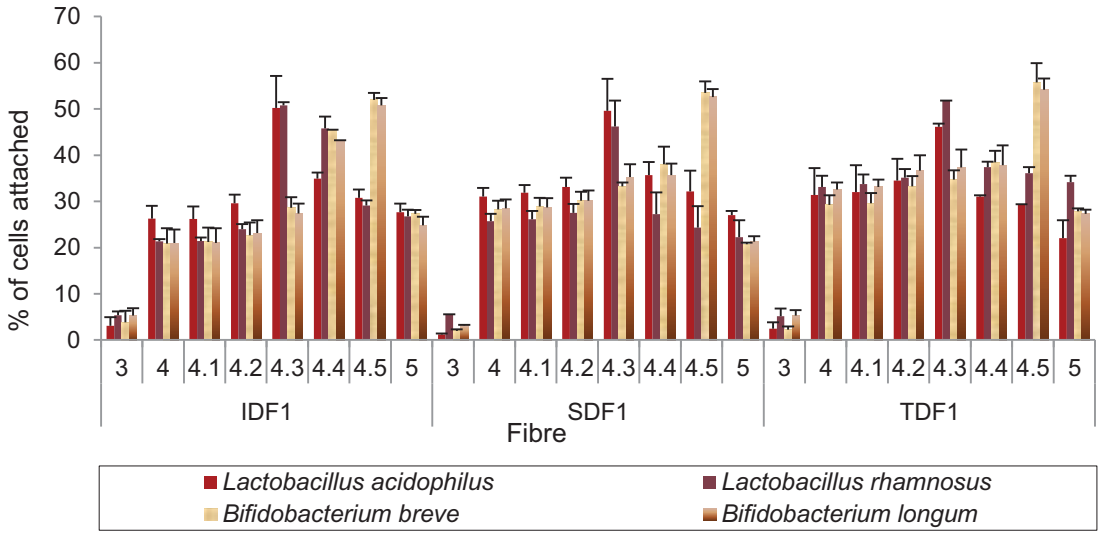

Figure 11.7.A. Effect of pH on fibre from RR1 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. pH3-3, pH4-4, pH4.1-4.1,pH4.2-4.2 pH4.3-4.3, pH4.5-4.5, pH5-5

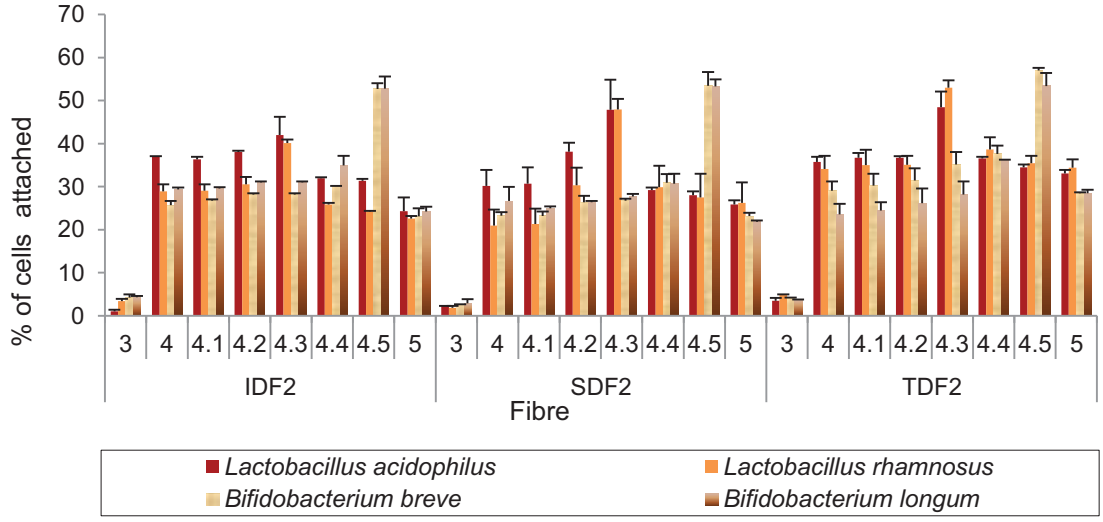

Figure 11.7.B. Effect of pH on fibre from RR2 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. pH3-3, pH4-4, pH4.1-4.1,pH4.2-4.2 pH4.3-4.3, pH4.5-4.5, pH5-5

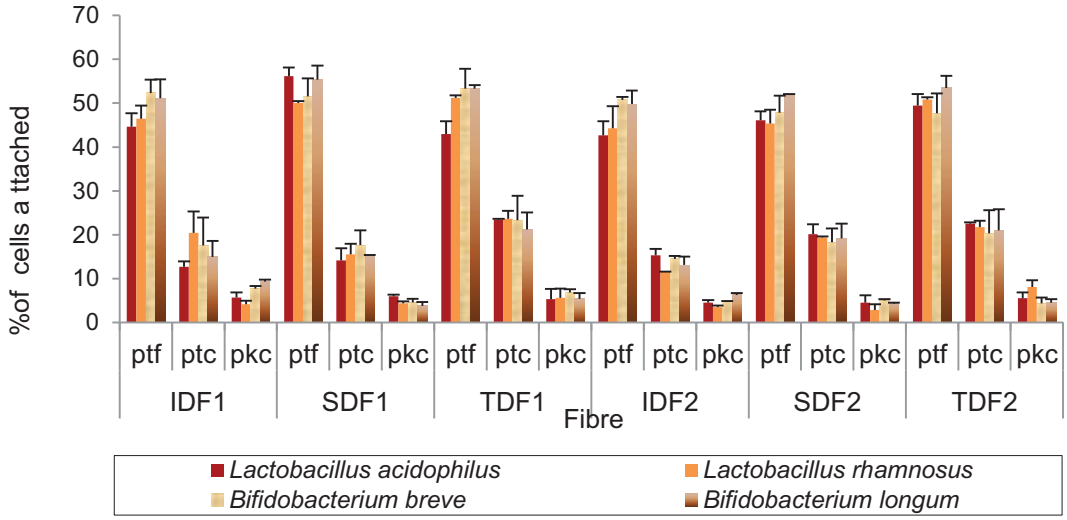

Figure 11.8 Effect of pepsin and proteinase on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Pepsin treated fibre-ptf, Pepesin treated cells – ptc and Protenase K treated cells-pkc

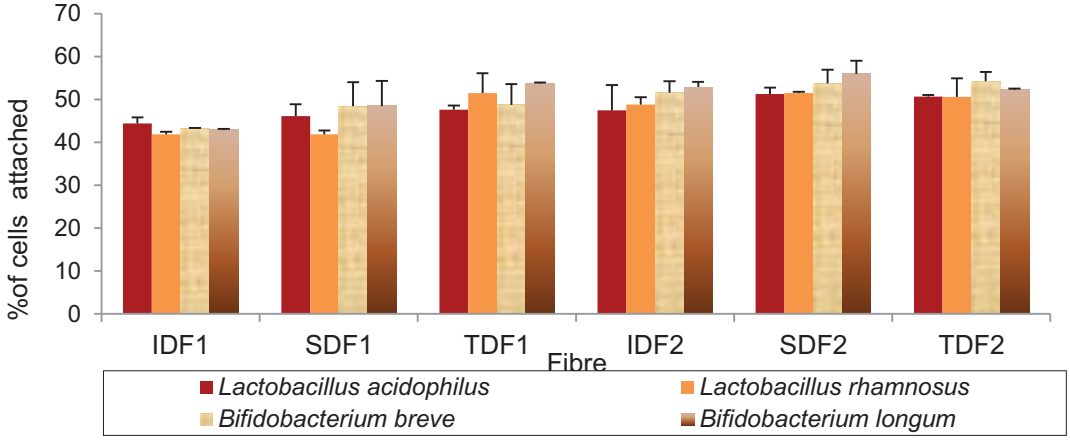

Figure 11.9Adhesion of bacteria to rice fibre fractions in the presence of glucose.

Results are expressed as the mean value of two trials±standard error.

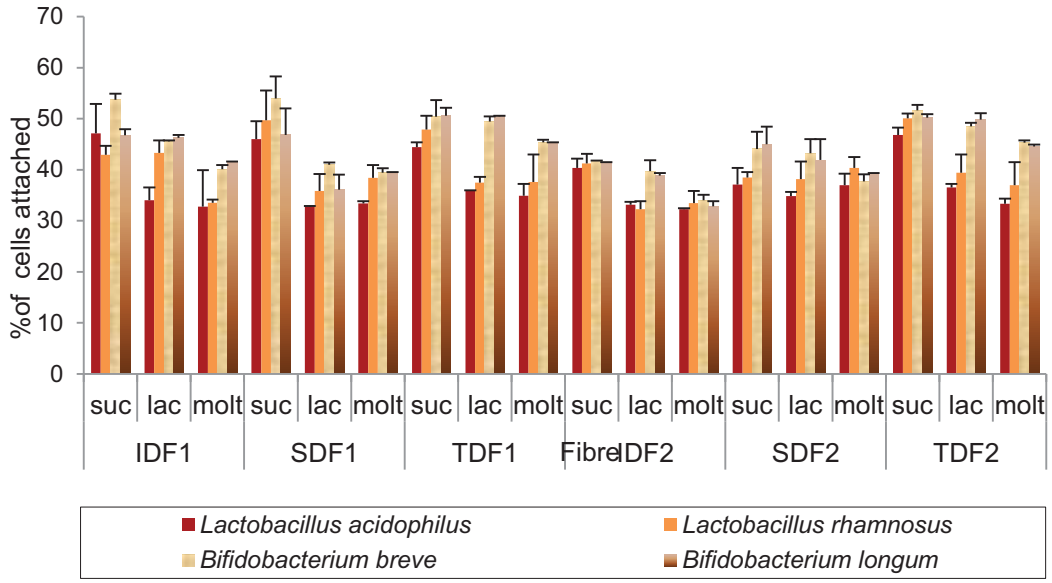

Figure 11.10A Adhesion of bacteria to fibre fractions in the presence of Sucrose, Lactose and Maltose.

Results are expressed as the mean value of two trials±standard error. Sucrose-suc, Lactose-lac, Maltose-molt.

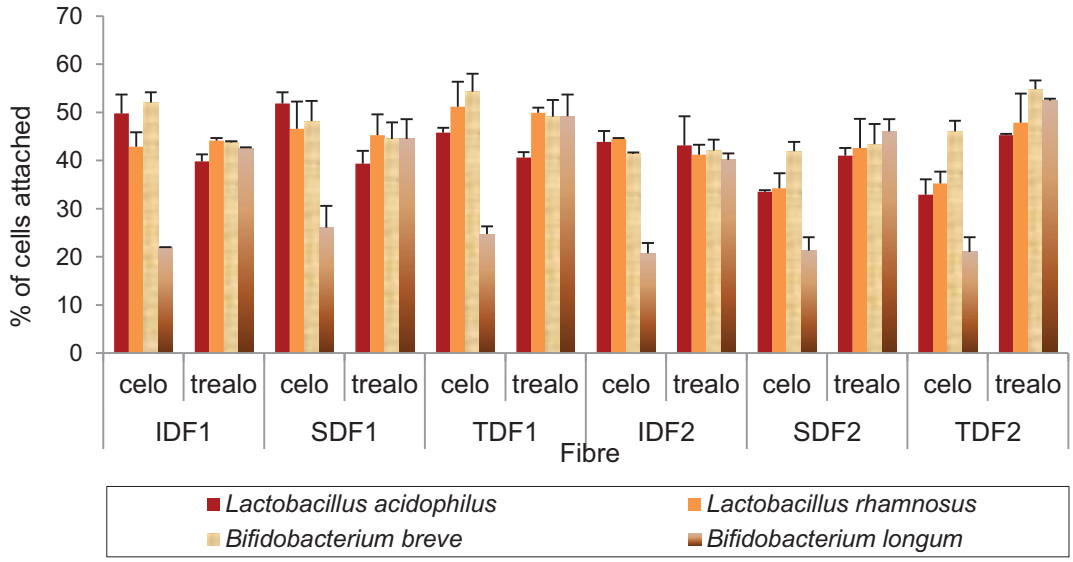

Figure 11.10B. Adhesion of fibre fractions in the presence of Cellobiose, Trehalose.

Results are expressed as the mean value of two trials±standard error. Cellobiose-celo, Trehalose-trealo.

The adhesion capacity of cells grown on several different carbon sources was compared. Glucose, sucrose, lactose, maltose, cellobiose, trehalose, amylopectin, motodextrin, amylose, maltose, and cellobiose, were used in this examination (Fig 11.2, 11.3, 11.4). The cells of all pure cultures grown on polysaccharides were significantly ($p<0.05$) weaker in their attaching ability to fibre than those grown on glucose or disaccharides.

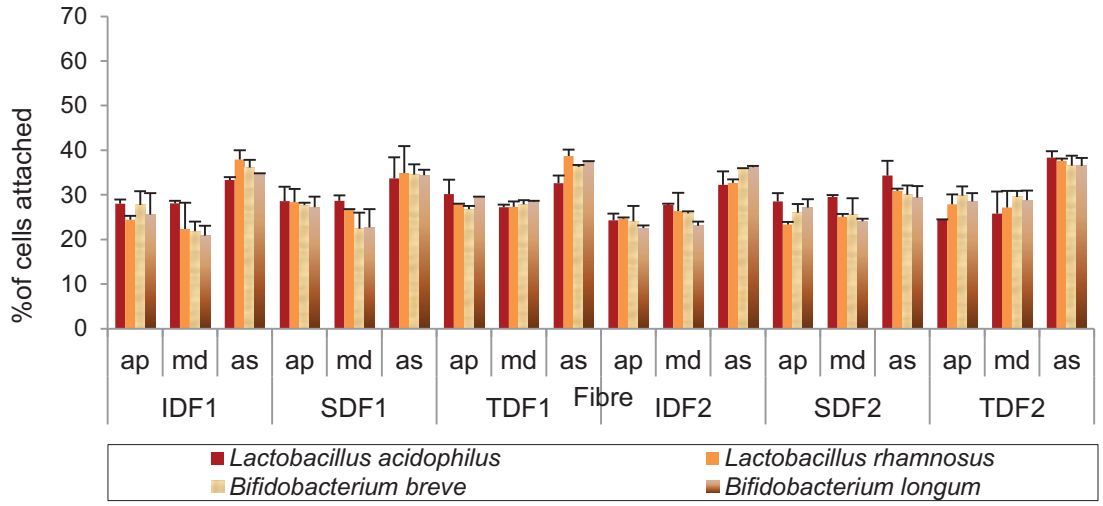

Figure 11.11 Adhesionof fibre fractions in the presence of (Amylopectin, motodextrin, Amylose.

Results are expressed as mean the mean value of two trials±standard error.

Amylopectin-ap, motodextrin-md, Amylose-as

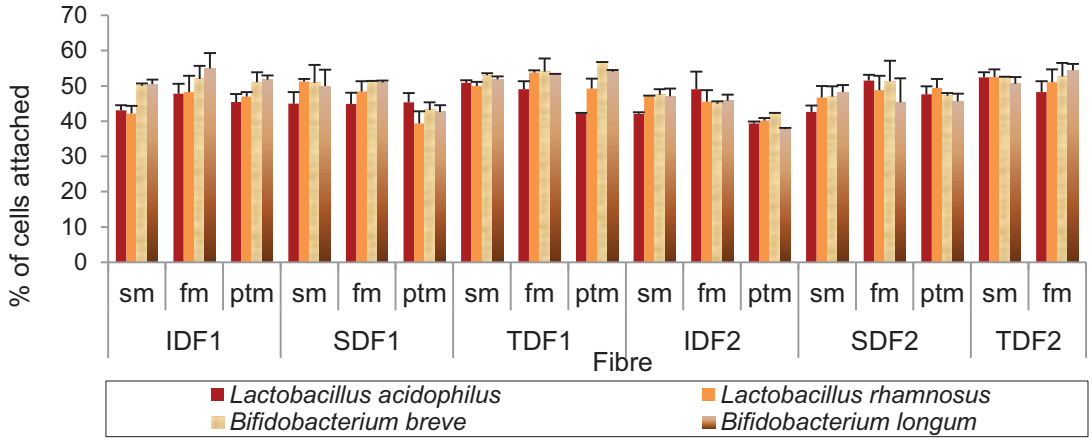

Figure 11.12 Effect spent medium, fresh medium, Pepsin treated medium on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Spent medium-sm, fresh medium-fm. Pepsin treated medium-ptm

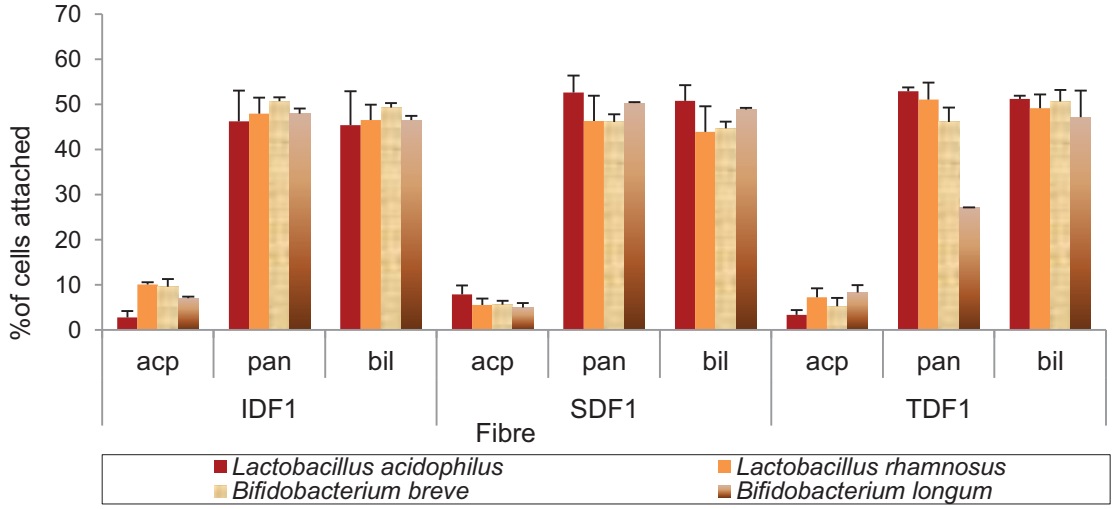

Figure 11.13.A. Effect of simulated gastrointestinal conditions on fibre from RR1 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Acid pepsin solution-acp, Pancreatin-pan, Bile-bi)

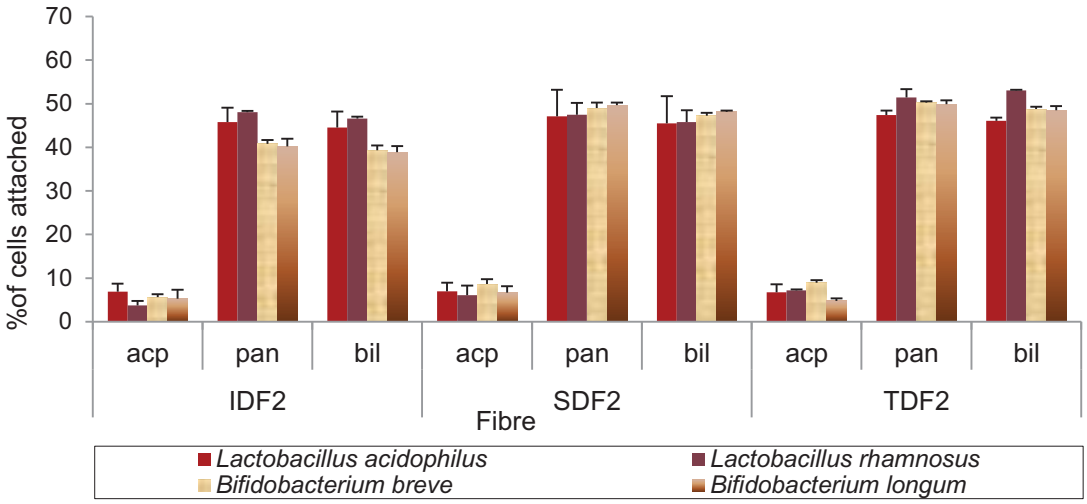

Figure 11.13.B. Effect of simulated gastrointestinal conditions on fibre from RR2 on bacterial adhesion to different rice fibre fractions.

Results are expressed as the mean value of two trials±standard error. Acid pepsin olution-acp, Pancreatin-pan, Bile-bil

11.5 Discussion

Bifidobacterium and *Lactobacillus* species adhered to dietary fibre fractions within 15 to 30 minutes of exposure to the fibre (Fig 11.1). Bifidobacteria adhered well to rice fibre with a total of 40-60% of the cells adhering. Lactobacilli adhered less well with less than 40<% of the bacteria adhering (Fig 11.2). Previous work observed 30-60% of the bacteria adhering to apple fibre (Saarela *et al.,* 2006).

Increasing the concentrations of the substrate would increase the surface area available for binding bacteria (Fig11.3). However, the present study did not observe a significant difference ($p<0.05$) in adhesion with an increase in the concentration of fibre in the medium. This suggests that the substrate surface area is not a limiting factor in this experiment.

Enzymes, amylase and cellulase are important for the digestion of dietary fibre by micro-organisms (Reeves *et al.,* 1997; Tancula *et al.,* 1992). Adhesion may facilitate

microbial digestion of fibre by bringing the microorganisms and their associated enzymes in closer contact with the substrate. Adhesion is known to be a prerequisite for some micro-organisms to ferment dietary fibre such as cellulose (Bhat *et al.,* 1990; Pell & Schofield. 1993; Reeves *et al.,* 1997; Tancula *et al.,* 1992). Previous work on rice fibre showed a high percentage of digestibility by the organisms used in current study (Fernando *et al.,* 2010). Therefore, adhesion to rice fibre does not appear to be important for the micro-organisms tested in this study to digest rice fibre.

High temperatures will deactivate the enzymes of cells and affect their attachment to substrates (Roger *et al.,* 1990). This may explain a reduction seen in the percentage of cells in our trials adhering to fibre at 100°C compared with 37°C and room temperature (Fig11. 4). As high temperatures will kill micro-organisms this observation suggests that live and metabolically active cells are more likely to bind with fibre than dead cells. This is supported to some extent by the fact that maximum adhesion occurred during the rapid growth of bacterea (Fig 11.5) as enzyme activity reaches a maximum. Adhesion was highest at 37 °C in line with the optimum growth temperature for these micro-organisms and body temperature.

Sodium chloride did not influence adhesion of our micro-organisms to rice fibre (Fig 11.6). Sodium chloride is required for metablolic activity by many micro-organisms for such activities as glucose uptake (Briczinski *et al.,* 2008). Research has not been done to understand the direct importance of NaCl on adhesion of bacteria to food particles so far.

The present study did not observe significant difference in adhesion with fibre by selected probiotics following treatment with Tween 80 (Fig11.6). Previous studies suggest that Tween 80 has a capacity to increase (Hwang *et al.,* 2008) and decrease (Lee *et al.,* 2007) the bacterial adhesion to fibre in animal feed. Tween 80 belongs to a

class of food additives (Hwang *et al.,* 2008) and is used in ice creams, pickles, vitamins/mineral preparations, shortenings, whipped toppings, gelatin desserts, cottage cheese, barbecue sauce, etc.

The effect of the conditions of the GI tract on bacterial adhesion to rice fibre was examined. The pH was shown to affect the adhesion of bacteria to rice fibre. The optimal pH for the adhesion of *Lactobacillus* species was 4.3 and for bifidobacteria was 4.5 (Fig 11.7). The pH may influence bacterial adhesion by changing the surface characteristics of both the bacteria (including surface proteins and surface charge) and the fibre substrate (Merritt & An, 2000). Other reports show no effect of pH on the adhesion of bifidobacteria to Hylon VII starch (Crittenden *et al.,* 2001) and pH >4 had no effect on the adherence of *V. alginolyticus* to chitin (Pruzzo *et al.,* 1996). Gastric pH is highly acidic (range 1.0-2.5) and the mean pH in the proximal small intestine is 6.6±0.5 (Evans *et al.,* 1988).

The treatment of the fibre with the protease (pepsin) did not significantly reduce adhesion ($p<0.05$) (Fig11.8). This suggests that adhesion does not involve proteins or peptides associated with the fibre. However, treatment of cells with pepsin and pancreases significantly reduced the adhesion. Pancreases and pepsin may cleave cell surface receptors that affect adhesion (Fig11.8). This indicates that the fibre binding mechanisms of the adherent *Bifidobacterium* and *Lactobacillus* species involve cell surface proteins or glycoproteins. This finding was supported by the very low adherence of heat treated cultures where proteins are likely to be denatured (Fig11.4). Studies with *Bifidobacterium* species (Bernet, 1993) and *Lactobacillus* species (Chauviere, 1992; Bernet1992; Imam & Harry-O'Kuru, 1991) have reported that the cell surface recognition is mediated by cell surface proteins. The involvement of cell surface

proteins in attachment appears to be a universal phenomenon in bacterial adhesion to many surfaces (Palmer *et al.,* 2007).

The effect of monosaccharides, disaccharides and polysaccharides on bacterial adhesion to rice fibre is variable (Figs 11.9,10,11).

Adhesion was not inhibited by glucose, maltose, cellobiose or trehelose. However, the adhesion percentage differed in the presence of monosaccharides and disaccharides. Polysaccharides inhibited adhesion by (20-30%) compared with monosaccharides and disaccharides (Fig 11.11). This suggests that the binding protein (or adhesin) has an affinity for starch-like carbohydrates. The higher degree of inhibition with longer starch polymer chains indicates that the adhesive proteins may have a stronger affinity for larger molecules, thereby prevent the bonding between adhesins of the cells and the fibre particles. These data agree well with the results of Imam and Harry-O'Kuru (1991) who described the adhesion of *Lactobacillus* amylovorus with derivatives of corn starch (Imam & Harry-O'Kuru, 1991). Many systems have been observed where the cell surface recognition is mediated by carbohydrate binding proteins (Bar-Shavit *et al.,* 1977; Dazzo 1981; Iman *et al.,* 1984). This may be due to steric resistance of larger molecules that can prevent chemical reactions with smaller molecules (Pophristic & Goodman 2001).

The present study did not observe any change in adhesion of the micro-organisms to fibre when the spent growth medium was treated with pepsin (Fig 11.12). This suggests that extracellular proteins do not play an important role in the adhesion of these bacteria. Extracellular components have been observed to be involved in the adhesion of *Lactobacillus fermentum* and for *Lactobacillus acidophilus* to human intestinal cells (Coconnier *et al.,* 1992; Conway & Kjelleberg, 1989).

Studies simulating the conditions of gut, indicated that acidic (as in Fig11.7) and protease rich environments significantly prevented adhesion ($p<0.05$) (Fig11.13). Therefore this study suggests that the adhesion of micro-organisms to fibre will be not in the human stomach. However, the higher pH in the proximal colon will be more favorable to the adhesion than the stomach.

Probiotics have an ability to deconjugate bile in the human gut and to reduce serum cholesterol (Corzo *et al.,* 1999a; Topping, 1991). In contrast, deconjugated bile (free bile salt) inhibits probiotics. Previous studies have noticed that free bile has an ability to adhere to soluble fibre (Cuesta-Alonso & Gilliland 2008; Story & Kritchevsky 1976). However, in the present study in the presence of bile, the micro-organisms had a moderate affinity for fibre and not only for soluble fibre but for total and insoluble fibre (Fig11.13). Previous studies have observed the enhanced survival of bifidobacteria at low pH, in bile and through the intestinal tract of rats in the presence of resistant starch (Wang *et al.,* 1999). Adhesion to rice fibre could similarly enhance the survival of probiotic bacteria.

Adhesion to fibre can be considered as a possible mechanism for the survival of *Lactobacillus rhamonsus, Lactobacillus acidphillus and Bifidobacterium breve* and *Bifidobacterium longum* through the digestive tract. Gastrointestinal survival of *Bifidobacterium* and *Lactobacillus* species has been studied by incorporating microbial cells into calcium alginate beads or granular starch (Lee & Heo, 2000). Generally, microbial cells are immobilized in food matrices through adhesion to solids (Fleet, 1999). The present study suggests that the survival of *Bifidobacterium* and *Lactobacillus* species in the human gut can be enhanced by the attachment of these micro-organisms to the dietary fibre of rice. Previous studies with *Lactobacillus* species have observed the effect of many of the factors studied in this present

investigation in the adherence of probiotics to human epithelial cells (Cook *et al.,* 1988; Lin & Savage, 1984) but not with dietary fibre. Previous studies of *L. acidophilus* and *L. fermentum* Rl indicated that the adherence factors were regulated by chromosomal genes (McCarthy, 1988).

11.6 Conclusion

Bacterial adhesion is complex, involving the bacteria, substrate and environment. Among the probiotics tested, *Bifidobacterium s*pecies had a greater ability than the *Lactobacillus* species to adhere to fibre fractions. Adhesion to rice fibre by *Bifidobacterium* and *Lactobacillus* species is not required for these micro-organisms to use rice fibre. Cell surface proteins bind with rice fibre fractions. The micro-organisms tested showed similar preferences in their binding to fibre fractions. The moderate to poor adhesion of *Bifidobacterium* and *Lactobacillus* species respectively seems to be a trait of these micro-organisms. Adhesion to fibre may not be influenced by bile but the binding capacity was reduced by low pH and acid pepsin solutions.

CHAPTER 12

Environmental factors affecting the adhesion of combinations of probiotics to insoluble, soluble and total dietary rice fibre

12.1 Abstract

Nine co-cultures of the *Bifidobacterium* and *Lactobacillus* species were tested for their ability to adhere to insoluble dietary fibre (IDF1, IDF2), soluble dietary fibre (SDF1, SDF2), and total dietary fibre (TDF1, TDF2) from two rice varieties (RR1 and RR2). Combinations of the same genus (BB+BL and LA +LR) showed 30 – 40 % (poor) adhesion and combinations of different genera showed 40 – 50 % (moderate) adhesion, which is significantly higher ($p<0.05$) than the combinations of same genus. The increase in adhesion with species from different genera suggests some synergistic activity. The microbial combinations had the ability to adhere to dietary fibre fractions as early as 30 min and less than 45 min. Colonisation of rice fibre by bacterial cells was affected by the temperature, with adhesion being higher at 37 ^{0}C than room temperature. The optimal pH value for adhesion was 4.2 - 4.5. This study observed that the combinations tested had a moderate percentage of adhesion in the presence of bile, low pH (4.3-4.5) and pancreatin, irrespective of the type of co-culture. In addition, adhesion was not affected by an increase in NaCl and Tween 80. Adhesion was affected by disaccharides and polysaccharides. The amount of adhesion of co-cultures was not significantly affected by the substrate ($p>0.05$). Results indicated that rice fibre fractions are suitable hosts for the probiotics tested and adhesion is not a prerequisite for fibre digestion of co-cultures.

12.2 Introduction

The capability of bacteria to adhere to a substrate is important for the degradation of the substrate. This is a prerequisite for effective degradation of plant cell wall polysaccharides (Lynd *et al.,* 2002). Adhesion of microorganisms to a substrate can affect biochemical responses at both the cellular and molecular levels (Imam *et al.,* 1984; Roper & Koch, 1988). Many studies that have attempted to understand the adhesion process of combinations have concentrated on ruminal bacteria and focused on cellulose as the substrate. Few studies have focused on the adhesion of the *Bifidobacterium* and *Lactobacillus* species on food particles (Betoret *et al.,* 2003; Lavermicocca *et al.,* 2005). The present study is the first study aimed at understanding the adhesion of combinations of probiotic bacteria to rice fibre.

Studies with the *Fibrobacter succinogenes* and *Ruminoccocus flavefaciens* have shown that the properties of the bacterium had the greatest impact on bacterial adherence to cellulose (fibre) (Mosoni *et al.,* 1997). However, many factors can affect adhesion when bacteria work as a consortium to attach to a substrate. Previous evidence suggests that competition amongst the bacteria (Miron *et al.,* 2001) as well as the antagonistic activity of bacteria may limit the adhesion to substrate (Odenyo *et al.,* 1994b). The antagonistic effect is more prominent when bacteria produce bacteriocins (Odenyo *et al.,* 1994b) that affect other organisms in the combination.

The adhesion of microorganisms is affected by their density on a substrate, with those present at high density most likely to be affected by competition. Some microorganisms release peptides that can act as a inhibitors to other organisms (Pettipher & Latham, 1979), or accumulated by- products can act as inhibitors to microorganisms. The number of viable cells in the gut also has a major contribution towards adhesion. Previous investigations with corn starch granules indicated that live

or metabolically active cells have a higher affinity than dead cells to bind with the substrate (Imam & Harryokuru, 1991). Symbiosis among the microorganisms may increase the adhesion to the substrate (Lavermicocca *et al.,* 2005).

The effect of physiochemical factors such as pH, temperature, NaCl, etc (Gaudet & Gaillard, 1987; Roger *et al.,* 1990) on the adhesion of probiotics to cellulose has been widely studied. A series of experiments were conducted in the current study to understand the coexistence of the *Bifidobacterium* and *Lactobacillus* species on rice fibre fractions and the physiochemical factors associated with these combinations. This study explores the mechanisms of adhesion of co-cultures of probiotics to dietary fibre.

12.3 Materials and methods

Sample collection, extraction of soluble, insoluble and total dietary fibre, chemicals, co-cultures, preparation of cell suspensions, preparation of growth medium, and statistical evaluation are described in 3.1, 3.3, 3.6.1, 3.6.3, 3.6.4, 3.6.5, and 3.12, respectively. Adhesion to fibre, effect of growth phase on adhesion, study of adhesion mechanisms, and adhesion under conditions that simulate the stomach and small intestine conditions of humans are described in 11.3.2,11.3.3,11.3.4 and 11.3.5, respectively.

12.4 Results

This study used two rice varieties (RR1 and RR2). Fibre was extracted from these rice varieties as SDF, IDF and TDF and fibre fractions from rice variety RR1 were labelled IDF1, SDF1 and TDF1, and fibre fractions from rice variety RR2 were labelled IDF2, SDF2, and TDF2.

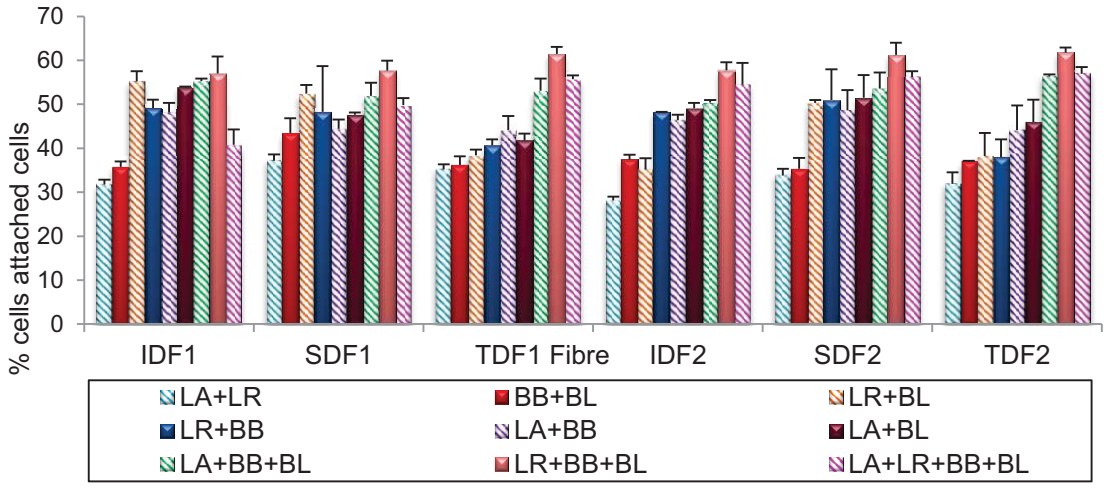

Figure 12.1 Adhesion of bacterial combinations to rice fibre

Results are expressed as the mean value of two trials ± standard error. All the combinations of microorganisms tested were able to adhere to rice fibre. Combinations consisting of more than one genus adhered in greater numbers than combinations of a single genus.

Monosaccharides, disaccharides and polysaccharides affect the adhesion of co-cultures to rice fibre fractions. (Fig 12.2, 12.3, and 12.4).

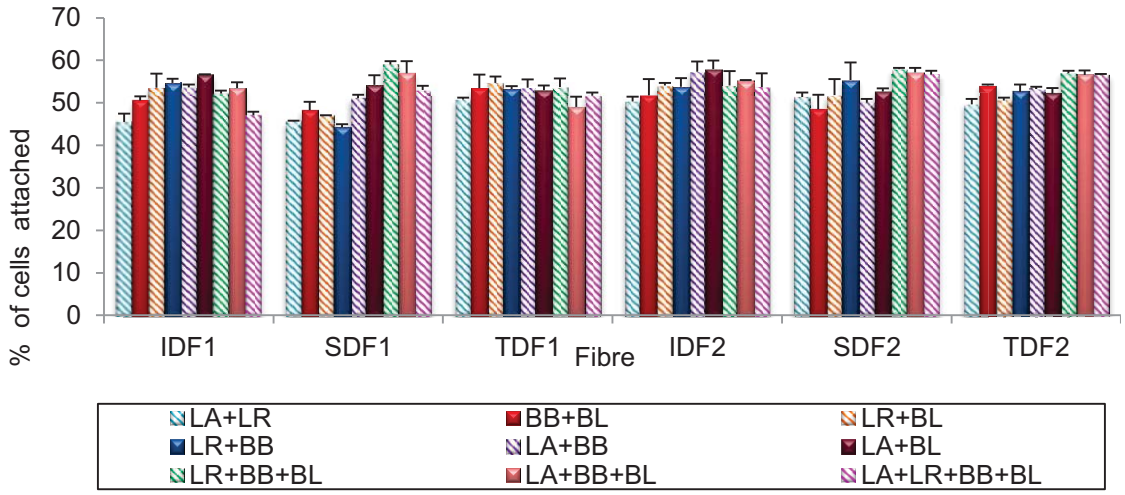

Figure 12.2 Adhesion of bacterial combinations to rice fibre fractions in the presence of glucose.

Results are expressed as the mean value of two trials ± standard error

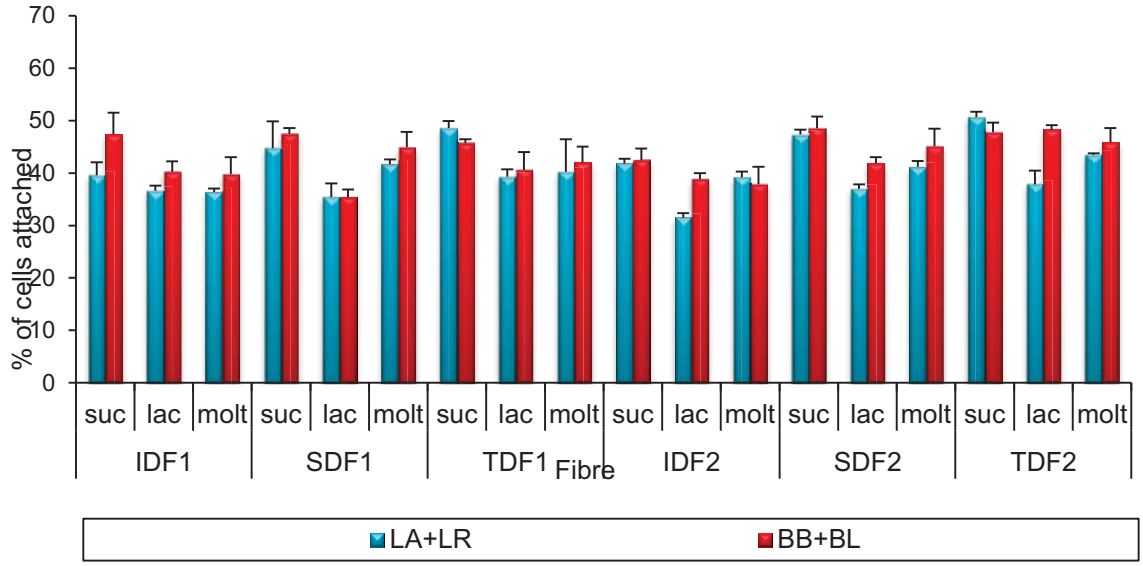

Fig 12.3 A) LA+LR, BB+BL

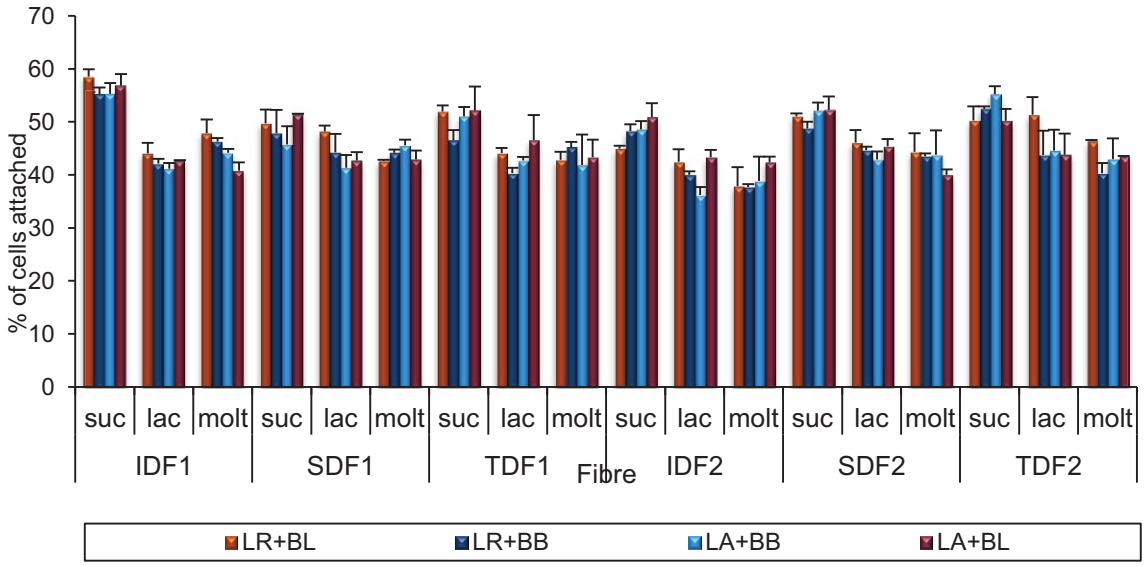

Fig 12.3 B) LR+BL, LR+BB, LA+BB,LA+BL

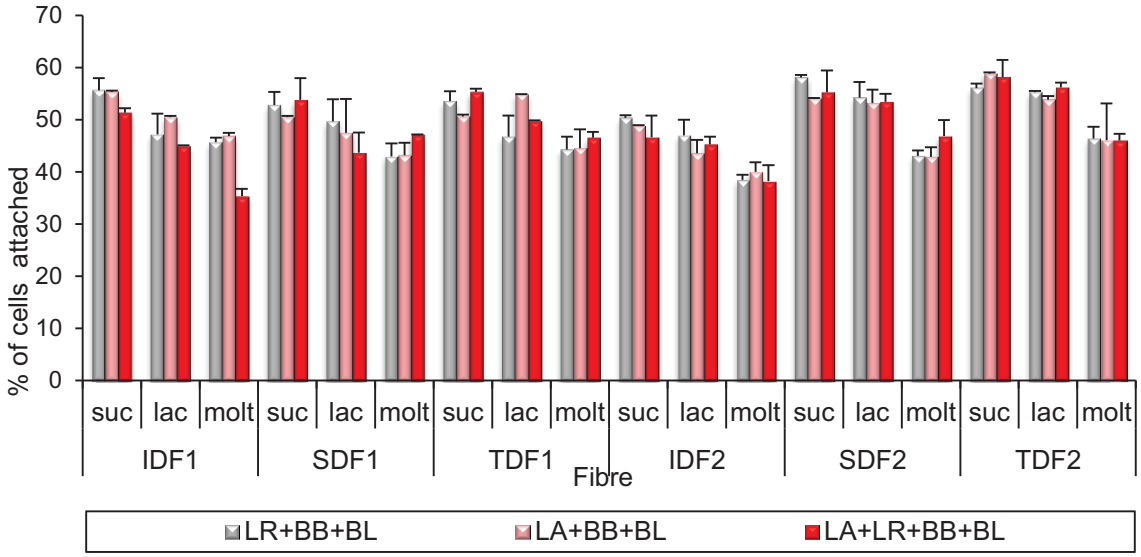

Fig 12.3 C) LA+BB+BL,LA+BB+BL, LA+LR+BB+BL

Figure 12.3 A, B, C Adhesion of bacterial combinations to rice fibre fractions in the presence of Sucrose, Lactose, and Maltose.

Results are expressed as the mean value of two trials ± standard error. Sucrose-suc, Lactose-lac, and Maltose-molt

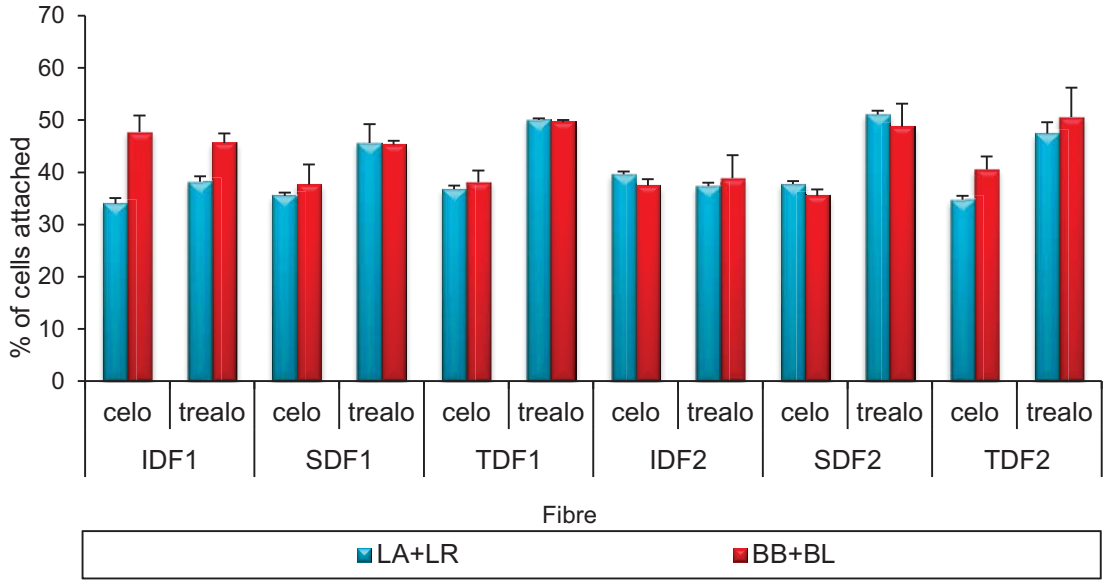

Fig 12.3 D) LA+LR, BB+BL

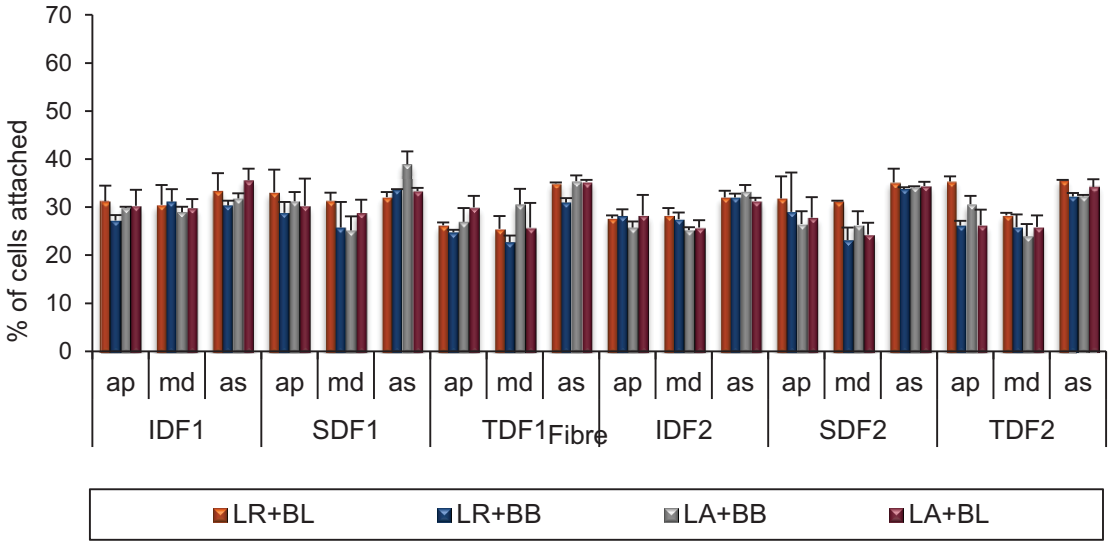

Fig 12.3 E) LR+BL, LR+BB, LA+BB,LA+BL

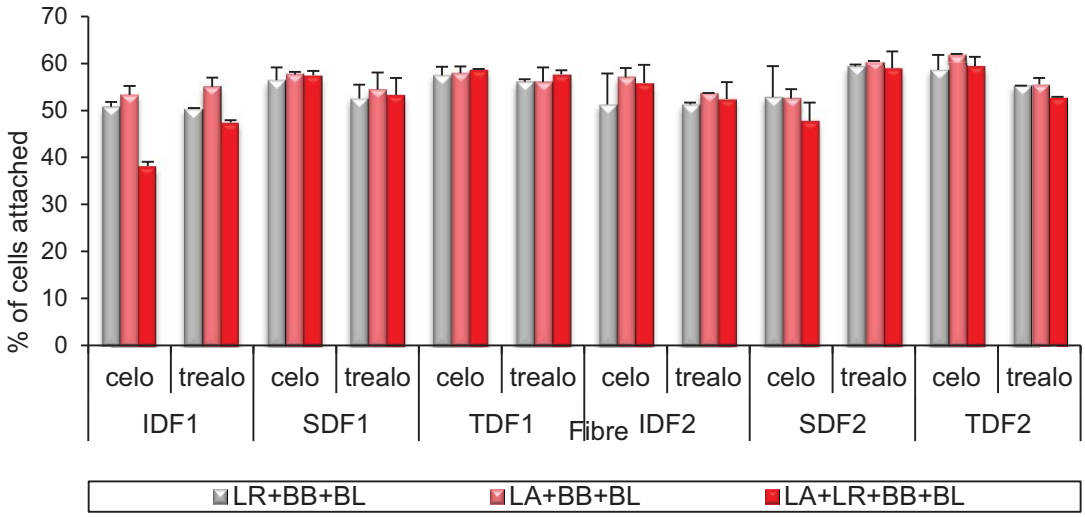

Fig 12.3 F) LA+BB+BL,LA+BB+BL, LA+LR+BB+BL

Figure 12.3 D, E, and F Adhesion of bacterial combinations to rice fibre fractions in the presence of Cellobiose, Trehalose.

Results are expressed as the mean value of two trials ± standard error. Cellobiose-celo, Trehalose-trealo.

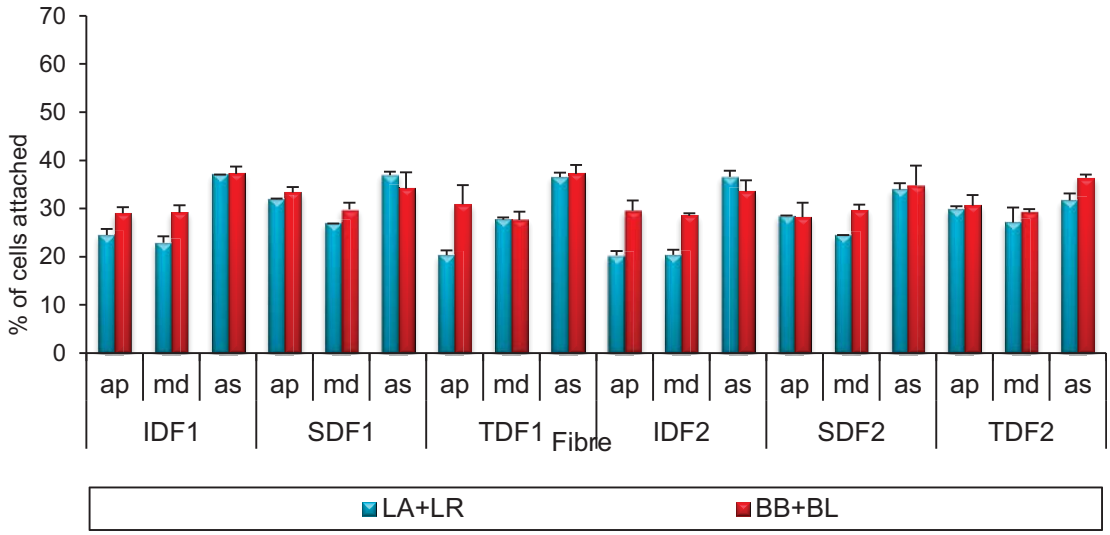

Fig 12.4 A) LA+LR, BB+BL

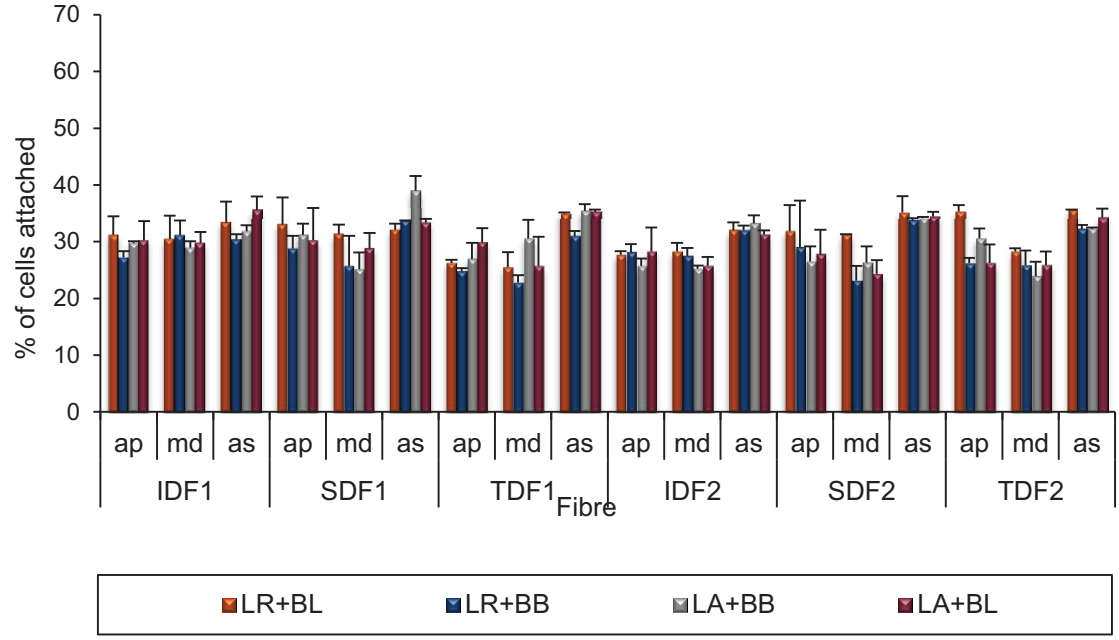

Fig 12.3 B) LA+LR, LR+BB, LA+BB, LA+BL

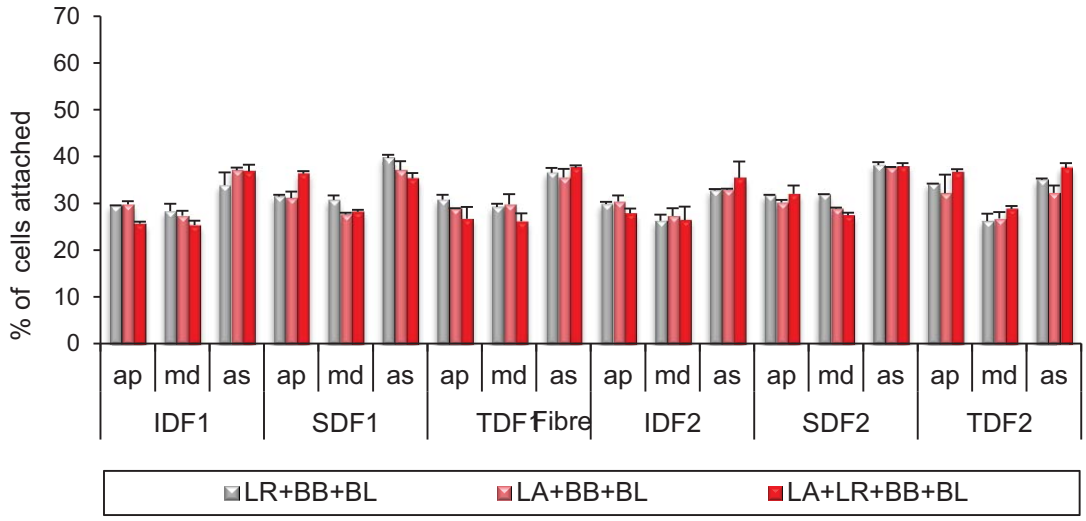

Fig 12.3 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

12.4 A, B, C Adhesion of bacterial combinations to rice fibre fractions in the presence of Amylopectin, moltodextrin, Amylose.

Results are expressed as the mean value of two trials ± standard error. Amylopectin-ap, moltodextrin-md, Amylose-as.

Glucose, sucrose, lactose, maltose, cellobiose, trehalose, amylopectin, motodextrin, amylose, maltose, and cellobiose, were used in this examination (Figs 12.2, 3 and 4). The numbers of cells adhering to polysaccharide, were significantly ($p<0.05$) less than those attaching to fibre, glucose or disaccharides. The adhesion of cells of bacteria to rice fibre was significantly ($p<0.05$) inhibited by amylopectin, moltodextrin, amylase.

NaCl, Tween 80 and phosphate buffer effect on the adhesion of co-cultures to fibre fractions (Fig 12.5 and 12.6).

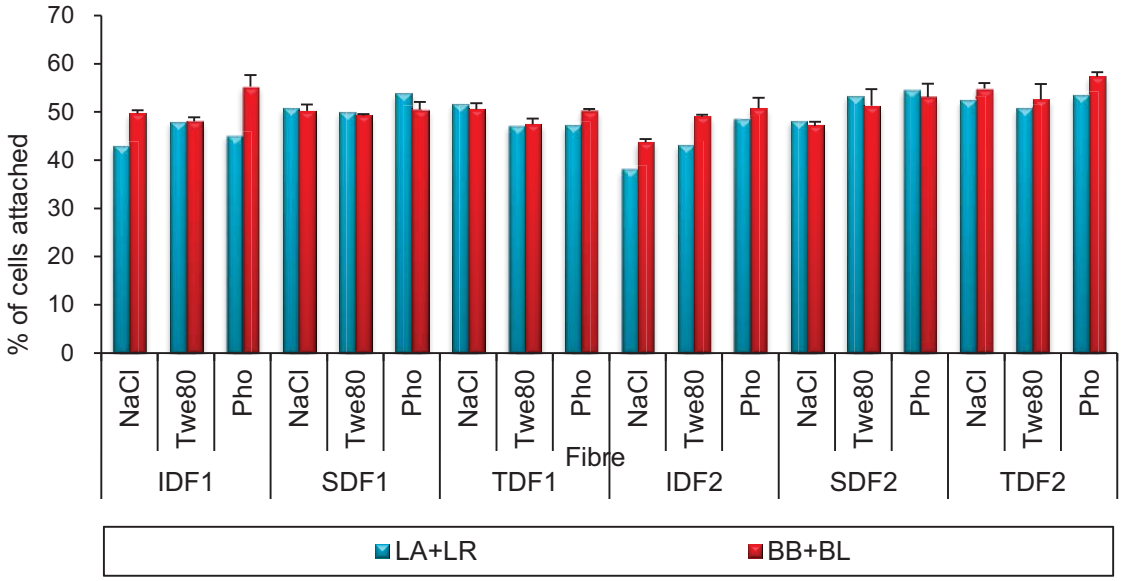

Fig 12.5 A) LA+LR, BB+BL

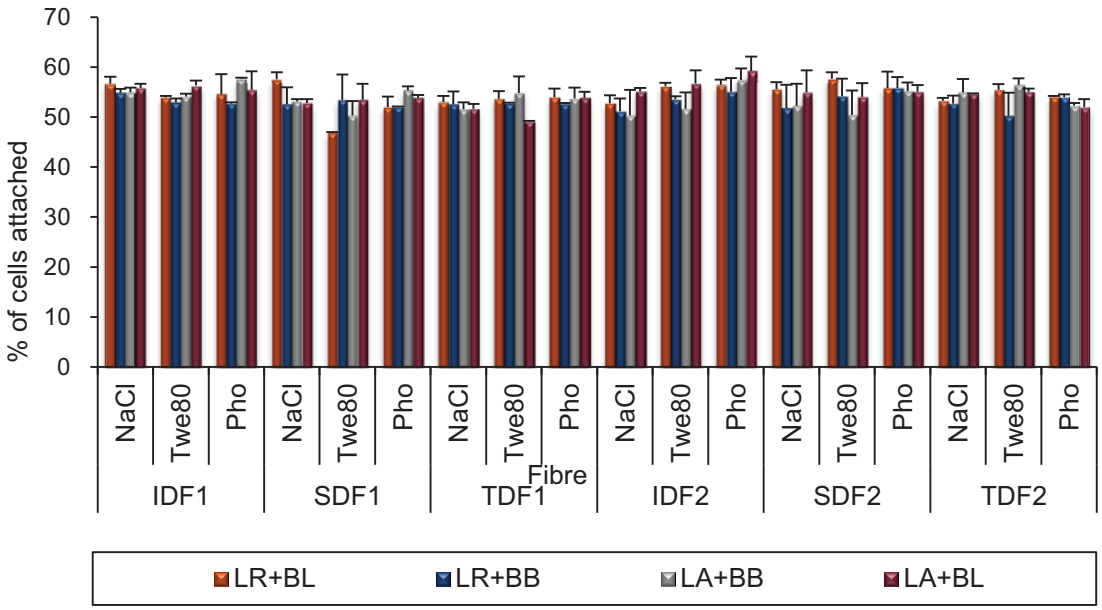

Fig 12.5 B) LR+BL, LR+BB, LA+BB, LA+BL

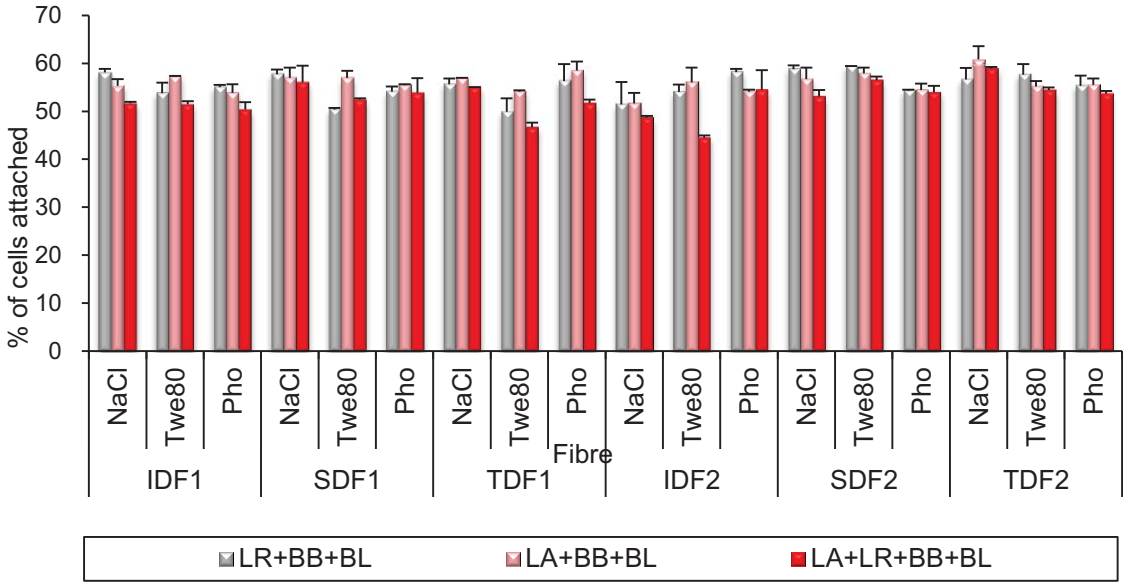

Fig 12.5 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

Figure 12.5 A, B, C Effect of Chemicals, NaCl, Tween 80, and Phosphate buffer.

Results are expressed as the mean value of two trials ± standard error. Tween 80-Twe80, and Phosphate buffer-Pho.

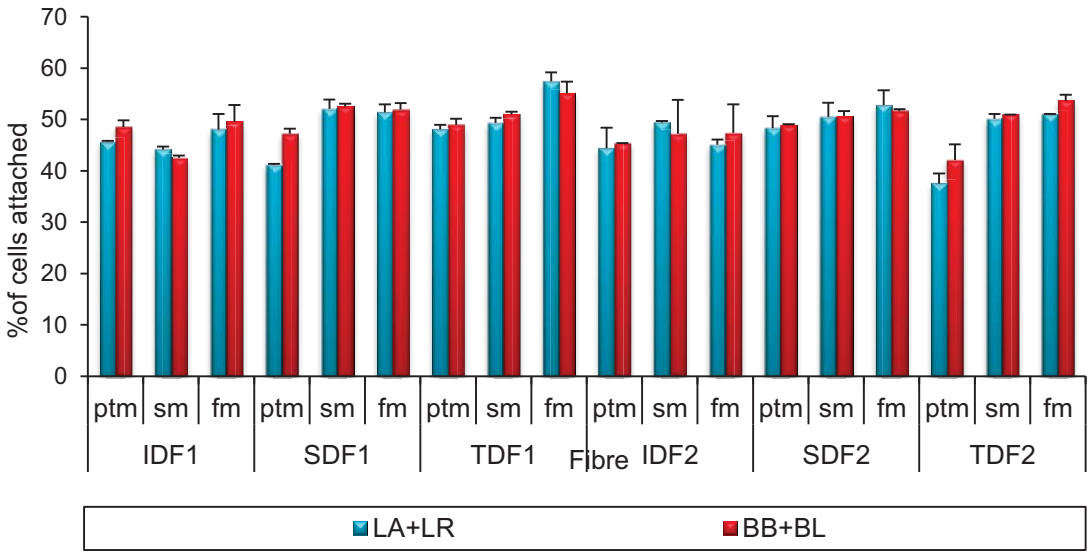

Fig 12.6 A) LA+LR, BB+BL

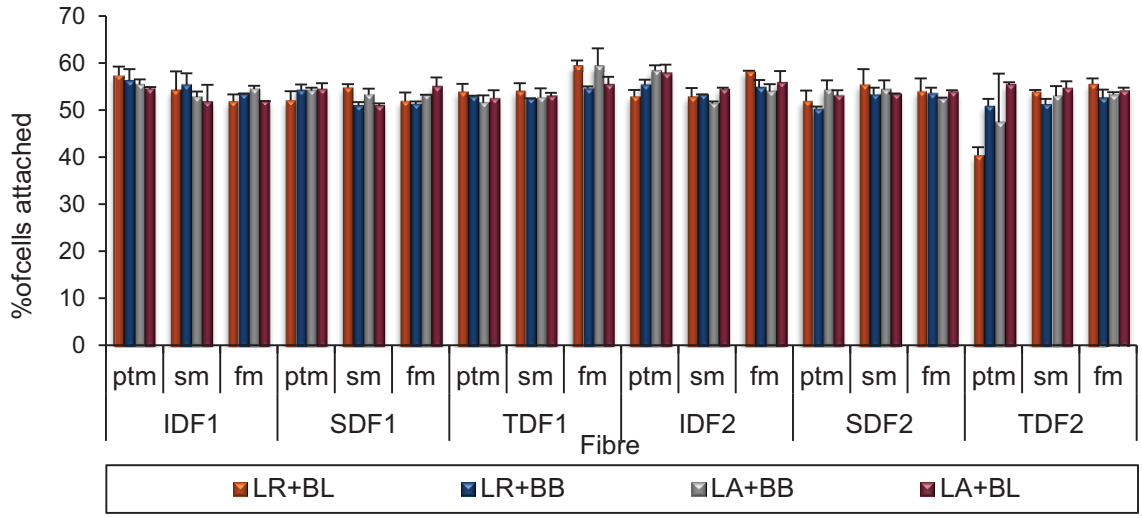

Fig 12.6 B) LR+BL, LR+BB, LA+BB, LA+BL

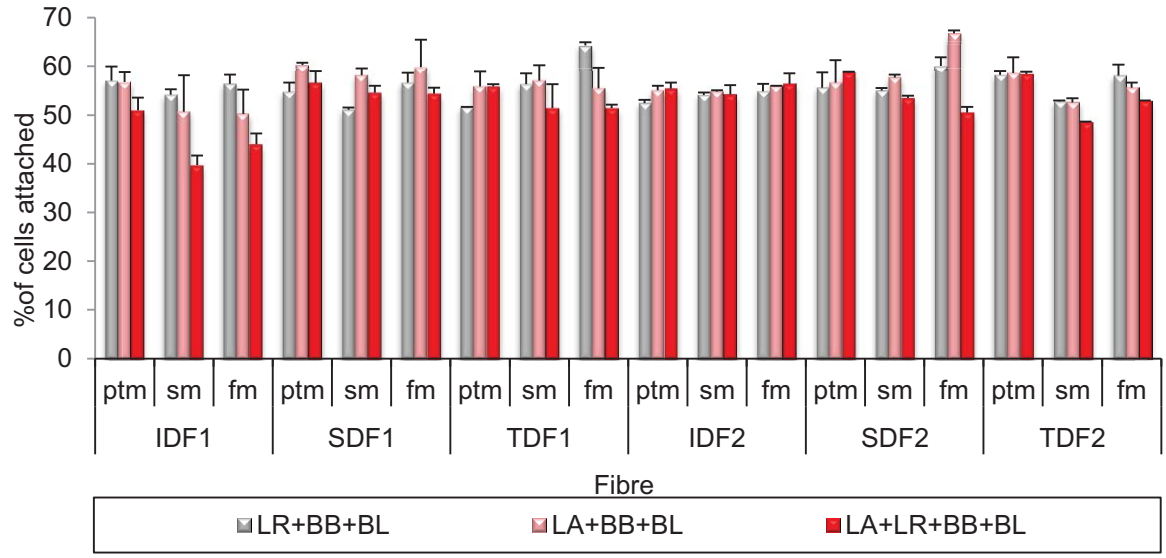

Fig 12.6 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

Figure 12.6 A, B, C Effect of spent medium, fresh medium, Pepsin treated medium on the adhesion of bacterial combinations to rice fibre fractions.

Results are expressed as the mean value of two trials ± standard error. Spent medium-sm, fresh medium-fm, Pepsin treated medium-ptm

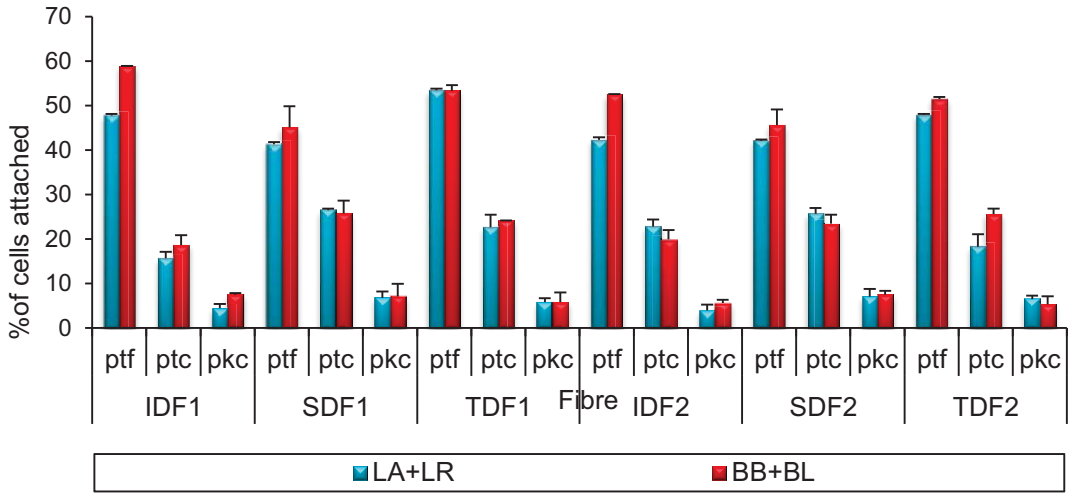

Fig 12.7 A) LA+LR, BB+BL

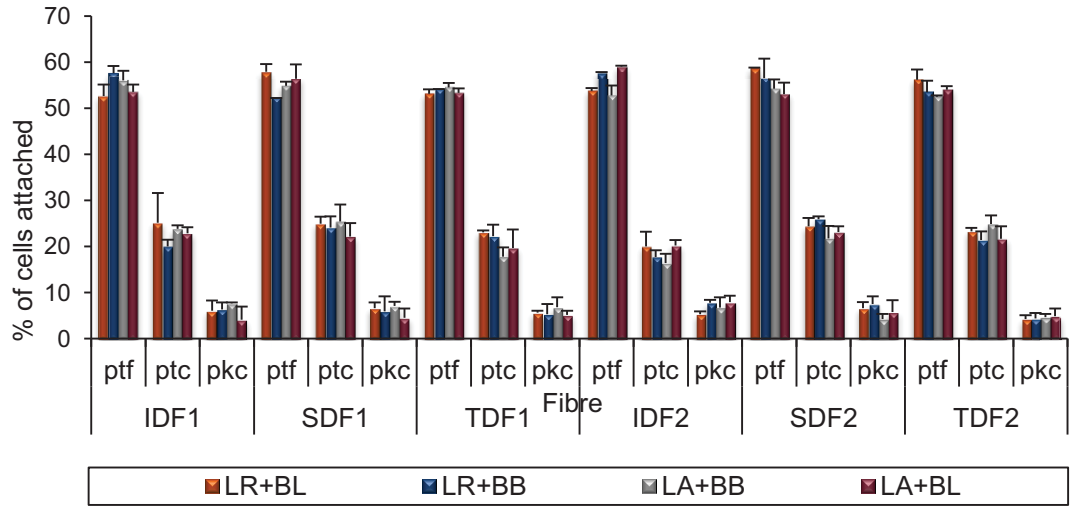

Fig 12.7 B) LR+BL, LR+BB, LA+BB, LA+BL

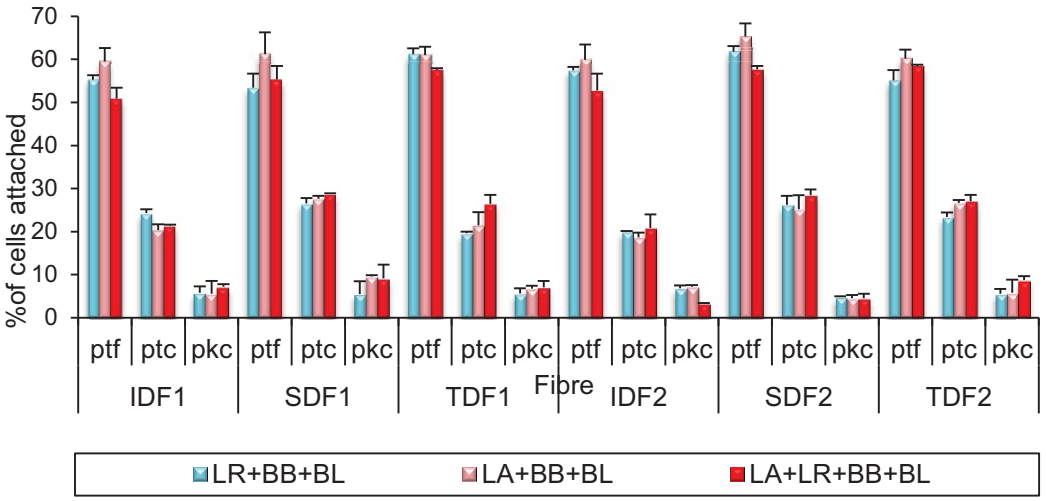

Fig 12.7 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

Figure 12.7 A, B, C Effect of pepsin and proteinase on the adhesion of bacterial combinations to rice fibre fractions.

Results are expressed as the mean value of two trials ± standard error. Pepsin treated fibre-ptf, Pepesin treated cells -PTC, Protenase K treated cells-pkc

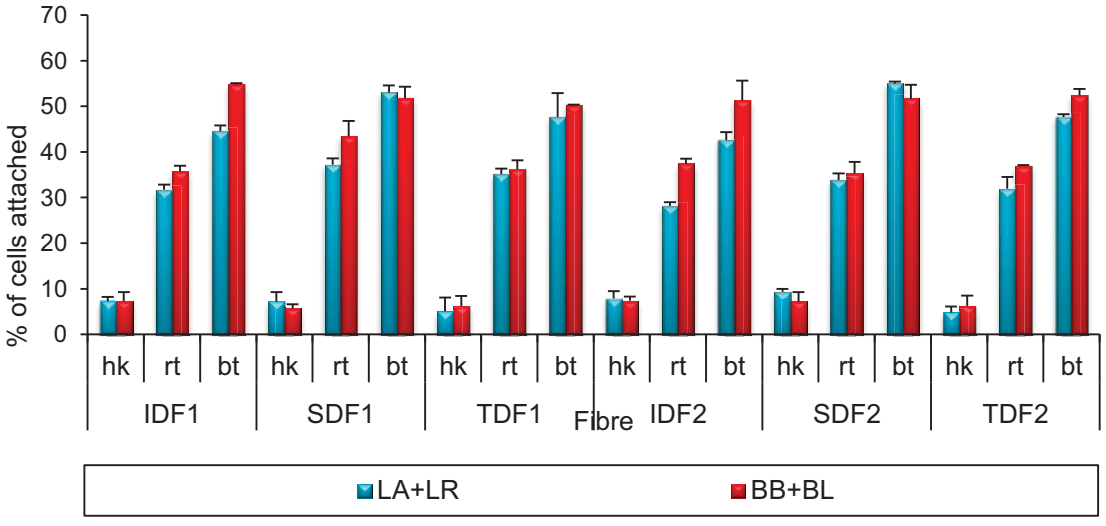

Fig 12.8 A) LA+LR, BB+BL

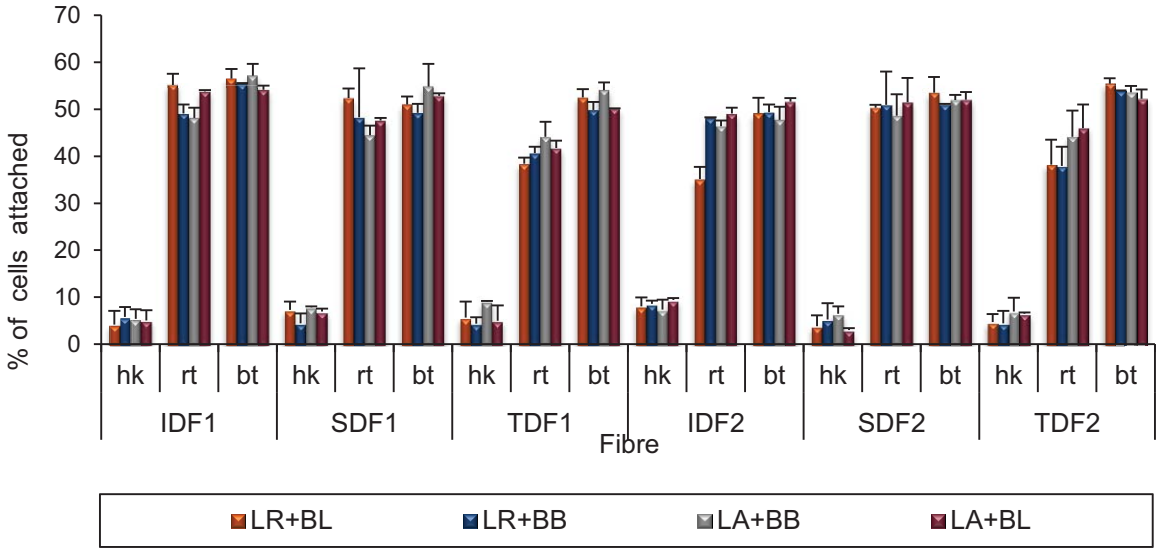

Fig 12.8 B) LR+BL,LR+BB,LA+BB,LA+BL

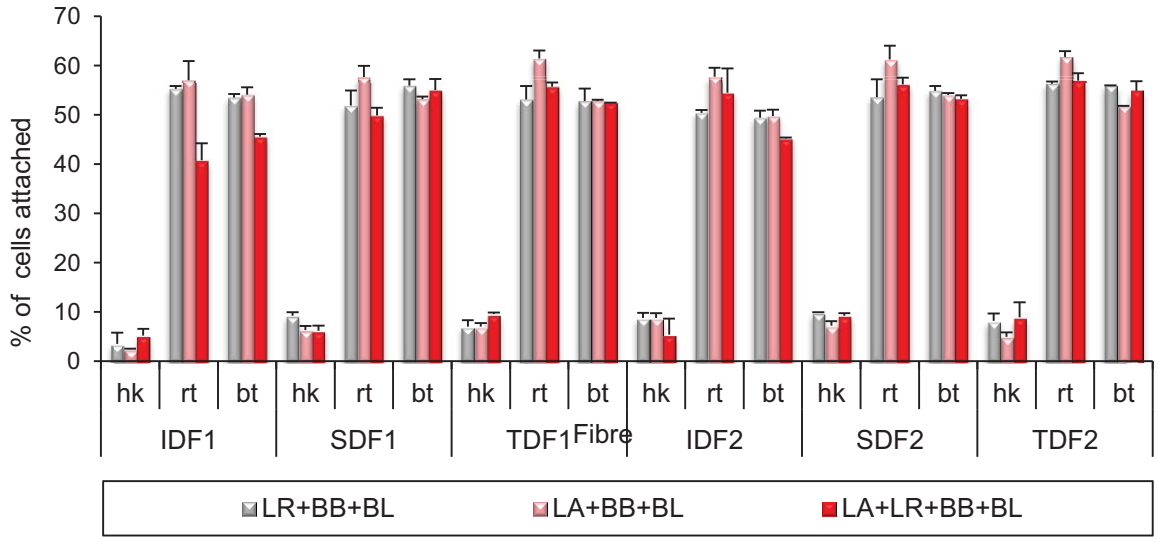

Fig 12.8 C) LR+BB+BL,LA+BB+BL,LA+LRBB+BL

Figure 12.8 A, B, C Effect of Tempreture (on the adhesion of bacterial combinations to rice fibre fractions.

Results are expressed as the mean value of two trials ± standard error. Heat killed-hk, room tempreture -RT, body tempreture-bt

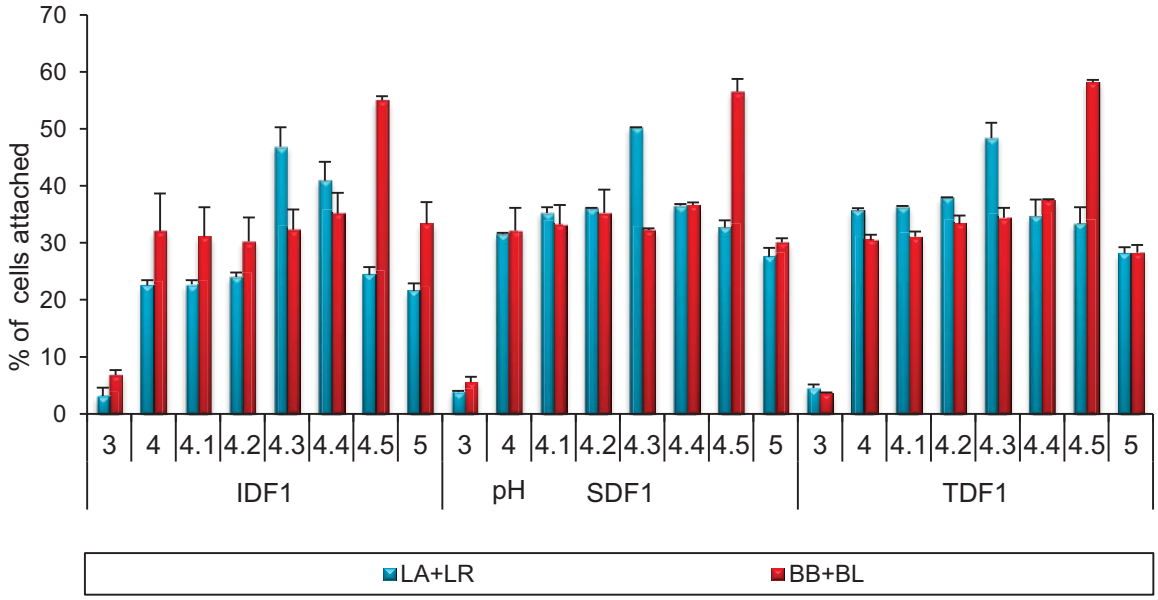

Fig 12.9 A) LA+LR, BB+BL

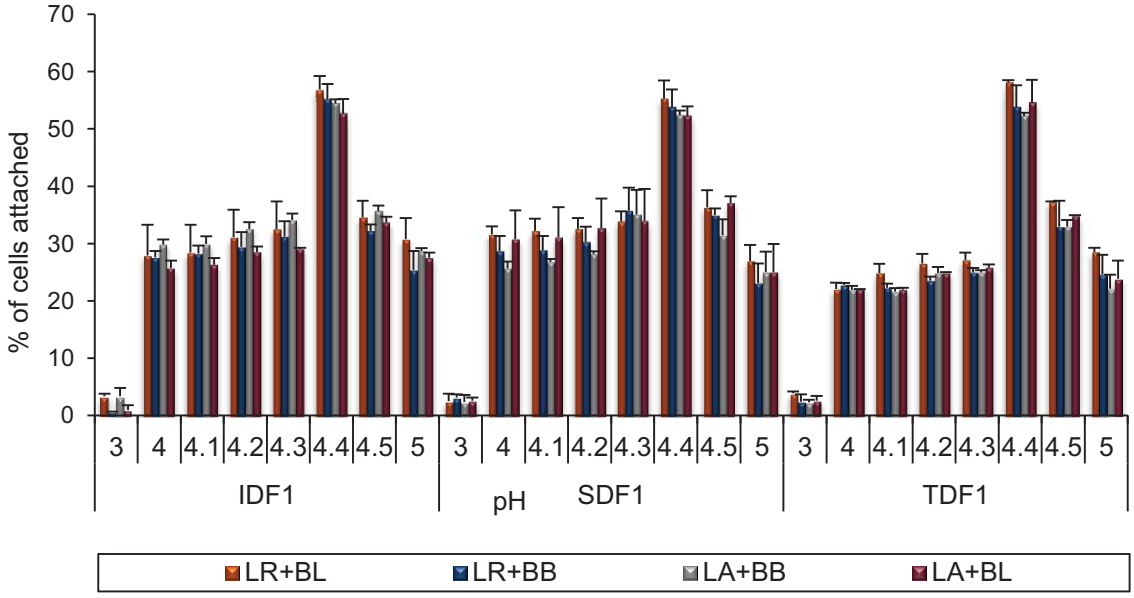

Fig 12.9 B) LR+BL, LR+BB, LA+BB, LA+BL

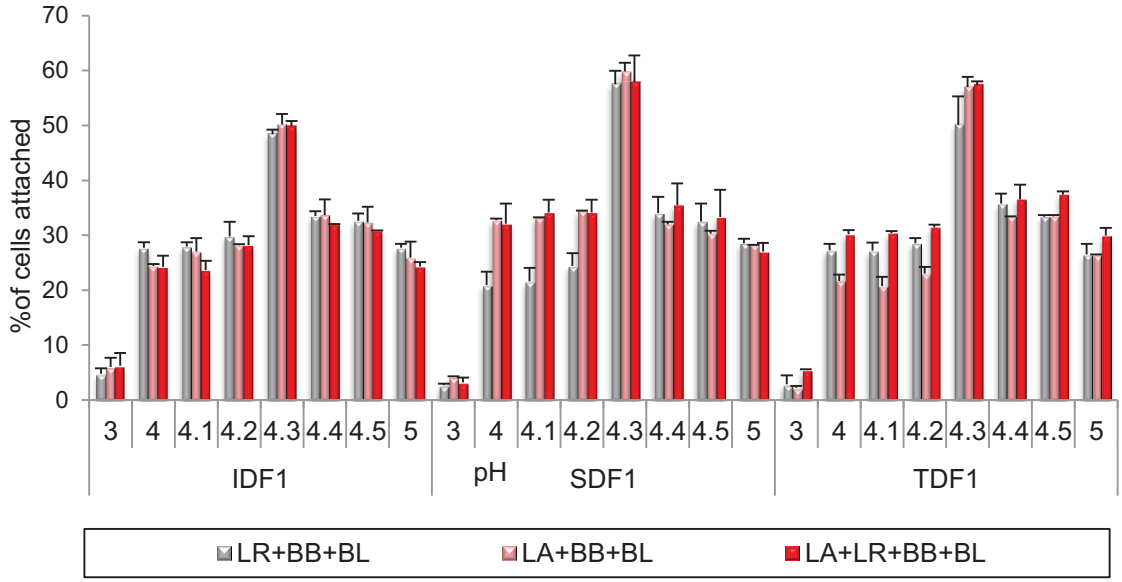

Fig 12.9 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

Figure 12.9 A, B, C Effect of pH on fibre from RR1 (on the adhesion of bacterial combinations to rice fibre fractions.

Results are expressed as the mean value of two trials ± standard error

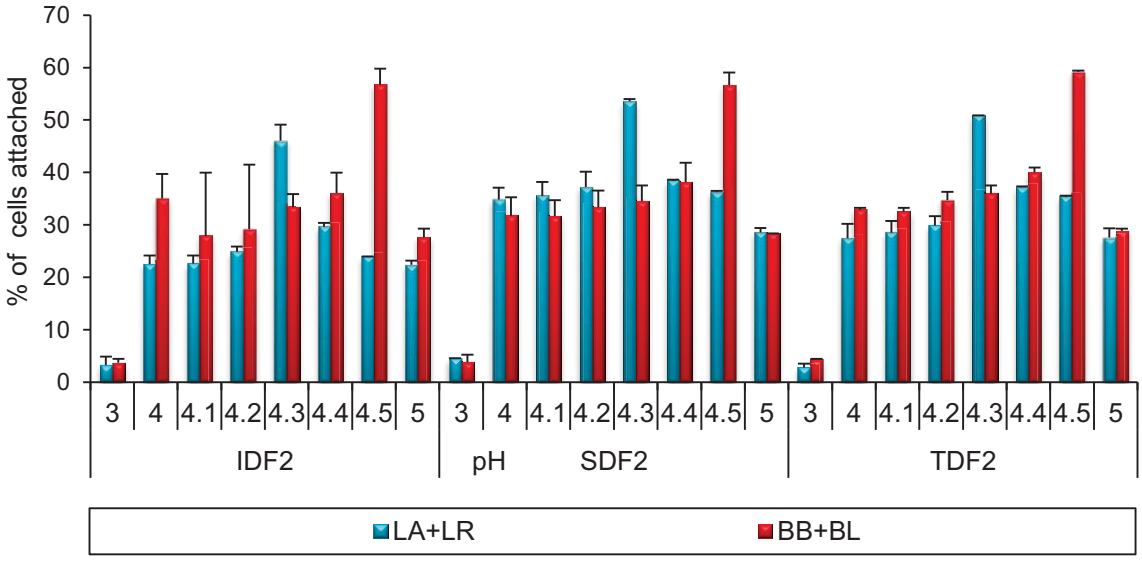

Fig 12.9 D) LA+LR,BB+BL

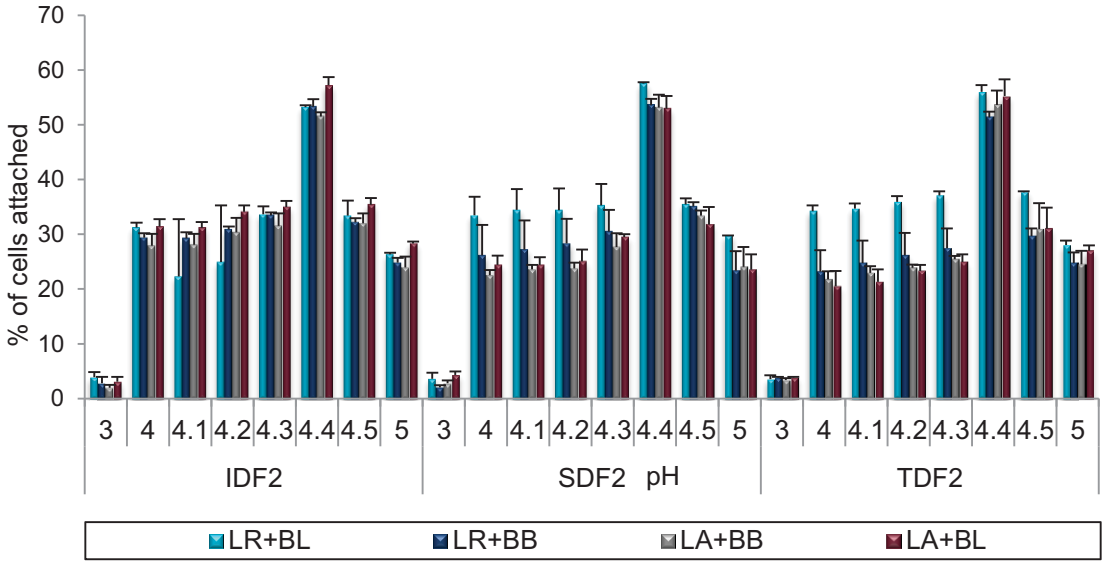

Fig 12.9 E) LR+BL,LR+BB,LA+BB,LA+BL

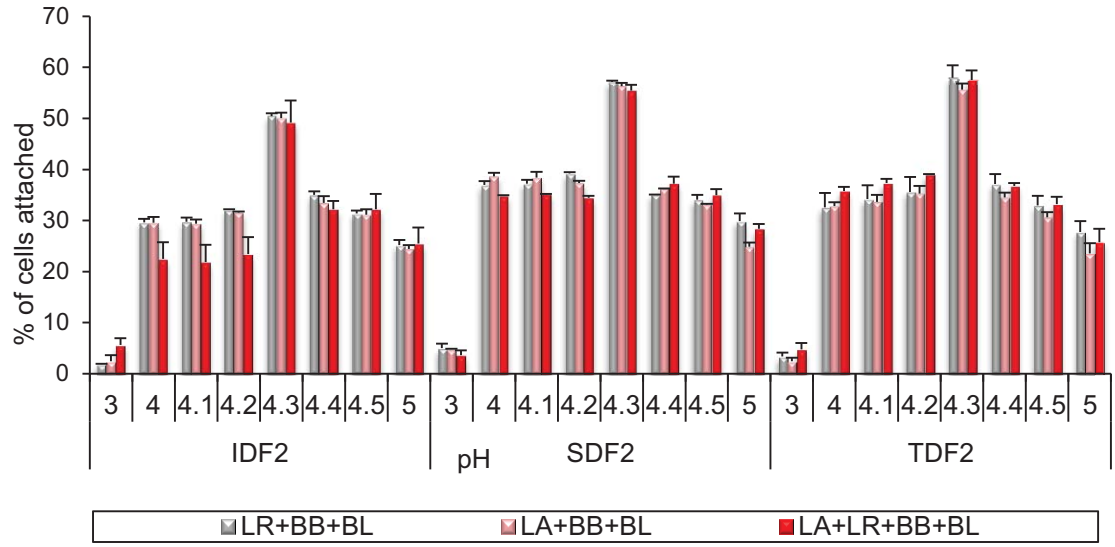

Fig 12.9 F) LA+BB+BL, LR+BB+BL, LA+LR+BB+BL

Figure 12.9 D, E, F Effect of pH on fibre from RR2 on the adhesion of bacterial combinations to rice fibre fractions.

Results are expressed as the mean value of two trials ± standard error.

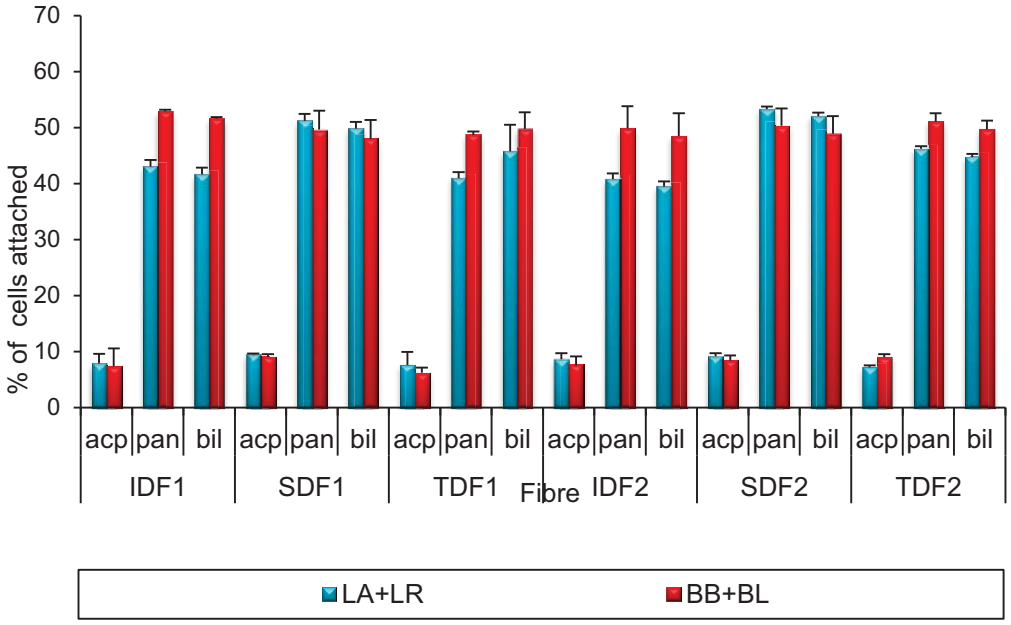

Fig 12.10 A) LA+LR,BB+BL

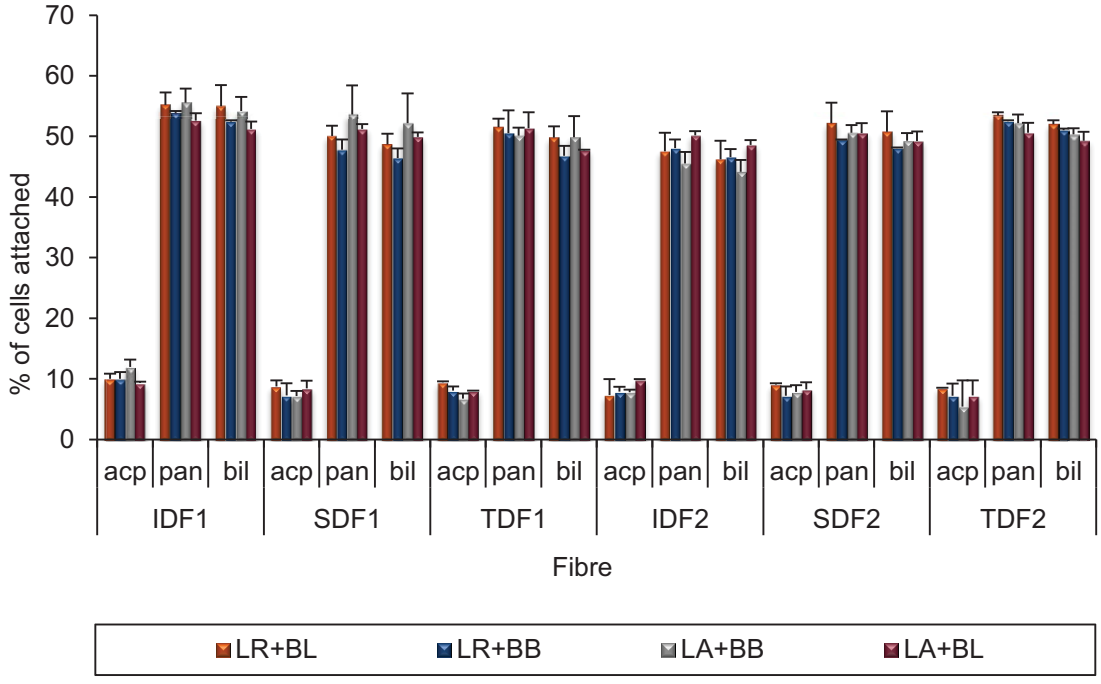

Fig 12.10 B) LR+BL, LR+BB, LA+BB, LA+BL

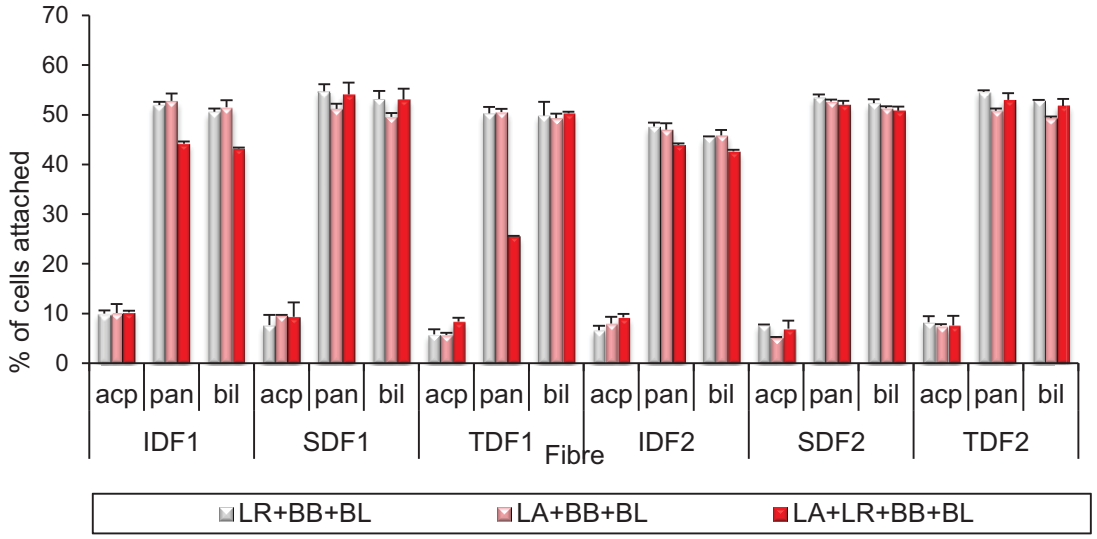

Fig 12.10 C) LR+BB+BL, LA+BB+BL, LA+LR+BB+BL

Figure 12.10 A, B, C Effect of simulating the gastrointestinal conditions on the adhesion of fibre fraction of rice (Acid pepsin solution-acp, Pancreatin-pan, Bile-bil).

Results are expressed as the mean value of two trials ± standard error. Acid pepsin solution-acp, Pancreatin-pan, Bile-bil.

The effect of substrate concentration on the adhesion of bacterial combinations to rice fibre fractions is shown in Fig 12.11.

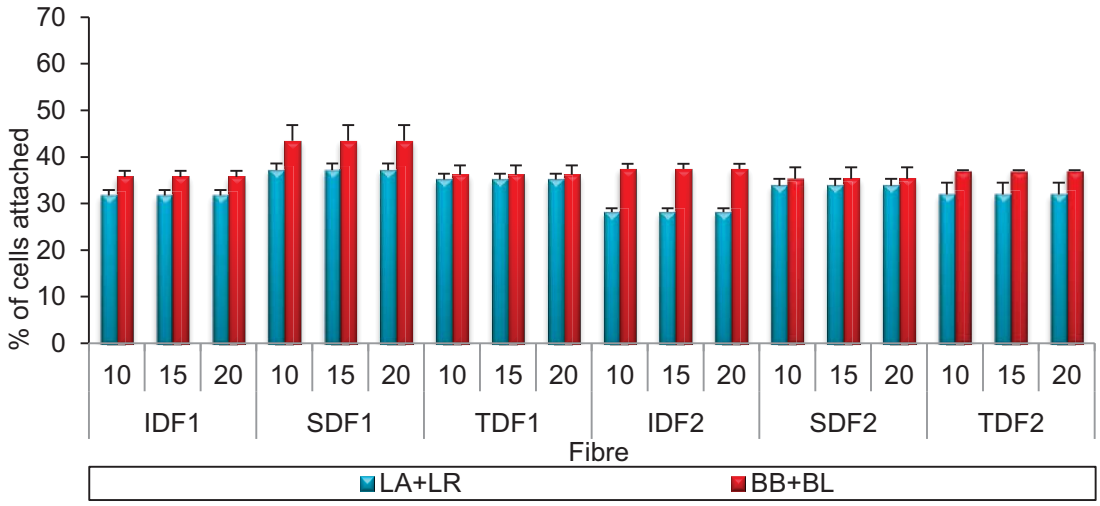

Fig 12.11 A) LA+LR,BB+BL

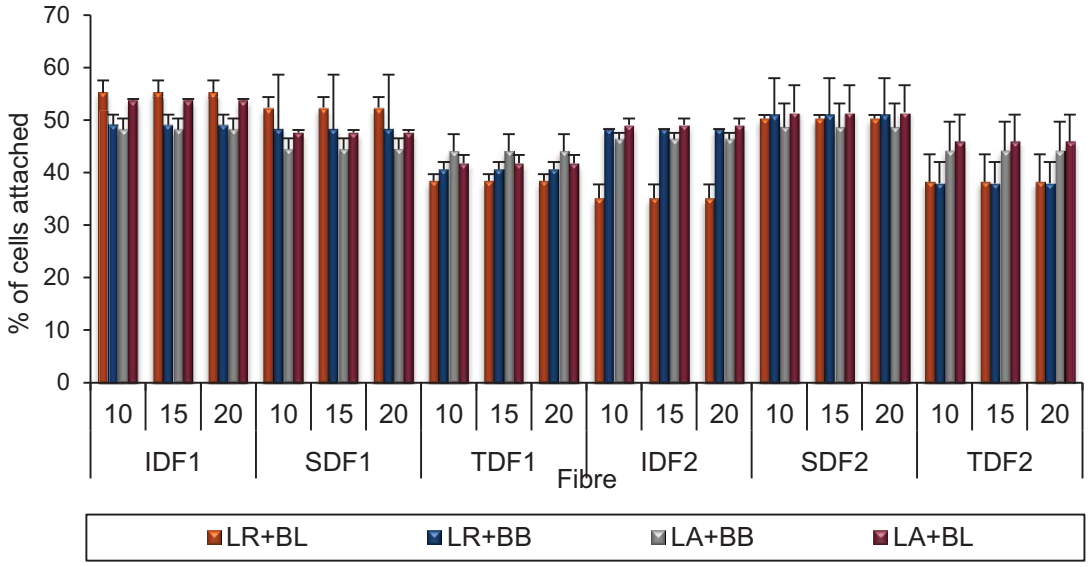

Fig 12.11 B) LR+BL, LR+BB, LA+BB, LA+BL

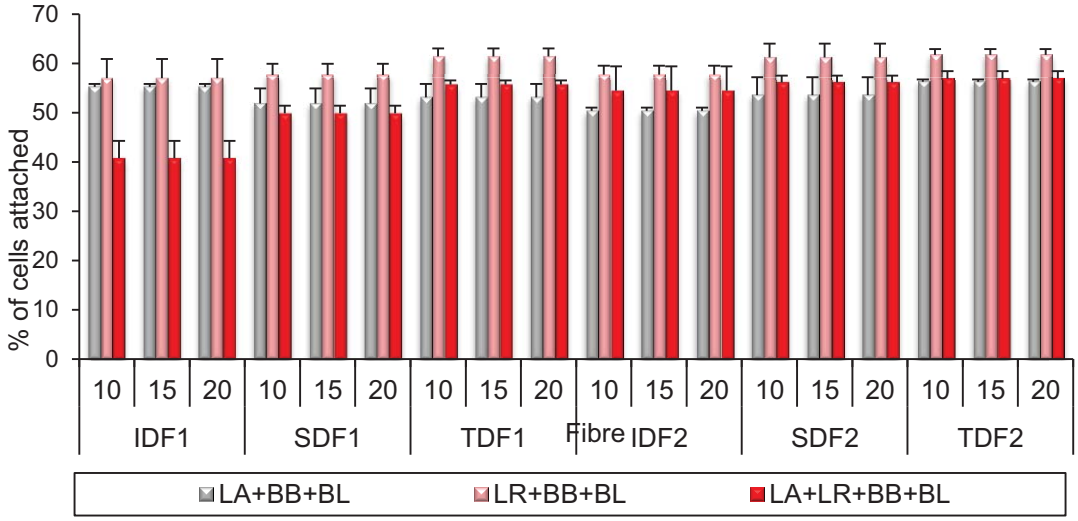

Fig 12.11 C) LA+BB+BL, LR+BB+BL, LA+LR+BB+BL

Figure 12.11 A, B, C. Effect of concentration of substrate on the adhesion of bacterial combinations to rice fibre fractions. (10g/l-10, 15g/l-15, and 20g/l).

Results are express as the mean value of two trials ± standard error.

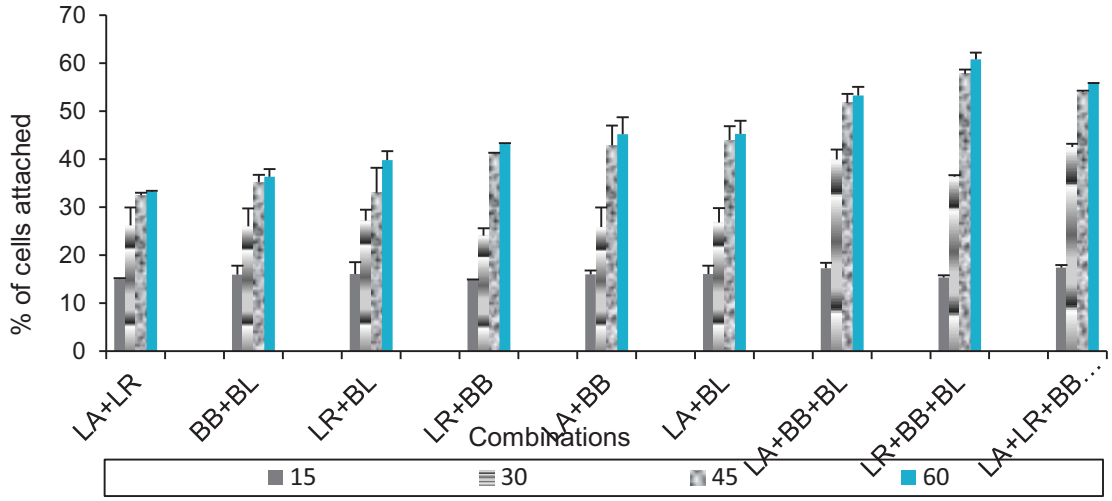

Figure 12.12 Effect of time on the adhesion of bacterial combinations to rice fibre fractions (average of fibre fractions of RR1 and RR2).

Results are expressed as the mean value of two trials ± standard error.

Most adhesion occurred between 0 and 40 minutes, but continued to at least 60 minutes.

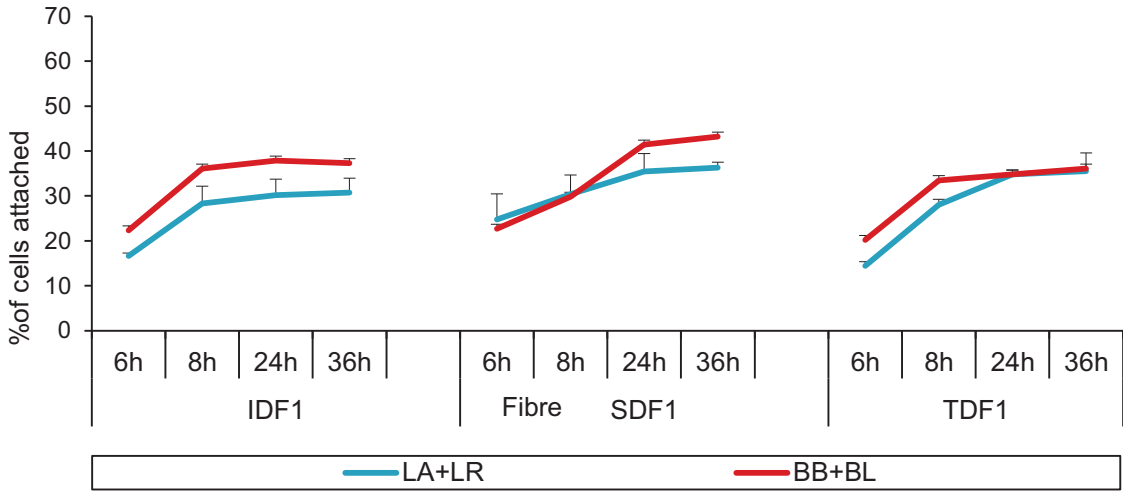

Fig 12.13 A) LA+LR,BB+BL

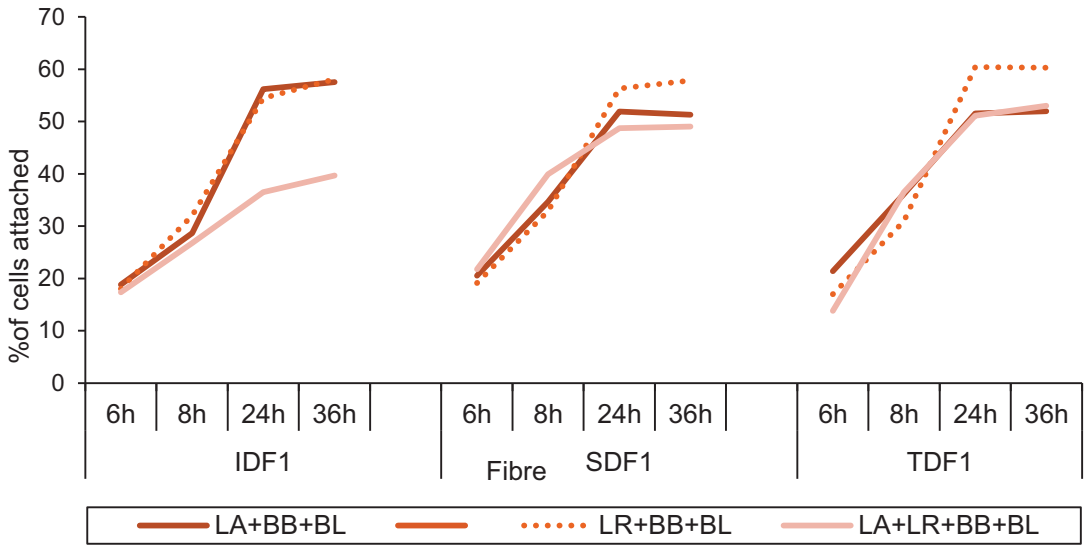

Fig 12.13 B) LA+BB+BL,LR+BB+BL,LA+BB+BL+LA

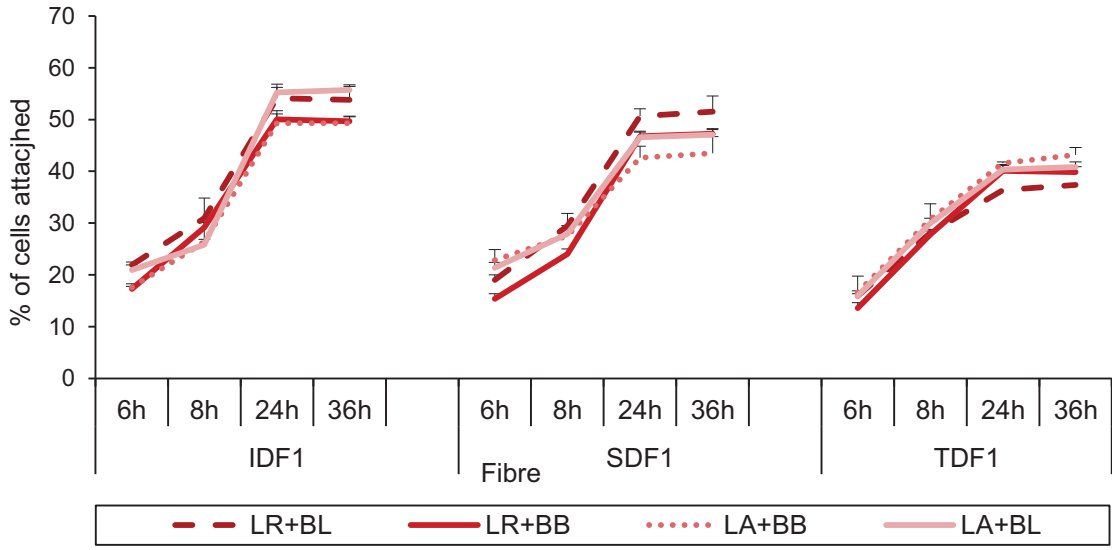

Fig 12.13 C) LR+BL, LR+BB, LA+BB, LA+BL

Figure 12.13 A, B, C. Effect of growth on the adhesion of bacterial combinations to rice fibre fractions RR1.

Results are expressed as the mean value of two trials ± standard error**.**

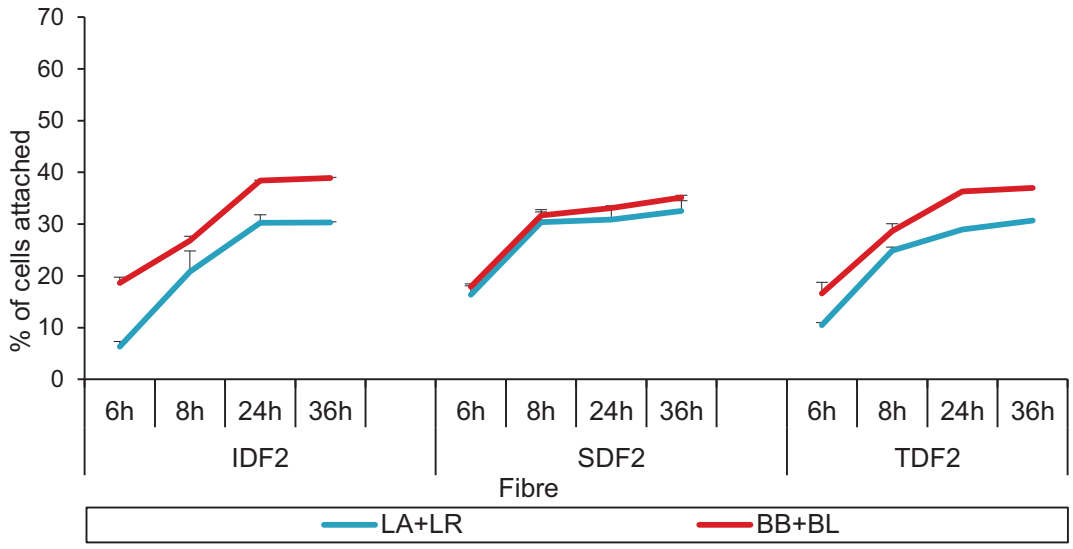

Fig 12.13 D) LA+LR,BB+BL

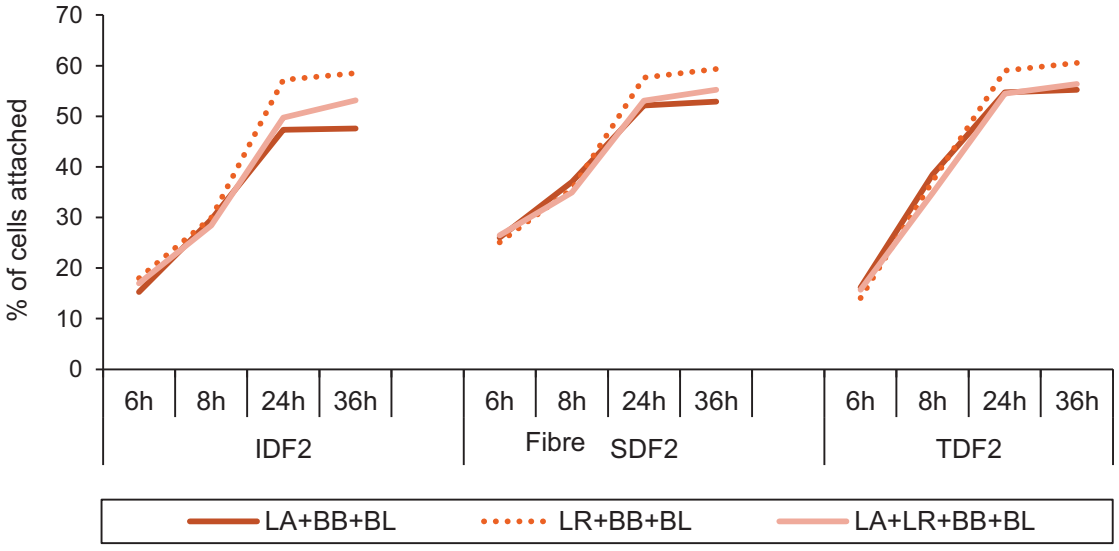

Fig 12.13 E) LA+BB+BL, LR+BB+BL, LA+BB+BL+LA

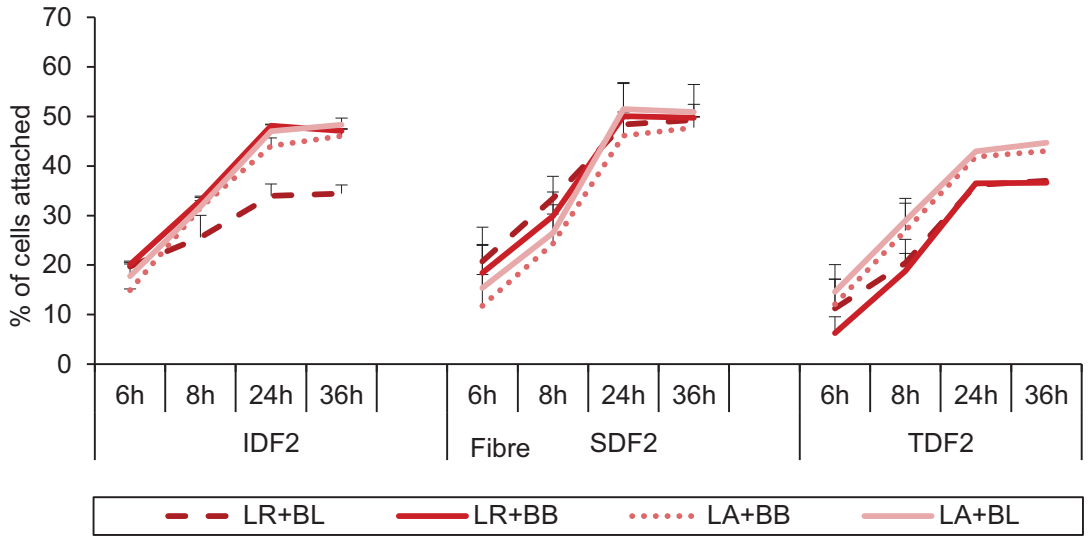

Fig 12.13 F) LR+BL, LR+BB, LA+BB, LA+BL

Figure 12.13 D,E,F. Effect of growth on the adhesion of bacterial combinations to rice fibre fractions RR2.

Results are expressed as the mean value of two trials ± standard error

12.5 Discussion

Microorganisms in the human gut behave as a consortium. Adhesion may be especially important in an ecosystem such as the human gut, which is continuously flowing, where bacteria that adhere to and colonise dietary fibre have an advantage in a competitive microbial environment.

In addition, the ability of fibre digesting bacteria to adhere to substrates (fibre) may assist in successful digestion and enable the products of microbial degradation to be directly available to the bacteria. However, it has not been identified to what extent adhesion of bacterial combinations is a prerequisite for the digestion of rice dietary fibre.

The adhesion of co-cultures of microorganisms to rice fibre fractions led to the identification of two clearly distinct types of adhesion. Combinations of the same genus

(BB+BL and LA+LR) that showed 30 - 40 % (poor) adhesion and combinations of different genus that showed 40 - 50 % (moderate) adhesion, which was significantly higher ($p<0.05$) than the combinations of same genus (Fig 12.1). Studies of adhesion on cellulose with the *Ruminoccocus* species have shown less competition for adhesion where there are different strains from different genera, whereas, adhesion was less when organisms belonged to the same genus (Mosoni *et al.,* 1997). The current study also suggests that combinations of different species of the *Bifidobacterium* and *Lactobacillus* species enable these probiotics to attach well to rice fibre which provides them with an ecological advantage in the human gut. As previously described, the attachment of bacteria to a solid may be important to ensure maximum retention time in the human gut (Morris & Cole, 1987; Miron *et al.,* 2001). Usually liquid digested in the gut would have a shorter retention time than solids. The attachment of bacteria to solid material in the gut will therefore, increase the likely retention time of the bacteria in the gut, therefore, those bacteria that are able to attach to material in the gut will dominate this environment. Based on the previous work on pure cultures, this study suggests the *Bifidobacterium* and *Lactobacillus* species are more likely to dominate in the human gut as combinations rather than as individual strains. However, this needs to be confirmed with other strains of the *Bifidobacterium* and *Lactobacillus* species.

The enhanced adhesion of probiotic bacteria to fibre when different species are combined may be related to the adhesion site on the fibre. Members of the same genus are likely to have a preference for the same adhesion sites, therefore, compete for attachment. Members of different genus are likely to have preference for different adhesion sites, Therefore, their competition will be less. This was demonstrated in our study where adhesion was more or less similar for all the dual combinations of different genus (LA+BB, LA+BL, LR+BB, and LR+BL) (Fig 12. 1 to Fig 12.12). This suggests

that when members of two different genera were simultaneously added to rice fibre, the microorganisms were not competing for attachment sites. This hypothesis is supported by the attachment of ruminal cellulolytic bacteria on cellulose (Bhat *et al.,* 1990). The present study expected to produce a higher percentage of adhesion from probiotic combinations consisting of more than two probiotics since these combinations had more microorganisms than other combinations. However the percentage of adhesion in these multiple combinations was closely related to dual combinations of different genera. This may be explained by the likelihood that in combinations of more than two probiotics (LA+BB+BL, LR+BB+BL and LA+LR+BB+BL), two bacteria of the same genus would compete for the same site. A third microorganism, belonging to a different genus, would be less likely to compete for this site and attach to a different site. Therefore the percentage of adhesion is more or less similar to LA+BB, LA+BL, LR+BB, and LR+BL (Fig12.1). The competition of probiotics for adhesion sites on dietary fibre has not been reported previously. However, the results from the adhesion of the triple combinations, showed a moderate adhesion percentage, suggesting some overall synergistic activity to enhance adhesion to fibre (Fig 12.1).

Whether competition between species occurs at all may depend upon the availability of substrate. Competition in substrate limited environments is likely to be greater than in substrate rich environments, and competition is likely to be greatest where there are more than two competing species. The triple combinations of bacteria showed an increased percentage of cells adhering to rice fibre compared with single and dual combinations. Members of the different species used in these combinations may have variations in their substrate requirements and in the metablolites produced that may benefit the total population, demonstrated by the “moderate” adhesion. This

synergism of different microbial metabolisms may help these combinations compete for nutrients (Odenyo *et al.,* 1994a).

The higher numbers of cells adhering in combinations of BB+BL+LR compared with BB+BL+LA suggests that BB+BL compete less with LR than with LA for adhesion sites (Fig 12.1 to 12.4). Previous work with cellulolytic bacteria (Odenyo*et al.,* 1994b) has shown the production of a bacteriocin which affects the initial adhesion of other organisms (Geis *et al.,* 1983; Greene & Klaenhammer, 1994). Mutual and antagonistic interactions during initial adhesion to fibre of the three bacterial species might have led to the observed differences in the percentage of adhesion.

Attachment of probiotic bacteria to fibre provides the bacteria with a survival advantage. When carbohydrates are limited in the environment, attachment to fibre will enhance survival. It may partly be due to the attachment of these bacteria to dietary fibre that members of the genera *Bifidobacterium* and *Lactobacillus* are able to constitute the dominant flora in the human gut.

NaCl (0.5M) did not affect the adhesion of the microbial combinations to fibre (Fig 12.5). Previous studies noted that glucose uptake of *Bifidobacterium animalis* was stimulated by the presence of NaCl (Briczinski *et al.,* 2008). Roger *et al.,* (1990) observed that Na^+ was essential for the adhesion of *Fibrobacter succinogenes* and not essential for *Ruminococcus flavefaciens*. In addition, the presence of NaCl with rice powder decreased the amount of *Lactobacillus* species (Bautista-Gallego *et al.,* 2008) in the medium. Previous results and the current study suggest that microorganisms may develop a resistance to NaCl when they are combined. Although it has yet to be demonstrated, inhibition of adhesion would probably become more pronounced as the concentration of NaCl increases. When NaCl increases, the effect of charge interactions between the bacteria and the substrate will be less (Fig 12.5). Sodium chloride was

chosen not only because of possible inhibitory effects, but also due to nutritional significance. Studying the effect of NaCl on the adhesion on microbial growth will provide an increased understanding of microbiological food safety issues (Sleator & Hill, 2007) by removing the spoilage organisms.

The present study investigated the effect of Tween 80 as a surfactant and as a food additive on the adhesion of combinations of probiotics to rice fibre. The mechanism of the action of Tween 80 is unknown, but it may not have a significant ($p>0.05$) effect on the accessibility of cells to the substrate, according to the finding of this study (Fig 12.5).

The moderate adhesion in the presence of spent culture medium (Fig 12.6) and the low adhesion with cells treated with pepsin (Fig 12.7), heat treated cultures (Fig 12.8) and in the presence of polysaccharides, monosaccharides and disaccharide, gives some indication of the adhesion mechanisms for these microorganisms (Fig 12.1 to Fig 12.4). The adhesion of culture combinations are similar to individual strains (Chapter 11). Microbial cell proteins and cell viability appear to be important for the adhesion of these bacteria.

The probiotic combinations had a significantly low adhesion when the medium was acidic (pH=3). The optimal pH for the adhesion of microbial combinations was 4.3 to 4.4 (Fig 12.9). The findings of this study show the combinations of bacteria tested had a moderate percentage of adherence in the presence of bile, and with pancreatin, irrespective of the type of co-cultures (Fig 12.10). Further studies should determine adhesion in the presence of other factors associated with the human gastrointestinal tract such as the presence of pathogens and other probiotics (Fig 12.10). An important function of probiotics is to prevent the colonisation of the gastrointestinal tract by pathogenic bacteria (Conway, 1996). It would be useful to know if the probiotic

combinations tested in the present trial will prevent pathogen colonisation of the gastrointestinal tract.

Variations in the concentration of rice fibre, (10/gL, 15/gL, and 20g/L), did not affect the adhesion of microbial combinations, with only a slight increase in the percentage of adherent bacteria observed in the presence of 15/gL and 20g/L, even though this would produce an increase in the surface area available for binding (Fig 12.11). There are reasonable grounds to assume that the variations in the adherence of combinations of same genus and different genera are a trait of these organisms, not substrate limitation. The increased substrate concentrations would provide excess nutrients in the environment and an opportunity for more bacteria to adhere. The fact that this was not observed in the current trial suggests that substrate surface area is not a limiting factor in microbial adhesion to rice fibre.

In this study, combinations of probiotics from different genera attached faster than combinations of the same genus. This has implications for competition in the human gut. A high rate of adhesion is likely to provide the probiotic combinations with a competitive advantage over other bacteria (Fig 12.12).

The colonisation of rice fibre with bacterial cells is affected by temperature, with an optimum temperature of 37°C in these trials (Fig 12.8). Since adhesion was highest during the rapid growth (Fig 12.13) and heat-killed cells had significantly ($p<0.05$) less adhesion to fibre (Fig 12.5), live cells, as well as the enzymes they produce, may play a major role in adhesion.

This trial has demonstrated a model system for studying bacterial adhesion of defined mixed cultures with a specific substrate.

12.6 Conclusion

The adherence process appears to vary for different combinations of the same species and for combinations of different species. Competition between combinations of the same species may explain the low adhesion observed on fibre, and the synergism between combinations of different species may explain the higher adhesion observed on fibre. Adhesion of probiotic combinations to fibre is affected by the temperature, pH, and protein digestive enzymes, but not affected by bile and NaCl. The microbial combinations appear to have similar mechanisms of adhesion as the individual probiotics. Studies of intestinal colonisation with animal models are needed to confirm these *in-vitro* observations. This study observed enhanced adhesion for BB+BL and LA+LR when these combinations were together as BB+BL+LA+LR. This is useful for food manufacturers in designing probiotic combinations for foods. The current study suggests that rice fibre fractions (IDF, SDF and TDF) can be used as a vehicle to deliver microorganisms since combinations of probiotics adhere well to rice fibre.

CHAPTER 13

General Discussion

Dietary fibre research is mainly limited to wheat, rye and maize. Dietary fibre includes many complex substances, each having unique chemical structure and physical properties. Dietary fibre is fermented by anaerobic saccharolytic microflora producing short-chain fatty acids (SCFA), which are absorbed and physiologically important to human health (Lim *et al.,* 2005; Liu *et al.,* 2003; McBurney *et al.,* 1986; McBurney & Thompson, 1987, 1990; Mortensen *et al.,* 1988; Titgemeyer *et al.,* 1991; Tedelind *et al.,* 2007). Fibre obtained from different sources varies in the rate and extent of fermentation (McBurney *et al.,* 1986) and in the amount of SCFA produced. Rice is consumed as a staple food in many parts of the world. Therefore, to understand the impact of the digestion of rice fibre on human health is internationally important. However, the data currently available on rice composition, nutrient content and the effect of rice on human nutrition are limited (Chapter 2).

13.1 Composition of dietary fibre in rice

The nutritional composition of rice varies depending on the type and the variety of the grain. Most of the previous work on the analysis of nutrition composition, including dietary fibre content of rice, was on milling grade as brown or white rice (type), both cooked or raw (Unnevehr *et al.,* 1992; Kennedy & Burlingame, 2003). A large number of rice varieties (such as Basmati 370, Basmati 198) have been examined for protein, zinc and, amylose because these influence consumer preference (Unnevehr *et al.,* 1992; Kennedy & Burlingame, 2003) for rice varieties. Knowledge of the

quantity of dietary fibre in different varieties of rice provides consumers with information to select the more nutritious rice varieties for consumption. Dietary fibre content varies between rice varieties as shown in this study. This variation was demonstrated by studying the IDF, SDF and TDF, rather than crude fibre. Most of the work on dietary fibre of rice has been limited to different layers of rice (bran and germ) (Sera *et al.,* 2005) as in oat and rye (Härkönen *et al.,* 1997; Nilsson *et al.,* 1997a). However, rice is consumed mainly as "whole grain". Therefore, "whole grain" was selected for analysis of fibre in this study.

The rice varieties used in the current study showed higher total dietary fibre content compared with previous research. The total dietary fibre content varied between 2-16g/100g (Table 4.1) compared with previous studies claiming 2-8g/100g (Eggum *et al.,* 1982; Juliano, 1985a; Pedersen & Eggum, 1983; Sera *et al.,* 2005). In the current study, purified fibre was used whereas previous studies used crude fibre. Purified fibre was determined on the basis of rice varieties whereas previous work was based on the rice types. Environmental and post harvest factors, such as solar radiation, irrigation, milling, preparation and cooking, also have contributed towards differences in results. Awareness of the dietary fibre content of rice varieties and the factors that affect the quantity of fibre in rice is important to be able to identify varieties likely to be superior in the diet.

13.2 Growth of faecal inocula, pure cultures and combinations on rice fibre

The current study used *in-vitro* fermentation of rice fibre with fresh faeces as the inocula and as a suitable model to simulate colonic fermentation of dietary rice fibre in the human gut, so as to understand the effect of dietary fibre derived from rice on colonic microorganisms. Differences in the human colonic microorganisms influenced the fermentation of the fibre. This was also observed by Kabel *et al.,* (2002) for the

fermentation of xylo-oligosaccharides. However,in all fermentations of rice fibre, populations of total *Lactobacillus* and total *Bifidobacterium* increased. This suggests that increased microbial counts of these beneficial microbes can be achieved by the addition of rice fibre to the diet. The relationship between rice fibre fractions (IDF, SDF and TDF), and the changes in the proportions of the genus *Bifidobacterium* and the genus *Lactobacillus* was difficult to establish. The genera *Bifidobacterium* and *Lactobacillus* contain large number of species, therefore, it is difficult to determine which specific species are favoured in rice fermentation. The carbohydrate metabolism of bifidobacteria is unusual, using the bifidus path way as explained in Chapter 2 (page 51-53). *Lactobacillus* can use the homo and hetero fermentative pathways. Enhancement of the growth of the genera *Bifidobacterium* and *Lactobacillus* from faecal inocula occur due to cross-feeding or synergistic relationships with other faecal microorganisms such as *Bacteroids* (Wexler, 2007) using glycolytic, bifidus pathways and with different carbon sources. Therefore, the growth of *Bifidobacterium* and *Lactobacillus* species in the faecal inocula is unlikely to have been solely due to the fermentation of rice fibre. In order to understand the fermentation of fibre in the human gut, most previous research on fibre fermentation has used faecal inocula (Chapter 2). Fibre degradation and fermentation in the colon and also *in-vitro* fermentation with human faecal inocula is a co-operative process involving consortia of different bacterial species. To be able to determine which microorganisms are favoured for rice fermentation, pure cultures of probiotics and specific combinations were used in this study. This type of study has not been carried out before and knowledge of the fermentative capacity of individual species and the combinations within the intestinal microorganisms will assist in greater understanding of the mechanisms of dietary fibre fermentation in the human colon.

Although *in-vitro* studies differ from *in-vivo* conditions, laboratory based fermentations can produce useful information on the colon ecosystem. Cocultures of different species produced a significant ($P<0.05$) increase in the total CFU at each fermentation sampling time (6, 24 and 48 h) compared with pure cultures. Individual species within these cocultures fermentations also reached greater numbers, after 24 h fermentation, than the same cultures as single species fermentations. This was particularly apparent for cocultures of more than two probiotics. These results suggested there were synergistic interactions affecting microbial growth in these fermentations and that the microorganisms must be using same fermenative pathways (Chapter 2). This may take the form of inhibitory metabolic products which accumulate in the pure culture fermentations being removed during the growth in the mixed cultures, thus enabling greater viable counts in mixed cultures.

Pure cultures as well as mixed cultures demonstrated similar growth kinetics on rice substrates. No preference was demonstrated for a particular rice variety. Both pure cultures and cocultures demonstrated a preference for TDF than other rice fibre fractions. However, most of the pure cultures and combinations produced higher growth on TDF than on glucose. Faster fermentation using faecal inocula on glucose or glucose-containing polymers compared with xylose or xylose-containing polymers has been reported (Barry *et al.,* 1989; Englyst & Hudson, 1987; Lebet *et al.,* 1998a; van Laere *et al.,* 2000). Results of this work indicated that TDF may have the potential to be a useful dietary fibre for the probiotics *Bifidobacterium longum* and *Bifidobacterium breve* strains in symbiotic combinations, rather than the *Lactobacillus* species.

Co-culture combinations had a higher rate of digestion than pure cultures. The ability to consume a variety of rice dietary fibre fractions efficiently indicated that co-culture combinations are able to compete well with other microorganisms in the

environment. The co-culture demonstrating the most efficient digestibility was BB+BL+LR. This indicates that the efficiency of rice fibre fermentation by *Bifidobacterium* is increased by combining it with *Lactobacillus*. Co-cultures may be more effective than individual microorganisms in fermentation through combined enzyme activity cleaving glycosidic bonds and degrading the resulting monosaccharides. Results from the present work clearly indicate that rice fibre degradation and fermentation in the colon is definitely a collaborative work of the probiotics in the colon. Therefore, food products should contain compatible combinations of probiotics to produce the maximum benefit from rice digestion.

The efficiency of fermentation of pure cultures significantly declined when the pure cultures were combined with species of the same genus. A better understanding of negative and positive contributions of pure cultures and their combinations would help in the preparation of functional foods containing probiotics.

13.3 SCFA formation on fibre

The production of SCFA in the fermentation of rice fibre followed the consumption of dietary fibre: the higher the rate and quantity of fibre consumption, the higher the rate and quantity of SCFA production.

The primary SCFA by-products of colonic bacterial fermentation of fibre are acetate, propionate and butyrate.

This study concentrated on three types of fermentations. The quantity of SCFA from the fermentations was the highest for faecal cultures, followed by co-cultures then pure cultures with acetate being the main fatty acid produced, followed by propionate and butyrate. This is similar to the fermentation of other cereals such as rye, oats and maize (Aura *et al.,* 2005, 2006; Kim & White, 2009) with faecal inocula. Co- cultures produced significantly ($P<0.05$) higher concentration of total SCFA than the pure

cultures after 24 h of incubation. This difference was mainly due to higher acetate production with no significant differences ($P>0.05$) in propionate and butyrate between co-cultures and pure cultures. There was a correlation between acetate production and culture growth in the fermentations using co-cultures. Butyrate and propionate levels in co-cultures fermentations were similar to the levels observed in pure culture fermentations. One explanation for this may be that these SCFAs may have been used as an energy source in the co-culture fermentations. The significantly ($P<0.05$) higher concentrations of SCFA produced from rice fibre fermentations using faecal cultures compared with the specific co-cultures, indicated that there are many bacterial combinations in nature, more effective than the bacterial combinations tested in this trial (Gibson, 1998). Many saccharolytic bacterial species, including the *Bifidobacterium* and *Lactobacillus* species, are effective in fibre digestion (Blatch & Woods, 1993). *Bacteroides* species are included in this group of known fibre consumers, are present in faeces, and this may explain the higher SCFAs produced from faecal cultures compared with the known cultures used in this fermentation trial. The pH after 24 h fermentation reflected the amount of SCFA produced. Differences in pH and acetate production were mainly observed in fermentations with co-cultures of the *Bifidobacterium* and *Lactobacillus* species rather than co-cultures made up of the same genus.

Higher concentrations of total SCFA (acetate, propionate, and butyrate) were produced from fermentations of TDF compared with IDF and SDF, irrespective of the fibre source. This corresponded to the increased growth of microorganisms on TDF compared with IDF and SDF. This indicates that the levels of SCFA formed in the human gut could be increased through improved microbial fermentation by selecting fibre sources in the human diet.

The health effects of dietary fibre are believed to be related to butyric acid followed by acetate and propionate produced during fermentation (Cummings, 1997). Butyric acid may have a protective effect on colon cancer (Russo *et al.,* 1999). The molar ratio of acetate: propionate: butyrate was higher with faecal inocula than either combinations or pure cultures. Factors which determine the ratio of SCFA (acetate, propionate, and butyrate) are not fully understood. The properties of the substrates and the combinations of microorganisms may be involved in determining the SCFA profile of pure cultures, faecal cultures and combinations. The molar ratios of SCFA, however, are not the only important consequence of fermentation, as the amount of SCFA produced also plays an important role. It may be that a small amount of SCFA with a high molar ratio of butyric acid may have the same health effect as a high amount of SCFA containing a little butyric acid.

Results from this part of the study suggest that rice dietary fibre fractions have a significant prebiotic effect on pure cultures and combinations of the *Bifidobacterium* and *Lactobacillus* species. This study confirms that rice dietary fibre fractions stimulate the pure culture growth. Selecting specific strains of the *Bifidobacterium* and *Lactobacillus* species as mixed cultures to ferment rice fibre promote rapid fermentation, growth, and SCFA production, increasing the prebiotic nutritional value of dietary fibre fermentation in the human gut.

13.4 Adhesion on fibre

There are different hypotheses to explain how probiotics work with food in the human gut (Lin, 2003). Fermentation of fibre by pure cultures and cocultures does not reveal the mechanism of fibre digestion in the colon. Digestion can be carried out by microbial enzymes and may involve adhesion of the micro-organism to the fermentable

substrate. Enzyme activity is generally understood. However, the importance of microbial adhesion to fibre as part of fibre fermentation is not so well understood.

In this study, adhesion to rice fibre was greater for the *Bifidobacterium* species than the *Lactobacillus* species, both as pure cultures and in culture combinations. The *Bifidobacterium* species showed 40 - 50 % adhesion as pure cultures and in combinations of BB+BL. When these were combined with the *Lactobacillus* species, the adhesion ranged from 50 -60%. The high viable count of *Bifidobacterium* in the later stages of fermentation might be explained by *Bifidobacterium* adhering to fibre, therefore, escaping environmental stress. The moderate adhesion of pure cultures and the combinations of probiotics on fibre indicates that fibre can be used as the vehicle to transport the probiotics to the human gut. Fibre may also facilitate the incorporation of probiotics into foods.

This study found that combinations of microorganisms had better adhesion capacity than the individual organisms. Previous work found that individual organisms can enhance or inhibit the adhesion of the organisms in combinations (Lee & Puong, 2002). The higher adhesion of the combinations consisting of more than two species, rather than the individual species, might indicate some synergistic activities with the counterpart microorganisms. However, the results of the present study, could not determine whether this was due to *Bifidobacterium* or *Lactobacillus* or both, in the combinations. The reduced adhesion by the BB+BL and LA+LR combinations when compared with the individual microorganisms indicated that both have the potential to inhibit adhesion. Poor SCFA formation by these combinations might be linked with this reduced adhesion. The combinations of two microorganisms, consisting of one member from each genus, showed similar adhesion to fibre as the individual organisms. The combination of BB+BL+LR achieved complete digestion of SDF and TDF, although

the micro- organisms demonstrated only moderate adhesion to the fibre. A variety of factors such as the naturally low rate and extent of adherence to rice fibre, efficiency in using the soluble products of depolymerisation of fibre fractions during the adhesion process, and the production of substances produced by pure cultures and combinations that prevent adhesion, may have contributed to the low adhesion. It would be interesting to know whether adherent organisms formed proportionally higher SCFA than the non adherent organisms.

Adhesion of the probiotics to rice fibre was not dependent upon substrate differences, whereas SCFA, fibre digestion and growth kinetics was dependant on the substrate.

The present study indicated that the adhesion of pure cultures and combinations to rice fibre may not be influenced by electrostatic forces, since both demonstrated greater adhesion in the presence of Tween 80, which is a non ionic compound.

This study provides a scientific basis for the screening and selection of probiotics which have the capacity to adhere to dietary rice fibre as pure cultures and as combinations.

The results from this work suggest that the selection of the strains of the *Bifidobacterium* and *Lactobacillus* species as co-cultures to ferment rice fibre not only would have the advantage of rapid growth and the production of SCFA, but would likely increase the probiotic nutritional value of the dietary fibre fermentation in the human gut.

Most of the functional food is based on dairy produce. Rice may provide an alternative source of functional foods, particularly for those people with dairy allergies (Shanahan & McCarthy, 2000).

13.5 Limitations of the study

The major limitation in this study is the availability of accurate *in vivo* data. These results are important for the critical assessment of *in vitro* results obtained for fibre digestion.

In the present study, the *in vitro* methods, while based on "best practice" from published research, are unlikely to have truly represented the conditions in the human gut. Because, studying fermentation in the gastrointestinal (GI) tract is challenging as it is difficult to develop rapid and simple hindgut *in vitro* methods or models that represents this complex microbial environment. This means that the results from this study, need to be considered with this in mind and that the actual conditions of the human gut may influence some of the data reported in this thesis. For example, my results show interactions between rice fibre and pure cultures or combinations of cultures of probiotic bacteria towards understanding the digestion of rice fibre by probiotic bacteria, the actual substrate and micro flora present in the gut will be far more complex with many interactions not considered in this present study. Though the faecal cultures used in this study represent the human colon to an extent, a faecal inoculum will vary from individual to individual as well as in same individual in different days, making it difficult to obtain repeatable results.

There are difficulties with *in vivo* studies too. In the present study, the subjects regularly eat rice so are likely to respond differently to individuals from other populations that may not regularly eat rice. While I was able to demonstrate some differences resulting from introducing a specific heavy rice diet, this is likely to vary in different populations.

In this study, fibre was extracted from rice enzymatically. This predigesting procedure could change the fibre cell wall structure, from that naturally experienced in the human

gut. This could lead to the fibre behaving differently when exposed for the microorganisms used in my trials (Guillon *et al.,* 1992).

The technique for adhesion study was very laborious. Several washings prior to the assay could create environmental stress for tested bacteria probably lead to lower the adhesion capacity of large number of bacteria, leaving only the bacteria with the strongest adhering ability.

Apart from cheapness, small size, and ready availability of rice, percentage of fibre in rice is low. Therefore it is costly to extract fibre from rice for long term research like I did. This may prevent to use rice as a wide experimental tool related to dietary fibre in future.

Sample sizes were limited (four rice samples, four humans, four pure cultures). Such limitations are necessary to manage costs and time however small sample sizes have some limitations with respect to the analysis and data analysis.

CHAPTER 14

Future Developments and Final Conclusions

The following are suggested future investigations to extend the results from the present study.

14.1 Compositional analysis of IDF, SDF, and TDF

This study examined the probiotic fermentation of IDF, SDF and TDF of rice fibre. The differences observed between the fibre fractions may be able to be explained by comparing the sugar composition of the individual fibre fractions, extracting those specific sugars and examining their role in probiotic fermentation leading to possible extraction for incorporation into food formulations. The effects of hull, bran and germ fractions of rice on fermentation and SCFA formation would identify fractions that are commercially extractable, and optimal for probiotic growth.

14.2 Sampling

Variations in the size of the inocula and the concentrations of substrate used in this study would determine the optimum conditions for rice fibre fermentation. This may help in formulating diets and foods containing probiotics. Increasing the frequency of sampling may allow a more accurate interpretation of the growth data for fermentation.

14.3 Microbial characterisation

Characterising the actual species from human microflora that are involved in rice fibre fermentation would be useful in confirming the results from the fermentation trials. Any special features associated with these isolates and their ability to ferment rice fibre could be used to design products containing probiotics that would be most

effective in rice fibre fermentation. Characterisation of the probiotics used in this work through DNA arrays would allow the gene expression of different probiotics to be compared.

14.4 Physiological changes

The effect of probiotics on human physiology may assist in understanding the SCFA formation in different sections of the human colon, and in formulating foods for different sectors of the population. The human requirement for rice fibre and specific probiotics may vary with age, gender, and race.

14.5 Adhesion of organisms on fibre

Identification of the properties of microbial cells and rice fibre involved in microbial adhesion to fibre in the environment of the human colon would help in selecting the specific microbial/fibre combinations to optimize the fermentation of rice fibre.

Studying the adhesion of probiotics and fibre with human colon carcinoma cell line HT-29, Caco2, and HT29-MTX under *in-vitro* conditions could reveal the adhesion mechanism of rice fibre and probiotics to human colon cells.

14.6 Extending studies on dietary fibre

Studies similar to those in this thesis may examine the fermentation of other dietary fibres such as rye, barley, maize, oat, etc, and would allow a comparison with the results from this study and some conclusions on the relative value of different dietary fibre to the human diet.

14.7 Application in medicine

The effects of dietary fibre on energy expenditure, body weight control and the biomarkers of coronary diseases and the possibility of adhering and eliminating cancer cells are topics in which there is much interest, but little information. Determining the

most effective probiotic or combinations of probiotcs with rice fibre may assist in treatment or prevention of gastrointestinal infections, certain bowel disorders, allergy, and urogenital infections, and may be useful in controlling these diseases.

14.8 Final Conclusions

This study showed that dietary fibre carbohydrates of rice can be divided into two groups: 1) fermentable, soluble carbohydrates that are rapidly fermented, 2) fermentable insoluble carbohydrates that are fermented slowly. Soluble fibre of rice was fermented and digested rapidly, while insoluble dietary fibre carbohydrates were fermented and digested slowly, under *in-vitro* conditions. Though soluble fibre is more important in terms of rate and extent of fermentation than insoluble fibre, the percentage of soluble fibre in rice was low. Therefore, total dietary fibre of rice, or as whole grain rice, might serve as a more balanced source of dietary fibre than either readily fermentable, isolated, soluble poly- and oligosaccharides, or insoluble fractions. This implies that differences of solubility, structure of the polysaccharides and the cell-wall matrix of the extracted fibre fractions might have an effect on the fermentation rate of rice fibre in the colon.

The most important rice fibre fraction for fermentation, based on rate and extent of SCFA formation, substrate degradation and microbial growth, was TDF. According to the rate and the extent of the fermentation of the rice fibre fractions tested in this study, the soluble dietary fibre of rice would most likely be fermented in the upper part of the colon (proximal and transverse colon), and, the insoluble dietary fibre of rice would most likely be fermented in the sigmoid colon. The non fermentable percentage of rice fibre would have a bulking effect in the colon. Total dietary fibre can be a substrate for organisms in the lower and upper parts of the colon.

In this study the rate and extent of SCFA formation differed between the cultures and the substrates used. Acetate, followed by, propionic and butyric acids, were produced during the fermentation of rice fibre. Differences in the fermentation rates of rice fibre fractions by pure cultures and combinations, suggest that propionic and butyric acids would be produced in many areas of the colon. Though the formation of butyrate was significantly lower than acetate, an adequate supply of rice fibre would ensure a high butyrate concentration along the entire length of the large intestine. This would maintain mucosal health in the colon, which is believed to play a role in the prevention cancer in the distal colon (German, 2007).

IDF, SDF, and TDF were fermented *in-vitro* by *Bifidobacterium longum*, a numerically dominant *Bifidobacterium* species in the adult human colon. This ensures fermentation of rice fibre fractions in the human colon effeciently. *Bifidobacterium breve, Lactbacillus acidophyllus* and *Lactobacillus rhamnosus* were also found to ferment rice fibre. Pure cultures and combinations of *Bifidobacterium* had a higher fermentation rate on rice fibre fractions than pure cultures and combinations of *Lactobacillus* species. Therefore, it is preferable to use the *Bifidobacterium* species instead of the *Lactobacillus* species as probiotics in foods. The type of rice fibre entering the colon is likely to have an impact on the metabolism and composition of colonic bacterial species, thus effecting the formation of SCFAs, the balance of which is important to maintain gut health.

Specific microbial combinations grew faster and produced more SCFA than pure cultures. This was believed to be due to synergistic interactions. Microbial combinations made up of the same genus had less capacity for growth and SCFA formation than combinations with more than one genus. The BB+BL+LR combination produced the best fermentation results. Propionic and butyric acid production was

favoured by the *Bifidobacterium* species, in particular, *Bifidobacterium breve,* as pure cultures and in combinations.

The adhesion of pure cultures and the combinations of probiotics onto rice fibre indicated that rice fibre can be used as a vehicle to transport the probiotics to the human gut and facilitate the incorporation of probiotics in foods. The ability to adhere to rice fibre in conditions that would be found in the gut is believed to help in the survival and fermentation of rice fibre in the colon.

Fibre fractions of rice, which consist of rapidly and slowly fermentable carbohydrates, and non-fermentable carbohydrates, all have their specific effects on the human colon with potential health benefits. Rice fibre fractions may be a useful prebiotic, but the effects need to be confirmed in human studies. Rice is an important natural source of dietary fibre.

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APPENDIX A

- **Preparation of 0.1 mol/L sodium phosphate buffer (Ph-7)**

One hundred and thirty eight grams of (138g) monobasic, monohydrate ($NaH_2PO_4.H_20$ (MW is 138g)) was dissolved in one litre of distilled water to prepare the 1m stock solution, and 142g of anhydrous form of Na_2HPO_4 (MW is 142g) was dissolved in 1 litre of distilled water, to have stock solution of 1M. Stock solutions were autoclaved (121ºC for 15 min) and stored at room temperature. Fifty seven and seven points mililitres of (57.7mL) of stock solution of $NaH_2PO_4.H_20$ was mixed with the 42.3mL of stock solution of $NaH_2PO_4.H_20$ to prepare the 0.1M of sodium phosphate buffer of pH 7 (protocol of fisher scientific U.K.).

- **Wilkins-chalgren broth**

Formula / Liter (Final pH: 7.1 ± 0.2 at 25°C)

Enzymatic Digest of Casein	10 g
Enzymatic Digest of Gelatin	10 g
Yeast Extract	5 g
Sodium Chloride	5 g
Dextrose	1 g
L-Arginine	1 g
Sodium Pyruvate	1 g
Hemin	0.005 g
Vitamin K	0.0005 g

Thirty three grams of (33 g) of the medium (powder form) was dissolved in one litre of distilled water. Solution was heated frequently with agitation and boiled for one minute

to dissolve the medium completely. The medium was autoclaved at 121°C for 15 minutes.

- **Nutrient Agar 1.5% (Difco)**

Formula / Liter **(Final pH: 7.1 ± 0.2 at 25°C)**

Beef Extract	3.0 g
Peptone	5.0 g
Sodium Chloride	8.0 g
Agar	15.0 g

Thirty one grams of (31 g) of the medium (powder form) was dissolved in one litre of distilled water and heated frequently with agitation, and boiled for one minute to dissolve the medium completely. The medium was autoclaved at 121°C for 15 minutes.

- **Wilkins-Chalgren Agar**

Formula / Liter (Final pH: 7.1 ± 0.2 at 25°C)

Enzymatic Digest of Casein	10 g
Enzymatic Digest of Gelatin	10 g
Yeast Extract	5 g
Sodium Chloride	5g
Dextrose	1g
L-Arginine	1g
Sodium Pyruvate	1g
Hemin.	0.005g
Vitamin K	0.0005g
Agar	15g

Thirty three grams (33 g) of the medium (powder form) was dissolved in one litre of distilled water and heated frequently with agitation, and boiled for one minute to dissolve the medium completely. The media was autoclaved at 121°C for 15 minutes.

- **Rogosa Agar**

Formula / Liter (Final pH 5.4 ± 0.2 at 25°C)

Tryptone	10g
Yeast extract	5g
Glucose	20g
Sorbitan mono-oleate `Tween 80'	1mL
Potassium dihydrogen phosphate	6g
Ammonium citrate	2g
Sodium acetate, anhydrous	17g
Magnesium sulphate	0.575g
Manganese sulphate	0.12g
Ferrous sulphate	0.034g
Agar	20g

Eighty two grams (82 grams) of medium was suspended in 1 litre of distilled water and boiled to dissolve completely. One and thirty two point's mililitres of (1.32 mL) glacial acetic acid was added and mixed thoroughly. Solution was heated to 90-100°C for 2-3 minutes with frequent agitation. Solution was distributed into Petri dishes. Medium was not autoclaved.

- **Beerens' agar**

Formula / Liter (Final pH 7.4 ± 0.2 at 25°C)

Colombia blood agar base (Difco)	17.6 g
Glucose	2.0 g
L-cystein HCl H2O	0.2g
Distilled water	400mL

Medium was heated to 100ºC, for 30 minutes. Two mililitres (2.0 mL) of propionic acid was added to medium after cooling to 55ºC. pH was adjusted to 5.0, using 4N-NaOH. The medium was poured to petri dishes immediately. Contamination was checked by incubating dry agar surface at 37ºC, for 24 h. The medium was prepared without sterilization. (This medium was easy to prepare for 400 mL than preparing 1000mL).

- **Lactobacilli MRS(de mann rogasa sharpe) Agar/Broth**

Formula / Liter (Final pH 5.7 ± 0.2 at 25°C)

Proteose Peptone	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g
Agar	15.0 g

- **Clostridial Agar (Rainforced Clostridial Medium, RCM)**

Formula / Liter (Final pH 6.8 ± 0.2 at 25°C)

Yeast extract	3.0 g
Meat extract	10.0 g
Meat peptone	10.0 g
Starch	1.0 g
D(+)-Glucose	5.0 g
L-Cysteine hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g

pH 6.8±0.2

- **Digested horse blood**

Sodium Chloride. 0.85% solution.	150mL
Pepsin (1:10000) ...granulated	1 g
Hydrochloric Acid, 12 N	6mL
Defibrinated horse blood	50 mL
Sodium Hydroxide, 20% (10N)	12 mL

Final pH 7.3 ± 0.2 at 25°C

Salt solution, HCL, blood, pepsin was added to a sterile glass stoppered bottle and shake well. This mixture was kept in water bath at 55ºC for 2-24 hours till digest. Sodium hydroxide (12 mL) was added and final pH was adjusted as pH 7.3 ± 0.2 at 25°C. Store at 2-8 ºC.

APPENDIX B

Table 1-App B Fermentation of *rice* dietary fibre with probiotics leads to formation of short chain fatty acids (SCFA, m moles/100mL, A-E) (Chapter 8)

A) O h

RICE	H	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.34	0.04	0.03	0.28±0.01	0.04	0.03	0.32±0.03	0.32	0.04	0.31±0.02	0.04	0.03
	SDF1	0.31±0.02	0.04	0.03	0.31±0.01	0.04	0.03	0.32±0.01	0.32	0.03	0.29±0.02	0.04	0.03
	TDF1	0.33±0.02	0.04	0.03	0.29±0.01	0.04	0.03	0.34±0.01	0.34	0.03	0.30±0.01	0.04	0.03
RR2	IDF2	0.31±0.02	0.04	0.03	0.27±0.01	0.04	0.03	0.28±0.01	0.28	0.03	0.29±0.01	0.04	0.03
	SDF2	0.30±0.01	0.04	0.03	0.31±0.01	0.04	0.03	0.28±0.01	0.28	0.03	0.30	0.04	0.03
	TDF2	0.30±0.03	0.04	0.03	0.30±0.01	0.06	0.03	0.30±0.01	0.30	0.03	0.28±0.01	0.04	0.03
	GLUCOSE	0.29±0.01	0.05	0.04	0.30±0.01	0.05	0.03	0.27±0.01	0.27	0.03	0.32±0.03	0.04	0.03
	CONTROL	0.30±0.03	0.04	0.03	0.36±0.01	0.05	0.03	0.31±0.02	0.31	0.03	0.30±0.01	0.04	0.03

B) 6 h

RICE	H	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.45±0.01	0.05	0.03	0.72±0.03	0.05	0.04	0.58±0.01	0.13	0.08	0.47	0.11	0.04
	SDF	0.52	0.05	0.04	0.79±0.01	0.05	0.03±0.01	0.54±0.03	0.14	0.09	0.55	0.12	0.05
	TDF1	0.67±0.02	0.06	0.04	0.62±0.05	0.05±0.01	0.03	0.55±0.01	0.16	0.10	0.62	0.13	0.06
RR2	IDF2	0.43±0.01	0.05	0.03	0.81±0.04	0.05	0.04	0.56	0.13±0.01	0.08	0.44	0.10	0.04
	SDF2	0.48±0.01	0.05	0.04	0.75±0.03	0.05	0.03	0.57±0.01	0.14	0.08	0.54±0.01	0.11	0.04
	TDF2	0.60	0.05	0.04	0.68±0.04	0.06	0.04	0.58±0.03	0.15	0.10	0.58	0.12	0.05
	GLUCOSE	0.42±0.02	0.06	0.04	0.69±0.04	0.05	0.03	0.59±0.06	0.16	0.09	0.48	0.15	0.06
	CONTROL	0.30±0.01	0.04	0.03	0.32	0.05	0.03	0.31±0.01	0.04	0.03	0.32	0.04	0.03

C) 12 h

RICE	H	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.51±0.05	0.04	0.03	0.77±0.04	0.05	0.04	0.59	0.14	0.08	0.56±0.04	0.11	0.04
	SDF1	0.51	0.05	0.04	0.84±0.03	0.05	0.03	0.50±0.01	0.16	0.08	0.66±0.03	0.12	0.05
	TDF1	0.72±0.05	0.06	0.04	0.76 ±0.3	0.05	0.03	0.70	0.14	0.1	0.70±0.03	0.13	0.06
RR2	IDF2	0.49±0.05	0.05	0.03	0.8±0.04	0.05	0.04	0.62±0.05	0.15	0.08	0.53±0.01	0.1	0.04
	SDF2	0.53±0.03	0.05	0.04	0.84±0.03	0.05	0.03	0.63±0.03	0.14	0.08	0.60±0.03	0.11	0.04
	TDF2	0.62±0.02	0.05	0.04	1.06±0.01	0.06	0.04	0.67±0.02	0.16	0.09	0.66±0.04	0.12	0.05
	GLUCOSE	0.68±0.05	0.06	0.04	0.72±0.05	0.05	0.03	1.34±0.08	0.16	0.09	0.53	0.15	0.06
	CONTROL	0.30±0.01	0.04	0.03	0.32	0.05	0.03	0.31	0.04	0.03	0.32±0.01	0.04	0.03

D) 18 h

RICE	H	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.48±0.04	0.05	0.04	078±0.01	0.05	0.04	0.59	0.14	0.10	0.56±0.04	0.13	0.04
	SDF1	0.57±0.02	0.05	0.05	0..82±0.01	0.05	0.04	0.60	0.17	0.09	0.66±0.04	0.13	0.06
	TDF1	0.71±0.05	0.06	0.07	0..851±0.05	0.05±0.01	0.03±0.01	0.70	0.15	0.10	0.70	0.13±0.01	0.06
RR2	IDF2	0.46±0.03	0.05	0.04	0.76	0.05	0.05	0.62±0.04	0.16	0.09	0.53±0.01	0.11±0.01	0.05
	SDF2	0.51±0.01	0.05	0.05	0.86±0.02	0.05	0.05	0.63±0.03	0.15	0.09	0.60±0.01	0.14	0.04
	TDF2	0.61±0.01	0.05	0.04	0.94±0.04	0.05	0.04	0.67±0.07	0.17	0.10	0.66±0.04	0.15	0.05
	GLUCOSE	0.78±0.02	0.06	0.05	1.52±0.05	0.05±0.01	0.03	1.34±0.04	0.16	0.10	0.53±0.04	0.18	0.07
	CONTROL	0.3±0.01	0.04	0.04	0.32	0.05	0.03	0.31	0.04	0.03	0.32±0.01	0.04	0.03

E) 48 h

RICE	H	LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
		Acetate	propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	propionate	Butyrate	Acetate	propionate	Butyrate
RR1	IDF1	0.56±0.01	0.15	0.06	0.40±0.01	0.15	0.06	0.64±0.05	0.22	0.23	0.60±0.01	0.21	0.16
	SDF1	0.61	0.16	0.07	0.51±0.02	0.16	0.06	0.85±0.01	0.23	0.24	0.65±0.03	0.24	0.17
	TDF1	0.72±0.03	0.17	0.08	0.58	0.19	0.08	1.07±0.02	0.24	0.34	0.93±0.05	0.28	0.18
RR2	IDF2	0.55	0.14	0.06	0.52±0.04	0.14	0.05	1.08±0.04	0.21	0.24	0.49	0.19	0.14
	SDF2	0.59	0.14	0.06	0.60	0.15	0.06	0.91±0.01	0.22	0.25	0.51	0.20	0.15
	TDF2	0.67	0.15	0.07	0.67	0.17	0.06	1.06	0.22	0.26	0.59±0.06	0.21	0.15
	GLUCOSE	0.72±0.01	0.19	0.09	0.48±0.02	0.21	0.09	1.68±0.09	0.31	0.33	0.56	0.18±	0.16
	CONTROL	0.32±0.01	0.04	0.03	0.29	0.04	0.03	0.33	0.04	0.03	0.39	0.00	0.03

Results are expressed as the mean value of two trials±srandard error. Units are mmoles /100mL.

Table 2-App B Relative percentage of SCFA formation by probiotics (A-E)

A) 0 h

RICE		Acetate%	Propionate %	Butyrate%	Acetate%	Propionate %	Butyrate%	Acetate %	Propionate %	Butyrate%	Acetate%	Propionate %	Butyrate %
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF1	81.79±0.02	10.49±0.04	7.73±0.06	79.80±0.38	12.39±0.23	7.80±0.15	80.37±1.14	10.63±0.94	9.00±0.20	80.40±0.39	11.31±0.21	8.29±.0.17
	SDF 1	80.95±0.66	10.99±0.41	8.06±0.24	80.54±0.27	11.85±0.19	7.61±0.07	80.77±0.48	10.75±0.29	8.48±0.20	80.83±0.75	11.05±0.42	8.13±0.33
	TDF 1	81.96±0.71	10.40±0.41	7.64±0.29	79.63±0.37	12.42±0.16	7.95±0.21	82.14±0.24	9.99±0.18	7.87±0.06	78.75±0.95	12.20±0.54	9.05±0.41
RR2	IDF 2	80.91±0.69	10.96±0.41	8.13±0.29	78.49±0.26	12.90±0.24	8.61±0.02	79.45±0.36	11.52±0.20	9.04±0.16	80.03±0.32	11.52±0.20	8.44±0.12
	SDF2	80.34±0.36	11.28±0.20	8.37±0.16	80.68±0.33	11.87±0.20	7.45±0.13	78.52±0.74	12.07±0.45	9.41±0.29	80.10±0.32	11.49±0.18	8.41±0.14
	TDF 2	80.40±1.03	11.24±0.58	8.36±0.45	77.67±0.75	14.64±0.52	7.69±0.23	80.33±0.15	10.98±0.07	8.69±0.07	80.22±0.17	11.42±0.09	8.36±0.08
	GLUCOSE	75.24±0.14	14.32±0.07	10.45±0.43	78.00±1.05	13.96±0.47	8.04±0.27	78.64±0.39	12.01±0.18	9.36±0.21	79.29±0.27	11.96±0.15	8.75±0.12

B) 6 h

RICE		Acetate%	Propionate %	Butyrate %	Acetate%	Propionate%	Butyrate %	Acetate %	Propionate %	Butyrate%	Acetate%	Propionate %	Butyrate%
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF 1	9.28±0.44	84.22±0.08	6.50±0.35	89.34±0.17	6.31±0.05	4.34±0.12	73.04±0.15	16.64±0.09	10.32±0.06	75.44±0.25	17.69±0.19	6.87±0.06
	SDF 1	8.36±0.23	85.69±0.25	5.95±0.02	90.31±0.49	5.79±0.07	3.89±0.42	70.15±0.85	18.31±0.52	11.53±0.33	76.61±0.23	16.49±0.16	6.91±0.07
	TDF 1	7.48±0.2	87.72±0.11	4.81±0.09	88.31±0.02	7.63±0.2	4.06±0.23	67.86±0.13	19.53±0.09	12.61±0.03	76.59±0.2	15.91±0.07	7.50±0.27
RR2	IDF 2	9.71±0.02	83.72±0.26	6.57±0.28	89.88±0.56	5.74±0.35	4.39±0.22	73.03±0.53	17.17±0.6	9.80±0.07	76.31±0.05	17.49±0.03	6.20±0.01
	SDF 2	8.67±0.1	84.87±0.06	6.46±0.04	89.81±0.15	6.15±0.19	4.04±0.34	71.76±0.17	17.68±0.23	10.56±0.07	77.90±0.1	15.93±0.05	6.17±0.15
	TDF 2	7.10±0.05	87.60±0.03	5.30±0.02	86.96±0.53	7.79±0.29	5.25±0.24	70.19±0.83	18.00±0.37	11.81±0.46	77.56±0.01	15.82±0.01	6.62
	GLUCOSE	11.13±0.05	81.78±0.31	7.09±0.19	89.51±0.15	6.78±0.01	3.71±0.16	69.73±1.21	19.05±0.63	11.22±0.58	68.61±1.93	22.20±1.36	9.19±0.56

C)12 h

RICE		Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF 1	83.75±0.12	9.56±0.11	6.69±0.23	89.23±0.16	0.04±0.05	4.39±0.12	72.46±0.15	17.47±0.1	10.07±0.37	75.11±0.37	17.93±0.28	6.96±0.09
	SDF 1	85.64±0.40	8.39±0.35	5.96±0.05	90.40±0.43	5.75±0.03	3.86±0.39	70.25±0.89	20.10±0.9	9.65±0.01	76.64±0.08	16.44±0.07	6.92
	TDF 1	87.49±037	7.62±0.21	4.89±0.16	88.34±0.1	7.62±0.28	4.04±0.18	69.41±0.58	18.00±0.80	12.59±0..22	76.84±0.30	15.73±0.08	7.42±0.39
RR2	IDF 2	83.80±.10	9.66±0.21	6.54±0.31	89.70±.62	5.84±0.38	4.47±0.24	70.89±0..5	19.04±0.75	10.07±0.26	75.83±0.03	17.85±0.02	6.32±0.01
	SDF	84.95±0.09	8.62±0.21	6.43±0.04	89.63±.36	6.24±0.07	4.12±0.43	73.07±0.11	16.63±0.35	10.3±0.25	77.72±0.12	16.14±0.01	6.14±0.13
	TDF 2	87.59±0.15	7.11±0.05	5.30±0.06	87.17±0.53	7.67±0.29	5.17±0.24	70.30±0.1	18.54±0.08	11.16±0.18	77.40±0.48	15.93±0.34	6.67±0.14
	GLUCOSE	81.26±0.52	11.45±0.09	7.29±0.2	89.89±0.36	6.53±0.13	3.58±0.23	69.88±1.66	18.74±1.06	11.37±0.6	69.08±1.68	21.60±0.92	9.32±0.75

D) 18 h

RICE		Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF 1	82.94±0.29	9.15±0.19	7.91±0.10	88.29±0.13	6.26±0.07	5.45±0.2	71.26±0.09	17.25±0.31	11.49±0.22	74.38±0.11	19.23±0.09	6.39±0.2
	SDF 1	84.56±0.24	8.13±0.29	7.32±0.05	89.59±0.56	6.13±0.25	4.28±0.31	69±1.07	20.33±0.84	10.67±0.22	75.90±0.06	16.64±0.44	7.46±0.51
	TDF 1	82.81±0.09	7.52±0.07	9.67±0.15	87.33±0.36	7.74±0.14	4.94±0.22	69.71±0.59	17.98±0.86	12.31±0.27	76.84±0.43	16.25±0.14	6.91±0.58
RR2	IDF 2	81.78±0.09	9.81±0.37	8.42±0.22	88.81±0.62	5.81±0.11	5.39±0.52	70.16±0.34	19.12±0.58	10.72±0.24	74.07±0.94	17.53±0.24	8.40±1.18
	SDF 2	83.87±0.15	8.30±0.06	7.82±0.01	87.63±0.04	6.28±0.11	6.09±0.16	70.97±0.83	17.69±1.12	11.34±0.29	75.62±2.47	18.36±2.67	6.03±0.2
	TDF 2	87.06±0.04	6.94±0.19	6.00±0.03	86.48±0.48	8.02±0.25	5.50±0.23	69.71±0.62	19.21±0.37	11.09±0.25	74.24±2.11	18.83±1.94	6.93±0.18
	GLUCOSE	81.11±0.23	10.58±0.72	8.31±0.38	88.90±0.29	6.85±0.05	4.25±0.34	69.71±1.60	18.97±0.37	11.31±0.25	66.11±2.47	24.49±2.12	9.40±0.36

E) 48 h

RICE		Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %	Acetate %	Propionate %	Butyrate %
		LA	LA	LA	LR	LR	LR	BB	BB	BB	BL	BL	BL
RR1	IDF1	71.40±0.19	21.02±0.13	7.58±0.06	65.77±0.19	24.74±0.11	9.49±0.08	61.48±1.22	28.28±0.6	10.24±0.62	61.37±0.47	22.05±0.3	16.58±0.17
	SDF 1	73.78±0.04	18.88±0.02	7.34±0.02	69.40±0.43	21.72±0..32	8.88±0.12	71.26±1.10	21.08±0.07	7.66±0.03	61.74±0.88	22.36±0.51	15.90±0.37
	TDF 1	75.23±0.66	17.56±0.35	7.20±0.31	68.75±0.55	22.25±0.39	9.00±0.16	70.06±0.17	22.66±0.13	7.28±0.04	66.83±0.93	20.13±0.57	13.05±0.37
RR2	IDF 2	71.76±0.12	20.29±0.08	7.95±0.03	73.01±0.07	19.92±0.13	7.07±0.2	74.39±0.48	18.87±0.22	6.74±0.26	59.53±0.24	23.30±0.14	17.16±0.10
	SDF 2	72.28	20.31	7.41	74.19±1.08	18.65±0.8	7.16±0.28	76.26±0.28	17.64±0.05	6.11±0.24	59.72±0.03	23.22±0.02	17.06±0.01
	TDF 2	74.44	18.99	6.58	74.37	18.45	7.19	75.79±0.04	18.08±0.02	6.13±0.02	61.62±1.71	22.17±0.99	16.21±0.72
	GLUCOSE	69.12±0.10	21.55±0.07	9.33±0.09	62.12±0.04	26.84±0.03	11.04±0.19	75.73±0.8	17.32±0.44	6.95±0.36	61.95±0.03	20.01±0.02	18.04±0.02

Results are expressed as the mean value of two trials±srandard error, units are mmoles /100mL.

APPENDIX C

Table 1App C-Fermentation of rice dietary fibre with combinations leads to formation of short chain fatty acids (SCFA, m moles/100mL, A-F) (Chapter10)

A) BL+LA

Hours	0	6	24	48	0	6	24	48	0	6	24	48
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF1	0.28±0.01	1.31±0.01	3.91±0.01	4.38±0.03	0.04	0.05	0.05	0.05	0.03	0.04	0.03	0.03
SDF1	0.27±0.02	1.44±0.03	4.26±0.05	4.66±0.03	0.04	0.05	0.06	0.05	0.03	0.04	0.03	0.03
TDF1	0.29±0.02	2.09±0.03	4.43±0.03	4.68±0.03	0.04	0.05	0.06±0.01	0.05	0.03	0.04	0.03	0.03
IDF2	0.31±0.02	1.56±0.01	2.05±0.01	2.99±0.01	0.04	0.05	0.05±0.01	0.05	0.03	0.04	0.03	0.03
SDF2	0.28±0.03	1.59±0.04	3.95±0.04	3.38±0.04	0.04	0.05	0.05	0.05	0.03	0.04	0.04	0.03
TDF2	0.30±0.01	1.82±0.05	4.04±0.04	4.13±0.04	0.04	0.05	0.04	0.05	0.03	0.04	0.03	0.03
GLU	0.28±0.01	1.76±0.03	3.93±0.03	3.64±0.01	0.04	0.05	0.04	0.05	0.03	0.04	0.04	0.03
CON	0.23±0.02	1.21±0.02	1.18±0.04	1.19±0.01	0.04	0.05	0.04	0.05	0.03	0.04	0.03	0.03

B) BB+LA

Hours	0	6	24	48	0	6	24	48	0	6	24	48
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.28±0.01	1.18±0.01	3.66±0.01	2.83±0.03	0.04	0.05	0.05	0.05	0.03	0.03	0.04	0.04
SDF-1	0.27±0.02	1.36±0.03	4.18±0.03	2.92±0.01	0.04	0.05	0.05	0.05	0.03	0.03	0.04	0.03
TDF-1	0.30±0.01	1.82±0.04	8.07±0.23	3.70±0.02	0.04	0.06	0.06	0.05	0.03	0.03	0.04	0.03
IDF-2	0.28±0.03	1.20±0.05	2.97±0.02	2.82±0.02	0.04	0.05	0.05	0.05	0.03	0.03	0.04	0.03
SDF-2	0.27±0.02	1.60±0.05	2.99±0.04	2.87±0.04	0.04	0.05	0.05	0.05	0.03	0.03	0.03	0.03
TDF-2	0.30±0.01	1.72±0.04	3.68±0.04	3.31±0.04	0.04	0.05	0.06	0.07	0.03	0.03	0.04	0.03
GLU	0.31±0.02	1.43±0.04	3.10±0.03	3.48±0.03	0.04	0.05	0.05	0.05	0.03	0.03	0.03	0.03
CON	0.23±0.01	1.17±0.01	1.19±0.03	1.20±0.02	0.02	0.05	0.05	0.05	0.03	0.03	0.03	0.03

C) BL+LR

Hours	0	6	24	48	0	6	24	48	0	6	24	48
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.28±0.01	1.44±0.04	3.92±0.02	4.36±0.03	0.04	0.05	0.05	0.05	0.03	0.04	0.04	0.03
SDF-1	0.32±0.01	1.63±0.05	4.37±0.14	4.67±0.06	0.04	0.05	0.06	0.05	0.03	0.04	0.04	0.03
TDF-1	0.27±0.02	2.13±0.04	4.47±0.04	4.68±0.02	0.04	0.06	0.06	0.05	0.03	0.04	0.04	0.04
IDF-2	0.29±0.02	1.56±0.03	2.10±0.02	2.99±0.03	0.04	0.18±0.12	0.05±0.01	0.05	0.03	0.04	0.04	0.03
SDF-2	0.33±0.01	1.72±0.05	3.97±0.02	3.43±0.06	0.04	0.06	0.06	0.07±0.01	0.03	0.04	0.04	0.03
TDF-2	0.32±0.03	1.94±0.03	4.13±0.02	4.22±0.06	0.04	0.05	0.07	0.05	0.03	0.04	0.04	0.04
GLU	0.31±0.02	1.93±0.02	3.93±0.05	3.71±0.05	0.04	0.05	0.05	0.05	0.03	0.04	0.05	0.04
CON	0.23±0.03	1.18±0.01	1.17±0.01	1.24±0.04	0.04	0.05	0.05	0.05	0.03	0.04	0.03	0.03

D) LA+LR

Hours	0	6	24	48	0	6	24	48	0	6	24	48
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.26±0.01	1.57±0.02	1.76±0.03	1.41±0.04	0.04	0.06	0.08	0.05	0.03	0.04	0.04	0.03
SDF-1	0.29	1.77±0.04	1.96±0.01	1.60±0.05	0.04	0.06	0.09	0.06	0.03	0.04	0.04	0.04
TDF-1	0.30±0.01	1.92±0.01	2.02±0.02	1.69±0.02	0.04	0.06	0.10	0.06	0.03	0.04	0.04	0.03
IDF-2	0.29±0.04	1.44±0.04	1.60±0.06	1.40±0.02	0.04	0.06	0.10	0.06	0.03	0.04	0.04	0.03
SDF-2	0.27	1.75±0.02	1.85±0.01	1.57±0.04	0.04	0.06	0.10	0.06	0.03	0.04	0.04	0.03
TDF-2	0.30±0.01	1.92±0.05	1.90±0.02	1.66±0.04	0.04	0.06	0.10	0.06	0.03	0.04	0.04	0.03
GLU	0.26±0.01	2.11±0.02	2.24±0.01	1.41±0.03	0.04	0.06	0.10	0.06	0.03	0.03	0.04	0.03
CON	0.23±0.01	1.20±0.03	1.21±0.01	1.19±0.03	0.04	0.05	0.05	0.05	0.03	0.03	0.03	0.03

A) BB+BL+LA

Hours	0	6	24	48	0	6	24	48	0	6	24	48
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.30±0.01	1.27±0.08	3.90±0.02	3.39±0.01	0.04	0.04	0.06	0.07±0.01	0.03	0.03	0.03	0.05
SDF-1	0.31±0.02	1.81±0.04	5.25±0.08	3.53±0.05	0.04	0.04	0.06	0.06±0.01	0.03	0.03	0.04	0.05±0.01
TDF-1	0.30±0.01	1.99±0.03	5.74±0.02	4.60±0.02	0.04	0.04	0.10	0.08	0.03	0.03	0.04	0.06
IDF-2	0.30±0.01	1.52±0.04	4.28±0.08	2.32±0.01	0.04	0.04	0.05	0.05	0.03	0.03	0.04	0.03
SDF-2	0.30±0.03	1.80±0.01	6.01±0.06	4.36±0.01	0.04	0.05	0.06	0.06	0.03	0.03	0.04	0.04
TDF-2	0.27±0.02	1.84±0.01	8.21±0.07	4.85±0.05	0.04	0.04	0.06	0.06	0.03	0.03	0.04	0.04
GLU	0.26±0.01	2.61±0.07	6±0.04	6.18±0.03	0.04	0.05	0.06	0.05	0.03	0.03	0.04	0.04
CON	0.23±0.01	1.19±0.05	1.16±0.02	1.21±0.04	0.04	0.05	0.05	0.05	0.03	0.03	0.03	0.03

F) BB+BL+LR+LA

Hours	**0**	**6**	**24**	**48**	**0**	**6**	**24**	**48**	**0**	**6**	**24**	**48**
	Acetate	Acetate	Acetate	Acetate	Propionate	Propionate	Propionate	Propionate	Butyrate	Butyrate	Butyrate	Butyrate
IDF-1	0.32±0.03	1.64±0.08	2.95±0.02	2.10±0.03	0.04	0.04	0.06	0.05	0.03	0.03	0.04	0.04
SDF-1	0.34±0.02	1.66±0.08	3.88±0.06	2.58±0.05	0.04	0.04	0.08±0.01	0.06	0.03	0.03	0.06	0.04
TDF-1	0.30±0.01	2.00±0.05	6.20±0.06	4.44±0.03	0.04	0.04	0.10	0.05	0.03	0.03	0.06	0.04
IDF-2	0.34	1.75±0.06	3.56±0.06	2.04±0.01	0.04	0.04	0.06	0.05	0.03	0.03	0.04	0.03
SDF-2	0.30±0.01	1.73±0.02	3.51±0.06	1.99±0.02	0.04	0.04	0.07	0.05	0.03	0.03	0.04	0.04
TDF-2	0.31±0.02	2.13±0.04	5.71±0.06	3.89±0.04	0.04	0.04	0.06	0.07±0.01	0.03	0.03	0.05	0.02
GLU	0.28±0.01	2.06	4.70±0.06	4.45±0.5	0.04	0.04	0.09	0.04	0.03	0.03	0.05	0.03
CON	0.23±0.06	1.21±0.02	1.15±0.01	1.22±0.02	0.04	0.04	0.05	0.05	0.03	0.03	0.03	0.03

Results are expressed as the mean value of two trials±srandard error, units are mmoles /100mL.

Table 2 AppC-Molar fraction of acetate: propionate: butyrate (A-F)

A)BL+LA

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	80.51±0.51	11.55±0.3	7.94±0.21	94.03±0.04	3.42±0.02	2.56±0.02	97..95	1.21	0.84	98.21±0.02	1.03±0.01	0.76±0.01
SDF-1	79.94±1.08	11.88±0.64	8.18±0.44	94.48±0.05	3.13±0.06	2.39	97.92±0.03	1.31±0.02	0.77±0.01	98.46±0.01	0.97±0.01	0.57
TDF-1	80.97±0.97	11.27±0.57	7.76±0.4	95.98±0.14	2.38±0.16	1.64±0.02	98.00±0.01	1.26±0.01	0.74	98.24±0.08	1.05±0.08	0.71
IDF-2	81.90±0.88	10.72±0.52	7.38±0.36	94.70±0.28	3.15±0.27	2.16±0.01	96.05±0.3	2.38±0.3	1.57	97.63±0.01	1.49	0.88
SDF-2	80.40±1.54	11.61±0.91	7.99±0.63	94.79±0.13	3.09±0.18	2.12±0.05	97.84±0.24	1.26±0.16	0.90±0.08	97.65±0.02	1.57±0.02	0.78±0.01
TDF-2	81.48±0.46	10.97±0.27	7.55±0.19	95.64±0.132	2.50±0.07	1.87±0.05	98.11±0.02	1.08±0.01	0.81±0.01	98.00±0.06	1.29±0.01	0.72±0.07
GLU	80.51±0.51	11.55±0.3	7.94±0.21	95.49±0.07	2.58±0.04	1.93±0.03	97.89±0.01	1.11±0.01	1.00±0.01	98.04	1.23	0.73
CON	77.51±1.35	13.32±0.8	9.17±0.55	93.57±0.09	3.68±0.05	2.75±0.04	93.80±0.18	3.54±0.1	2.66±0.08	94.26±0.04	3.61±0.03	2.12±0.02

B) BB+LA

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	80.51±0.51	11.55±0.3	7.94±0.21	93.64±0.05	3.76±0.03	2.61±0.02	97.72±0.08	1.29	0.98±0.09	97.15±0.14	1.57±0.01	1.28±0.13
SDF-1	79.94±1.08	11.88±0.64	8.18±0.44	94.6±0.1	3.29±0.06	2.04±0.04	97.92±0.01	1.14±0.01	0.94±0.01	97.32±0.26	1.67±0.14	1.01±0.11
TDF-1	81.48±0.46	10.97±0.27	7.55±0.19	95.52±0.08	2.94±0.06	1.54±0.03	98.54±0.05	0.85±0.03	0.60±0.02	97.88±0.01	1.32±0.11	0.80±0.09
IDF-2	80.40±1.54	11.61±0.91	7.99±0.63	93.98±0.21	3.72±0.13	2.30±0.08	97.11±0.02	1.58±0.01	1.31±0.01	97.26±0.02	1.58±0.01	1.16±0.01
SDF-2	79.94±1.08	11.88±0.64	8.18±0.44	95.21±0.13	2.83±0.08	1.96±0.05	97.33±0.03	1.58±0.02	1.09±0.01	97.13±0.07	1.84±0.03	1.03±0.1
TDF-2	81.48±0.46	10.97±0.27	7.55±0.19	95.29±0.33	2.88±0.29	1.83±0.03	97.51±0.06	1.51±0.02	0.97±0.08	97.24±0.09	1.97±0.1	0.79±0.01
GLU	81.90±0.88	10.72±0.52	7.38±0.36	94.66±0.13	3.15±0.08	2.19±0.05	97.46±0.02	1.49±0.01	1.05±0.01	97.95±0.02	1.29±0.01	0.76±0.01
CON	76.88±0.72	13.7±0.42	9.42±0.29	93.59±0.09	3.78±0.05	2.63±0.04	93.63±0.13	3.74±0.08	2.63±0.06	94.30±0.08	3.59±0.05	2.11±0.03

C)BL+LR

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	80.51±0.51	11.55±0.3	7.94±0.21	94.12±0.04	3.37±0.19	2.50±0.15	97.80±0.01	1.21	0.99±0.01	98.19±0.01	1.04±0.01	0.76
SDF-1	82.36±0.42	10.45±0.25	7.19±0.17	94.83±0.37	3.02±0.32	2.15±0.05	97.77	1.34±0.03	0.89±0.03	98.27±0.07	0.99±0.04	0.73±0.03
TDF-1	79.94±1.08	11.88±0.64	8.18±0.44	95.87±0.07	2.53±0.04	1.60±0.03	97.82±0.05	1.32±0.06	0.87±0.01	98.18	1.00±0.03	0.82±0.03
IDF-2	80.97±0.97	11.27±0.57	7.76±0.4	88.37±0.17	9.61±0.27	2.01±0.11	95.87±0.32	2.32±0.3	1.81±0.01	97.23±0.11	1.68±0.09	1.08±0.02
SDF-2	83.16±0.38	9.98±0.23	6.87±0.16	94.9±0.15	3.11±0.09	1.97±0.06	97.46±0.03	1.57±0.03	0.97	97.21±0.14	1.85±0.13	0.93±0.01
TDF-2	82.28±1.26	10.50±0.75	7.22±0.51	95.89±0.05	2.35±0.03	1.76±0.02	97.51±0.02	1.55±0.01	0.93	97.78±0.1	1.22±0.02	1.00±0.08
GLU	81.90±0.88	10.72±0.52	7.38±0.36	95.87±0.04	2.36±0.02	1.77±0.02	97.66±0.13	1.19±0.02	1.15±0.14	97.78±0.03	1.26±0.07	0.96±0.1
CON	76.70±2.17	13.80±1.28	9.50±0.88	93.43±0.05	3.76±0.03	2.81±0.02	93.52±0.03	3.81±0.03	2.67±0.02	94.45±0.15	3.49±0.1	2.05±0.06

D) LA+LR

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	77.49±0.61	13.27±0.36	9.25±0.25	94.25±0.06	3.37±0.04	2.38±0.03	93.33±0.07	4.38±0.07	2.30±0.14	94.12±0.18	3.63±0.11	2.25±0.07
SDF-1	79.18	12.27	8.55	94.43±0.11	3.46±0.07	2.12±0.04	93.57±0.18	4.35±0.01	2.07±0.16	94.12	3.70±0.12	2.18±0.13
TDF-1	79.68±0.49	11.98±0.29	8.35±0.2	95.25±0.02	2.78±0.01	1.97±0.01	93.72±0.13	4.42±0.015	1.86±0.02	94.60±0.05	3.51±0.04	1.89±0.02
IDF-2	78.98±1.09	12.39±1.23	8.64±0.86	93.72±0. 2	3.93±0.37	2.35±0.17	91.96±0.29	5.73±0.21	2.31±0.08	93.83±0.19	3.91±0.23	2.26±0.03
SDF-2	78.09	12.91	9.00	94.55±0.12	3.50±0.04	1.96±0.16	93.18±0.17	4.80±0.18	2.02±0.01	94.21±0.16	3.76±0.1	2.02±0.05
TDF-2	79.68±0.49	11.98±0.29	8.35±0.2	95.25±0.11	2.78±0.06	1.97±0.04	93.33±0.26	4.70±0.24	1.97±0.01	94.73±0.36	3.34±0.32	1.92±0.04
GLU	77.49±0.61	13.27±0.36	9.25±0.25	95.95±0.03	2.55±0.02	1.50±0.01	94.14±0.32	4.18±0.33	1.68±0.01	93.86±0.37	3.90±0.34	2.25±0.03
CON	74.77±0.76	14.87±0.45	10.36±0.31	93.71±0.13	3.71±0.08	2.57±0.05	93.65±0.04	3.75±0.03	2.59±0.02	94.26±0.12	3.62±0.08	2.13±0.05

E)BB+BL+LA

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	81.48±0.46	10.97±0.27	7.55±0.19	94.71±0.32	3.13±0.19	2.17±0.13	97.87±0.02	1.44±0.02	0.69	96.82±0.19	1.89±0.14	1.30±0.05
SDF-1	81.90±0.88	10.72±0.52	7.38±0.36	96.20±0.07	2.22±0.04	1.58±0.03	98.05±0.07	1.19±0.06	0.77±0.01	97.18±0.42	1.56±0.18	1.26±0.24
TDF-1	81.48±0.46	10.97±0.27	7.55±0.19	96.41±0.06	2.14±0.03	1.45±0.02	97.54±0.9	1.76±0.91	0.70	96.94±0.11	1.72±0.05	1.34±0.06
IDF-2	81.48±0.46	10.97±0.27	7.55±0.19	95.41±0.11	2.70±0.07	1.89±0.05	97.84±0.15	1.22±0.13	0.94±.02	96.63±0.26	2.07±0.17	1.30±0.09
SDF-2	81.39±1.39	11.03±0.87	7.59±0.57	95.96±0.03	2.43±0.02	1.60±0.01	98.42±0.01	0.91±0.01	0.67±0.01	97.77±0.04	1.24±0.03	0.99±0.07
TDF-2	79.94±1.08	11.88±0.64	8.18±0.44	96.04±0.03	2.33±0.02	1.63±0.01	98.71±0.01	0.77±0.03	0.52±0.03	98.08±0.02	1.13±0.04	0.79±0.02
GLU	79.43±0.57	12.18±0.32	8.38±0.23	97.06±0.07	1.72±0.04	1.23±0.03	98.35±0.01	0.96±0.01	0.69±0.01	98.45±0.07	0.87±0.01	0.68±0.08
CON	76.88±0.72	13.70±0.42	9.42±0.29	93.76±0.27	3.64±0.16	2.60±0.11	93.42±0.09	3.89±0.06	2.69±0.04	94.20±0.06	3.71±0.01	2.09±0.08

F) BB+BL+LA+LR

Hours	0	0	0	6	6	6	24	24	24	48	48	48
	Acetate%	Propionate%	Butyrate%	Acetate %	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%	Acetate%	Propionate%	Butyrate%
IDF-1	82.28±1.26	10.50±0.75	7.22±0.51	95.61±0.21	2.52±0.12	1.87±0.09	96.71±0.13	1.91±0.05	1.39±0.08	96±.26	2.37±0.08	1.64±0.18
SDF-1	83.50±0.73	9.77±0.43	6.7±0.30	95.60±0.1	2.56±0.02	1.84±0.08	96.68±0.27	1.92±0.16	1.40±0.11	96.22±0.39	2.17±0.21	1.61±0.18
TDF-1	81.48±0.46	10.97±0.27	7.55±0.19	96.37±0.09	2.09±0.06	1.54±0.03	97.59±0.03	1.50±0.05	0.91±0.08	98±0.07	1.15±0.04	0.86±0.03
IDF-2	83.46	9.84	6.70±	95.88±0.14	2.37±0.09	1.75±0.06	97.16±0.05	1.66±0.12	1.19±0.07	96.19±0.08	2.22±0.07	1.60±0.01
SDF-2	81.48±0.46	10.97±0.27	7.55±0.19	95.84±0.04	2.39±0.02	1.76±0.02	96.95±0.08	1.88±0.03	1.17±0.05	95.79±0.29	2.46±0.11	1.75±0.17
TDF-2	81.90±0.88	10.72±0.52	7.38±0.36	96.59±0.06	1.97±0.03	1.45±0.03	98.14±0.09	1.06±0.08	0.80±0.01	97.83±0.12	1.64±0.13	0.53±0.01
GLU	80.51±0.51	11.55±0.3	7.94±0.21	96.48	2.03	1.49	97.24±0.1	1.81±0.08	0.94±0.03	98.38±0.02	0.87±0.01	0.75±0.01
CON	78.24±3.70	12.89±2.19	8.87±1.51	94.15±0.08	3.37±0.03	2.48±0.03	93.38±0.05	3.92±0.05	2.71±0.02	94.38±0.08	3.54±0.05	2.08±0.03

Results are expressed as the mean value of two trials±srandard error, units are mmoles /100mL.

APPENDIX D

Table 1 App D-Stock solutions (standards)

Preparation of 100mL stock solutions reagent for each SCFA

Chemicals	Con .mM	Mol/L	Mol/ 100mL	Mw g/mol	Purity %	gram	Density g/mL	mL	µL
Acetic acid	500	0.500	0.050	60.05	100.0	3.0025	1.0492	2.86170	2861.7
Propionic acid	200	0.200	0.020	74.08	99.0	1.4816	0.9930	1.49204	1492.0
Butyric acid	200	0.200	0.020	88.12	99.5	1.7624	0.9577	1.84024	1840.2

Table 2 App D Standard mix

Preparation of 100 ml standard mix reagent from the stock solutions

Chemicals	Con.mM	Wanted concentration in standard mix	Times to be diluted	Number of mL to a total of 100mL*
Acetic acid	500	20	25	4
Propionic acid	200	10	20	5
Butyric acid	200	10	20	5