Fermentation of resistant starch :

Implications for colonic health in the monogastric animal

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April 1999

A thesis submitted to The Robert Gordon University in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy

Sponsored by the Boyd-Orr Research centre : The Robert Gordon University; School of Food and Consumer Studies and The Scottish Agricultural College, Aberdeen; Microbiology Division.

Abstract

Retrograded starches are commonly found in foods due to the production and/or processing conditions they have received prior to consumption. These resistant starches escape digestion in the small intestine and are fermented in the colon by the microflora present, to produce gases and SCFA in varying amounts. These are utilised by the host animal as an energy source, with a low gut pH being maintained by the production of SCFA. The fermentation of carbohydrates within the colon is beneficial to the health of the gut, as the beneficial bacterial species such as *Lactobacillus* and *Bifidobacterium* spp. are maintained, and a low pH reduces the activity of potentially harmful species such as the coliforms. The production of toxic metabolites from the breakdown of proteins will be reduced if these resistant starches persist further along the colon as a carbohydrate source. This is particularly important in the distal region of the colon, where the carbohydrate source usually becomes limited. The fermentation of both native and retrograded starches from various botanical sources containing varying amounts of the major components amylose and amylopectin, was examined. This was carried out using pure cultures of starch-degrading bacterial species (namely *Clostridium butyricum*, *Bifidobacterium suis* and *Fusobacterium* sp), a simulation of the monogastric colon using a single-stage system containing a mixed population of colonic flora, and a monogastric animal study in which various native and retrograded starches were fed to pigs. In particular the effects on bacterial fermentation of variations in the ratios of amylose and amylopectin in starch, and of treatments such as retrogradation and/or pancreatin digestion was examined.

In pure cultures the different starch-degrading species exhibited preferences which varied according to starch source or the processing treatment. High levels of gas and butyrate were produced by *Cl. butyricum*, with the highest levels produced from the retrograded pancreatin digested (RPD) form of the waxy maize starch. The major product from the fermentation of resistant starches by *B. suis* and *Fusobacterium* sp. was acetate. A significant increase ($P<0.05$) in the *Lactobacillus* population, a significant decrease ($P<0.01$) in the *Bacteroides* population, and an increase in *Bifidobacterium* spp. was observed, when untreated waxy maize starch was added to the *in vitro* colon simulator. The levels of acetate and propionate detected from the high amylose maize (HAM) starch were significantly lower ($P<0.05$) than the levels detected from the waxy maize starch. Colon samples from animals fed the retrograded waxy maize starch showed the highest numbers of *Lactobacillus* and *Bifidobacterium* spp., and the lowest numbers of *Bacteroides* spp. after three weeks on the diets. The highest Lactobacillus : coliform ratio along the length of the colon was detected with the retrograded waxy maize starch. An increase in the levels of total SCFA between the mid and distal colon was observed with the retrograded maize, waxy maize and mixture starches, with the increase from the waxy maize starch being significantly higher ($P<0.05$) than the level detected from the native maize starch. An increase in the production of butyrate was observed throughout the length of the colon with the retrograded maize starch. The proportion of isoacids in the distal region of the colon was significantly higher ($P<0.05$) with the retrograded maize starch than that observed with the retrograded waxy maize starch.

This thesis shows that both the amylose : amylopectin ratio and the treatment the starch has received, will alter the way that starch is fermented by the microflora both *in vitro* and *in vivo*. The various forms of starch which enter the colon will be fermented to different extents by the bacteria present. This will reduce the competition for carbohydrate in the gut. The potential for manipulation of the beneficial species of microflora, a reduction in the coliform population, a stable production of SCFA and a reduction in the fermentation of proteins, is evident when retrograded waxy maize is included in the diet.

Acknowledgements

This thesis would not have been completed, had it not been for the help and much appreciated support, from a number of special people. I would therefore like to take this opportunity to thank those individuals.

Firstly, a huge thank you to my two long-suffering supervisors, **Dr Colin Henderson** and **Dr Kevin Hillman** for their never-ending support and guidance. I very much appreciate all the help, effort and hard work you have both contributed to this thesis. I would also like to thank **Dr Vernon Fowler**, who, although retiring during the first year of the project, was always there to offer guidance, assistance and assurance. I have learned a great deal throughout my PhD and for this I thank you all very much.

I would also like to thank my family, in particular my mum **Jean** and my sister **Nicola**, as well as my friends (both old and new). You have all been there for me along the way, and I thank you all for the much appreciated help and support. I owe you all a very large drink!

I would also like to say a big thank you to the many technical staff at SAC Aberdeen (both the Microbiology and Chemistry departments), The Robert Gordon University and Tillycorthie farm for their help and assistance in the running of the practical work. A big thank you also goes to **Dr Vernon Fowler** and **Dr Sandra Edwards** for their help in designing the animal diets, and for general assistance in planning the animal trial. I would also like to thank **Dr Andrew Lynn** of SAC Auchincruive for supplying the starches tested in Chapter 3, and the team at the Rowett Research Institute for carrying out the starch analysis described in Chapter 4.

Last, but by no means least, I would like to say a very big thank you to my biology teacher at Dumfries High School, **Mr Graham Herbert.** If it had not been for his enthusiasm and passion for both biology and microbiology, I might have become a chemist !

Table of contents :-

List of Figures :-

Chapter 1 :-

Chapter 2 :-

List of Tables :-

Chapter 1 :-

Chapter 2 :-

CHAPTER 1

Overview of literature

1.1 Starch composition

Starch is the major form of stored carbohydrate in higher plants (Shannon & Garwood, 1984), and is laid down in the plant as water-insoluble granules which vary in size and shape depending on characteristics such as plant species (Hoseney, 1994). The starch granules from rice tend to be small and very angular, and the granules from potato tend to be large, smooth and oval (Fitt & Snyder, 1984). **Table 1.1** shows certain characteristics of some of the more common starches. It is also possible to find more than one size and/or shape of granule from the same plant, such as in wheat. As starch is a biologically produced material, the size and shape of the starch granules can vary from the same plant from one year to the next, depending on the environmental conditions in which the plant was grown.

Starch is described as a homopolymer composed of glucose molecules linked together. This was discovered by Kirchoff in 1811, when upon hydrolysing wheat and potato starches with sulphuric acid, he obtained a "sweet tasting product" (Whistler & Daniel, 1984). Since this discovery, the nature of the composition of starch has been of great interest, and research has since shown that starch was not simply composed of a single polysaccharide as first thought. Starch is now known to be comprised of two principal polymers, amylose and amylopectin, which are mainly composed of glucose residues linked together with α(1,4) bonds. (Manners, 1974). **Figure 1.1** shows the relative structures of the two principal polymers, amylose and amylopectin.

Amylose is described as the simpler of the two polymers with a molecular weight typically reported to be between 10^5 and 10^6 (Galliard & Bowler, 1987), and can be composed of anywhere between 500 and 10,000 glucose units (Galliard & Bowler, 1987; French, 1984; Cummings & Englyst, 1992). Amylose was initially described as a purely linear polymer until Peat *et al.* (1952) discovered that potato starch was not completely hydrolysed by β-amylase alone, indicating the presence of α(1,6) branch points (Hizukuri *et al.* 1981). A series of experiments were conducted by Hizukuri *et al.* (1981) to determine the degree of this α(1,6) branching within the amylose polymer. With the use of various methods, it was deduced that amylose which had been isolated from potato starch contained anywhere between 9 and 20 α(1,6) branch points.

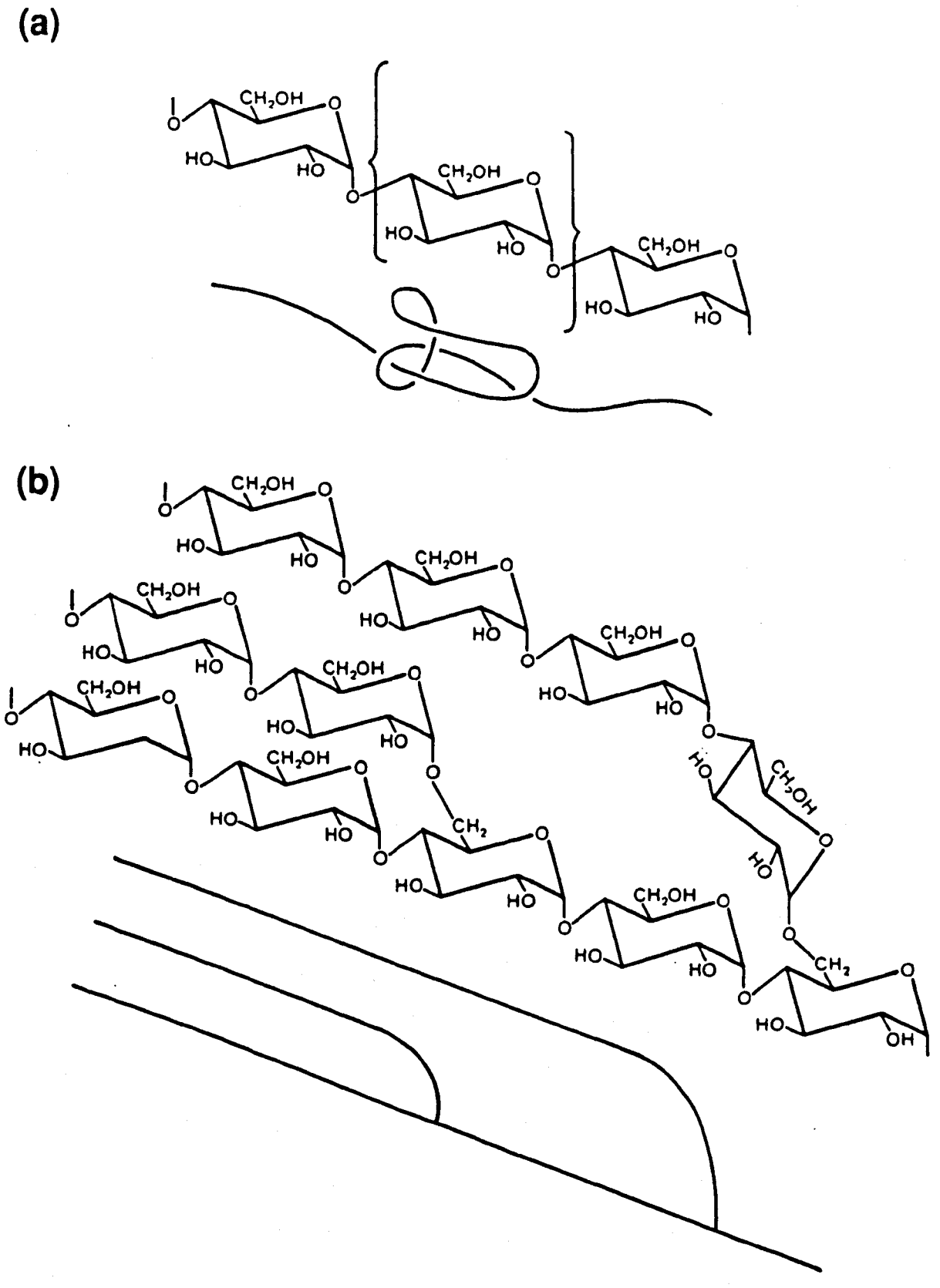
Amylopectin has been variously reported as having a molecular weight in the region of 10^6, and between 10^7-10^9 (Galliard & Bowler, 1987; Kennedy *et al.* 1987). The

Table 1.1 :-
Various characteristics associated with some of the common starches

	Characteristics of starches			
Starch source :-	**Gelatinization temperature**	**Granule size (μm)**	**Granule shape**	**Amylose content (%)**
Potato	59-68	15-100	oval	23
Maize	62-72	5-25	polyhedral	28
Waxy maize	63-72	5-15	round	1
Oat	53-59	5-15	polyhedral	23-27
Wheat	58-64	20-35	lenticular or round	26-31
High amylose maize	67-80	25	round, irregular sausage shaped	52-80
Rice	68-78	3-8	polyhedral	14-32
Tapioca (manihot)	52-64	5-35	round	17
Barley	51-60	20-25	round or lenticular	22-29

Reproduced from Lineback (1984); Blanshard (1987) and Rapaille & Vanhemelrijck (1992).

Figure 1.1 :-
Structural diagrams of the main polymers found in starch granules, (a) amylose, (b) amylopectin. Reproduced from Morris (1990).



molecular weight of this molecule is said to be difficult to measure accurately due to its variable nature (Kennedy *et al.* 1987). Amylopectin contains the same α(1,4) linkages found in amylose, but is also highly branched, containing approximately 5% α(1,6) branch points (Galliard & Bowler, 1987; Greenwood, 1979; Manners, 1974), which leads to short chains of between 20-25 glucose residues in length (Greenwood, 1979; Manners, 1974). Further study into the amylopectin polymer between 1935-1940, using methylation analysis, revealed three different types of chain, denoted as A, B and C. The A-chains were shown to be linked to the polymer only by the reducing group; B-chains can have one or more A-chain linked to them, and C-chains contain the only reducing group present in the polymer (Stark & Lynn, 1992). This finding led to research being carried out to determine how these chains were arranged within the amylopectin polymer. This led to the proposal of various models, the first ones being from Haworth and Staudinger which were denoted as "single branching", and "comb-type" structures respectively (Manners, 1974). These models were proposed before specific techniques for the exploration of molecular structure became available (Manners, 1974), and hence were soon modified once these techniques were made available. Among the new models proposed was that by Meyer which featured equal numbers of A and B chains in a multiple branched structure (Manners, 1974). This was re-drawn in 1970 by Gunja-Smith and co-workers to produce a "tree-like structure", after they carried out debranching studies on amylopectin using β-amylase and phosphorylase (Manners, 1974). The ratio of A : B chains present in the amylopectin molecule was found to be important and can be estimated by the release of maltose and maltotriose from a β-limit dextrin (Manners, 1985). These sugars can only come from the A-chains in the molecule and various techniques have been used to estimate them, such as charcoal-celite column chromatography, paper chromatography, gel filtration chromatography and high performance liquid chromatography (HPLC) (Manners, 1985). These studies showed that there had to be either equal numbers of A and B chains, or more A chains than B chains in the amylopectin polymer (Manners, 1985). The models to date which appear to incorporate all the previous findings and which best explain certain properties of the polymer, are those which are based on cluster structures such as those proposed by French, Robin *et al.* and Manners & Matheson (Manners, 1985).

1.2 The starch granule

The major components amylose and amylopectin are packaged together into starch granules. The levels of each of these components can vary within starch granules from different plant species, but amylose is usually 20-30% of the total on average (Lineback, 1984; Young, 1984). **Table 1.1** details the levels of amylose and amylopectin found in some of the more common starches. However, the ratio of amylose : amylopectin within starch granules can be altered by genetic modification of the plant, to produce starches such as amylomaize which can contain up to 80% amylose and waxy starches which contain little or no amylose (Young, 1984; Kennedy *et al.* 1987). These two polymers constitute the majority of the starch granule, but other minor components are also known to be present. A third component, often referred to as the intermediate fraction, has been reported in some types of starch, such as those from cereal origin (Greenwood, 1979). This fraction has been reported to contribute between 5-10% of the starch granule (Greenwood, 1979), although it may be as high as 20% of the starch granule (Lineback, 1984; Greenwood, 1979; Manners, 1985). Although this intermediate fraction contributes such a large part to the starch granule, its structure still appears to be uncertain (Lineback, 1984; Greenwood, 1979; Manners, 1985) but it has been reported to be different from both the amylose and amylopectin components as far as chain length and chain number are concerned (Manners, 1985; Stark & Lynn, 1992). It has also been described as an "amylopectin with a lower degree of branching" (Young, 1984) and a "less branched amylopectin or a slightly branched amylose" (Whistler & Daniel, 1984). Starch granules also contain minor components such as proteins, lipids, ash, phosphorus and pentosan in varying amounts (Galliard & Bowler, 1987; Biliaderis, 1991). The protein component can be between 0.05 and 0.5% depending on the starch source, and can be found both internally and on the surface of the granule (Stark & Lynn, 1992). The lipids have been more extensively studied, particularly the internal lipids which are associated within the granule matrix itself, with the amount and type of lipid found being variable (Galliard & Bowler, 1987). Internal lipids are known to complex with the amylose polymer by forming a core which runs through the centre of the amylose helix (Galliard & Bowler, 1987).

It is not quite known how the major components are arranged within the granules (Hoseney, 1994), but the amylopectin polymers appear to be arranged radially within the starch granules, angled at 90° to the granule surface, possibly with the amylose polymers interspersed among them (Jane, 1992; Hoseney, 1994). The branched outer chains of amylopectin appear to form double helix crystalline structures (Lineback, 1984; Jane, 1992). It has been suggested by Lineback (1984) that the ends of the amylopectin chains may protrude from the surface of the granule to form a "hairy billiard ball" appearance. The ends of the amylose chains may protrude from the surface in the same way (Stark & Lynn, 1992). The original growth point, known as the hilum, can be found at the centre of the starch granule (French, 1984).

Starch granules appear to exhibit a layered structure which is easier seen when the granules have been degraded by enzymes (Kennedy *et al.* 1987) or in large hydrated starch granules such as those found in potato (French, 1984). Starch granules appear to contain both crystalline and amorphous regions (French, 1984; Manners, 1985; Kalichevsky & Ring, 1987) which were identified by X-ray diffraction studies (French, 1984). Starch is said to be only partially crystalline in nature, with the crystalline regions contributing approximately 30% of the starch granule (Hoseney, 1994). It appears that during attack by enzymes, the crystalline regions are more resistant to attack than the amorphous regions (French, 1984; Manners, 1985; Gallant *et al.* 1992). It now appears that the crystalline regions within the granules occur as a result of the amylopectin polymer, as starches such as waxy maize which contain no or virtually no amylose, contain the same birefringence and X-ray pattern as normal maize starch (Greenwood, 1979; Lineback, 1984; Stark & Lynn, 1992). In 1937 Katz employed X-ray diffraction techniques to the study of the intact starch granule. From this it was revealed that three different types of crystalline structure existed, which were designated as A, B, and C (Greenwood, 1979), with the type of pattern exhibited usually dependant on the botanical source of the starch. The A pattern is the most thermodynamically stable and is usually associated with cereal starches. The B pattern is usually found in tuber and fruit starches such as potato and banana, and the C pattern, which is said to be intermediate of the A and B patterns, is associated with legume starches such as pea and bean (Greenwood, 1979; French, 1984; Lineback, 1984; Cummings & Englyst, 1992). The size and crystalline nature of the starch granules is known to influence

their susceptibility to pancreatic enzymes, with those showing crystallinity patterns B and C being more resistant to enzyme attack (Cummings & Englyst, 1992). Starch granules are also said to be birefringent i.e. can reflect light differently from different planes, and exhibit "maltese cross" patterns under polarized light, due to the high degree of molecular order (Greenwood, 1979; Blanshard, 1987; Kennedy *et al.* 1987).

1.3 Starch in the diet

Nutritionally, starch in one form or another, represents the major component of the human diet. It has been recommended that 55% of the available energy from the diet should come from carbohydrates (Bender, 1993). The average diet in the UK yields 27% of available energy from starch alone, but this figure would be higher if the consumption of various starch derivatives was also included (Galliard, 1987).

In order to release this energy, the starch has first to be digested. The starch-containing food is taken into the mouth and chewed where it mixes with saliva which contains ptyalin, more commonly known as salivary α-amylase. The salivary α-amylase is present at high concentrations, 400mg of pure enzyme per litre, but it was originally thought that salivary α-amylase was unlikely to play an appreciable part in the digestion of starch (Gray, 1992). This is due to the short time the enzyme is in contact with the food and the fact that α-amylase present in the saliva can be rapidly degraded in the acidic environment of the stomach (Gray, 1992). However, studies carried out by Rosenblum and co-workers (1988) have suggested that salivary α-amylase may be protected from the stomach acid (Gray, 1992). When food is chewed it is formed into a bolus by the tongue and swallowed. This bolus structure allows the starch and/or its breakdown products to bind to the active sites of the salivary α-amylase which then holds the enzyme in a position which is said to be favourable for its protection (Gray, 1992).

The breakdown of starch depends on a number of factors such as its physical form, crystallinity and any processing/cooking which it has encountered (Biliaderis, 1991; Cummings & Englyst, 1992). The size of the food particles can affect the extent of starch digestion due to the surface area to volume ratio, with those particles which are smaller being digested more quickly than those which are larger (Annison & Topping, 1994). The

rate at which starch can be broken down will depend on factors such as the botanical source of the starch (Williamson *et al.* 1992). It has been reported that rice and wheat starches can be degraded at least six times faster than starch from bananas, and at least twenty times faster than potato starch, using porcine pancreatic α-amylase (Williamson *et al.* 1992). It has also been noted that cereal starches are hydrolysed more easily than potato starches. This may be due to potato starch granules containing more crystalline, and hence less amorphous, regions than are present in cereal starches, and the fact that α-amylase hydrolyses the amorphous regions in the starch granule first (Williamson *et al.* 1992). Cooked foods are also easier to digest than intact starch granules, as cooking results in the loss of structured order of the starch (Colonna *et al.* 1992). This loss in order can be observed due to the absence of the characteristic maltese cross which is seen in semi-crystalline intact starch granules.

Once the starch-containing food leaves the stomach, it mixes with pancreatic secretions which contain various enzymes such as proteases, lipases and α-amylase (Schneeman, 1993). The major starch polymers amylose and amylopectin will both be hydrolysed, but not completely, by the endo-action of pancreatic α-amylase (Annison & Topping, 1994). The products from the digestion of the essentially linear amylose are maltose and maltotriose (Gray, 1992). As α-amylase cleaves only the α(1,4) linkages, the breakdown products from amylopectin are noted as maltose and maltotriose as above, but also α-limit dextrins due to the presence of α(1,6) linkages (Gray, 1992). The rate and extent at which α-amylase digests can be due to a number of factors, such as the starch granule may be surrounded by cell wall components or proteins as in the case of processed foods, which may limit the access of α-amylase, and the size of the particles the α-amylase encounters may limit the rate of breakdown by the enzyme (Heaton *et al.* 1988; Annison & Topping, 1994). The amylose portion of the starch can also form complexes with the lipids present, which can also reduce degradation by the enzyme (Annison & Topping, 1994). Naturally occurring proteinaceous α-amylase inhibitors are also found in starches, but are more important in legume rather than cereal starches, but are usually destroyed once the starch is cooked (Colonna *et al.* 1992). Non-proteinaceous inhibitors such as tannins and phytic acid may also be present in the diet and can affect α-amylase activity (Colonna *et al.* 1992). The products of starch digestion by α-amylase are incapable of being absorbed into

the bloodstream via the small intestine as they are still too large at this stage. They are further degraded by enzymes such as maltase, which are present in the small intestine brush border, to yield free glucose (Bender, 1993; Gray, 1992). This glucose can then be absorbed and utilised by the host as an energy source.

1.4 Resistant starch (RS) in the diet

Until the early 1980's, the breakdown of starch in the small intestine was thought to be complete. This assumption was based on the presence of excessive levels of pancreatic α-amylase (Englyst & Cummings, 1985; Englyst & Macfarlane, 1986; Faisant *et al.* 1993), and studies which showed that after digestion of a meal no starch could be detected in faecal material (Siljeström & Björck, 1990). It is now known and widely accepted that some starch in the diet will resist digestion in the small intestine and enter the colon. Indigestible starch is now know to exist naturally in starch-containing foods such as potatoes, legumes, wheat flour, cornflakes, bread and bananas (Englyst & Cummings, 1985; 1986; 1987; Stephen *et al.* 1983; Asp & Björck, 1992; Faisant *et al.* 1993). This component of the diet has been termed "resistant starch" (RS). Starch can resist digestion in the small intestine for various reasons, and it has always been present normally in cooked starch-containing foods (Englyst & Macfarlane, 1986). Factors which contribute to resistance can be both intrinsic and extrinsic; intrinsic where the starch is encased in seed coats such as in grains (whole or partly milled), or in other plant cell or tissue structures (Englyst & Kingman, 1990; Asp & Björk, 1992). Starch granules which produce X-ray diffraction patterns B and C tend to be very resistant to digestion, as are raw starch granules (Englyst & Kingman, 1990; Asp & Björk, 1992; Annison & Topping, 1994). Extrinsic factors are mainly those which are not related to the structure of the starch granule such as extent of chewing, transit time along the small intestine, the amount of starch present and the presence of any other food components which may interfere with starch digestion (Englyst & Kingman, 1990).

1.5 The production of resistant (retrograded) starch

Starch granules are described as "cold water insoluble", but when heated in the presence of water, starch granules swell (Greenwood, 1979). This swelling is reversible until a certain temperature, denoted as the "gelatinization temperature" is reached (Manners, 1974; Miles *et al.* 1985a; Cummings & Englyst, 1992). The gelatinization temperature of starch is usually in the range of 60°C-70°C, but this varies with the botanical source of the starch (Miles *et al.* 1985a). Once the gelatinization temperature is reached, the granular order and structure is irreversibly lost, and the amylose component of the starch is preferentially solubilised and leaches out of the granules (Greenwood, 1979; Miles *et al.* 1985a; Ring *et al.* 1988). If the temperature is maintained below 100°C, the granules will remain swollen and hydrated, and intact with the amylopectin still inside (Ring *et al.* 1988). When the temperature is decreased to around room temperature or lower, the long amylose chains will reassociate by hydrogen bonding to form a gel structure (Schoch, 1969; Filer, 1988). This process is termed "retrogradation" (Miles *et al.* 1985b; Filer, 1988; Ring *et al.* 1988; Schoch, 1969). If the concentration of starch is high enough, it will form an opaque elastic gel, which will become firmer during storage (Miles *et al.* 1985a; 1985b). Over a period of time, a B-type X-ray diffraction pattern will slowly develop, which is actually reported as the retrogradation procedure as the gels return to a granular state (Miles *et al.* 1985a; 1985b). These retrograded gels tend to be highly resistant to enzymatic hydrolysis (Ring *et al.* 1988). The retrogradation process is mainly attributed to the amylose component of the starch, but it has been shown that the amylopectin component is also capable of retrogradation (Filer, 1988). However, this type of "recrystallization" is reversible on heating to between 70-100°C (Miles *et al.* 1985a; Ring *et al.* 1987). This process can occur when starch-containing foods are produced or prepared, such as when potato is cooked and then cooled prior to consumption.

1.6 Man-made resistant starch

Resistant starch can be produced in starch-containing foods during the initial production. In these cases, the resistant starch present will usually be due to retrograded amylose. The amount of resistant starch formed during processing will depend on the amylose/

amylopectin ratio, processing temperature and time, water content and number of heating-cooling cycles (Saura-Calixto *et al.* 1992; Pomeranz, 1992; Sievert & Pomeranz, 1989; Asp & Björck, 1992). Pomeranz (1992) showed that when maize starch containing 70% amylose was autoclaved, the amount of resistant starch produced was affected by the autoclaving temperature, the starch/water ratio and the number of autoclaving and cooling cycles it had received. The resistant starch level was increased from 21% to over 40% when the number of autoclaving and cooling cycles was increased to 20. The level of resistant starch produced was also correlated to the amount of amylose present in the starch (Pomeranz, 1992). Rabe & Sievert (1992) carried out a number of studies examining the effect of baking and pasta production on the formation of resistant starch. Baking of wheat starch bread (without the influence of protein and non-starch polysaccharides) resulted in the formation of 2% resistant starch with approximately 1.7% resistant starch being present in the crust (Rabe & Sievert, 1992). They concluded that this resistant starch formation was due to the retrogradation of amylose and not amylopectin (Rabe & Sievert, 1992). The cooked pasta they examined appeared to contain more resistant starch, at approximately 2.2%. This may be due to the presence of more water in the cooking of pasta than in the process used to make bread (Rabe & Sievert, 1992). It therefore appears that the amount of resistant starch can be altered depending on the nature of the processing that it receives (Rabe & Sievert, 1992).

1.7 Physical and chemical modification of starch

As well as modification by heating and cooling, starches can be modified either physically or chemically to produce starches with desired properties for use in various industries, such as the food and textile industries. One type of physical modification results in pregelatinised starches, which are produced by cooking the starch in water and drying the resulting paste either by drums or spray drying before retrogradation occurs (Jane, 1992; Schoch, 1969; Rapaille & Vanhemelrijck, 1992). This process can be carried out using both native and chemically modified starches, and can also be carried out using extrusion techniques instead, but only on those starches where high viscosity is not required (Rapaille & Vanhemelrijck, 1992). Extrusion techniques require severe conditions such as high temperature and shear

regimes. Pregelatinization techniques produce starches which are described as cold-water soluble and have a loss of granule integrity, a reduction in paste viscosity and a reduction in gelling properties (Jane, 1992; Colonna *et al.* 1987). To produce granular cold water starches, an injection and nozzle-spray drying proocess was developed by Pitchon and coworkers (Jane, 1992). This process produces a 100% gelatinized starch which is uniformly cooked, but has been produced with the minimum amount of heat and shear damage (Jane, 1992). These pregelatinized starches are usually used in instant pudding mixes as they produce a smooth, non-grainy texture with a desired mouth feel (Jane, 1992). They are also used in any instant food where rehydration with water is required (Schoch, 1969).

The majority of starch modification methods which are applied involve the use of chemicals. Modification with chemicals is carried out to alter the way the polysaccharide chains in the starch granules interact with each other (Galliard, 1987). Reactions such as oxidation, alkylation or esterification of the hydroxyl groups in the glucose will alter properties such as the hydrogen bonding, charge interactions and hydrophobic properties (Galliard, 1987; Rapaille & Vanhemelrijck, 1992).

Esterification and etherification of starches are carried out in an alkaline slurry reaction at between 30°C-50°C. In these reactions, some of the hydrogen atoms of the hydroxyl groups are replaced by a substituent group, which means that these starches are less likely to retrograde when cooked and gel pastes become more transparent (Rapaille & Vanhemelrijck, 1992).

Modification with acid is one of the oldest methods of starch modification and was first carried out by Lintner & Naegeli in the 1800's, hence they are now known as lintnerised starches (Radley, 1976; Hoseney, 1994). Acid-thinned starches result from the partial hydrolysis of granular starch suspensions. The hydrochloric acid used penetrates the amorphous regions of the starch granule and hydrolyses glucosidic bonds. This results in the production of chains which are shorter than normal, have an increased range in gelatinization temperatures, are much more soluble upon gelatinization, have a low viscosity, and are more likely to retrograde due to the shorter chain lengths present (Hoseney, 1994; Rapaille & Vanhemelrijck, 1992). This type of modification results in the

formation of a rigid gel and is used in the confectionery industry in gums, pastilles and jelly beans (Hoseney, 1994; Rapaille & Vanhemelrijck, 1992).

Oxidised starches are prepared by treatment with hypochlorite, to produce starches with chain lengths shorter than normal starches. This technique produces soft gels of high clarity due to the hydrogen bonding being affected (Hoseney, 1994; Radley, 1976; Rapaille & Vanhemelrijck, 1992). These modified starches are mainly used in non-food applications such as the laundry, paper industries and sizing in the textile industry (Radley, 1976; Hoseney, 1994). Other oxidising agents can be used and this produces modified starches for use as thickeners. Diluted solutions of starches which have been highly oxidised are used in some canned soups (Rapaille & Vanhemelrijck, 1992).

Cross-linked starches are usually produced by bonding two starch molecules together by way of covalent bonds to produce a larger molecule. This can also occur within the amylopectin polymer (Hoseney, 1994; Rapaille & Vanhemelrijck, 1992). The degree of cross-linking will depend on the ratio of the number of bridges used, to the number of glucose units (Rapaille & Vanhemelrijck, 1992). The more cross-linking the starch contains, the higher the required gelatinization temperature becomes (Hoseney, 1994). Starches which are cross-linked will also have a short paste i.e spoons cleanly, and will have a reduced tendancy to retrograde due to the presence of longer chains, and hence the changing of the starch gel to an opaque one is also delayed (Hoseney, 1994). Those starches which have a high degree of cross-linking are used for purposes such as dusting powder for surgeon's gloves, as the starch can be sterilised, and will be digested with no ill effects if it enters wounds accidentally (Hoseney, 1994). Only a very small number of cross-linked groups needs to be introduced to considerably modify the starch (Rapaille & Vanhemelrijck, 1992). Those starches for use in the food industry usually have a small amount of cross-linking (Hoseney, 1994).

Substituted starches are usually produced from waxy maize starches and are prepared by forming a monoester of phosphoric acid on starch (Hoseney, 1994). These starches are susceptible to thinning at high temperatures, but are less likely to retrograde and are less likely to turn opaque (Hoseney, 1994). They are usually used in the making of instant puddings and pie fillings (Hoseney, 1994).

Other uses for modified starches have been the proposal of small granule starches (granule diameter ~2μm which is similar to that of lipid micelles) as substitutes for fat (Jane, 1992; Hoseney, 1994; Lucca & Tepper, 1994). This proposal is based on the ability of starch-based fat mimics to produce products with a much higher water content (Hoseney, 1994). This higher level of water in products will give many of the same properties as fat provides (Hoseney, 1994). Three types of starch-based fat replacers are known. These are short-chain dextrins, long-chain starch molecules and microcrystalline particles produced as a result of acid-hydrolysed starches (Hoseney, 1994). Small granule starches are also being used as dusting powder for the face, a stabiliser in baking powder, a stiffening agent for laundry and a filler in biodegradable plastic films (Jane, 1992). **Table 1.2** details some of the uses of the modified starches described, and others which are commonly manufactured.

1.8 Fate of resistant starch in the monogastric animal

When resistant starch and undigested starch products reach the colon, they become available for fermentation by the bacteria present (Miller & Wolin, 1979; McBurney *et al.* 1990; Cummings & Macfarlane, 1991). The amount of resistant starch entering the colon varies greatly. It has been estimated at between 2-30% (Chapman *et al.* 1985), at least 20g a day for those on a Western diet (Cummings, 1983), and possibly as much as 40g a day for those on a Western diet (Stephen *et al.* 1983). This latter value matches those reported by Cummings & Macfarlane, who estimated that 10% of the Western diet (8-40g) per day could escape small intestine digestion (Cummings & Macfarlane, 1991).

It has been estimated that some 400 different species of bacteria inhabit the monogastric colon (Moore, 1978; Finegold *et al.* 1983), resulting in approximately 10^{11}-10^{12} organisms per gram (Simon & Gorbach, 1980; Finegold *et al.* 1983; Gorbach & Goldin, 1990; Macfarlane, 1991). These bacteria obtain their energy for growth from the substrates entering the colon. When specific anaerobic culture techniques became available it soon became apparent that the anaerobic bacterial species outnumbered the facultative species, such as *Escherichia coli* and *Lactobacillus* spp., by as much as 10^2-10^4 in the adult animal (Moore, 1978; Simon & Gorbach, 1981). The major anaerobes which predominate in the colon include *Bacteroides* spp., *Bifidobacterium* spp., *Fusobacterium* spp.,

Table 1.2 :-
Some of the more common treatments of starches and their applications

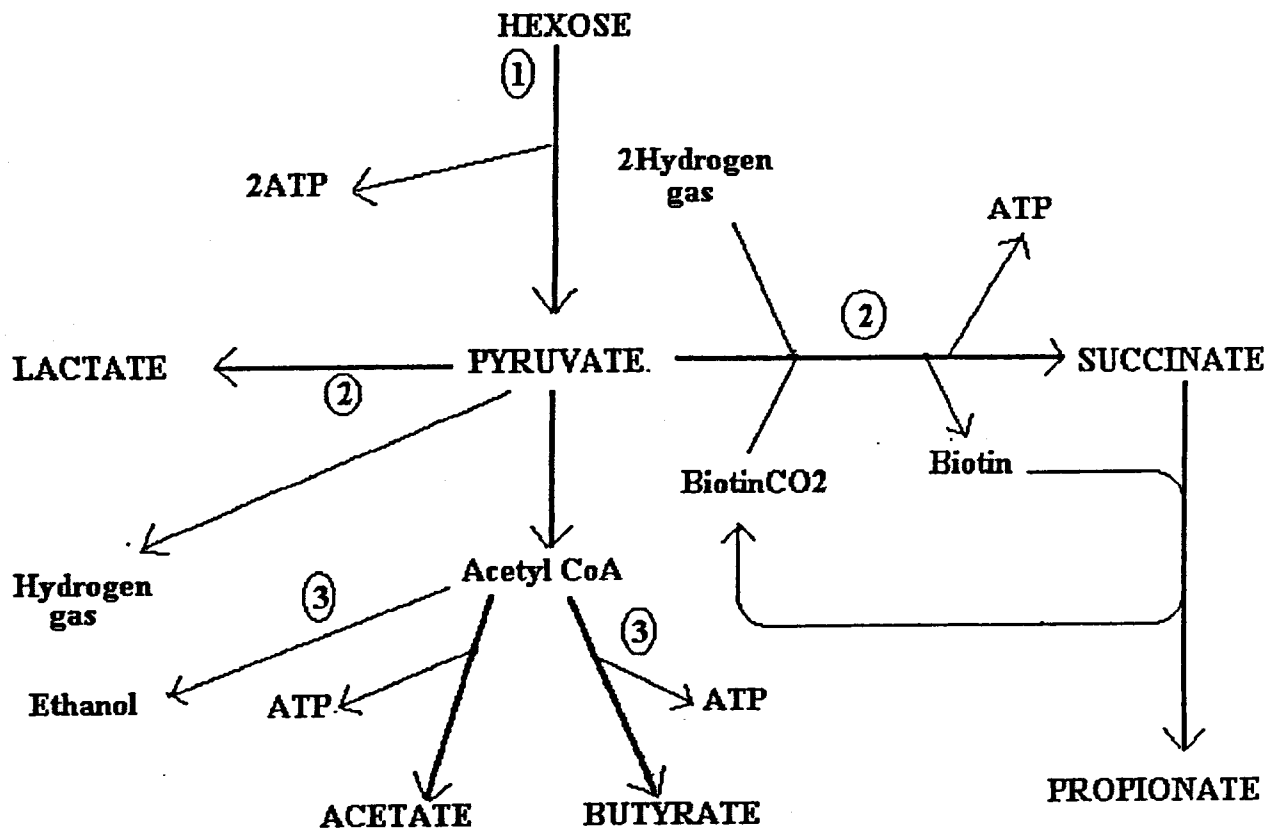
Modification process	Treatment	New property	Uses in Food industry	Uses in industry
Pregelatinized	Heating in water	Cold-water soluble	Pie filling, instant products	Mining, oil drilling
Acid-thinned	Acid	Low viscosity, form rigid gels	Gums, pastilles, jellies	Textiles, laundry
Oxidised	Hypochlorite	Increased clarity	Gravies, sauces, thickeners	Paper, textiles, spray starch
Etherification	Propylene oxide	Increased clarity, increased stability	Salad dressings, pie fillings	Paper and textile industries
Esterification	Acetic anhydride	Increased clarity, forms films	Instant foods, frozen foods	Textiles, paper, film, packaging
Monophosphates	Phosphoric acid	Increased stability for freeze/thaw cycles	Frozen foods, infant foods	Paper, textiles
Cross-linked, di-starch phosphate	Phosphorus oxychloride	Increased stability to heat and pH	Canned and frozen foods	Paper, metal sequestrants

Reproduced from Galliard (1987) and Hoseney (1994).

Eubacterium spp., and *Clostridium* spp. (Gorbach & Goldin, 1990). The bacterial species present are able to make use of the dietary components which enter the colon, with some degrading proteins but the majority will make use of the various carbohydrate components available. The number of anaerobes in faecal material which have the ability to degrade starch has been detected as between $1.1x10^{10}$ - $3.3x10^{12}$ per gram (Macfarlane & Englyst, 1986). The experiment also identified 120 different starch hydrolysing species, with the main ones being from the genera *Bifidobacterium*, *Bacteroides*, *Fusobacterium* and *Butyrivibrio* (Macfarlane & Englyst, 1986). Studies on the colonic microflora are generally carried out by examining the microflora present in faecal material (Hill & Drasar, 1975; Borriello, 1986). The micro-organisms isolated from a particular individual appear to be stable, but variation is known to exist from person to person, due mainly to diet, age and sex (Coates *et al.* 1988; Simon & Gorbach, 1980; Hill, 1990; Borriello, 1986). Other genera of anaerobic or facultative species which exist in the human colon include *Streptococcus* spp., *Lactobacillus* spp., coliforms, Enterobacteriaceae, Eubacteria and *Propionibacterium* spp. (Clarke, 1977; Borriello, 1986; Drasar, 1988). *E. coli*, *Enterococcus* spp. and *Lactobacillus* spp. are the most frequently isolated of the aerobic or facultative species, and Gram-negative organisms such as *Klebsiella*, *Proteus* and *Pseudomonas* species can be found in approximately 50% of faecal specimens (Simon & Gorbach, 1980).

The bacteria which inhabit the monogastric colon ferment the resistant starch and starch products which enter to produce short-chain fatty acids (SCFA). **Figure 1.2** shows the pathway of formation for the main breakdown products. The principal SCFA are acetate, propionate and butyrate, with acetate being the major one formed (Roediger, 1980; Cummings *et al.* 1987; Breves & Stück, 1995; Macfarlane & Gibson, 1995). Various gases also result from this bacterial fermentation such as carbon dioxide, hydrogen which acts as an electron sink, and in some cases methane (Cummings & Bingham, 1987; Hiele *et al.* 1991; Macfarlane & Gibson, 1995). The amount of SCFA produced in the monogastric colon is thought to be in the range of 175 mmol l^{-1} (Cummings, 1991), but has been estimated to be greater than 300 mmol l^{-1} (Bugaut & Bentéjac, 1993). The SCFA are produced on average in the ratio of acetate : propionate : butyrate of 60 : 20 : 20 (Floch, 1990; Cummings, 1991). The amounts and types of SCFA produced can differ depending

Figure 1.2 :-
Major products from fermentation of carbohydrates by human colonic bacteria.



1=> $2NAD \rightarrow 2NADH_2$, 2=> $NADH_2 \rightarrow NAD$, 3=> $2NADH_2 \rightarrow 2NAD$. Reproduced using information taken from Macfarlane (1991).

on the substrate type and amount fermented, the microflora present and host factors such as transit time (Cummings, 1991).

1.9 Fate of digestion end products

The hydrogen gas produced during fermentation can follow one of several paths. It can be transferred to the lungs and excreted or it can be metabolised by the bacteria in the production of methane, hydrogen sulphide or acetate (Gibson *et al.* 1990). It appears that a small amount of hydrogen is excreted by the lungs, with the majority being metabolised to hydrogen sulphide if sufficient sulphate is present. If the sulphate concentration is low, production of methane and acetate predominate (Gibson *et al.* 1990). The remaining gas is expelled via the anus, but as little gas is released through this route, it appears that the majority of the gas is effectively metabolised by the bacteria present (Gibson, 1994). The production of acetate is an important process in individuals whose floras do not produce methane (Gibson *et al.* 1990). The other products of bacterial fermentation, the SCFA, are known to be absorbed from the human colon (McNeil *et al.* 1978). Man is not unique in this absorption with various animal species, such as the pig, horse and goat also reported to absorb SCFA (Cummings, 1981). Of the SCFA produced, a very small amount (~10 mmol l^{-1}) has been reported in the faecal material (Bugaut & Bentéjac, 1993) indicating that approximately 95-99% of the total SCFA produced is absorbed by the host (Engelhardt *et al.* 1991).

The SCFA acetate and propionate are reported to be taken up by the liver where they may be further metabolised, such as the incorporation of acetate into higher lipids (Bugaut, 1987; Rémésy *et al.* 1995). These SCFA may also have an effect on certain processes within the monogastric animal such as the ability of propionate to induce gluconeogenesis (Todesco *et al.* 1991) and the ability of acetate and propionate to influence the production of cholesterol (Miller & Wolin, 1996). The presence of propionate will also inhibit the use of acetate for cholesterol synthesis (Wolever *et al.* 1991).

Of the major SCFA produced, the most studied in recent years is butyrate. It was demonstrated by Roediger (1982) that butyrate is the preferred fuel for isolated rat colonocytes, even in the presence of acetoacetate, L-glutamine or D-glucose. This finding

explains why very little butyrate has been found in the portal blood to be delivered to the liver (Wolever, 1995). Butyrate has also recently been documented as having a possible protective effect against cancer of the colon. These studies have been carried out using cultured tumour cell lines (Young, 1991; Young & Gibson, 1995). Butyrate has been said to :

> "Inhibit DNA synthesis and arrest *in vitro* cultured tumour cells in the G_1 phase of the cell cycle" (Young, 1991). This slows the growth of the cultured cancer cells (McIntyre *et al.* 1993).
>
> "Induce differentiation of tumour cells which produces a phenotype associated with the normal mature cell" (Young & Gibson, 1995).

Another possible therapeutic use of butyrate is in the treatment of blood disorders such as sickle-cell anaemia. This is due to numerous studies which have shown that butyrate has the ability to elevate the levels of foetal haemoglobin (Kruh *et al.* 1995).

Small amounts of branched short-chain fatty acids are also produced from microbial fermentation, but of proteins instead of carbohydrates. These SCFA are isobutyric and isovaleric (Rémésy *et al.* 1995).

1.10 Energy value of SCFA

The physiology of the digestive tract of the ruminant animal is different from that of the monogastric, as the ruminant animal has a stomach consisting of four compartments (Fahey & Garleb, 1991), which allows the breakdown of carbohydrates by the micro-organisms present (Gäbel, 1995). This carbohydrate fermentation is essential to the ruminant animal as, unlike the monogastric animal, the products of fermentation are the principal energy source, producing approximately 80% of the animals energy needs (Bugaut, 1987; Gäbel, 1995). It was originally thought that the undigested carbohydrate component of a typical monogastric diet was of no energy value to the host. Royall *et al.* (1990) estimated from the amount of SCFA released from the breakdown of resistant starch, that the process could yield significant energy for the host. This may be particularly beneficial in patients with carbohydrate malabsorption. Livesey & Elia (1995) calculated that from a typical western diet where approximately 28g of carbohydrates were available for bacterial fermentation,

approximately 2.5% of energy requirements would come from this SCFA production alone. This value could of course be higher in diets where more undigestible carbohydrate was consumed (Livesey & Elia, 1995). In a study carried out in the early 1980's by Cummings, it was estimated that 7% of the energy requirements for man could come from the SCFA produced in the large intestine (Bugaut, 1987). A similar study carried out by McNeil (1984) estimated this value to be between 5-10%.

1.11 Research in the field

Some of the first studies carried out on dietary components were carried out on the fibre component. The definition of "crude fibre" was established by Henneberg & Stochmann in the 1860's and was taken as the:-

> "sum of all those organic compounds of the plant cell membrane and supporting structures, which in chemical analysis of plant foodstuffs remain after removal of crude protein, crude fat and N-free extractives" (Mangold, 1934).

The Weende method for the estimation of crude fibre which was based on treatment with sulphuric acid followed by treatment with potash, water, alcohol and ether (1.25%), was also developed by Henneberg & Stochmann (Mangold, 1934). An earlier method had previously been described by Einhof in 1806 (Cummings, 1976). The majority of the early crude fibre research appeared to be based on cellulose but was later extended to incorporate hemicellulose, lignins and pectins (Mangold, 1934). This early research was related to the crude fibre in the feed of ruminants and other farm animals (Mangold, 1934). Since this early research the importance of fibre in the diet of man and other animals has become apparent.

The main benefit of fibre was that it was found to alter bowel function. This concept however, is not a new one (Eastwood, 1978). Williams and Olmstedt in 1936 carried out experiments which showed that stool weight could be increased by feeding plant sources such as carrot > cabbage > sugar beet pulp > peas > wheat bran (Eastwood, 1978). In the 1970's nutritionists started to research into food components in the intestinal tract, particularly the role of fibre in the prevention and treatment of certain large intestinal

diseases (Stephen, 1991). This led to a change of definition and a new term, where it was renamed "dietary fibre" by Trowell. This was defined as :-

> "the residue derived from plant cell walls that is resistant to hydrolysis by human alimentary enzymes" (Trowell, 1976).

The interest in dietary fibre research was sparked off by the observations that incidences of colonic disease, particularly colonic cancer, varied throughout the world. The varying factor in this appeared to be diet. Colonic cancers were found to be high in the Western world where high fat, animal protein and less vegetable material was consumed (Nagengast *et al.* 1993). African and Asian countries which have a higher consumption of vegetable material appeared to have less incidence of colonic cancers (Burkitt,1978; Eastwood, 1978). This led to the theory that more fibre and less protein should be consumed in the diet for colonic health. Through research it became apparent that someone from a low risk group could develop the same high risk cancer status associated with Western diets, by moving there and adopting a Western-style diet (Burkitt, 1978). This led to the suggestions by Burkitt that dietary fibre could protect against colon cancer as it has the following attributes:-

-Decrease stool transit time through the gut
-Improved the consistency and increased the weight of the stool
-Dilution of carcinogenic material in the colon (Eastwood, 1987).

A study carried out by Stephen *et al.* (1987) showed that reducing the transit time through the gut will increase the bacterial mass within the stool and hence increase the stool weight. When the transit time was increased the reverse was observed. This showed that transit time plays an important role in the bacterial turnover which may have an influence on colonic disease (Stephen *et al.* 1987). The main source of carcinogens present in the colon appears to be from the bacterial breakdown of proteinaceous material (Aries *et al.* 1969). A study comparing the faeces from English people living in a high cancer risk area with faeces from people living in a low cancer risk area- Uganda, was carried out, with those on the English diet consuming a mixture of animal and vegetable material, whereas the Ugandan diet consisted mainly of vegetable material (Aries *et al.* 1969). The results showed that the

same group of bacteria from both the English and Ugandan faecal materials were able to breakdown bile salts, but that the bile salts from the English faecal material was broken down to a greater extent than that from the Ugandan material. This may indicate that the English faecal material may be more likely to become carcinogenic. The faecal material also revealed higher numbers of *Bacteroides* and lower numbers of *Streptococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp. in the English faecal material (Aries *et al.* 1969). Findings such as these suggested that colonic diseases such as colon cancer were diet related and made suggestions that there may be a correlation with animal protein. This led to further suggestions that animal protein and fat should be reduced in favour of higher fibre (Maier *et al.* 1974). High numbers of *Bacteroides* species and low numbers of *Enterococcus* spp., *Streptococcus* spp., and *Lactobacillus* spp. were being increasingly linked to high cancer risk (Hill *et al.* 1971). Thornton (1981) hypothesised that a "high colonic pH promotes colorectal cancer". It was proposed that as well as the above benefits of dietary fibre documented by Burkitt, it may also reduce colonic pH which would in turn reduce the formation of bacterial carcinogens from bile salts (Thornton, 1981). A high fat diet apparently raises the faecal excretion of bile salts and in turn would increase the production of the bacterial enzyme which breaks it down to the carcinogenic substance. However, this enzyme is not active below pH 6.5 (Thornton, 1981). Protein in the diet does not appear to have this effect. It was postulated that a high fat diet may be less harmful if sufficient quantities of dietary fibre were included to acidify the colon sufficiently (Thornton, 1981). It was discovered that as well as providing faecal bulk, some of the dietary fibre was being degraded in the colon by the bacteria and was not simply passing straight through (Vercellotti *et al.* 1978). This was then taken over in the 1980's by the finding that starch from the diet may also act in the same way as the dietary fibre component (Stephen, 1991). The term "resistant starch" was first used by Englyst, Wiggins and Cummings in 1982 when they discovered that after treatment with amylase/pullulanase on non-starch polysaccharides (NSP), a fraction remained, which could be solubilised in potassium hydroxide and then hydrolysed to glucose using amyloglucosidase (Asp & Björck, 1992). In 1987, Englyst and Cummings defined three categories of starch present in foods. These were classified as :-

1. **Readily digestible starch (RDS)**- completely digested in the small intestine
2. **Partially resistant starch (PRS)**- partly escapes digestion in the small intestine
3. **Resistant starch (RS)**- totally escapes digestion in the small intestine

(Englyst & Cummings, 1987).

The first two definitions were later altered to :-

1. **Rapidly digestible starch (RDS)**- rapid, complete digestion (fresh cooked starchy foods)
2. **Slowly digestible starch (SDS)**- slow but complete digestion (raw cereals)

(Englyst *et al.* 1992).

Within the resistant starch (RS) category, three individual types were also found :-

RS_1- physically inaccessible starch such as in partly milled grains and seeds

RS_2- resistant starch granules such as in raw potato and bananas

RS_3- retrograded starch such as in cooled, cooked potato, bread and cornflakes

(Englyst *et al.* 1992).

Discussions arose as to whether or not RS_3 could be included in the calculations of dietary fibre. It was observed that the amount of RS_3 present in a food at any one time would depend on the processing which the food had passed through. Any subsequent processing such as reheating or freezing could subsequently alter the previous amount of RS_3 and hence make it difficult to include in food tables (Englyst & Kingman, 1990). This has led to RS measurement being an entity in its own right, and hence methods have been reported by Englyst, Kingman and Cummings (1992), to measure these RS classifications.
The actual definition of resistant starch was proposed by EURESTA (European concerted action on Resistant Starch) at a meeting in June 1990 as:-

> "The sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Muir *et al.* 1994).

1.12 Methods of RS study

Some of the earlier experiments involving dietary fibre analysis used hydrogen and methane measurements to assess dietary fibre breakdown (Tadesse & Eastwood, 1978). Breath hydrogen was also used in later studies by some researchers examining resistant starch fermentation, such as in the study using oats which had been subjected to various treatments

by Lund and Johnson (1991). Various other researchers have used this method during the early years of RS research (McBurney, 1991). It has been estimated that as much as 13% of the H_2 gas produced may be excreted from the lungs (Grimble, 1989). The method however was thought to be unsuitable in some cases as although hydrogen excretion could only be a result of microbial fermentation, certain factors such as smoking and oral carbohydrate breakdown may actually produce H_2 and hence give false results (McBurney, 1991). However, other researchers have demonstrated that breath hydrogen is an adequate technique for assessing the microbial breakdown of unabsorbed carbohydrates, as breath hydrogen was shown to increase linearly when lactulose was ingested (McBurney, 1991). This is one of the more preferred techniques for measuring non-absorbed carbohydrate, as it is non-invasive, and it allows frequent and repeatable measurements (McBurney, 1991; Perman, 1991).

Another technique employed for the measurement of unabsorbed carbohydrate breakdown is to measure the amount of acetate present in venous blood. Rumessen *et al.* (1992), tested a dose-response relationship between lactulose and venous acetate, and showed that the method was satisfactory for determining malabsorbed carbohydrates, although as in breath hydrogen measurements, problems can exist with the method (Rumessen *et al.* 1992). The most accurate way of measuring the levels of resistant starch which enter the colon has been carried out using intubation or ileostomate subjects. These experiments involve collection and analysis of the effluent from the end of the small intestine and have provided valuable information as to the amount of starch from various foods which escape digestion in the small intestine. At the forefront of the intubation studies are Stephen *et al.* and Flourie *et al.* (Cummings & Englyst, 1991). These two groups fed liquidized meals containing either 20 and 60g of starch from rice bananas and potato, or 100 and 300g of starch from white bread, potato and noodles (McBurney, 1991). These researchers recovered between 5-10% of the starch from these meals at the end of the ileum (McBurney, 1991). Ileostomates vary from intubated patients in that thay have had their large intestines completely removed, and the terminal ileum brought out onto the anterior abdominal wall to provide a fistula (McBurney, 1991). At the forefront of this type of research is Hans Englyst and John Cummings (1985; 1986; 1987). Cummings and Englyst also carried out experiments comparing the hydrogen breath test, blood acetate values and

ileostomate effluents in the measurement of starch fermentation in the large intestine and showed that the study of ileostomates provides the most accurate results (Cummings & Englyst, 1991). Ileostomate studies have also been favoured by several other researchers (Chapman *et al.* 1985; Schweizer *et al*,.1990; Faisant *et al.* 1993; Muir & O'Dea, 1993). Other invasive techniques which have been used to study fermentation reactions in the human large intestine, such as SCFA production, has been the study of sudden death victims (Macfarlane *et al.* 1992). These studies have been used to determine the amount of various SCFA's present in various areas of the gut. The above experiments led to information concerning the amount of starch which escapes digestion in the small intestine, and ways that it could be measured. Faisant *et al.* (1993) examined the structure of the starch which escapes small intestine digestion when bean and potato flakes were fed to ileostomates. It was observed that the fragments of starch which escaped small intestine digestion were oligosaccharides, retrograded amylose and high-molecular weight α-glucans (Faisant *et al.* 1993). Tomlin & Read (1990) conducted experiments where they assessed the effect on colon function of feeding supplements of cornflakes and rice krispies. No actual difference in colon function was observed (Tomlin & Read, 1990). Later experiments by the same team compared the effects of resistant starch and lactulose on colon function and again no difference in colon function was observed (Tomlin & Read, 1992). As well as research into the cereal starches, legume starches were also being studied. Starches from vegetable origin had already been recommended for inclusion in the diets of diabetics due to their low postprandial glucose and insulin responses (Wong *et al.* 1985). A diet which contains less readily available carbohydrates, such as glucose and sucrose, and more complex carbohydrates such as starch has always been advocated for diabetics (Crapo *et al.* 1976). The beneficial effects of high-fibre diets in the diabetic patient had already been documented by Trowell (1978). Researchers looked at the effect of processing on starches from legumes and discovered that, as with other starches, raw legumes contained more resistant starch than cooked, while extrusion cooking of legume starches reduces the amount of resistant starch present (Lintas & Cappelloni, 1992). Studies by Saura-Calixto *et al.* (1992) showed that the protein present in bean starches appeared to complex with the starch to produce an indigestible starch-protein complex. These and other such findings with lipids raised the observations that as well as the already known factors which could influence the

amount of starch escaping digestion, other dietary components present may also exert an effect.

As well as human studies, animal models have also been employed for the study of resistant starch, such as the rat used by Tovar *et al.* (1992) and Faulks *et al.* (1992) to study the digestibility of legume starches and digestion and absorption sites of resistant starch respectively. Other animal models, such as the rat and pig, have been employed to study the colonic microflora (Miller & Ullrey, 1987; Rumney & Rowland, 1992). Due to the expense of animal studies and the relative difficulty of obtaining the animals, *in vitro* simulation devices such as continuous culture systems have been employed to study the microflora of the monogastric large intestine. These systems have benefits over the use of other invasive systems in that manipulation of the flora and addition of potentially toxic metabolites can be carried out without causing harm. A single-stage and a five-stage fermenter has been used by Allison *et al.* (1989) to study the microbial population from the human intestine. A three-stage system was employed by Macfarlane *et al.* (1989) to determine whether or not pancreatic enzymes from the gut could be a potential substrate for the microflora. Duncan & Henderson (1990) also employed a semi-continuous fermenter system to study the fermentation of dietary fibre.

Research into the various SCFA produced from bacterial fermentation was carried out and butyrate emerged as the preferred fuel for colonocytes, and also emerged as having anti-cancer properties. This sparked off research into the levels of production of butyrate from various types of resistant starch. Weaver *et al.* (1992) carried out experiments which showed that butyrate production from cornstarch by both human and rat faecal bacteria was higher than from the fermentation of cabbage fibre. It became apparent that carbohydrate fermentation, and the resulting production of SCFA was beneficial to the gut. It also emerged that protein fermentation was also being carried out, particularly in the distal regions of the colon (Smith & Bryant, 1979). It emerged that large amounts of protein (possibly between 3-25g) were available for fermentation in the gut and that this produced toxic metabolites such as ammonia, amines, phenols and indoles (Gibson *et al.* 1989; Macfarlane *et al.* 1986; Wrong, 1988). Several studies which were carried out concerning the microbial breakdown of proteins, identified *Bacteroides* sp. as one of the major species involved (Macfarlane *et al.* 1986; Macfarlane *et al.* 1988). These findings also correlated

with studies carried out in the rumen by Allison (1978) which showed that a *Bacteroides* sp. from the rumen was responsible for the highest amount of protein fermentation through the production of branched fatty acids.

Researchers then examined the role of the bacteria in the health of the gut, and outlined beneficial species of bacteria, which led to the idea of bacterial manipulation i.e to select a more beneficial gut microflora. This introduced the new age of "probiotics" which entailed the addition of these beneficial bacteria into the colon by means of "live" yoghurts, fermented milks and even ice-cream (Hekmat & McMahon, 1992; Lee & Salminen, 1995). These beneficial bacteria tended to be from both the *Lactobacillus* and the *Bifidobacterium* genera. These bacterial groups ferment carbohydrates instead of protein which maintains a low colonic pH, and hence reduces colonisation from potentially harmful species such as the coliforms. Most of the bacterial species which inhabit the monogastric large intestine require some form of carbohydrate source (Salyers, 1979). This has led to the latest area of research within gut health which is to supplement the diet with carbohydrates such as inulin or oligofructose which will be fermented by, and hence select for, beneficial species such as *Bifidobacterium* spp. (Gibson & Wang, 1994; Gibson *et al.* 1995). Supplementing the diet with the beneficial microorganism as well as the carbohydrate supply utilised by that same species has also been carried out (Fuller & Gibson, 1997).

1.13 Research carried out in this thesis

The research which has been carried out in the area of resistant starch and gut health has established ways in which to maintain gut health and hence lead a healthier lifestyle, with the major area of change being the diet. It has been shown that a reduction in items such as red meat, animal fat, salt and sugars together with an increase in dietary fibre can benefit the health of the gut, by cutting down the risks of diet-related cancers and other gut disorders. Although diet has a major effect on gut health, the microflora present have also been shown to play a major part. To maintain a healthy gut, a stable microbial population is required, consisting of mainly normal gut inhabitants such as *Lactobacillus* and *Bifidobacterium* spp., to the exclusion of gut pathogens such as those from the coliform group. This finding led to the idea of gut manipulation and hence the introduction of probiotics, which is the

addition of desirable gut bacteria to the large intestine in the form of live yoghurts and fermented milks. As well as helping to displace the undesirable gut pathogens, these bacterial groups also help to lower the gut pH due to the metabolism of dietary carbohydrates. This is also another factor which helps to exclude pathogenic species, as these bacterial groups prefer a slightly higher pH. This led to the addition of desirable bacteria to the gut together with the substrates which they prefer to ferment, giving the normal gut inhabitants even more of an edge against the pathogens. These two methods have been used to alter the gut microflora, but it is not certain if these methods will maintain the population for a short time only, or if the bacterial supplements have to be administered continuously to maintain the desired effect. Another way to maintain a healthy bacterial population has been shown by the addition of a dietary component only, which preferentially selects members of the normal gut microflora. This has been carried out recently using substances such as the oligosaccharides, in particular oligofructose, which are naturally occurring carbohydrates found in plants such as onion and garlic. It has been established that butyrate, which is a bacterial product of resistant starch fermentation, is not only a preferred fuel for the colonocytes, but also appears to have anti-cancer properties. This SCFA has also been shown to be produced in high levels from dietary starches, rather than any other form of dietary fibre. The microflora have also been shown to ferment large quantities of protein, which has been shown to be potentially harmful to the gut. This protein fermentation also appears to be linked to particular bacterial species in the gut, and appears to take place mostly, when the carbohydrate component of the diet becomes exhausted.

This thesis extends the previous work by using a normal dietary component, **resistant starch** (resistant to host enzyme degradation), as a bacterial substrate which may contribute to the health of the gut. One type of resistant starch, known as retrograded starch, is found naturally in small quantities in cooked or processed foods which are consumed in the monogastric diet, and therefore may be already consumed in high amounts. These starches are fermented by the normal gut bacteria to produce gases and SCFA which help to keep the gut pH low. As the amount of these starches decreases along the length of the large intestine, the bacteria will turn to the fermentation of proteinaceous material to provide energy, which results in the production of toxic metabolites. If these resistant

starches were able to travel further along the large intestine as a bacterial carbohydrate source, the need for protein breakdown would be reduced. The aim of this thesis is to examine the breakdown of these retrograded starches, using starches from various botanical sources in the monogastric gut, using both simulations of the monogastric gut and an actual monogastric animal model. In particular, the effect of different ratios of the major components amylose and amylopectin in the retrograded starches will be examined, in terms of bacterial fermentation products and the overall effect on the monogastric gut population. These findings should give a further insight into the potential effects that resistant starches from different botanical sources can have on the health of the monogastric gut.

CHAPTER 2

Fermentation of various treated starches by pure cultures of *Cl. butyricum*, *B. suis* and *Fusobacterium* sp.

2.1 Introduction

The monogastric colon is known to be densely populated by approximately 400 different bacterial species, of which at least 120 have been identified as starch-hydrolysers (Moore, 1978; Finegold *et al.* 1983; Macfarlane & Englyst, 1986). Among the species identified are anaerobes belonging to the genera *Bifidobacterium*, *Bacteroides* and *Fusobacterium* (Macfarlane & Englyst, 1986). Another starch degrading organism identified by Baker *et al.* (1950) is *Clostridium butyricum*. This organism was identified as a major degrader of starches in the porcine large intestine at this time, resulting in the production of gas and short-chain fatty acids (SCFA), with butyrate being the principal one.

Starches which are not broken down by the host's own enzymes in the small intestine become a potential substrate for bacterial degradation in the large intestine (Wolin & Miller, 1993; Granfeldt *et al.* 1993). These starches which enter the large intestine are termed "resistant starches" of which several forms are known. Starches can resist small intestine digestion for various reasons, with the processing which the starch has received being an important factor. Starches from different botanical sources and even from plant to plant vary in composition and in their physical properties, which means that they not only have different inherent resistances as raw starches, but also react differently to processing procedures (Moore *et al.* 1984; Biliaderis, 1991; Stark & Lynn, 1991). The main difference in the composition of starch granules is the proportion of the major components amylose and amylopectin which they contain. The relative proportions of amylose and amylopectin are approximately 25 : 75 respectively, but plants can be specifically bred to alter the proportions of these components in the starch (Young, 1984). Plants have been bred to produce starches such as waxy maize starch which contains as little as 1% amylose, or high amylose maize starch which contains approximately 52% amylose.

One form of processing which alters the breakdown of starch in the gut is the heating/cooling procedures the starch receives i.e. cooking and cooling prior to consumption. Heating starch granules above the gelatinisation temperature (characteristic of the starch type, but usually >60°C) in the presence of water causes the granules to swell and burst, releasing amylose and amylopectin into solution. Cooling of this solution allows

the long amylose chains to form hydrogen bonds with water molecules to form a gel. A proportion of this gel then forms more solid bonding by cross-linking of amylose chains in a process known as retrogradation. The amount of resistant starch produced in this way is dependent on several factors with a major one being the proportion of amylose and amylopectin present (Saura-Calixto *et al.* 1992). This process occurs more readily with the amylose component and renders the starch resistant to mammalian enzymes but not necessarily resistant to bacterial ones. As little as 1-3% of starch in food may be resistant in this way, but as many monogastrics diets, such as pigs and humans, are based mainly on foods containing high levels of starch, this may represent a significant amount of bacterial substrate in the colon. This may in turn have a significant effect on the health of these monogastric guts.

This chapter examines the breakdown of native (untreated, extracted starches) and resistant (those which have been processed using the heating-cooling method to produce retrograded fractions) starches by pure cultures of three known starch-degrading bacterial species, namely *Clostridium butyricum*, *Bifidobacterium suis* and an unidentified species of *Fusobacterium*. The experiments involved starches from various botanical sources containing varying amounts of amylose and amylopectin i.e. average and higher/lower than average anounts. The effect of different proportions of amylose and amylopectin on bacterial degradation, as well as the effect of processing (heating/cooling) these starches was assessed. To simulate the bacterial breakdown of starch fractions which have the potential to enter the monogastric large intestine, digestion with pancreatic enzymes was carried out.

2.2 Materials

2.2.1 Bacterial Cultures

Bifidobacterium suis	(20211 DSM)
Clostridium butyricum	(NCIMB 7423)
Fusobacterium sp.	(NCIMB 12177)

The culture of *Bifidobacterium suis* was obtained from the Rowett Research Institute, Aberdeen; the other two cultures were obtained from the NCIMB, Aberdeen.

2.2.2 Preparation of culture media :-

(I) The following were prepared as per manufacturer's instructions:

de Man, Rogosa, Sharpe (MRS) agar

Maximum recovery diluent (MRD)

Ringers solution

(II) Brain-heart infusion (BHI) agar

BHI agar was prepared and sterilised as per manufacturer's instructions. Prior to pouring the plates, 10% (v/v) defibrinated horse blood was added to the agar and mixed.

(III) Brain heart infusion (BHI) broth

BHI broth was prepared as per manufacturer's instructions and the procedure followed as for MRS broth for dispensing, sterilising and pressure-equalising the Hungate tubes. Prior to inoculation 1ml defibrinated horse blood was added aseptically to each Hungate tube.

(IV) de Man, Rogosa, Sharpe (MRS) broth

MRS broth was prepared as per manufacturer's instructions, heated to disperse the powder and reduced while cooling by bubbling a mixture of 50% CO_2 : 50% N_2 through it. The broth was dispensed into Hungate tubes in 5ml amounts under the same gas mixture, and sterilised by autoclaving at 121°C for 20 minutes. All tubes were equilibrated to atmospheric pressure prior to inoculation with the same gas mixture as above

(V) Cooked meat medium

Nutrient broth was prepared as per manufacturers instructions and 1% (w/v) potato starch added. The medium was heated and reduced while cooling using the same gas mixture as above. The medium was dispensed in 5ml amounts under the gas mixture into Hungate tubes containing 1g cooked meat granules. The medium was sterilised by autoclaving at 121°C for 20 minutes. All tubes were equilibrated to atmospheric pressure prior to inoculation using the same gas mixture.

(VI) Trypticase-Phytone-Yeast extract (TPY) medium (Scardovi, 1986)

Prepared as follows:

	$g\ l^{-1}$
Trypticase	10
Phytone	5
Glucose	5
Yeast extract	2.5
Tween 80	$1ml\ l^{-1}$
Cysteine hydrochloride	0.5
K_2HPO_4	2
$MgCl_2 \cdot 6H_2O$	0.5
$ZnSO_4 \cdot 7H_2O$	0.25
$CaCl_2$	0.15
$FeCl_3$	trace

The above components were mixed in distilled water, boiled and reduced using a 50% CO_2 : 50% N_2 gas mixture. The medium was dispensed in 5ml amounts into Hungate tubes and sterilised by autoclaving at 121°C for 20 minutes. All tubes were pressure equalised prior to inoculation using the same gas mixture.

2.2.3 Starch azures used

Amylose azure (potato)

Amylopectin azure (potato)

Pure amylose (potato)

Pure amylopectin (potato)

2.2.4 Preparation of starches :-

2.2.4.1 Starches used

Amylopectin (corn)

Maize

Waxy maize

High amylose maize

Tapioca

Potato

Wheat

2.2.4.2 Native starch preparation :-

(I) Medium for *Clostridium butyricum*

Nutrient broth powder was dissolved in distilled water and the appropriate amount of starch powder from above added (w/v) to produce the required starch concentration i.e. 0.5% and 1%. The components were dissolved by heating and reduced using a mixture of 50% CO_2 : 50% N_2. The medium was dispensed in 5ml amounts under the same gas mixture into Hungate tubes which were sealed before being sterilised by autoclaving at 121°C for 20 minutes. All tubes were equilibrated to atmospheric pressure using the same gas mixture prior to inoculation. Sets of tubes containing both concentrations of each of the native and retrograded starches were prepared. Porcine pancreatin (2.5mg) was also added to a set of tubes as above.

(II) Media for *Bifidobacterium suis* and *Fusobacterium* sp.

TPY medium was prepared as above except the glucose was omitted and replaced with the appropriate amount of each starch powder from above (w/v) to produce the required starch concentration i.e. 0.25%, 0.5% and 0.75%. The components were mixed in distilled water, dissolved by heating and reduced using a mixture of 50% CO_2 : 50% N_2. The medium was dispensed in 9ml amounts under the same gas mixture into Hungate tubes and sterilised by autoclaving at 121°C for 20 minutes. All tubes were equilibrated to atmospheric pressure using the same gas mixture as above prior to inoculation.

2.2.4.3 Retrograded starch preparation

Each of the above starches was dissolved in distilled water at a concentration of 10% (w/v). The starches were heated for 20 minutes at 121°C to gelatinize and then cooled for 4 days at 4°C to allow retrogradation to take place as storage at <4°C contributes to the process (Leloup *et al.* 1992; Muir & O'Dea, 1992). To incorporate into the media (TPY and cooked meat), the starches were mechanically broken up and softened by heating in a water bath at approximately 65°C. The TPY and cooked meat broths were prepared as for the native starches, except the starch component was substituted for the retrograded starches. The amount of water added to the broths was altered to compensate for the retrograded starches already being hydrated. The media were heated and reduced using a mixture of 50% CO_2 : 50% N_2 and dispensed in Hungate tubes as for the native starches. The tubes were sterilised as for the native starches and stored at 4°C until required. All tubes were equilibrated to atmospheric pressure prior to inoculation using the same gas mixture.

2.2.4.4 Pancreatin digested starches (Native-NPD and Retrograded-RPD)

The native forms of the starches were added to water (10% w/v) and the retrograded forms of the starches were prepared as detailed in **Section 2.2.4.3**, as far as being softened in the water bath. Both forms of the starches were digested using porcine pancreatin at 40°C for 30 minutes to ensure digestion had gone as far as possible. The starches were spun at 6000 x *g* and washed in distilled water to remove sugars and any excess pancreatin and then the pellets re-suspended in distilled water (10% w/v). After this stage the starches were incorporated into the TPY and cooked meat media as described in **Section 2.2.4.3** to prepare media containing the desired starch concentrations i.e. 0.5 and 1% for the cooked meat broth and 0.25, 0.5 and 0.75% for the TPY media). The amount of water used in preparation of both broths was altered to compensate for both starch forms already being in a hydrolysed state. All media were reduced using the 50% CO_2 : 50% N_2 gas mixture, dispensed in Hungate tubes and sterilised as described previously. Once sterilised, the tubes containing the RPD starches were stored at 4°C until required. All tubes were equilibrated to atmospheric pressure using the same gas mixture as above prior to inoculation.

2.2.5 Chemicals used :-

2.2.5.1 Deproteinising reagent

Metaphosphoric acid (20g) was dissolved in 50ml distilled water and 2.2ml sulphuric acid added. A solution of 2-ethyl-n-butyric acid was prepared as an internal standard by dissolving 0.25g in 25ml distilled water. The two solutions were then mixed together and made to 100ml with distilled water.

The suppliers of the media and chemicals used is detailed in **Appendix 1**

2.3 Methods

2.3.1 Experiments involving *Cl. butyricum*

Each of the cooked meat media tubes containing the various starches and starch forms (0.5 and 1%) was inoculated with 0.2ml of a 24 hour culture of the organism (containing approximately 10^7 cfu), which had been grown up in the cooked meat media containing 1% potato starch (w/v). All tubes were incubated for 48 hours at 39°C during which gas production was recorded after 18, 24, 42 and 48 hour by inserting a needle and 20ml glass syringe into the rubber septum in the Hungate tube. A final pH reading was taken after 48 hours and a sample was taken for short-chain fatty acid (SCFA) analysis.

2.3.2 Experiments involving *B. suis* and *Fusobacterium* sp.

Each of the TPY media tubes containing the various starches and starch forms (0.25, 0.5 and 0.75%) were inoculated with 0.2ml of 4 day cultures. The *B. suis* culture had been grown up in MRS broth and had an inoculum size of approximately 10^7 cfu, and the *Fusobacterium sp.* had been grown up in BHI broth and had an inoculum size of approximately 10^6 cfu. All incubations were carried out at 39°C for 4 days, after which time gas production was measured as detailed before and samples taken for SCFA and bacterial counts. The bacterial samples were serially diluted to 10^{-8} in MRD and plated in an anaerobic cabinet under a gas mixture of 10% CO_2 : 10% H_2 : 80% N_2 using a modification of the Miles & Misra (1938) technique. MRS agar was used for *B. suis* and BHI agar containing horse blood was used for *Fusobacterium* sp. All plates were incubated under anaerobic conditions at 39°C for 4 days

2.3.3 SCFA analysis

Deproteinising reagent (0.2ml) was added to 1ml of sample and left at room temperature for approximately 30 minutes. The samples were centrifuged for 10 minutes at 6000 x *g* and the supernatant taken off. Analysis of the samples was performed by HPLC chromatography with a Bio-Rad HPX-87H column and a Shodex SE-61 detector. The mobile phase was sulphuric acid (5mmol l^{-1}) at 0.5 ml min^{-1}, at 50°C.

2.3.4 Statistical analysis

All the above experiments were carried out in triplicate and statistical analysis performed on the data using the MINITAB statistical package (Ryan *et al.* 1985). Statistical analysis of the triplicate data was carried out using one-way analysis of variance (ANOVA), where a comparison was made between each data set in a column. Mean and standard deviations were also carried out on the data obtained using the same MINITAB package, and are detailed in **Appendix 2.**

2.3.5 Starch azure experiments

A suspension of each of the amylose and amylopectin azures and the pure versions were made in sterile Ringers solution (20mg ml^{-1}). Each of the four components (0.5 ml) were added to test tubes containing 6ml sterile ringers solution. Aliquots of *Cl. butyricum* or the filtrate from a 24 hour culture of the organism (0.5ml) was added to the tubes. All working volumes were made to 7.5ml with sterile Ringers. The tubes were incubated at room temperature and after 0, 1, 2, 3, 4 and 24 hours, centrifuged at 6000 x *g* for 10 minutes and read on a spectrometer at 600 nm. Control tubes were also prepared containing either amylose or amylopectin azure only.

2.4 Results

Figures 2.1-2.16 show the gas production measured from pure cultures of *Cl. butyricum* grown in cooked meat medium containing either 0.5 or 1% of a particular starch type and form. In all cases the rate of gas production was greatest during the first 18 hours of incubation.

The greatest volume of gas produced over the 48 hours was observed with the native pancreatin-digested (NPD) form of the maize starch at both the 0.5 and 1% concentration (Figure 2.1). The gas produced from the incubations containing 1% maize starch was greater than that observed from the incubations containing 0.5% starch. The retrograded pancreatin-digested (RPD) form of the maize starch gave the smallest total volume of gas production which was significantly lower ($P<0.01$) than the volume of gas produced from all the other treatments of the maize starch at both concentrations.

With the waxy maize starch (Figure 2.11), the highest volume of gas was measured in the tubes containing the RPD form of the starch at both the 0.5 and 1% concentration. The lowest gas production was measured from the retrograded starch with added pancreatin (R+P) at the 0.5% concentration and the native starch at the 1% concentration. The gas produced from most treated forms of the waxy maize starch was similar with both concentrations of the starch. Doubling the amount of starch from 0.5 to 1%, independent of the treatment, increased both the rate and volume of gas produced within the first 24 hours.

The volume of gas produced from each treated form of the high amylose maize starch (HAM) were similar (Figure 2.12). The native starch with added pancreatin (N+P) treatment gave the greatest volume of gas ($P<0.01$) with both concentrations of starch. The initial rates of gas production observed with each treatment of this starch were slower than those observed with either the maize or waxy maize starches. The rate and extent of gas production with HAM starch was not increased when the starch concentration was doubled to 1%.

Figure 2.13 shows the gas production measured using various treated forms of amylopectin. At the 0.5% starch concentration the NPD form of the amylopectin produced a volume of gas significantly higher ($P<0.05$) than the other treatments, except for the retrograded form. The other four treated forms of the amylopectin produced very similar

volumes of gas with 0.5% starch. All the treated forms of the 1% amylopectin gave similar levels of gas, with that from the RPD treatment being significantly higher than the native and N+P treatments ($P<0.05$), and the NPD treatment ($P<0.01$). The rate and extent of gas production from the 1% starch concentration was higher than with the 0.5% starch concentration.

Figure 2.14 shows the amount of gas produced by *Cl. butyricum* with various forms of treated potato starch. More variation in the volumes of gas production was observed between the various treatments of potato starch at both concentrations. The NPD treatment produced the highest volume of gas at both concentrations and the RPD treated starch resulted in the lowest volume of gas. With the 0.5% starch, all treatments produced volumes of gas which were significantly different from each other ($P<0.01$). When the amount of starch was doubled to 1% little difference was observed in the volume of gas produced.

Figure 2.15 shows gas production by *Cl. butyricum* with various forms of tapioca starch. With the 0.5% concentration, the gas produced with each treated form was similar and low. With the 1% starch concentration the volumes of gas resulting from the retrograded and RPD starch forms were both similar and significantly lower ($P<0.01$) than from the other treated forms of the tapioca. Doubling the amount of starch from 0.5 to 1% increased both the rate and extent of gas production with most treatments.

Gas production from the various treated forms of wheat starch were similar with both the 0.5 and 1% concentrations, with the extent of gas production being only slightly higher for the 1% starches (Figure 2.16). The R+P treatment gave a significantly higher ($P<0.01$) volume of gas compared with the other treatments at both starch concentrations, except from the N+P treatment. The RPD treated form of wheat resulted in a significantly lower ($P<0.01$) volume of gas at both concentrations, compared with all other treatments.

Figure 2.2 shows the gas produced by *Bifidobacterium suis* after 4 days incubation in TPY medium containing various concentrations (0.25, 0.5 and 0.75%) and treatments of maize, waxy maize and high amylose maize (HAM) starch.

The volume of gas produced by this bacterial species was low reaching no higher than 2.5ml per tube, although some differences in gas volume were observed within each starch concentration, which was dependent on the type of starch and the treatment.

With the 0.25% starch concentration, the native form of each starch produced significantly lower gas volumes than any other treatment of that starch ($P<0.01$ maize and HAM; $P<0.05$ waxy maize), except the RPD treatment of HAM which was not significantly different. With the maize and waxy maize starches, there were no significant differences ($P>0.05$) observed between the amount of gas produced from the retrograded, NPD and RPD starch treatments. However, the amount of gas produced from the RPD treatment of the HAM starch was significantly lower ($P<0.01$) than from the retrograded and NPD treatments, but was not significantly different from the volume produced with the native form.

With the 0.5% starch concentration the bacterial breakdown of retrograded waxy maize produced the greatest volume of gas, but a significantly lower ($P<0.01$) gas volume was produced with the NPD treatment of the waxy maize starch (compared with the other treated forms of the waxy maize starch and the NPD form of the other two starches). The volume of gas produced from 0.5% HAM starch was similar for all treatments apart from the RPD treatment.

All three starches produced similar volumes of gas with the native, NPD and RPD forms of the starches at 0.75%. The retrograded form of the maize and waxy maize starches produced significantly higher ($P<0.01$) levels of gas from the 0.75% concentration when compared with the other three treatments of both starches.

Figure 2.3 shows the gas produced by a *Fusobacterium* sp. after 4 days incubation in TPY medium with various concentrations (0.25, 0.5 and 0.75%) and treatments of maize, waxy maize and high amylose maize starches. Gas production with this species did not exceed 7ml per tube.

Similar volumes of gas were produced with each treated form of all three starches at 0.25%. Gas production from the various treated forms of HAM starch showed the least variation with 0.25% starch, although the volume of gas from the native form of this starch was significantly lower ($P<0.05$) when compared to the other three treatments. The gas

production with the waxy maize starch was also similar for all treatments, with the native and retrograded treatments giving the highest volume of gas for this particular starch.

The lowest gas volumes for each starch were observed with the 0.5% concentration, with the retrograded form of each starch resulting in the lowest volume. The highest volume was observed with the NPD treatment for all three starches. The 0.5% starch concentration gave the largest range of gas volumes for all treatments of the three starches.

The gas volumes recorded with the 0.75% starch concentrations showed little variation between starch types or treatments. However, the lowest volume of gas production with each treatment was observed with the HAM starch, with the gas produced from the retrograded HAM starch being significantly lower ($P<0.05$) than from other treated forms of the HAM starch. With most of the treated forms examined, at each of the three starch concentrations, the greatest volumes of gas were observed with the waxy maize starch.

Figure 2.4 shows the bacterial counts of *B. suis* obtained in TPY medium with maize, waxy maize and high amylose maize starch at 0.5% concentration. With each of the treated starch forms studied, the highest count was observed with the HAM starch and the lowest count was observed with the waxy maize starch. The highest count obtained with the maize and waxy maize starch was observed with the RPD treated form and the highest count obtained with the HAM starch was observed with the native form. No significant difference was found with any of the bacterial counts.

Figure 2.5 shows the bacterial counts of *Fusobacterium* sp. obtained in TPY medium containing maize, waxy maize and HAM at a concentration of 0.5%. The general pattern of counts obtained for the native treatment of the three starches matched that obtained for the NPD form of the starches, as the pattern obtained for the retrograded form of the starch matched that obtained for the RPD form of the starch. The counts obtained were similar in general for all treatments apart from the RPD form of the starches. With this starch form, the count obtained with the waxy maize starch was significantly higher ($P<0.05$) than the native and retrograded forms of this starch, and the counts obtained with this form of the HAM starch were significantly higher ($P<0.05$) than the NPD treatment of the starch. The

counts obtained for the RPD form of the maize starch were not significantly different from those obtained from any other treatment of this starch.

Table 2.1 shows the SCFA data obtained from pure cultures of *Cl. butyricum* grown in cooked meat medium containing starches which had undergone various treatments. In all cases the major SCFA produced by the organism was butyrate followed, in general, by acetate. In most cases the lowest butyrate production was observed with the RPD treated form of the starches at 0.5%, although the RPD form of the waxy maize starch produced the highest level of butyrate for this starch, and the lowest level of butyrate observed with the amylopectin was obtained from the native form of the starch. A significant reduction in the production of butyrate was observed when all starches (except the waxy maize and amylopectin) were retrograded, when compared with that produced from the native forms of the starches at 0.5% ($P<0.01$ HAM, potato and wheat; $P<0.05$ tapioca and maize). Retrograding the waxy maize and amylopectin resulted in a slightly lower and slightly higher amount of butyrate being produced respectively (compared to the native form), but these were not significant. The treatment which produced the highest level of butyrate at 0.5% differed for each starch, and was found to be the NPD treated form for maize, amylopectin and potato; the R+P treated form for HAM, wheat and tapioca; and the RPD treated form for the waxy maize starch. The highest acetate production for each starch studied at 0.5% was produced from the same treated form of starch as the highest butyrate level. Increasing the starch from 0.5% to 1% increased the amount of SCFA produced, with the degree of increase being dependent on the starch type and the treatment it had received.

Tables 2.2 and 2.21 show the final pH recorded from the tubes after 48 hours incubation with *Cl. butyricum* and the various starches. The lowest pH for each treatment was found in incubations containing a different starch, with no one starch producing the lowest pH throughout. Retrograding the starches (except the amylopectin and tapioca) at 0.5% significantly increased the final pH ($P<0.01$ maize, HAM, potato and wheat; $P<0.05$ waxy maize) after incubation with *Cl. butyricum*. Doubling the starches to 1% (Table 2.21) lowered the pH, in the majority of cases, over that observed for the same treatment at 0.5%.

At the 1% concentration the lowest pH observed for each of the starches was observed with the N+P treatment.

Table 2.3 shows the breakdown of pure amylose and amylopectin by *Cl. butyricum,* and by the extracted enzyme from *Cl. butyricum*, measured using starch azures. *Cl. butyricum* broke down the amylose azure at the same rate and to the same extent whether or not the uncoloured amylopectin was present. There was no significant difference in the breakdown of the amylose azure by the organism or by the filtrate of the organism alone. The presence of the uncoloured amylose did not significantly affect the breakdown of the amylopectin azure, although it was degraded to a significantly greater extent ($P<0.01$) when the organism was present, compared to the filtrate alone. The amylose azure was degraded to a significantly greater extent ($P<0.01$) than the amylopectin azure by *Cl. butyricum.*

Tables 2.4-2.42 show the SCFA levels detected from pure cultures of *B. suis* grown in TPY medium containing either maize, waxy maize or HAM starches which were either untreated or had undergone one of three treatments. In all cases and at all three concentrations studied (0.25, 0.5 and 0.75%) the major SCFA produced was acetate.

At the 0.25% concentration (Table 2.4) the amount of acetate produced with the native form of the HAM starch was significantly higher ($P<0.01$) than that detected from any of the other three treatments. Each treatment of the maize starch produced acetate levels which were significantly different from each other ($P<0.01$). The amount of acetate produced from the native form of the waxy maize starch was significantly higher ($P<0.01$) than that from the NPD treatment, and the acetate detected from the retrograded form of the waxy maize starch was significantly higher ($P<0.01$) than that from the RPD treatment.

Table 2.41 shows the amount of acetate produced from 0.5% starch. The amount of acetate from the native HAM and maize was not significantly different from that detected in the incubations containing the other treatments of the starches. The native form of the waxy maize starch produced significantly higher levels of acetate and butyrate ($P<0.01$) than the RPD treated form of the starch.

The RPD form of the waxy maize starch, at 0.75%, produced significantly lower levels of succinate, lactate, acetate and propionate ($P<0.05$) compared with all the other

treated forms of this starch (Table 2.42). The lowest levels of all the SCFA detected were observed with the treated forms of the HAM starch. When the SCFA from the maize, waxy maize and HAM starches were compared, the major differences were observed in the productions of lactate and acetate at all starch concentrations.

Tables 2.5-2.52 show the SCFA levels detected from pure cultures of *Fusobacterium* sp. grown in TPY medium containing either maize, waxy maize or HAM starches which were either untreated or had undergone one of three treatments.

The levels of each SCFA produced were similar for each of the three starches (Table 2.5), with the SCFA being produced in the order acetate > propionate > butyrate. Virtually no lactate was detected in any of the incubations for all three starches. The production of acetate from the NPD waxy maize starch was significantly lower ($P<0.01$) than that from the native and retrograded treatments at 0.25%, and the production of propionate from the NPD waxy maize starch was significantly higher ($P<0.01$) than from any other treatment of this starch. No significant difference was observed in the production of acetate from any of the treated forms of maize starch at 0.25%.

With the 0.5% starches (Table 2.51), there was no one SCFA produced as the major SCFA for all the treated forms of the starches. For each starch, the levels of propionate produced from each treated form were significantly different from each other ($P<0.01$ and 0.05). With the waxy maize and HAM starches the highest levels of acetate and propionate were detected from the NPD treated forms of the starches, and with the maize starch, the highest levels of acetate and propionate were detected from the NPD and native starch forms respectively. Overall, the total amount of SCFA detected for each starch was low.

With the 0.75% starches (Table 2.52) the major SCFA produced from all treated forms of the starches was acetate, although significantly higher levels of propionate ($P<0.01$) were detected from the NPD form of the maize and waxy maize starches compared to other treatments of these starches. The levels of acetate detected from the 0.75% starches was similar to that observed with the 0.25% starches.

Figure 2.1 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and maize starch at 0.5 and 1%.

Figure 2.11 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and waxy maize starch at 0.5 and 1%.

Figure 2.12 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and high amylose maize starch at 0.5 and 1%.

Figure 2.13 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and amylopectin at 0.5 and 1%.

Figure 2.14 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and potato starch at 0.5 and 1%.

Figure 2.15 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and tapioca starch at 0.5 and 1%.

Figure 2.16 :-
Gas production measured at various intervals over 48 hours from a pure culture of *Cl. butyricum* in nutrient broth containing cooked meat and wheat starch at 0.5 and 1%.

For all above figures the following key applies :-

□	**Native starch**
■	**Native starch + porcine pancreatin**
○	**Retrograded starch**
●	**Retrograded starch + pancreatin**
Δ	**Native pancreatin-digested starch**
▲	**Retrograded pancreatin-digested starch**

Figure 2.1 :-

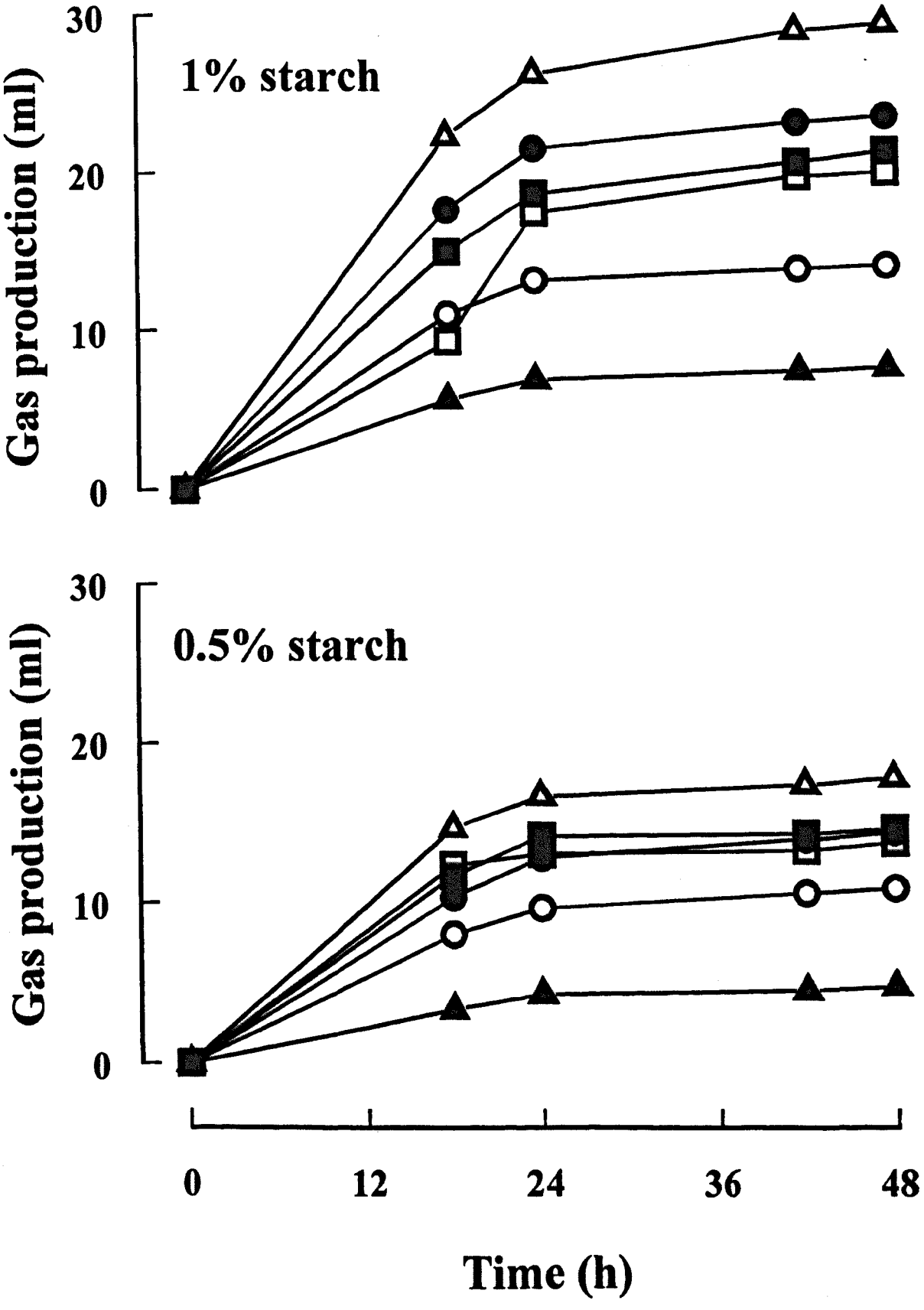

30
20
10
0
1% starch
Gas production (ml)
30
20
10
0
0.5% starch
Gas production (ml)
0
12
24
36
48
Time (h)

Figure 2.11 :-

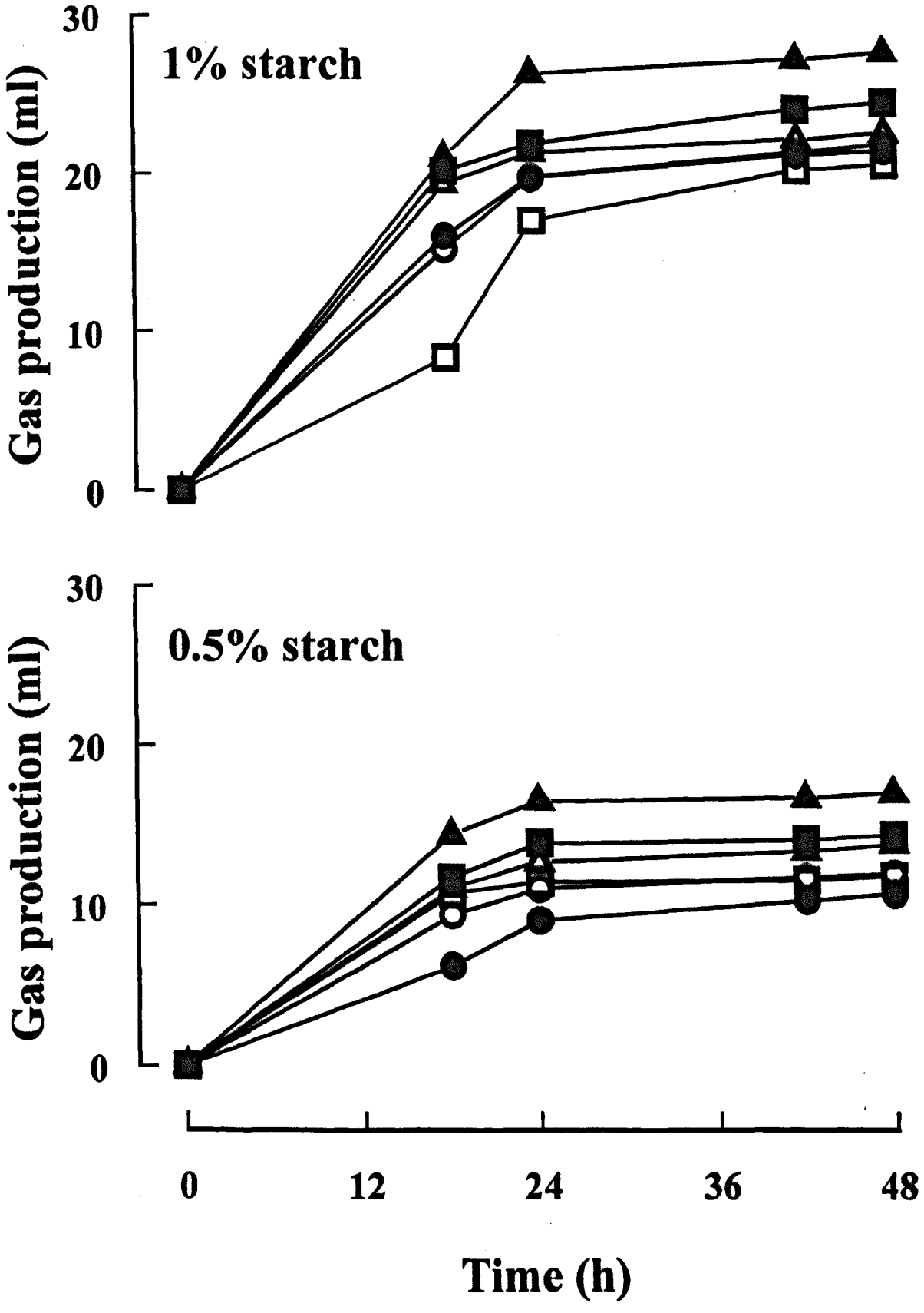

30
1% starch
Gas production (ml)
20
10
0
30
0.5% starch
Gas production (ml)
20
10
0
0
12
24
36
48
Time (h)

Figure 2.12 :-

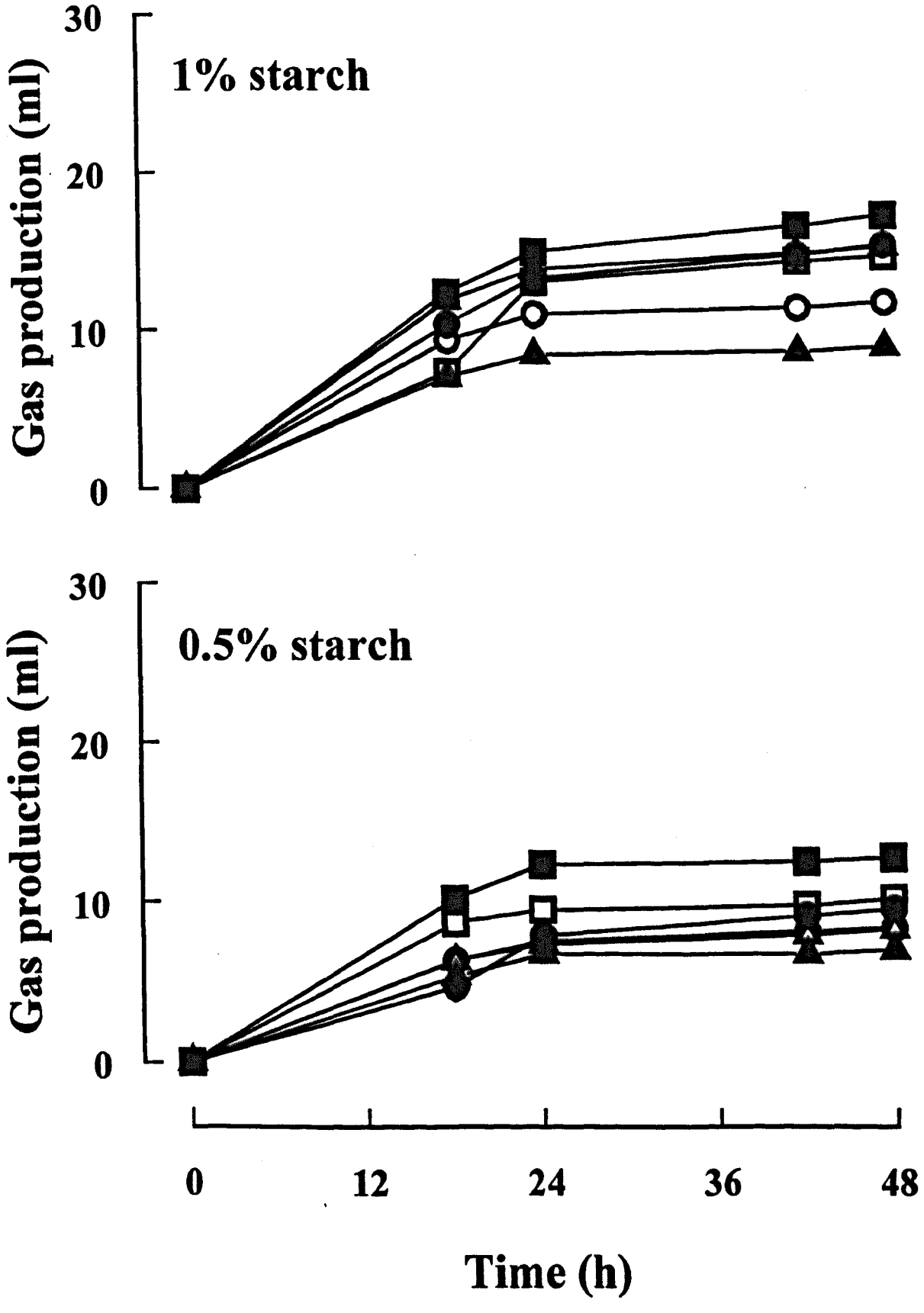

30
1% starch
20
10
0
Gas production (ml)
30
0.5% starch
20
10
0
Gas production (ml)
0
12
24
36
48
Time (h)

Figure 2.13 :-

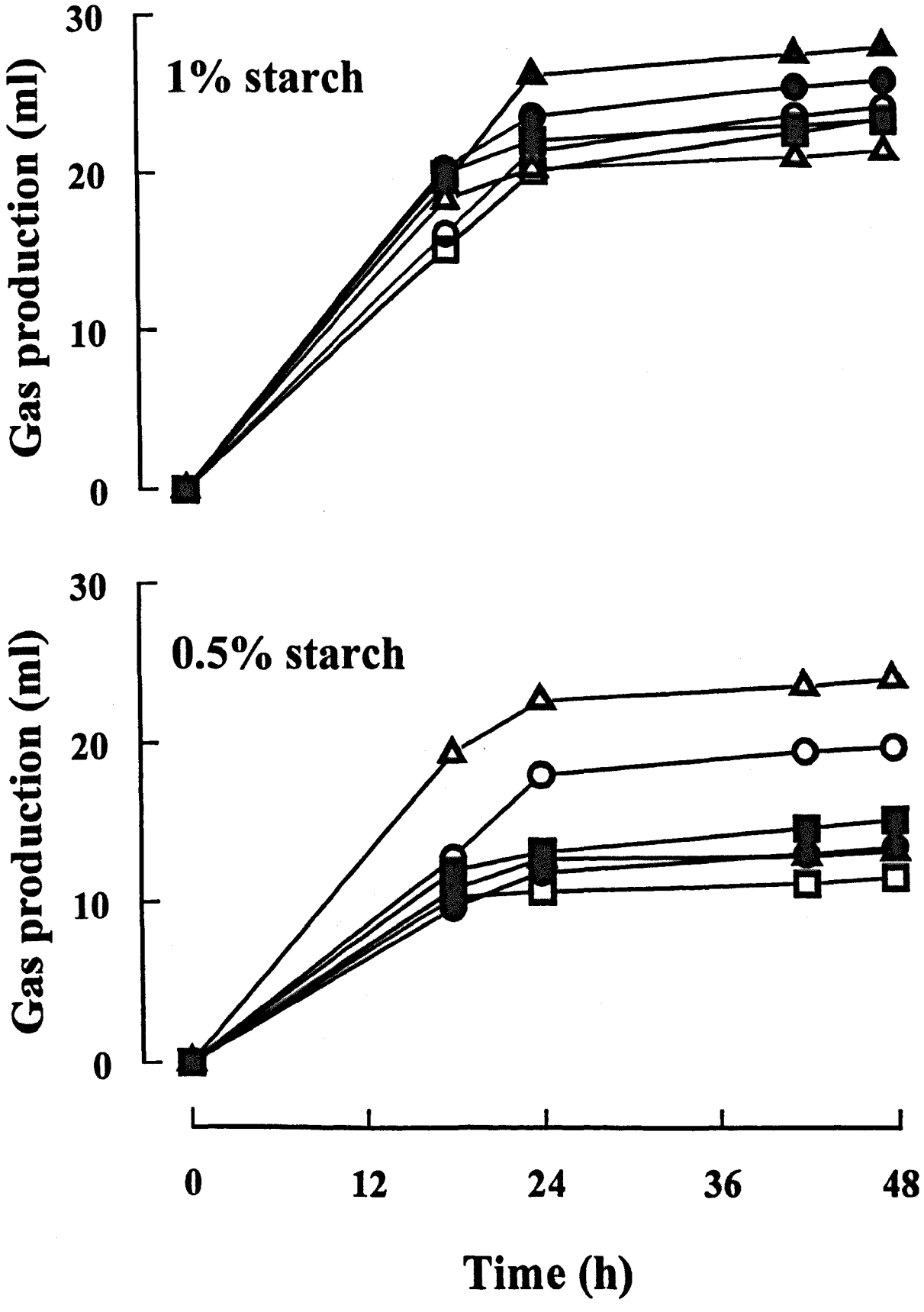

30
1% starch
Gas production (ml)
20
10
0
30
0.5% starch
Gas production (ml)
20
10
0
0
12
24
36
48
Time (h)

Figure 2.14 :-

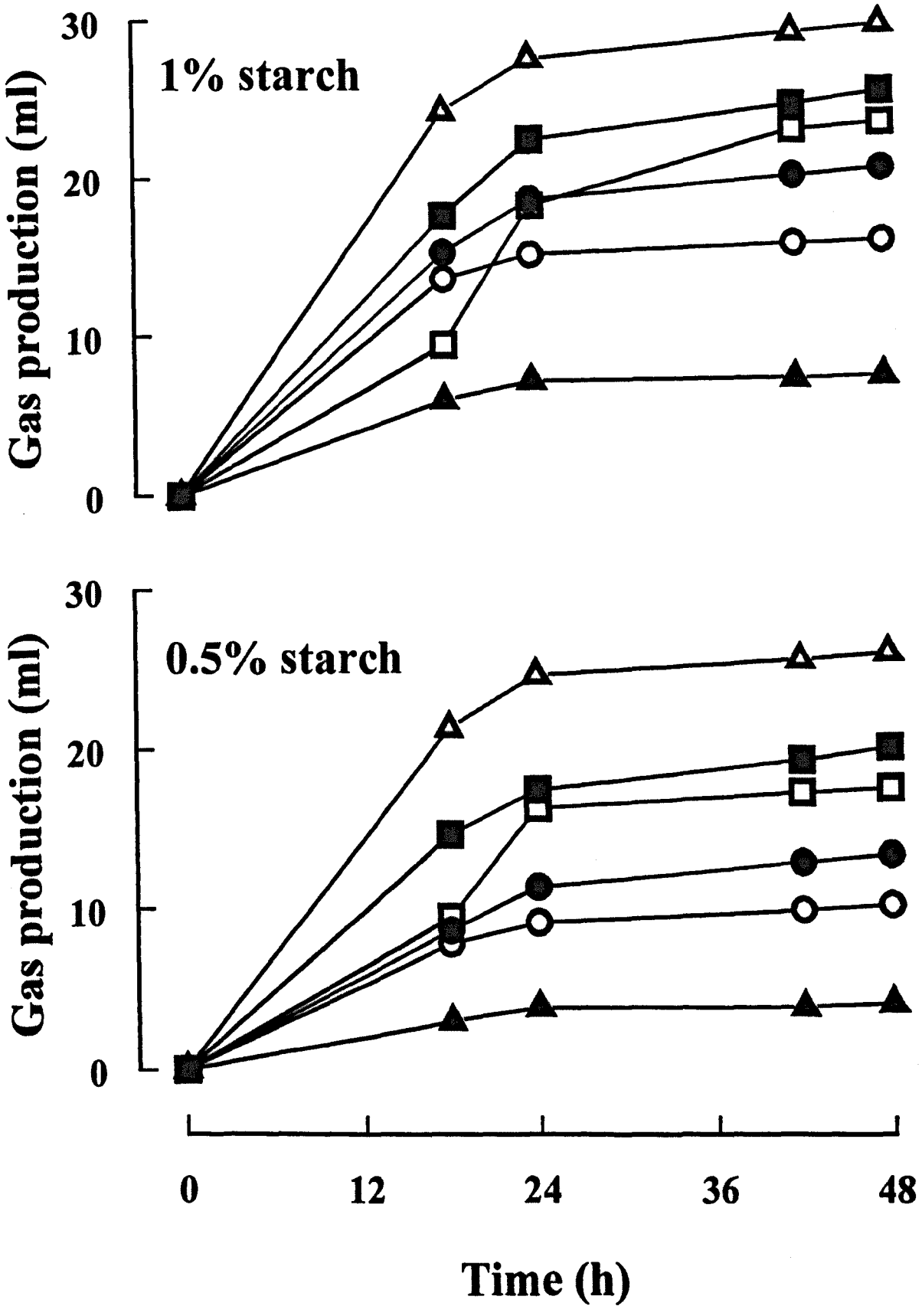

30
1% starch
20
10
0
Gas production (ml)
30
0.5% starch
20
10
0
Gas production (ml)
0
12
24
36
48
Time (h)

Figure 2.15 :-

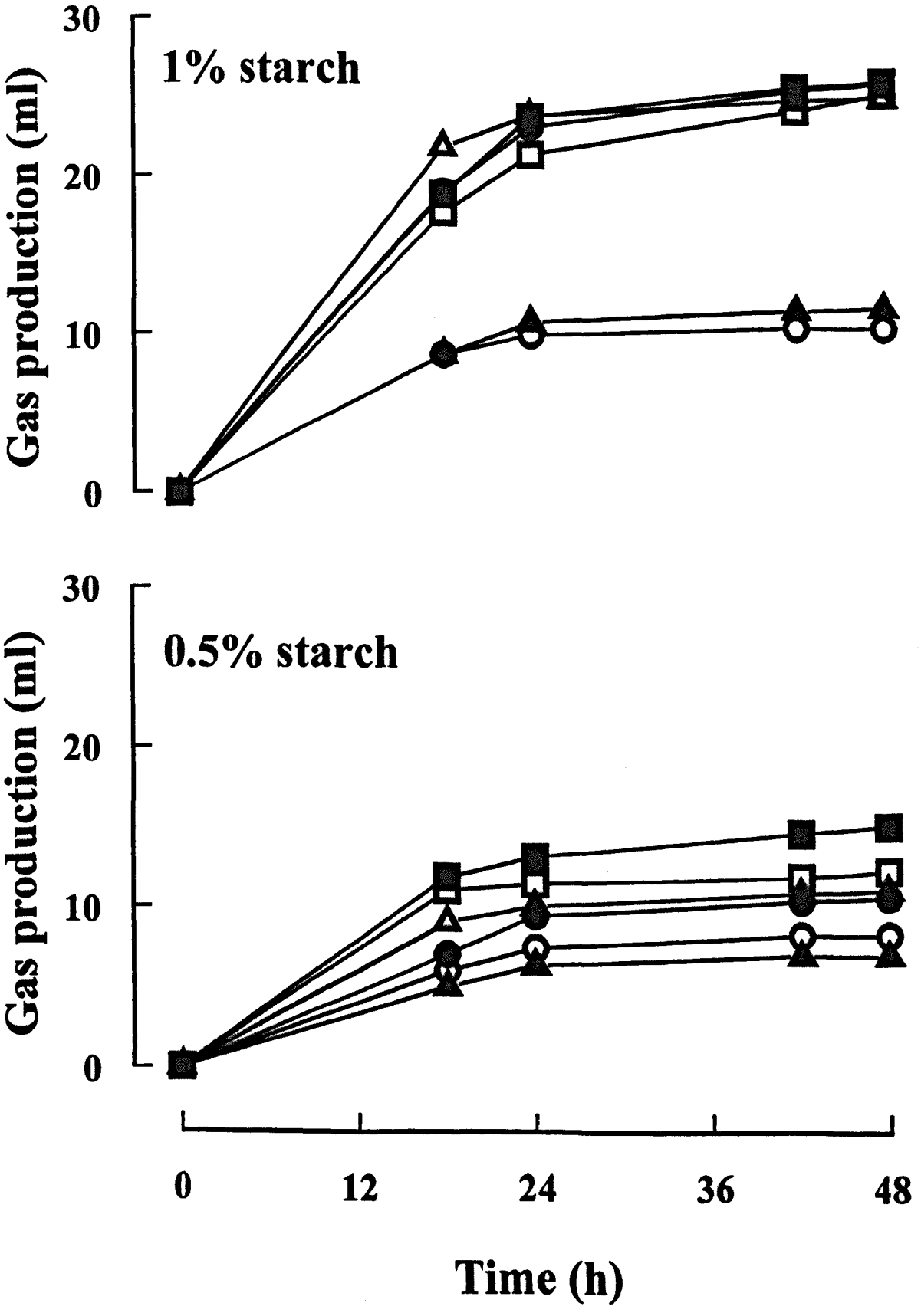

30
1% starch
20
10
0
Gas production (ml)
30
0.5% starch
20
10
0
Gas production (ml)
0
12
24
36
48
Time (h)

Figure 2.16 :-

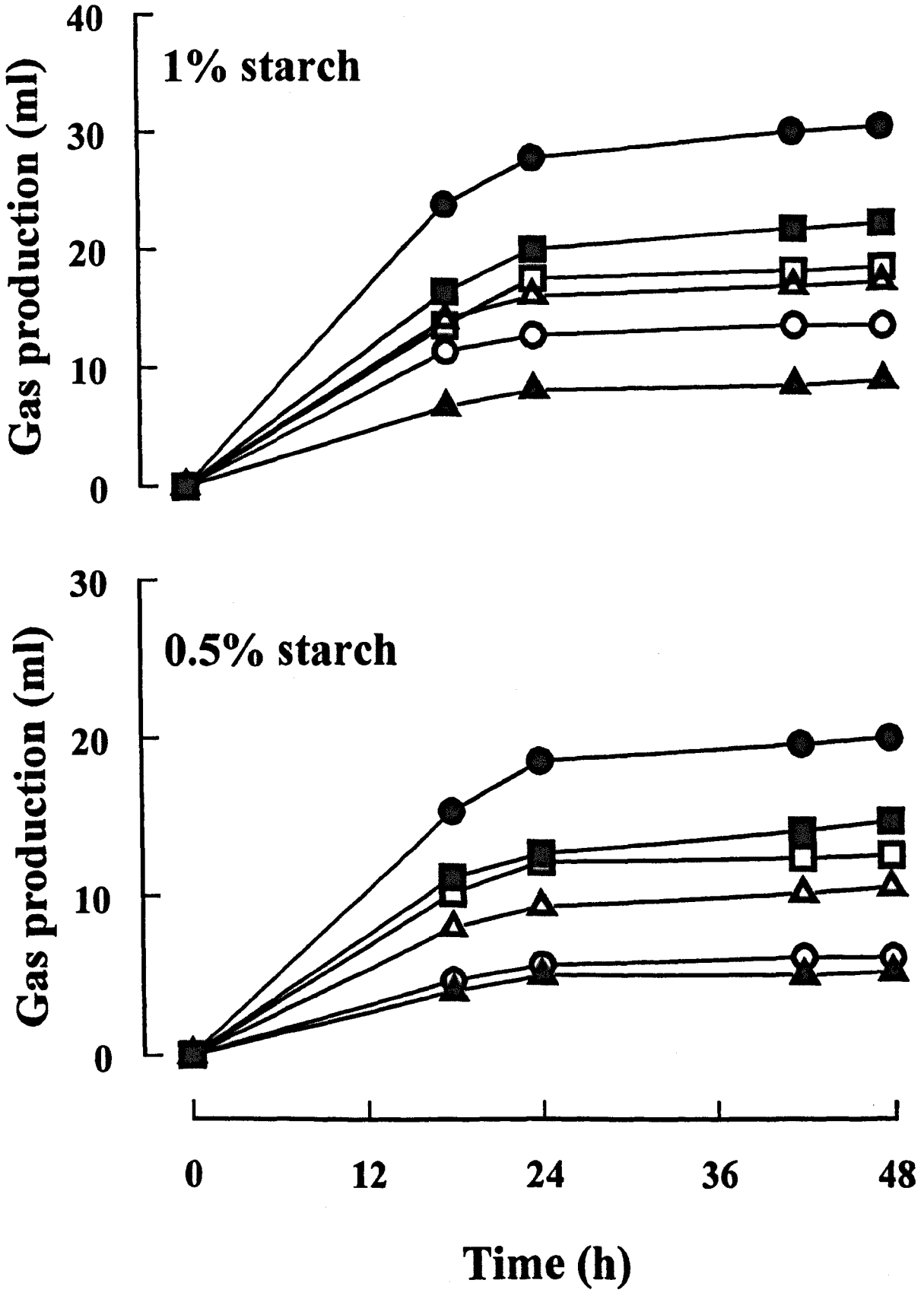

40
30
20
10
0
1% starch
Gas production (ml)
30
20
10
0
0.5% starch
Gas production (ml)
0
12
24
36
48
Time (h)

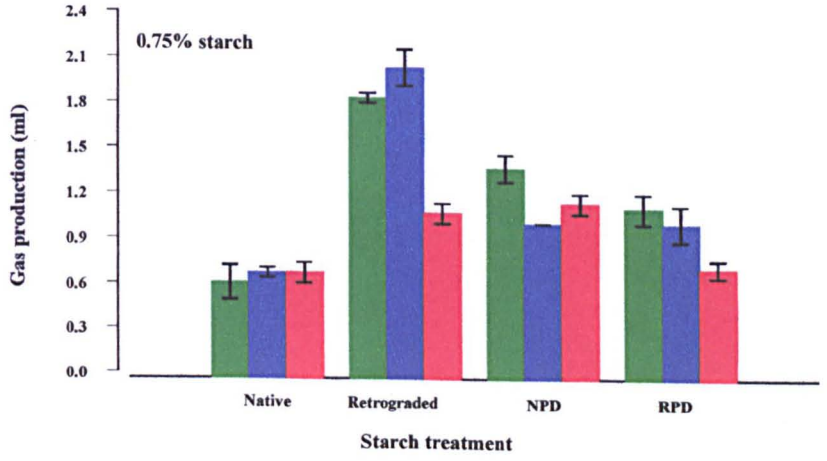
Figure 2.2 :-
Gas production (ml) measured after 4 days incubation of pure cultures of *Bifidobacterium suis* in TPY broth containing 0.25, 0.5 and 0.75% starch. Three starches under four different treatments were studied.

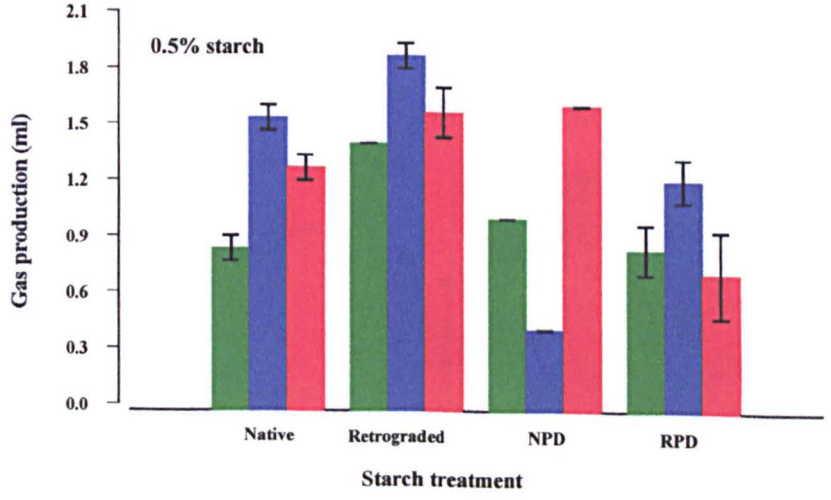
Figure 2.3 :-
Gas production (ml) measured after 4 days incubation of pure cultures of *Fusobacterium* sp. in TPY broth containing 0.25, 0.5 and 0.75% starch. Three starches under four different treatments were studied.

For the above two Figures the following key applies :-

- **Maize starch**
- **Waxy maize starch**
- **High amylose maize starch**

Figure 2.2 :-


0.75% starch
Gas production (ml)
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Native
Retrograded
NPD
RPD
Starch treatment


0.5% starch
Gas production (ml)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Native
Retrograded
NPD
RPD
Starch treatment

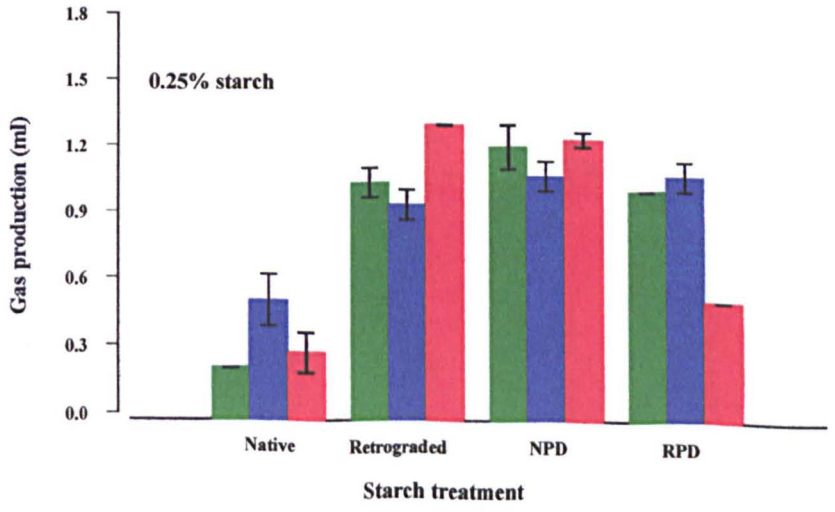
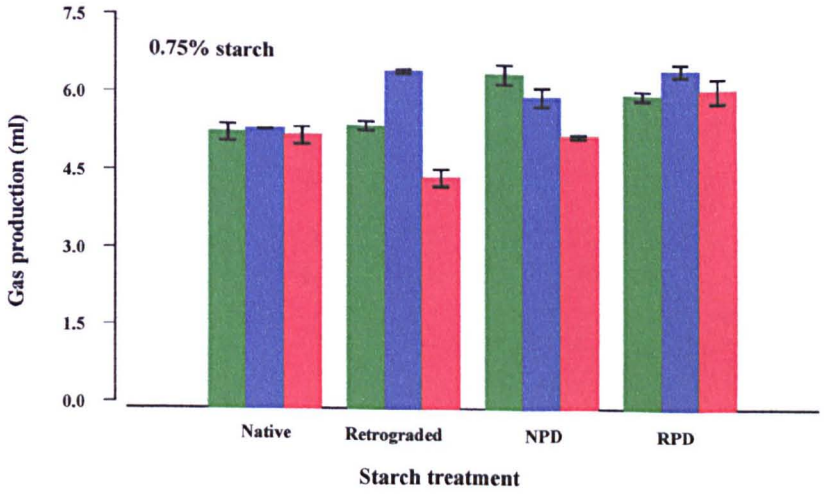
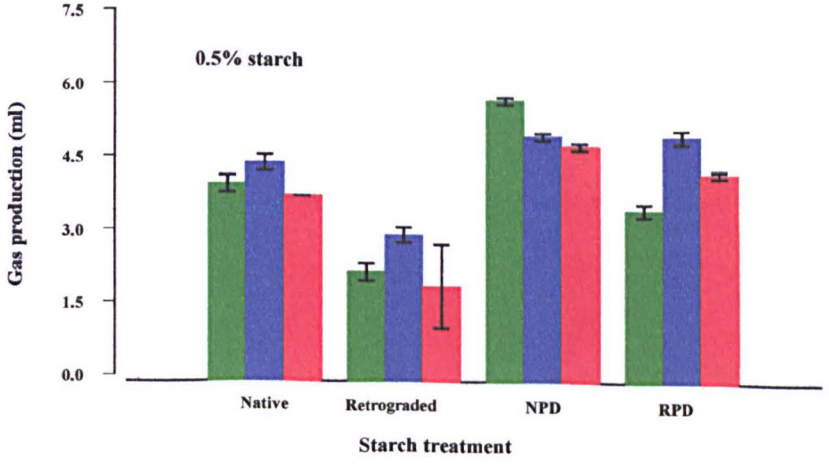

0.25% starch
Gas production (ml)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Native
Retrograded
NPD
RPD
Starch treatment

Figure 2.3 :-


0.75% starch
Gas production (ml)
7.5
6.0
4.5
3.0
1.5
0.0
Native
Retrograded
NPD
RPD
Starch treatment


0.5% starch
Gas production (ml)
7.5
6.0
4.5
3.0
1.5
0.0
Native
Retrograded
NPD
RPD
Starch treatment

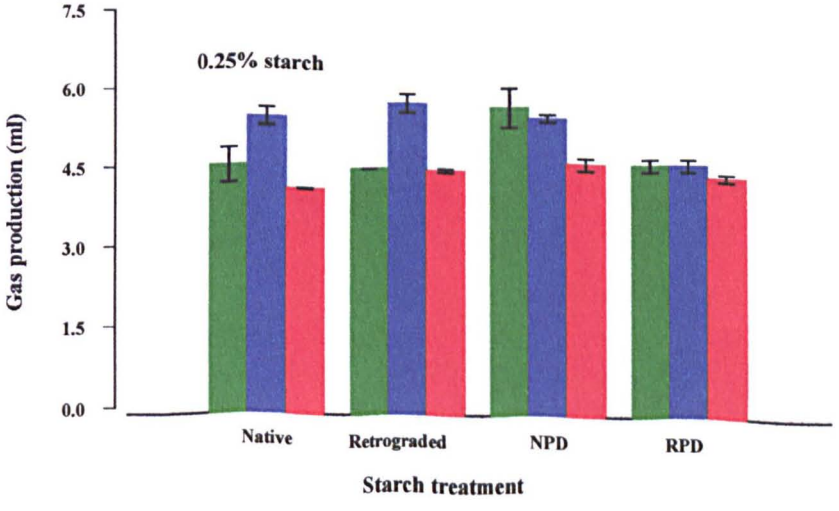

0.25% starch
Gas production (ml)
7.5
6.0
4.5
3.0
1.5
0.0
Native
Retrograded
NPD
RPD
Starch treatment

Figure 2.4 :-

Bacterial counts (cfu ml^{-1}) of *B. suis* obtained after 4 days growth in Hungate tubes containing TPY broth and 0.5% starch. Three starches under four different treatments were studied.

Figure 2.5 :-

Bacterial counts (cfu ml^{-1}) of *Fusobacterium* sp. obtained after 4 days growth in Hungate tubes containing TPY broth and 0.5% starch. Three starches under four different treatments were studied.

For the above two Figures the following key applies :-

- **Maize starch**
- **Waxy maize starch**
- **High amylose maize starch**

Figure 2.4 :-

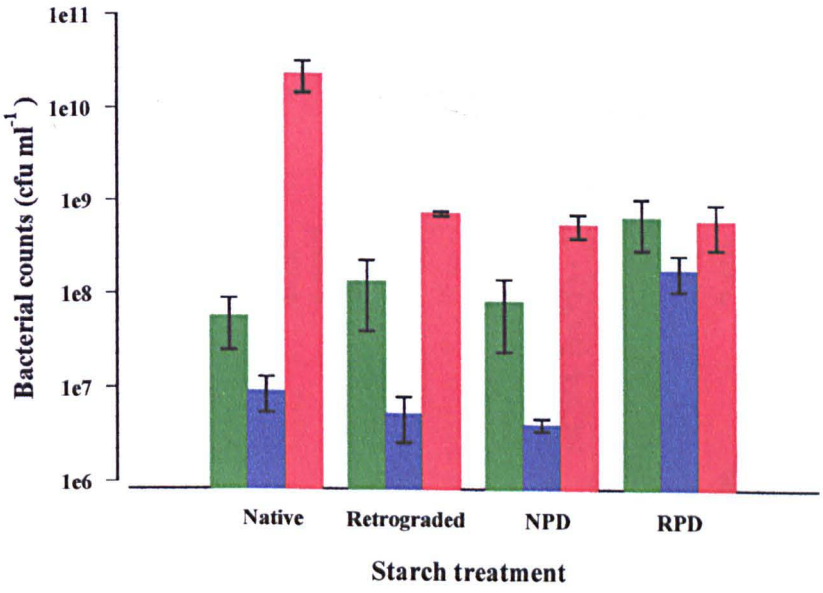


Figure 2.5 :-

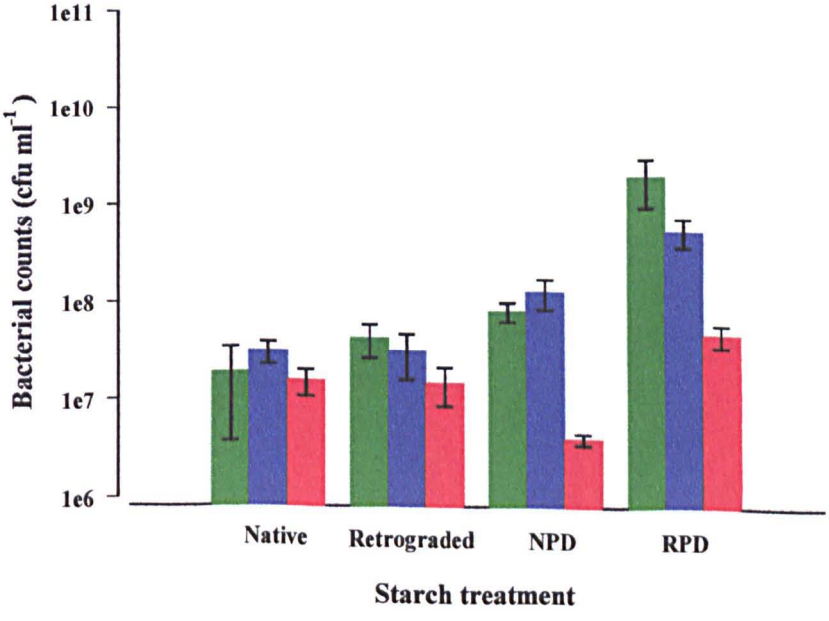


Table 2.1 :-

SCFA production (mM) measured from pure cultures of *Cl. butyricum* grown in Hungate tubes containing cooked meat media and either 0.5 or 1% starch, after 48 hours incubation at 39°C.

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Maize	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	8.17^{ab}	9.71^{a}	37.19^{ac}	15.87^{ab}	8.08^{a}	53.16^{ac}
N+P	10.84^{a}	8.32^{a}	33.11^{ab}	19.76^{a}	8.32^{a}	45.34^{a}
R	4.89^{b}	3.26^{c}	23.10^{b}	8.22^{b}	2.98^{b}	30.73^{b}
R+P	14.91^{ac}	4.25^{b}	37.60^{ac}	29.71^{ac}	4.29^{cd}	69.98^{c}
NPD	19.26^{c}	4.57^{b}	47.00^{c}	37.41^{c}	4.08^{c}	70.71^{c}
RPD	5.94^{b}	5.84^{d}	13.12^{d}	7.37^{b}	4.98^{d}	19.47^{d}

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Waxy maize	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	6.34^{a}	8.69^{a}	33.59	17.17^{a}	9.46^{a}	58.66^{ac}
N+P	14.19^{bde}	8.55^{ac}	29.98	23.99^{bc}	8.44^{a}	51.96^{ab}
R	7.00^{ae}	3.89^{bd}	29.97	13.45^{abc}	2.62^{c}	38.61^{ab}
R+P	12.10^{c}	5.50^{c}	31.80	26.41^{abc}	4.05^{b}	60.81^{abc}
NPD	12.99^{cd}	4.34^{bd}	33.36	23.04^{b}	3.76^{bc}	49.11^{b}
RPD	17.63^{ce}	4.56^{cd}	45.47	30.18^{c}	3.87^{b}	72.15^{c}

Table 2.1 continued :-

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
HAM	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	5.48^{ac}	10.48^{a}	30.06^{a}	7.38^{a}	7.05^{a}	34.61^{a}
N+P	9.77^{b}	9.94^{a}	27.59^{a}	13.39^{bc}	8.03^{ac}	33.85^{abde}
R	4.99^{c}	4.24^{b}	19.76^{b}	6.85^{a}	3.89^{b}	29.91^{b}
R+P	10.85^{b}	5.29^{bcd}	31.07^{a}	19.15^{b}	4.99^{bc}	48.69^{c}
NPD	7.65^{ab}	5.23^{c}	21.69^{b}	15.25^{b}	4.69^{bc}	38.76^{cd}
RPD	8.00^{b}	5.96^{d}	18.84^{b}	9.79^{c}	5.70^{c}	23.96^{e}

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Amylopectin	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	11.75^{a}	3.97^{ae}	31.35^{a}	18.61^{a}	5.57^{a}	46.96^{a}
N+P	11.20^{a}	4.30^{ac}	32.98^{a}	21.46^{a}	7.43^{b}	48.83^{ab}
R	13.90^{ab}	2.94^{ad}	37.70^{ab}	18.50^{ab}	2.63^{c}	47.09^{ab}
R+P	15.67^{a}	4.92^{bc}	43.16^{bc}	37.07^{ab}	3.52^{c}	78.50^{bc}
NPD	24.87^{b}	3.52^{d}	55.17^{b}	21.72^{a}	3.83^{c}	47.60^{a}
RPD	13.17^{a}	4.68^{ce}	35.58^{ac}	33.60^{b}	3.44^{c}	76.86^{c}

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Potato	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	8.50^{a}	9.08^{ac}	40.96^{a}	22.24^{a}	6.34^{a}	52.59^{a}
N+P	15.61^{b}	12.52^{a}	45.40^{a}	44.61^{b}	4.10^{ac}	88.88^{b}
R	4.60^{c}	3.40^{b}	23.57^{b}	8.43^{c}	3.27^{b}	36.17^{c}
R+P	14.72^{b}	6.42^{c}	49.72^{a}	33.77^{b}	4.23^{abcd}	76.51^{bd}
NPD	28.70^{d}	3.66^{b}	60.49^{c}	41.98^{b}	3.27^{bc}	72.44^{d}
RPD	6.27^{c}	5.90^{c}	14.22^{d}	7.89^{c}	5.55^{d}	20.66^{e}

Table 2.1 continued :-

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Tapioca	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	11.65^{a}	3.60^{a}	32.20^{ac}	22.61^{a}	5.35^{a}	47.96^{a}
N+P	11.85^{a}	4.24^{a}	34.20^{a}	26.26^{a}	6.85^{b}	55.23^{a}
R	3.24^{b}	3.97^{ab}	20.83^{bc}	6.38^{b}	3.60^{c}	13.94^{b}
R+P	10.22^{ac}	5.23^{abc}	34.63^{a}	29.41^{a}	4.08^{c}	72.54^{c}
NPD	10.54^{ac}	4.91^{bc}	30.00^{c}	25.19^{a}	3.26^{d}	52.03^{a}
RPD	7.71^{c}	5.37^{c}	17.58^{b}	10.99^{c}	4.90^{ad}	28.63^{d}

	SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Wheat	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	10.84^{a}	3.92^{a}	29.65^{a}	11.39^{a}	7.17^{a}	40.30^{a}
N+P	10.47^{ab}	4.71^{ab}	34.91^{ab}	18.37^{abc}	8.22^{a}	49.40^{a}
R	3.44^{c}	3.85^{a}	13.00^{c}	7.00^{b}	3.28^{b}	29.00^{b}
R+P	25.46^{d}	3.91^{a}	59.54^{d}	39.45^{d}	3.67^{bc}	83.10^{c}
NPD	8.31^{b}	4.82^{a}	26.88^{b}	15.80^{c}	4.20^{c}	40.76^{a}
RPD	6.23^{e}	5.77^{b}	13.73^{c}	7.72^{b}	5.68^{d}	22.91^{d}

Data represent mean of triplicate data. HAM = High amylose maize; N = native (untreated) starch; N+P = Native starch with added porcine pancreatin; R = Retrograded starch; R+P = Retrograded starch with added porcine pancreatin; NPD = Native pancreatin-digested starch; RPD = Retrograded pancreatin-digested starch. Values within a column bearing different superscript letters are significantly different ($P<0.05$).

Table 2.2 :-

pH values measured after 48 hours incubation for *Cl. butyricum* in cooked meat media with starches (0.5%) which have undergone various treatments and with the addition of porcine pancreatin (2.5mg tube^{-1}).

	Type of starch added to cooked meat media						
Starch treatment	**Maize**	**Waxy maize**	**HAM**	**Amylopectin**	**Potato**	**Tapioca**	**Wheat**
Native	5.10^{a}	5.20^{a}	5.43^{a}	5.00^{a}	5.00^{a}	4.80^{a}	4.93^{a}
Nat. + panc.	4.80^{b}	4.73^{b}	5.03^{b}	5.07^{ab}	5.10^{ac}	4.97^{a}	5.13^{ac}
Retrograded	5.67^{c}	5.53^{c}	5.83^{cd}	5.17^{abc}	5.67^{b}	5.23^{abcd}	5.87^{bd}
Ret. + panc.	5.20^{a}	5.37^{c}	5.40^{a}	5.20^{b}	5.23^{c}	5.40^{b}	4.87^{a}
NPD	5.00^{a}	5.20^{d}	5.57^{ad}	4.90^{c}	4.80^{a}	5.40^{c}	5.47^{c}
RPD	5.83^{d}	5.10^{abcd}	5.80^{d}	5.20^{abc}	5.90^{d}	5.70^{d}	5.70^{d}

Table 2.21 :-

pH values measured after 48 hours incubation for *Cl. butyricum* in cooked meat media with starches (1%) which have undergone various treatments and with the addition of porcine pancreatin (2.5mg tube^{-1}).

	Type of starch added to cooked meat media						
Starch treatment	**Maize**	**Waxy maize**	**HAM**	**Amylopectin**	**Potato**	**Tapioca**	**Wheat**
Native	4.83^{a}	4.87^{a}	5.03^{ad}	4.70^{a}	4.67^{ac}	4.60^{a}	5.00^{ac}
Nat. + panc.	4.60^{b}	4.43^{b}	4.77^{a}	4.37^{b}	4.73^{a}	4.27^{b}	4.60^{a}
Retrograded	5.33^{c}	5.17^{a}	5.50^{b}	4.97^{acd}	5.40^{b}	4.40^{c}	5.37^{b}
Ret. + panc.	4.97^{ad}	4.87^{a}	5.17^{cd}	4.73^{acd}	4.87^{c}	4.80^{d}	4.77^{a}
NPD	4.77^{d}	4.90^{a}	5.13^{d}	4.97^{c}	4.73^{a}	4.83^{d}	5.07^{c}
RPD	5.70^{c}	4.83^{a}	5.63^{b}	4.80^{d}	5.73^{d}	5.40^{e}	5.73^{d}

Data represent averages of triplicate data. HAM = High amylose maize starch; Nat. + panc.= native starch with porcine pancreatin added; Ret. + panc. = retrograded starch with porcine pancreatin added; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column bearing different superscript letters are significantly different ($P<0.05$).

Table 2.3 :-

Colourimeter readings at 600nm taken at various time intervals using amylose and amylopectin azure.

	Time of reading (hours)					
Experiment	**0**	**1**	**2**	**3**	**4**	**24**
***Cl. butyricum* + amylose azure + white amylop.**	0.59	0.97	1.15	1.26	1.32	1.37
***Cl. butyricm* + amylop. azure + white amylose**	0.06	0.09	0.12	0.17	0.17	0.17
Filtrate + amylose azure + white amylop.	0.61	0.90	1.07	1.13	1.20	1.36
Filtrate + amylop. azure + white amylose	0.01	0.02	0.01	0.01	0.01	0.01
***Cl. butyricum* + amylose azure**	0.58	0.87	1.03	1.07	1.17	1.23
***Cl. butyricum* + amylop. azure**	0.08	0.11	0.14	0.15	0.17	0.16
Filtrate + amylose azure	0.62	1.02	1.09	1.14	1.20	1.33
Filtrate + amylop. azure	0.04	0.04	0.03	0.03	0.03	0.02

Data represents mean of triplicate data. amylop. = amylopectin.

Table 2.4 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.25% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	1.02^{a}	13.17^{a}	27.06^{a}	7.92^{a}	0.38^{ab}	0.60^{ab}	0.18
R	0.90^{b}	8.05^{b}	15.24^{b}	7.62^{a}	0.25^{a}	0.47^{a}	0.21
NPD	0.89^{ab}	12.74^{a}	22.25^{c}	7.08^{ab}	0.25^{a}	0.48^{a}	0.14
RPD	0.85^{b}	8.78^{c}	14.40^{d}	6.88^{b}	0.19^{b}	0.36^{b}	0.17
Waxy maize							
N	1.10^{a}	14.15^{a}	31.73^{a}	7.97^{a}	0.65^{a}	0.51^{a}	0.04
R	1.12^{a}	14.37^{ab}	31.50^{a}	7.96^{a}	0.71^{a}	0.46^{ab}	0.13
NPD	0.83^{b}	11.90^{bc}	19.79^{b}	6.57^{b}	0.21^{b}	0.38^{b}	0.08
RPD	0.92^{b}	10.39^{c}	16.89^{b}	7.34^{c}	0.19^{b}	0.31^{ab}	0.09
HAM							
N	0.96^{a}	10.03^{a}	19.42^{a}	7.77^{a}	0.37^{a}	0.44	0.13
R	1.01^{a}	9.21^{b}	16.35^{b}	8.25^{b}	0.26^{b}	0.42	0.16
NPD	0.86^{b}	9.51^{ab}	16.00^{b}	7.07^{ac}	0.26^{b}	0.43	0.19
RPD	0.89^{b}	9.32^{b}	15.52^{b}	7.06^{c}	0.21^{b}	0.43	0.16

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

Table 2.41 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.5% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	0.99^{abc}	12.66^{a}	27.32^{ab}	7.69^{ab}	0.70^{abc}	0.34^{a}	0.13
R	0.96^{ac}	10.70^{a}	22.95^{a}	7.66^{a}	0.65^{a}	0.27^{b}	0.14
NPD	1.11^{b}	19.70^{b}	34.33^{b}	8.02^{ab}	0.38^{b}	0.26^{b}	0.16
RPD	1.01^{c}	12.26^{a}	18.00^{a}	8.21^{b}	0.26^{c}	0.10^{c}	0.14
Waxy maize							
N	1.14^{ab}	15.97^{a}	37.13^{a}	8.10^{a}	0.89^{a}	0.36^{a}	0.16^{a}
R	1.20^{a}	20.36^{b}	40.44^{a}	8.06^{a}	0.49^{abc}	0.37^{a}	0.03^{b}
NPD	1.08^{bc}	19.74^{b}	35.36^{a}	7.54^{b}	0.37^{b}	0.26^{b}	0.11^{ab}
RPD	1.03^{c}	11.96^{c}	19.73^{b}	7.85^{ab}	0.22^{c}	0.13^{c}	0.04^{ab}
HAM							
N	0.92	8.84^{a}	19.27	7.69^{ab}	0.62^{a}	0.30^{a}	0.10
R	0.93	9.68^{ab}	20.50	7.60^{a}	0.45^{abc}	0.25^{a}	0.08
NPD	0.97	12.01^{bc}	21.19	7.88^{b}	0.44^{b}	0.22^{b}	0.05
RPD	0.96	16.12^{c}	21.84	8.63^{ab}	0.26^{c}	0.43^{ab}	0.18

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

Table 2.42 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.75% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	1.10^{abc}	22.47^{a}	42.39^{a}	7.77	0.55^{a}	0.46^{ab}	0.18
R	1.01^{ac}	13.22^{b}	24.94^{b}	8.06	0.38^{a}	0.54^{ab}	0.15
NPD	1.17^{b}	25.97^{a}	42.94^{a}	7.67	0.24^{b}	0.58^{a}	0.20
RPD	1.00^{c}	13.47^{b}	22.18^{b}	7.64	0.21^{c}	0.48^{b}	0.19
Waxy maize							
N	1.35^{a}	31.21^{a}	58.86^{a}	8.89^{a}	0.71^{a}	0.66^{a}	0.24
R	1.35^{a}	33.09^{a}	54.82^{a}	9.02^{a}	0.33^{b}	0.62^{ab}	0.22
NPD	1.31^{a}	29.75^{a}	46.46^{a}	8.77^{a}	0.26^{b}	0.60^{a}	0.23
RPD	1.00^{b}	17.66^{b}	28.49^{b}	7.38^{b}	0.21^{b}	0.46^{b}	0.10
HAM							
N	0.95^{ac}	11.80^{a}	23.54^{a}	7.49	0.52^{a}	0.49^{a}	0.19
R	0.79^{b}	9.30^{b}	16.48^{b}	6.64	0.28^{b}	0.37^{b}	0.12
NPD	0.97^{c}	13.20^{a}	23.35^{a}	7.53	0.23^{b}	0.48^{a}	0.13
RPD	0.95^{c}	13.25^{a}	22.21^{a}	7.16	0.27^{b}	0.51^{a}	0.14

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

Table 2.5 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.25% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	0.13^{a}	0.03	26.02	10.47^{a}	2.04^{ab}	7.69^{ab}	0.54
R	0.02^{b}	0.08	26.40	9.50^{b}	1.03^{ab}	8.03^{a}	0.35
NPD	0.25^{c}	0.08	24.27	11.57^{c}	2.29^{a}	6.38^{b}	0.55
RPD	0.08^{d}	0.12	22.96	10.28^{abc}	1.07^{b}	7.25^{ab}	0.34
Waxy maize							
N	0.05^{a}	0	26.62^{a}	9.72^{a}	2.01^{ab}	7.42^{a}	0.38^{ab}
R	0.14^{a}	0	27.23^{a}	9.52^{a}	2.56^{a}	7.10^{a}	0.69^{a}
NPD	0.23^{b}	0	22.11^{b}	17.67^{b}	2.15^{a}	3.40^{b}	0.56^{a}
RPD	0.12^{a}	0	22.92^{b}	11.59^{c}	1.34^{b}	7.24^{a}	0.29^{b}
HAM							
N	0.05^{abc}	0.03	23.87^{ab}	9.86^{a}	1.89	7.59^{a}	0.54^{a}
R	0.05^{a}	0.02	24.41^{a}	8.88^{b}	1.18	7.92^{b}	0.38^{ac}
NPD	0.12^{b}	0	20.73^{b}	9.74^{ab}	1.11	6.80^{c}	0.28^{b}
RPD	0.07^{c}	0	21.77^{ab}	10.31^{a}	1.30	7.26^{abc}	0.27^{bc}

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

Table 2.51 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.5% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	1.50^{a}	0^{a}	10.82^{a}	19.14^{a}	1.13^{a}	0^{a}	0.25^{ac}
R	0.01^{b}	0.06^{b}	0.12^{b}	0.15^{b}	0.05^{b}	0^{b}	0^{a}
NPD	1.94^{c}	0.18^{c}	13.08^{c}	13.89^{c}	0.15^{c}	0.19^{c}	0.65^{b}
RPD	1.64^{a}	0.16^{c}	9.61^{a}	11.96^{d}	3.35^{d}	0.15^{d}	0.42^{c}
Waxy maize							
N	0.02^{a}	0^{a}	0.19^{a}	0.33^{a}	0.03^{a}	0^{a}	0.01^{a}
R	0.44^{a}	3.94^{b}	6.13^{b}	6.83^{b}	2.70^{b}	0^{a}	0^{a}
NPD	0.11^{c}	0.23^{c}	28.56^{c}	16.67^{c}	3.29^{c}	6.51^{b}	0.36^{ab}
RPD	1.38^{d}	0.46^{ac}	9.70^{d}	10.60^{d}	3.30^{bc}	0.14^{c}	0.27^{b}
HAM							
N	0.03^{a}	0^{a}	0.20^{a}	0.36^{a}	0.03^{a}	0^{a}	0.01^{a}
R	0.67^{b}	4.50^{b}	5.23^{b}	7.37^{b}	0.33^{ab}	0.08^{ac}	0.06^{a}
NPD	2.00^{c}	0.31^{c}	21.45^{c}	15.40^{c}	0.17^{b}	0.19^{b}	0.59^{b}
RPD	1.53^{d}	0.25^{c}	10.38^{d}	11.05^{d}	2.96^{ab}	0.14^{c}	0.36^{c}

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

Table 2.52 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.75% starch.

	SCFA (mM)						
Maize	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	0.13^{a}	0.03^{ac}	27.35^{a}	9.93^{a}	3.30^{a}	7.30^{a}	0.61^{a}
R	0.20^{b}	0.07^{abc}	26.17^{a}	9.65^{a}	2.42^{b}	7.33^{a}	0.68^{a}
NPD	0.37^{c}	0.25^{b}	20.80^{b}	22.85^{b}	1.73^{c}	1.31^{b}	0.57^{ab}
RPD	0.20^{b}	0^{c}	26.67^{a}	14.70^{c}	1.07^{c}	7.28^{a}	0.27^{b}
Waxy maize							
N	0.15^{ac}	0.02^{a}	27.27^{a}	9.87^{a}	2.87	7.38^{a}	0.38
R	0.16^{a}	0^{a}	27.39^{a}	9.81^{a}	2.21	7.55^{a}	0.58
NPD	0.38^{b}	0.37^{b}	22.20^{b}	25.16^{b}	1.86	1.57^{b}	0.45
RPD	0.22^{c}	0.15^{c}	24.24^{ab}	21.83^{b}	1.82	3.64^{c}	0.45
HAM							
N	0.07^{a}	0	26.90^{a}	10.98^{a}	1.95^{a}	8.17^{a}	0.53^{a}
R	0.11^{ab}	0.03	21.02^{b}	9.08^{b}	0.72^{b}	7.09^{ab}	0.23^{b}
NPD	0.15^{b}	0.03	21.59^{b}	10.08^{ab}	1.44^{b}	6.70^{b}	0.47^{ab}
RPD	0.13^{b}	0.03	23.13^{b}	12.88^{c}	1.50^{b}	6.36^{b}	0.29^{b}

Data represent mean of triplicate data. HAM = high amylose maize starch; N = native (untreated) starch; R = retrograded starch; NPD = native pancreatin-digested starch; RPD = retrograded pancreatin-digested starch. Values within a column (individual starch comparison only) bearing different superscript letters are significantly different ($P<0.05$).

2.5 Discussion

The fermentation of various starches and starch residues by known starch-degrading bacterial species was carried out to assess the effects of botanical source and various treatments on bacterial fermentation. As the experiments were carried out *in vitro* extrapolation of the actual bacterial counts and values of the end products should not be made to the monogastric gut, although, the trends observed in these fermentations may be applied. The range of end products from the breakdown of starch was similar for the three bacterial species studied, although the individual levels of each SCFA varied, showing that not all starch degrading species produce the same end products to the same extent. This indicates that the end products of bacterial starch fermentation available to the host for further metabolism or for gut health will be dependent on the bacterial species present within the large intestine of the monogastric animal. The starches studied had characteristics which could influence the way in which a particular starch was fermented. The major difference between the starches was the amylose/amylopectin ratio, with the wheat, maize, potato and tapioca containing typical amounts of each component (25% amylose), the high amylose maize starch (HAM) containing 52% amylose (higher than average) and the waxy maize containing only 1% amylose. The fermentation of the starches can also be affected by the size and shape of the starch granules i.e. potato contains large, smooth granules whereas wheat and tapioca contain smaller granules. The amylopectin studied was chemically extracted from potato starch and should contain no amylose and have no granule structure.

Cl. butyricum is not one of the most studied bacterial groups when it comes to monogastric starch degradation as others such as the anaerobic species *Bifidobacterium* sp. are now being studied. The products of bacterial starch fermentation are known to be the SCFA and gases such as carbon dioxide (CO_2) and hydrogen. The production of gas has been used as a marker of colonic fermentation in man, by the measurement of breath hydrogen (Cummings, 1983). *Cl. butyricum* produced large quantities of gas from starch fermentations, therefore the differences due to the type and treatment of the starch were more apparent. As the volume of gas production was used as a comparison of fermentation, the analysis of the gas produced by *Cl. butyricum* was not carried out.

However, as the dominant SCFA detected in most incubations with the organism was butyrate, it is probable that the gas would contain a mixture of both carbon dioxide and hydrogen. The production of hydrogen by organisms in pure culture (both aerobic and anaerobic) *in vitro* has been reported (Wrong *et al.* 1981). The Hungate tubes were equilibrated prior to inoculation with the organism, therefore all starting values were zero and any gas in the tubes must have resulted from the bacterial fermentation of the starch. With each starch studied the experimental conditions were the same and hence any differences can only be due to the properties of the starch. Experiments with *Cl. butyricum* were carried out using two different concentrations of starch to examine the effect of starch quantity on the bacterial fermentation products. Doubling the amount of starch in the incubations caused different effects depending on the starch type and treatment as observed from the gas data. Total gas production did not appear to increase with the HAM or potato starch when each treatment at the 1% level was compared with the 0.5% level, although the initial rate of gas production was increased for the potato starch. The tapioca and wheat starches showed increased gas levels with the 1% starches when compared to the 0.5% starches, with the rate of gas production also increasing with the majority of the treated forms of tapioca starch. The waxy maize starch (high amylopectin) and the amylopectin itself showed an increase in the rate and extent of gas production for all treatments at the 1% level compared to the 0.5% level. These results suggest that in the case of the amylopectin and the waxy maize starch, the initial substrate concentration (0.5%) was limiting the rate and extent of bacterial fermentation. However, with the high amylose starch (HAM) bacterial fermentation was limited by the rate of starch hydrolysis which was not increased when the substrate concentration was increased. Apart from the waxy maize starch, retrograding the starch and then digesting it with pancreatin (at both concentrations) did not appear to increase the gas production when compared to the retrograded form. This suggests that pancreatin digestion may have removed the accessible parts of the starches left after retrogradation, leaving the more resistant fractions, which may also be partially resistant to bacterial breakdown (Reid *et al.* 1998). This did not apply to the waxy maize starch as the retrogradation process does not occur as readily in the amylopectin portion of the starch (Filer, 1988). Digestion with pancreatin increased the availability of the waxy maize starch for *Cl. butyricum* breakdown (Reid *et al.* 1998). The findings from the gas

data for the RPD starches were reflected in the SCFA data. The remaining discussion will focus on the results obtained for the 0.5% starches unless otherwise stated.

Retrogradation was carried out to simulate the changes which could occur during the processing or production of a starch-containing food prior to consumption. The retrogradation process produces a gel which is partially resistant to the host's pancreatic enzymes, thus leaving a proportion of starch which is malabsorbed that becomes available to the colonic bacteria. Retrograding the starches reduced the amounts of gas and SCFA produced, compared with the native forms, from the starches which contained average or higher than average amounts of amylose, but either did not change or increased gas and acetate/butyrate production from the waxy maize and amylopectin starches respectively. This suggests that the breakdown of starch by bacteria is reduced once the starch forms a gel structure such as in a typical starch, but that the retrogradation process actually opens up high amylopectin starches to allow increased bacterial breakdown.

Pancreatin was added to the starches to simulate residual host enzymes which enter the large intestine and become a potential substrate for bacterial breakdown (Lepkovsky *et al.* 1966), and to determine whether or not the enzymes are active in the breakdown of starch, when starch-degrading bacteria are present. The addition of pancreatin caused different effects depending on the starch source. A slight increase was observed in the gas produced from the native starches with added pancreatin (N+P), and either a slight increase or slight decrease (waxy maize and amylopectin) in the gas production from the retrograded starches with added pancreatin (R+P), when compared to the native and retrograded forms of the starches respectively. The largest difference was observed in the increased gas production for the retrograded + pancreatin form of the wheat starch, when compared with the retrograded form. In the case of the SCFA, the native starches with added pancreatin (N+P) showed a significant increase in acetate and a decrease in butyrate levels from the waxy maize, HAM and potato when compared with the native forms. An increase in butyrate levels was observed for the N+P treatment of the amylopectin, potato, tapioca and wheat when compared with the native form, although the increases were not significant. The levels of acetate and butyrate from the retrograded + pancreatin form of the starches

(R+P treatments) were significantly higher than those from the retrograded form of the starches, in most cases. With HAM starch, the addition of pancreatin to the native form resulted in the highest production of gas for this starch, and the addition of pancreatin to the retrograded form of the starch resulted in the highest acetate and butyrate levels, when compared with all other treatments of the starch. With the R+P form of the waxy maize and amylopectin starches, slight increases were observed in the acetate and butyrate levels (slight decrease in butyrate with the waxy maize starch) when compared with the retrograded form, but these increases were not as high as observed with the other starches. The addition of pancreatin to the retrograded form of the waxy maize starch also resulted in the lowest production of gas for this starch. The results suggest that the addition of pancreatin to some of the starches studied, particularly HAM, may assist in the breakdown of the starch. This was observed with the addition of pancreatin to the retrograded starches, where the production of acetate and butyrate were increased significantly compared to the retrograded forms, in the majority of cases. The active pancreatin may be assisting starch breakdown by removing some of the more obstructive components of the retrograded gel. However, the addition of pancreatin to starches such as waxy maize appeared to interfere with the bacterial breakdown of the starch, possibly by competing with the bacterial enzymes for attachment sites, which would have an effect on bacterial breakdown.

Digestion of the starches was carried out using porcine pancreatin to simulate monogastric small intestine digestion and produce starch fractions which are more likely to be encountered by the microflora of the large intestine (porcine pancreatin contains a mixture of enzymes found in the pancreas). The retrograded pancreatin digested form (RPD) of the starches is more representative of the fractions which reach the colon as this form has undergone both retrogradation to simulate cooking and/or processing which happens to most starches prior to consumption, and simulated small intestinal digestion with pancreatic enzymes. The RPD form of most of the starches gave acetate and butyrate levels which were lower than those produced from the NPD form of the starches, although the RPD treated form of the waxy maize starch gave the highest level of acetate and butyrate when compared with the same treated form of any of the other starches. The NPD treated

form of amylopectin gave the highest level of gas, acetate and butyrate with the 0.5% concentration, when compared to other treatments of this starch. The same starch form also showed increased acetate levels from the maize and waxy maize starches and a significant decrease in butyrate levels from HAM starch, when compared with the native forms. These findings may be supported by Biliaderis (1991) who suggested that when the starch granules are disrupted during gelatinization and retrogradation, the amylose coats the outside of the granule. If pancreatin could disrupt this layer sufficiently the bacteria could gain access to ferment the less retrograded amylopectin underneath (Reid *et al.* 1998). In the case of the amylopectin and waxy maize, pancreatin digestion would make bacterial access easier as there is no amylose layer (or a very thin one) to disrupt. HAM starch contains higher than average amounts of amylose which will result in a thicker coating round the granules which may be too thick for the pancreatic enzymes to penetrate (Reid *et al.* 1998), and hence also reduce access to bacterial enzymes. Amylose gels have been shown to contain partially crystalline regions separated by amorphous regions (Leloup *et al.* 1992). These amorphous regions are easily hydrolysed by acid and enzymic treatments (Leloup *et al.* 1992) and so if these models are representative of the starch gels in this study, it may be possible for the pancreatin to break these amorphous regions to allow bacteria to enter into the amylopectin underneath. This model is in agreement with the findings from the RPD treated starches. The results suggest that digestion with pancreatin appeared to influence the way in which the starch was fermented (by altering the amounts of the products) and also appeared to accentuate the differences due to amylose/amylopectin ratio. Starches which have higher than average amounts of amylopectin (such as waxy maize) appear to survive heat treatment and are in a form which is more available to bacterial breakdown (Reid *et al.* 1996). These starches also appear to survive digestion with pancreatic enzymes and become more available to bacterial breakdown if the starch has been retrograded first. The amylopectin substrate appeared to follow the above proposal to a certain extent, although not to the same extent as the waxy maize. This may be due to the amylopectin being chemically extracted from corn rather than a naturally occurring starch, which will result in no apparent granule structure, and will alter the way the amylopectin is fermented.

From the above findings, it may be concluded that the amount of amylose and amylopectin which a starch contains may make a difference to the way the starch is fermented after treatment. In order to determine if *Cl. butyricum* preferentially degraded the amylose or amylopectin portion, starch azures were used to allow the breakdown of these components to be observed easily. Uncoloured amylose and amylopectin were also added to the incubations together with the starch azures to determine if the presence of both substrates altered the way in which one or the other was degraded by the bacteria. All four components were in the native form and when *Cl. butyricum* was added to the incubations it appeared that the amylose azure was degraded in preference to the amylopectin azure. The presence of the uncoloured amylopectin did not appear to alter the apparent bacterial preference. The filtrate of the bacterial enzyme was also added to the incubations instead of the micro-organism and again the amylose azure was degraded in preference to the amylopectin. These results are different from those obtained from the breakdown of the native starches by *Cl. butyricum,* where the order of the highest production of gas and SCFA was maize>waxy maize> HAM. However, the starch azures are pure forms which have been extracted from maize starch and covalently bonded to remazol brilliant blue, and will have a different structure to the actual starches studied in the initial experiment, affecting the way in which they are broken down. The findings from the starch azure experiments show that when the amylose and amylopectin are in pure, extracted form, the straight-chained amylose is degraded to a greater extent by *Cl. butyricum.* The findings also extend the theory that heating and cooling a starch (when whole) will reduce the breakdown by bacteria due to an outer coating of amylose, but will leave the amylopectin portion available for bacterial breakdown, particularly when pancreatic enzymes clear a path. The findings also suggest that the HAM starch may contain a higher proportion of amylose than can be degraded by *Cl. butyricum.*

The above results indicate that the difference in the amounts of amylose and amylopectin contained within a starch influences the way the starch reacts to treatment and bacterial breakdown. To examine this further, two other known starch-degrading bacterial species, *Bifidobacterium suis* and a *Fusobacterium* (of unknown species) were examined. More than one concentration of starch was used to examine the effect of substrate level on

starch breakdown. With the *B. suis,* the level of starch added to the incubations appeared to result in differences in the gas productions. When 0.25% starch was added, similar levels of gas were produced with the maize and waxy maize at the retrograded, NPD and RPD treatments. When the level was increased to 0.5%, the waxy maize starch produced the highest level of gas for the native, retrograded and RPD treatments, although there were no large differences between the gas values. Although this particular organism does not produce large quantities of gas, enough was produced to show differences in the bacterial breakdown of the particular starch forms. The breakdown of the RPD treated form (0.5%) by *B. suis* produced a similar result as for *Cl. butyricum,* with the waxy maize starch producing the highest level of gas compared to the other two starches. This suggests that the *Cl. butyricum* and the *B. suis* degrade starches which are high in amylopectin in a similar way when they are retrograded and then digested with pancreatin. With 0.75% starch, the gas level did not appear to increase above that observed for the starches and treatments at the 0.5% level, except in the case of the retrograded maize and waxy maize. This is similar to the results with *Cl. butyricum* in that the rate of fermentation of waxy maize may be limited by the initial substrate concentration, but with HAM the rate of starch hydrolysis is the limiting factor. The SCFA data do not appear to correlate with the above gas data. The highest gas level at 0.5% for all the starches was observed with the retrograded form, which correlates with the majority of the SCFA for waxy maize, but with the maize and HAM starches the highest acetate and lactate was observed with the NPD and RPD treatments respectively. This suggests that *B. suis* may be adopting a different pathway from *Cl. butyricum* for the fermentation of starches such as HAM. The gas produced by *B. suis* followed a similar pattern at each concentration, with the retrograded and NPD treatments producing the highest gas for these starches (apart from the NPD waxy maize at 0.5%), and the native and RPD treatments producing lower levels of gas. This suggests that the bacteria are able to ferment the starches to a similar extent whether retrograded or digested with pancreatin when in the native form. These two processes appear to make the starches easier to degrade by *B. suis.* However, retrogradation and pancreatin digestion together resulted in reduced bacterial degradation, as with the starches in the native form. *B. suis* appeared to ferment the waxy maize starch more effectively as judged by the amounts of SCFA produced, however, the bacterial counts indicated that *B. suis* appeared to increase in

numbers more effectively when HAM starch was added, independent of the treatment which it had received. This suggests that *B. suis* is able to grow better with a high level of amylose, but are able to ferment the amylopectin portion more effectively, which is similar to the results obtained with *Cl. butyricum*.

With *Fusobacterium* sp. the gas levels were higher than those observed with *B. suis* species. With 0.25 and 0.75% starch, there does not appear to be a large difference in the gas production for each treatment. The biggest differences were observed with 0.5% starch where the gas produced from all starches was lower when they were retrograded. The highest level of gas at this concentration was observed with the waxy maize starch at each treatment, except the NPD. Pancreatin digestion of the starches also produced high levels of gas with the highest level from 0.5% starch being from the NPD treatment with maize starch and from the RPD treatment with waxy maize. These results were also observed with *Cl. butyricum*. The highest levels of gas with each starch concentration were observed with waxy maize starch from the native and retrograded forms and maize starch from the NPD and RPD treated starches. The SCFA levels generally correlated with these findings but not on every occasion. The bacterial counts obtained for *Fusobacterium* sp. suggest that the bacteria increased in numbers more effectively with starches which contain retrograded or RPD maize, or native or NPD waxy maize starch. The bacteria appeared to grow less effectively with the starch which contains a high amount of amylose (HAM), which is similar to the results with *Cl. butyricum*. Both *Cl. butyricum* and *Fusobacterium* spp. were similar in that they were able to utilise the same starches more effectively than the others, although the amounts of the products differed.

The results suggest that the three known starch-degraders from the monogastric colon examined have different preferences for the type and form of starch which they are able to ferment and grow on, with no one starch appearing to be most effective for all bacteria. The bacterial species also appeared to follow different pathways of starch fermentation, producing similar end-products but in differing amounts. If these findings were extrapolated to the gut environment they suggest that there will always be a starch fraction from the diet which can be utilised by a particular bacterial group. This indicates

that many types of starch can be degraded in the gut by the bacteria present which will limit the competition between starch-degrading bacterial groups. These findings also imply that the microflora of the large intestine can be manipulated by the inclusion of different types and treatments of starch in the diet. The amounts of the major components amylose and amylopectin contained within the starches had an effect on the way these starches were fermented by bacteria, and also appeared to have an effect on the way the starches responded to the various treatments carried out.

CHAPTER 3

The use of a single-stage colon simulator to study the effect of retrogradation and amylose/amylopectin content on the growth and activity of the colonic microflora

3.1 Introduction

The study of pure cultures of micro-organisms *in vitro* can be employed to examine the metabolic activities associated with these species, such as the breakdown of various dietary components. These studies were carried out using batch culture techniques (Macfarlane *et al.* 1990; Hidayat *et al.* 1993), which are not suitable for long term or detailed examination of the intestinal micro-organisms. This is due to the rapid changes which take place in the system, which may negate comparisons made between results from these systems and the fermentation patterns and/or bacterial counts from the intestine itself (Khaddour *et al.* 1998).

Systems which are more suitable as simulators of the monogastric intestine are those which employ a continuous addition of fresh media, together with a system which removes spent media and bacterial cells. Such a system has been employed to simulate the microflora of the porcine ileum and the effects of various factors on these micro-organisms (Hillman *et al.* 1995). Such systems can be altered to simulate the colonic environment, by altering the pH, dissolved oxygen concentration and the nature of the dietary components fed into the system. The monogastric colon is known to consist of three different regions- the proximal, transverse and distal sections (Macfarlane *et al.* 1992). Each of these sections exhibits a slightly different pH, each is supplied by a nutrient source which varies and which also contains similar species of bacteria, but at slightly different numbers. A number of simulators of the colon have been employed using both single-stage and multiple-stage (up to three) vessels to study the addition of various components to mixed intestinal populations from human sources (Duncan & Henderson, 1990; Macfarlane *et al.* 1989a, 1989b; Gibson *et al.* 1988; Gibson *et al.* 1993; Gibson & Wang, 1994). These systems have been employed mainly for the examination of micro-organisms from human sources. Continuous culture systems allow studies to be carried out on micro-organisms from human or animal origin without encountering the problems of invasive techniques. They also allow microbial activity to be examined under stringent microbial containment conditions when toxic or potentially harmful micro-organisms or substances are added. Such a system has also been found to adequately simulate the fermentation patterns of various bacterial groups within a population of micro-organisms from the porcine colon (Khaddour *et al.* 1998).

The proximal colon is a nutrient-rich area which operates at an acidic pH where bacterial growth is fast (Macfarlane & Macfarlane, 1993). The distal colon, however, has a higher pH and is fed by a nutrient source which is depleting and hence contains slow-growing bacteria (Macfarlane & Macfarlane, 1993). The previous chapter focussed on the breakdown of native and retrograded starches from various botanical sources by pure cultures of known starch-degrading organisms. This chapter follows on to study the growth and activity of a mixed bacterial population taken from the colon of a monogastric animal, when various starches are used as the sole carbohydrate source. This was carried out in a single-stage continuous culture fermenter system which was set up to simulate the proximal region of the monogastric colon. The number of starches examined in this chapter has been reduced from the previous chapter, to focus mainly on the differences in bacterial degradation due to the proportions of amylose and amylopection.

3.2 Materials

3.2.1 Preparation of culture media :-

(I) The following media were prepared as per manufacturer's instructions:

MacConkey agar no. 3

de Man, Rogosa, Sharpe (MRS) agar

Maximum recovery diluent (MRD)

(II) Columbia blood agar (CBA)

Columbia agar base was prepared as per manufacturer's instructions. Prior to pouring the plates, 5% (v/v) defibrinated sheep blood was added aseptically to the agar.

(III) Wilkins-Chalgren blood agar (WCBA)

Wilkins-Chalgren anaerobe agar was prepared as per manufacturer's instructions. Prior to pouring the plates, 5% (v/v) defibrinated sheep blood was added aseptically to the agar.

(IV) Bifidobacterium agar (BIF)

Prepared as follows :-

Columbia agar	39g
Yeast extract	5g
Maltose	5g
Fructose	5g
L-cysteine hydrochloride	0.5g
Sodium formaldehyde sulphoxylate	0.3g
Distilled water	880ml
Haemin solution (0.5 mg ml^{-1})	10ml
Vitamin K_1 solution (1 mg ml^{-1})	1ml
Tomato juice	100ml
Kanamycin (10 mg ml^{-1})	5ml
Nalidixic acid (10 mg ml^{-1})	5ml

All ingredients except the haemin, vitamin K_1, tomato juice, kanamycin and nalidixic acid were dissolved in distilled water and sterilised by autoclaving at 115°C for 20 minutes. The

last four components were added aseptically prior to pouring the agar, once the media had cooled to 50°C (Drasar and Roberts, 1991).

(V) Kanamycin vancomycin agar (KV)

Prepared as follows :-

Brain-heart infusion agar	23.5g
Distilled water	450ml
Kanamycin (10 mg ml^{-1})	5ml
Vancomycin (1 mg ml^{-1})	3.75ml
Defibrinated horse blood	50ml

The kanamycin, vancomycin and horse blood were added aseptically prior to pouring the agar (Drasar and Roberts, 1991).

3.2.2 Starches incorporated into the fermenter media

Maize
Waxy maize
High amylose maize
Tapioca
Potato

The starches were kindly extracted and donated by Dr Andrew Lynn, SAC, Auchincruive

3.2.3 Preparation of Fermenter media (Hillman *et al.* 1994) :-

(I) Medium to stabilise the fermenter

The following components were mixed together in 5 litres of distilled water. For each of the stabilising runs set up prior to the addition of the test starches, the medium was prepared using potato starch from an alternate source (Sigma) :-

Xylan	**3g**
Pectin	**3g**
Amylopectin	**3g**
Arabinogalactan	**3g**
Starch*	**25g**
Casein	15g

Peptone water	15g
K_2HPO_4	10g
$NaHCO_3$	1g
NaCl	22.50g
$MgSO_4.7H_2O$	2.50g
$CaCl_2.2H_2O$	2.25g
$FeS0_4.7H_2O$	0.025g
Haemin	0.05g
Bile salts	0.25g
Antifoam A	2.50ml
Tween 80	10ml

The above were sterilised by autoclaving at 121°C for 20 minutes. The medium was placed on a stirrer while still hot to prevent the components settling out.

(II) Fermenter medium for experimental use

The medium was prepared as above except that the components listed in bold i.e. xylan, pectin, amylopectin, arabinogalactan and starch were omitted. These were combined together in a separate flask, added to approximately 150ml of 1M HCl (to pH 2) and left for 1 hour at 39°C. The pH of the mixture was adjusted to 7.55 using 1M KOH. The components were digested with porcine pancreatin (5g) for 30 minutes at 40°C in a shaking incubator. The mixture was centrifuged at 6000 x *g* for 15 minutes, the supernatant discarded, the pellet washed in distilled water and re-spun at the same speed. The supernatant was discarded and the pellet resuspended to 300ml with distilled water. These preparations were stored at refrigeration temperatures until required (these will be referred to as the pancreatin-digested mixtures). The starch component in this mixture was replaced using one of each of the starches being tested, for each run of the fermenter.

The remainder of the fermenter medium components were prepared in the same way as for the medium to stabilise the fermenter **(3.2.3 - (I))**, and sterilised by autoclaving at 121°C for 20 minutes.

3.2.4 Chemicals used :-

3.2.4.1 Vitamin solution

The following components were mixed together :-

	mg l^{-1}
Menadione	1
Biotin	2
Pantothenate	10
Nicotinamide	5
Vitamin B_{12}	0.5
Thiamine	4
Para-aminobenzoic acid	5

Once mixed, the solution was stored at 4°C until required.

3.2.4.2 Trace element solution

The following components were mixed together :-

	mg l^{-1}
EDTA	500
$FeSO_4.7H_2O$	200
$ZnSO_4.7H_2O$	10
$MnCl_2.4H_2O$	3
H_3BO_3	30
$CoCl_2.6H_2O$	20
$CuCl_2.2H_2O$	1
$NiCl_2.6H_2O$	2
$Na_2MoO_4.2H_2O$	3

Once mixed, the solution was stored at 4°C until required.

3.2.4.3 Half-strength Gram's iodine

The following were prepared as for Gram's iodine :-

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

The above solution was then diluted 1:1 with distilled water:

3.2.4.4 Deproteinising reagent

Prepared as detailed in **Chapter 2, 2.2.5.1.**

3.2.5 Measurements carried out on test starches

	Parameter		
Starches tested	Total blue value	Starch damage	S + TS lipids
Maize	0.46	0.94	511
Waxy maize	0.09	1.45	117
High amylose maize	0.88	0.74	721
Potato	0.47	0.16	60
Tapioca	0.40	0.78	58

S + TS lipids = surface and true starch lipids. All measurements were carried out on the starches by Dr Andrew Lynn from S.A.C. Auchincruive.

The suppliers of the media and chemicals used is detailed in Appendix 1

3.3 Methods

3.3.1 Construction of single-stage continuous culture system

A single-stage continuous culture fermentation was constructed to simulate the monogastric colon, based on the ileum simulator of Hillman *et al.* 1994. A diagram of the system is detailed in **Figure 3**. The fermenter vessel had a working volume of 500ml, and contained glass beads at the bottom with a stirring bar to mix the faecal material. The temperature of the vessel was set to 39°C using a heating jacket, and the pH was maintained at 6.0 using a pump linked to 0.5 mM KOH. The oxygen entering the system was set at 25 μM and regulated using an oxygen probe.

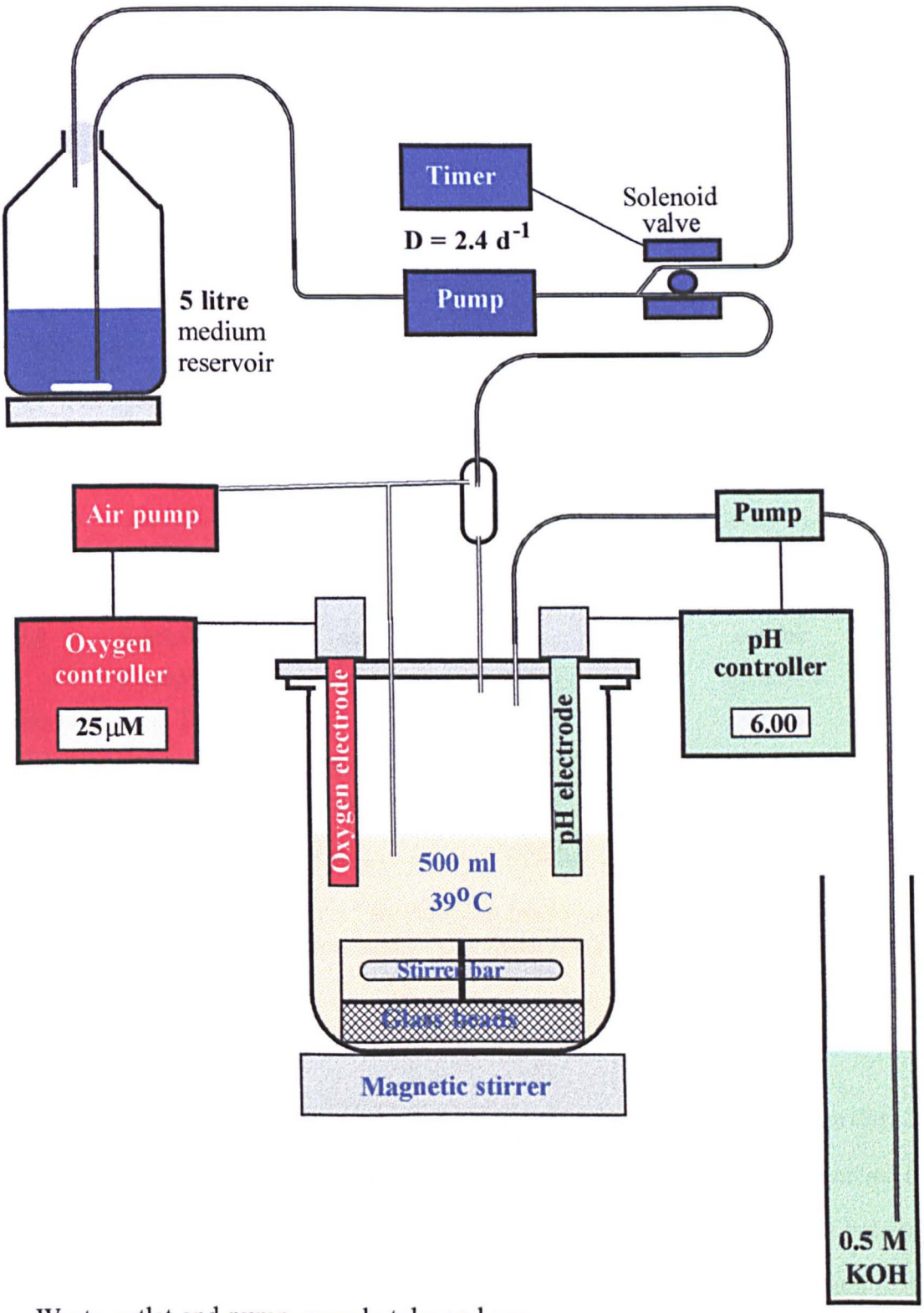
3.3.2 Stabilisation of the fermentation system

The fermentation vessel was inoculated with fresh porcine faecal material diluted 1 : 1 in MRD. The stabilising medium detailed in **Section 3.2.3 (I)** was connected to the system and pumped round the tubing to prevent the components settling out. When a fresh pot of media was connected to the system, vitamin solution (1ml l^{-1}) and trace element solution (2 ml l^{-1}) were added to the media via a 0.2 μm syringe filter. Twice a day the solenoid switched the valve and the medium was pumped into the fermentation vessel at a rate of 2.4 dilutions/day. At the same time, excess medium and spent bacterial cells were pumped out of the system and collected in a waste pot. The fermentation system was left to run for four days prior to the start of sampling. This stabilising was also carried out between the testing of each starch, when the system was also inoculated with fresh faecal material.

3.3.3 Experimental procedure

After the four day stabilising, the fermenter medium detailed in **Section 3.2.3 (II)** was connected to the system. Once a day prior to sampling, approximately 75ml of pancreatin-digested mixture was added to the fermenter vessel manually, via a hole in the top. A sample was taken from the fermentation vessel prior to the addition of the pancreatin digested mixture, and at 1, 2, 5, 7 and 24 hours after feeding. These samples were analysed for SCFA using the method outlined in **Chapter 2, Section 2.3.3.** Samples were also taken prior to feeding and 5 hours after feeding and used for bacterial analysis. KOH and O_2 readings were also taken 2, 7 and 24 hours after feeding.

Figure 3 :-
Schematic diagram of the single-stage fermenter system.



Waste outlet and pump, sample tube and gas outlet tube have been omitted for clarity.

	Sampling times (hours)					
Hours after feeding	0	1	2	5	7	24
SCFA	*	*	*	*	*	*
Bacterial counts	*			*		
KOH			*		*	*
O_2			*		*	*

3.3.3.1 Bacterial analysis of samples

Samples were serially diluted to 10^{-8} using MRD. Each sample was plated using a modification of the Miles & Misra (1938) method. Samples were plated out either on the bench, or where appropriate, in an anaerobic cabinet. All incubations were carried out at 39°C. The agars were subjected to the following conditions :-

	Conditions		
	Bacterial species	**Incubation time**	**Gas phase**
MAC	Coliforms	24h	aerobic
MRS	*Lactobacillus* spp. (aer)	48h	aerobic
	Lactobacillus spp. (tot)	48h	anaerobic
CBA	Total aerobes	48h	aerobic
WCBA	Total anaerobes	48h	anaerobic
BIF	*Bifidobacterium* spp.	4 days	anaerobic
KV	*Bacteroides* spp.	4 days	anaerobic

MRS aerobic = for the isolation of aerotolerant *Lactobacillus* spp., MRS anaerobic = for the isolation of total (aerotolerant and strictly anaerobic) *Lactobacillus* spp.

3.3.4 Statistical analysis

Each fermenter run was carried out over four days with the data obtained from days 2, 3 and 4 treated as a triplicate set. Statistical analysis of the triplicate data was carried out using one-way analysis of variance (ANOVA), where a comparison was made between each data set in a column. Mean and standard deviations were carried out on the data obtained using the MINITAB statistical package (Ryan *et al.* 1985) and are detailed in **Appendix 3.** Correlation co-efficients (r^2) were also calculated on data in **Table 3.1** to test for relationships between the bacterial fermentation products and the measured characteristics of the starches.

3.4 Results

Figures 3.1-3.16 show the bacterial counts of various species detected in the samples obtained from the single-stage fermentation system, prior to feeding and 5h after feeding a pancreatin-digested suspension containing one of five starches.

Figures 3.1 and 3.11 show the total aerobic and total anaerobic counts, respectively, obtained with each of the five starches. With all starches, the total anaerobic counts were higher than the total aerobic counts at the 5h postfeed sampling, but only in the case of the maize starch was the difference significant ($P<0.01$). The total anaerobic counts were also higher than the total aerobic counts in the samples prior to feeding with the maize and HAM starches, but only in the case of the maize starch was the difference significant ($P<0.01$). Little difference was detected in the total aerobic counts between the time zero and 5h postfeed samples. However, increases were observed in the total anaerobic counts, with the waxy maize, tapioca and potato starches, although these increases were not significant. There were no changes in the total anaerobic counts from the HAM and maize starch samples between the two sampling times.

Figure 3.12 shows the counts of coliforms obtained with each of the five starches from the single-stage fermenter system, using MacConkey agar. There appeared to be no change in the numbers of coliforms obtained from the 5h postfeed samples, when compared to the samples taken prior to feeding. The coliform counts obtained from the 5h postfeed maize and potato starch samples were significantly higher ($P<0.01$ and $P<0.05$ respectively) than those from the HAM starch, which resulted in the lowest count of coliforms at this sampling time.

Figures 3.13 and 3.14 show the counts of aerotolerant and total *Lactobacillus* spp. respectively, obtained from the single-stage fermentation system. The counts obtained from the time zero samples were similar for both groups of bacteria, as were those from the 5h postfeed samples. However, the aerotolerant *Lactobacillus* spp. counts obtained from the waxy maize time zero samples were higher than those from the total *Lactobacillus* spp., at the same sampling time. The highest counts for both groups

of bacteria were obtained throughout when the waxy maize starch was added to the fermenter system. The total *Lactobacillus* spp. counts (Fig. 3.14) obtained with the waxy maize starch were found to be significantly higher ($P<0.05$) than the counts obtained for the same bacterial group with the HAM starch, at both sampling times. However, the counts obtained from both groups of *Lactobacillus* spp. obtained from the HAM starch samples at 5h postfeed, were significantly higher ($P<0.01$) than those from the HAM starch time zero samples.

Figure 3.15 shows the counts of *Bifidobacterium* spp. obtained from the single-stage fermentation system when five different pancreatin-digested starches were added. An increase in *Bifidobacterium* numbers was observed with each of the five starches tested at the 5h post-feed sampling, when compared to the time zero samples. The only significant increase ($P<0.01$) in numbers between these two sampling times was observed with the tapioca starch. The largest increase in *Bifidobacterium* numbers between the two sampling times was observed with the waxy maize starch.

Figure 3.16 shows the counts of *Bacteroides* spp. which were obtained from the single-stage fermentation system when five different starches were added. The counts of *Bacteroides* spp. obtained from the waxy maize and HAM starches appeared to decrease slightly from the time zero to the 5h postfeed samples. The 5h postfeed samples from the maize starch appeared to show an increase in numbers of *Bacteroides* spp. of approximately 1 order of magnitude compared to the time zero samples. The *Bacteroides* spp. counts from the potato and tapioca samples remained unchanged at the 5h postfeed sampling, compared to the sampling at time zero. The counts of *Bacteroides* spp. from the 5h postfeed waxy maize starch samples were significantly lower ($P<0.01$) than those from both the potato and tapioca starches.

Figures 3.2-3.26 show the individual short-chain fatty acids (SCFA) obtained from the single-stage fermentation system, when various starches were added. With the majority of the SCFA, the highest level was detected from each starch 7 hours after feeding,

apart from the maize starch which appeared to show peak production of SCFA 5 hours after feeding.

Figure 3.2 shows the levels of succinate detected in the samples from the single-stage fermentation system. The levels of succinate detected in the samples from each starch were very low, with the highest levels (0.42 mmol l^{-1}) being detected in the samples taken one hour after feeding.

Figure 3.21 shows the levels of lactate detected in the samples from the single-stage fermentation system. The highest level of lactate production were detected in the samples taken two hours after feeding. The highest level of lactate detected in the 2h postfeed samples was detected in the samples using waxy maize starch. This level was significantly higher ($P<0.01$) than the lactate levels detected from any other starch at this time. No lactate was detected throughout in the samples from the potato starch. In the 24h postfeed samples, tapioca was the only starch in which lactate was detected.

Figures 3.22 and 3.23 show the levels of acetate and propionate respectively, detected in the samples from the single-stage fermentation system. With both SCFA, a similar pattern of production was observed, with the peak output for each starch detected in the 7h postfeed samples. However, the levels of acetate detected from the samples were higher than the levels of propionate. The highest levels of acetate and propionate were obtained from the waxy maize and tapioca starches. The lowest levels of acetate and propionate were from the HAM starch, which was significantly lower ($P<0.05$) than the levels obtained from the waxy maize starch. The levels of acetate and propionate detected from the 7h postfeed maize, waxy maize and HAM samples were significantly higher ($P<0.01$; $P<0.05$ waxy maize propionate) than the levels detected in the time zero samples. The levels of acetate were still significantly higher than the time zero samples at the 24h postfeed testing, but only for the maize ($P<0.01$) and waxy maize ($P<0.05$) starches. Both acetate and propionate levels were still detected in the 24h postfeed samples, with the highest levels being from the waxy maize and tapioca starches, respectively.

Figure 3.24 shows the levels of butyrate detected from the single-stage fermentation system when different pancreatin-digested starches were added. There appeared to be greater differences in the levels of butyrate produced from each of the starches. The lowest levels at the 7h postfeed sampling (peak output) i.e. <10 mmol l^{-1}, were detected from the HAM and tapioca starches. The maize and potato starch samples showed a butyrate production of no higher than 25 mmol l^{-1} at the time of peak output. The highest levels were detected throughout from the waxy maize starch samples, with the levels of butyrate from this starch being significantly higher ($P<0.01$) than from any other starch at the 7h postfeed sampling. With the maize, waxy maize and potato starches, butyrate levels of between 15-20 mmol l^{-1} were detected in the samples taken 24 hours after feeding.

Figure 3.25 shows the levels of valerate detected in the samples from the single-stage fermentation system. The levels detected from each starch were similar throughout the sampling period, not reaching higher than 15 mmol l^{-1}. However, the valerate levels detected from the waxy maize starch at the time of peak output, were significantly higher ($P<0.01$), at almost 3 times the levels detected from the other starches at this time. The levels of valerate detected in this starch were still significantly higher ($P<0.01$) than the levels detected from any other starch, 24 hours after feeding.

Figure 3.26 shows the levels of isoacids (isobutyrate and isovalerate) detected in the samples from the single-stage fermentation system. The highest level throughout was detected in the samples from the waxy maize starch, which produced significantly higher ($P<0.01$) levels than the maize and HAM starches at the 5, 7 and 24h samplings.

Figures 3.3-3.35 show comparisons made between the various measured characteristics of the starch granules and the SCFA data. Only the characteristics of the maize, waxy maize, and HAM starches have been compared. The comparisons were made using the SCFA data from the periods of peak output (mainly 5 and 7h postfeed, but 2h postfeed for the comparisons using lactate).

Figures 3.3-3.32 show the comparisons made between the reported blue values (measure of amylose content) of the starch and butyrate, total SCFA and lactate levels respectively. In all cases, there was a negative correlation between the total blue value and the total production of SCFA.

Figures 3.33-3.35 show the comparisons made between the reported starch damage for each starch and the level of total SCFA, lactate and isoacids produced respectively. The graphs indicate that as the level of starch damage increases, the production of each type of SCFA increases.

Figures 3.4-3.44 show the cumulative levels of 0.5M KOH required to maintain the pH of the single-stage fermentation system at 6.0, when various pancreatin-digested starches were added. Readings were taken at 2, 7 and 24h postfeed, over a four day feeding period. Each step in the graph represents one day of sampling.

Figure 3.4 shows the usage of KOH over the four day sampling period when waxy maize starch was added to the fermentation system. The graph indicates that in the first two days, approximately 100ml of KOH is added to the fermenter vessel by 2h postfeed. All of the KOH required was added within the first 7h postfeed each day, with no KOH required between the 7 and 24h postfeed samples. A change in the pattern of KOH requirement was observed after the second day of feeding this starch. The levels of KOH usage appeared to increase slightly over the four day sampling period.

Figure 3.41 shows the cumulative levels of 0.5M KOH required over the four day sampling period, when HAM starch was added to the fermenter vessel. Only a small amount of KOH was required to maintain the pH in the first seven hours of each sampling day, but KOH was still required between the 7 and 24h samplings. The usage of KOH each day did not appear to increase over the four day sampling period.

Figure 3.42 shows the addition of 0.5M KOH over the four day sampling period when maize starch was added to the fermenter vessel. KOH requirement over the four day sampling period was considerably less than with waxy maize starch, as was the

KOH usage each day. The majority of KOH was required within the first seven hours after feeding, with little or no addition of KOH between the 7 and 24h samplings.

Figure 3.43 shows the cumulative addition of 0.5M KOH to the single-stage fermentation system when tapioca was added to the vessel. The majority of KOH usage was used in the first 7h postfeed each day, with addition of KOH after 7h postfeed observed on day 2 only. The cumulative level of KOH over the four day period reached 440ml, which was higher than the cumulative KOH usage from waxy maize.

Figure 3.44 shows the cumulative KOH usage over four days when potato starch was added to the fermenter vessel. The majority of KOH usage was observed within the first 7h postfeed. The level of KOH required on day one of sampling was very low, but the level added appeared to increase steadily over the four day sampling period.

Table 3.1 shows the correlation co-efficients calculated from comparisons between the SCFA data and three of the measured characteristics from the starches. A correlation of r^2=0.999 was observed between the blue value and the production of lactate at 2h; while correlations of r^2=0.969 and r^2=0.997 was observed with the production of butyrate at 5h and 7h respectively, and correlations of r^2=0.993 and r^2=0.905 was observed with the total amount of SCFA detected in the 5 and 7h samples respectively. There appeared to be no correlation between total blue value and isoacid production. The best correlation (r^2=0.997 and r^2=0.882) with starch damage, was observed with propionate production at 5 and 7h postfeed respectively. Correlations between starch damage with butyrate and total SCFA production at the 7h postfeed sample, produced r^2 values of 0.948 and 0.999 respectively. There appeared to be no correlation between the amount of starch damage and acetate production (r^2=0.175 and r^2=0.777). With the surface lipid characteristic the best correlation was observed with the butyrate (r^2=0.975) and total SCFA (r^2=0.989) at the 7h postfeed sample.

Table 3.2 shows the rate of KOH usage for the first 2.5 hours after feeding. The table shows that the fastest rate of usage over the four days was observed with the waxy

maize starch, and the slowest rate was observed with the potato starch. The rate of KOH usage appeared to alter slightly over the four day sampling period with each of the starches.

Table 3.3 shows the ratios of Lactobacillus : coliforms, determined from the bacterial counts, (using the counts of the total *Lactobacillus* spp.) at both the time zero and 5h postfeed samplings. All starches showed an increase in the ratio of Lactobacillus : coliform bacteria at the 5h postfeed sampling, compared to the ratios at the time zero sampling, with that from the HAM starch being significant ($P<0.05$). The highest ratio at the 5h postfeed sampling compared to the time zero sample, was observed with the waxy maize starch. However, the largest increase between the two sampling times was observed with the HAM starch, where the ratio increased approximately 10 times.

Figure 3.1 :-
Total aerobic counts (cfu ml^{-1}) on CBA agar at time zero and 5 hours postfeed from the single-stage fermentation system, when various starches were added to the media.

Figure 3.11 :-
Total anaerobic counts (cfu ml^{-1}) on WCBA at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

Figure 3.12 :-
Counts of coliforms (cfu ml^{-1}) on MacConkey agar at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

Figure 3.13 :-
Counts of *Lactobacillus* spp. (aerotolerant) (cfu ml^{-1}) on MRS agar at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

Figure 3.14 :-
Counts of *Lactobacillus* spp. (total) (cfu ml^{-1}) on MRS agar at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

Figure 3.15 :-
Counts of *Bifidobacterium* spp. (cfu ml^{-1}) on BIF agar at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

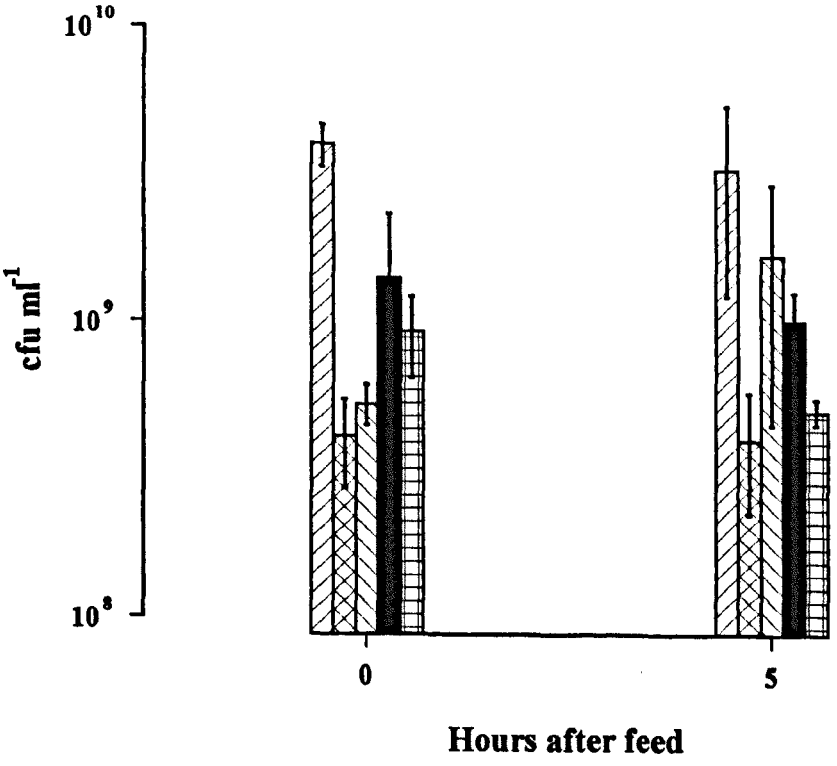
Figure 3.16 :-
Counts of *Bacteroides* spp. (cfu ml^{-1}) on KV agar at time zero and 5 hours postfeed from the single-stage fermentation system, when different starches were added to the media.

For all above figures the following key applies :-

▨	**Waxy maize**
▩	**High amylose maize**
▧	**Maize**
■	**Tapioca**
▦	**potato**

Data represent the mean ± SEM of triplicate determinations.

Figure 3.1 :-



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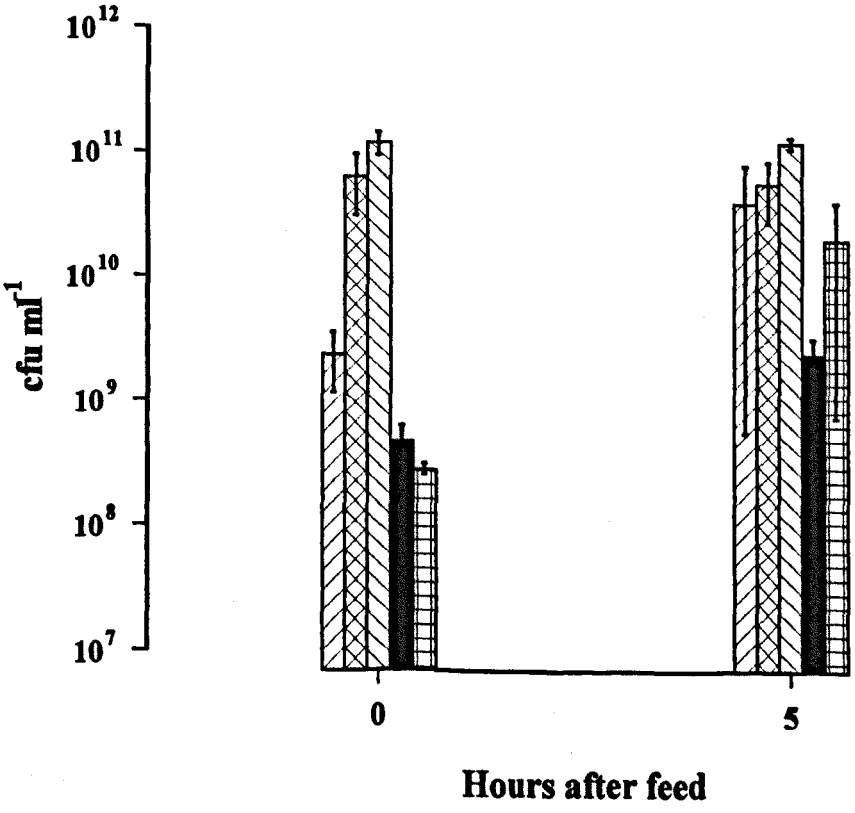

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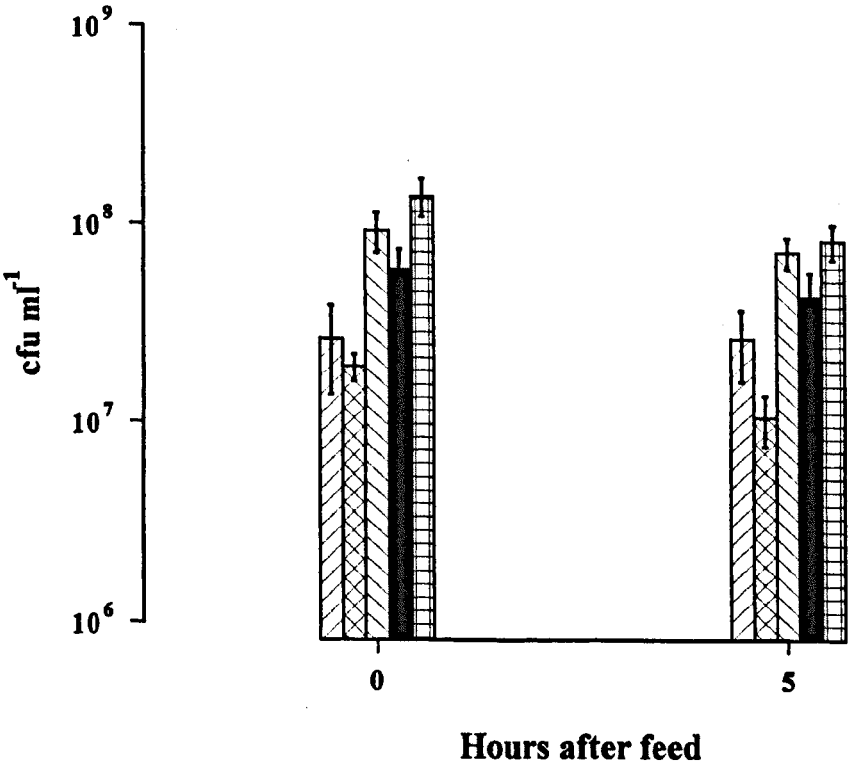

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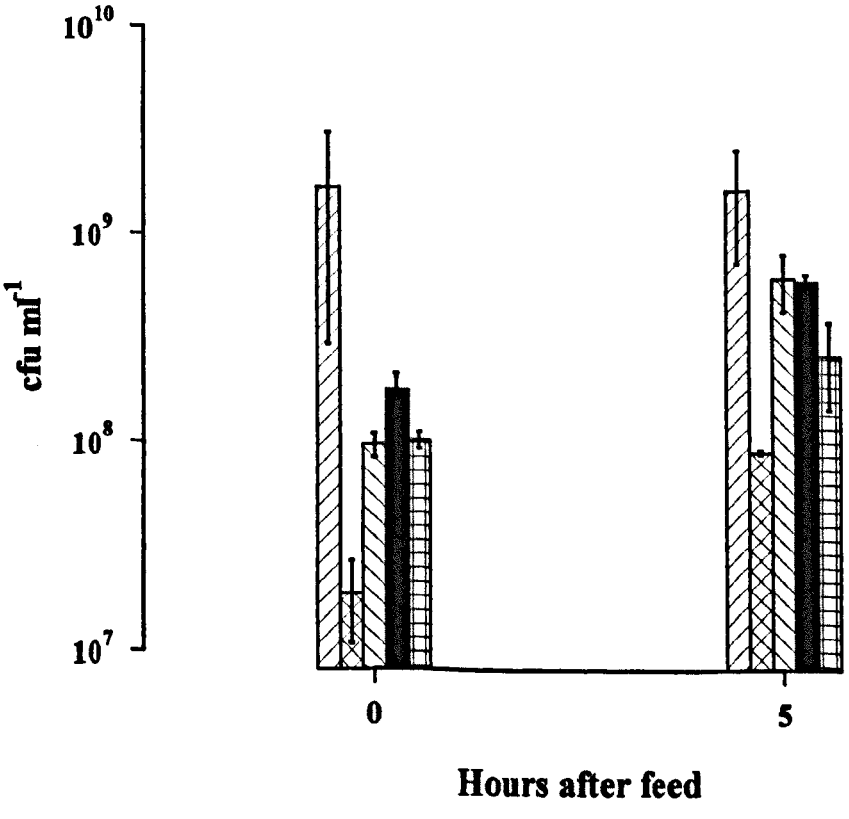

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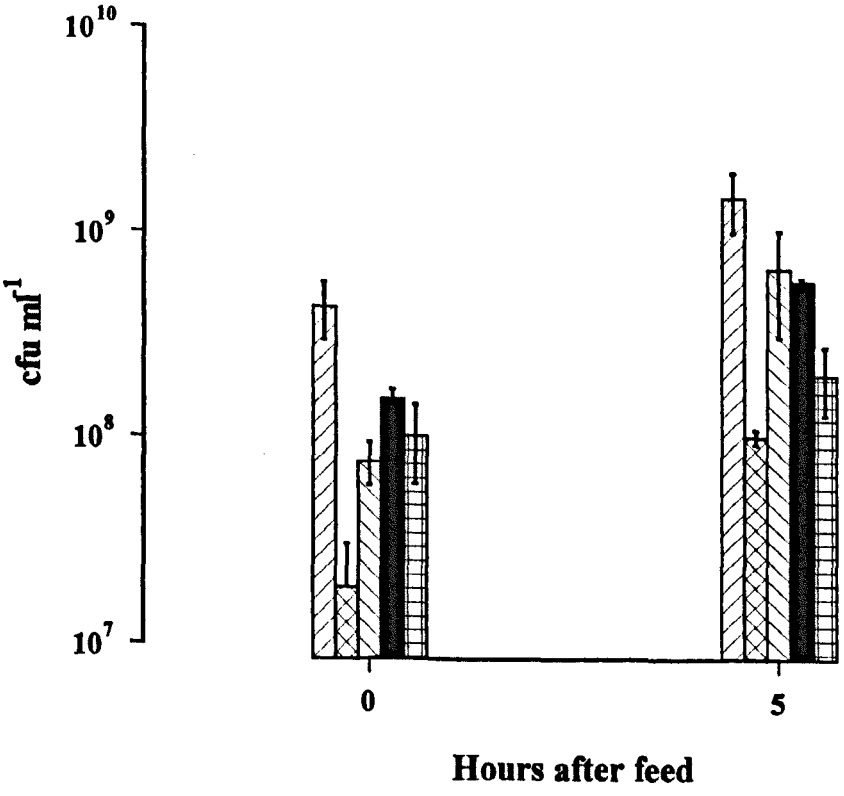

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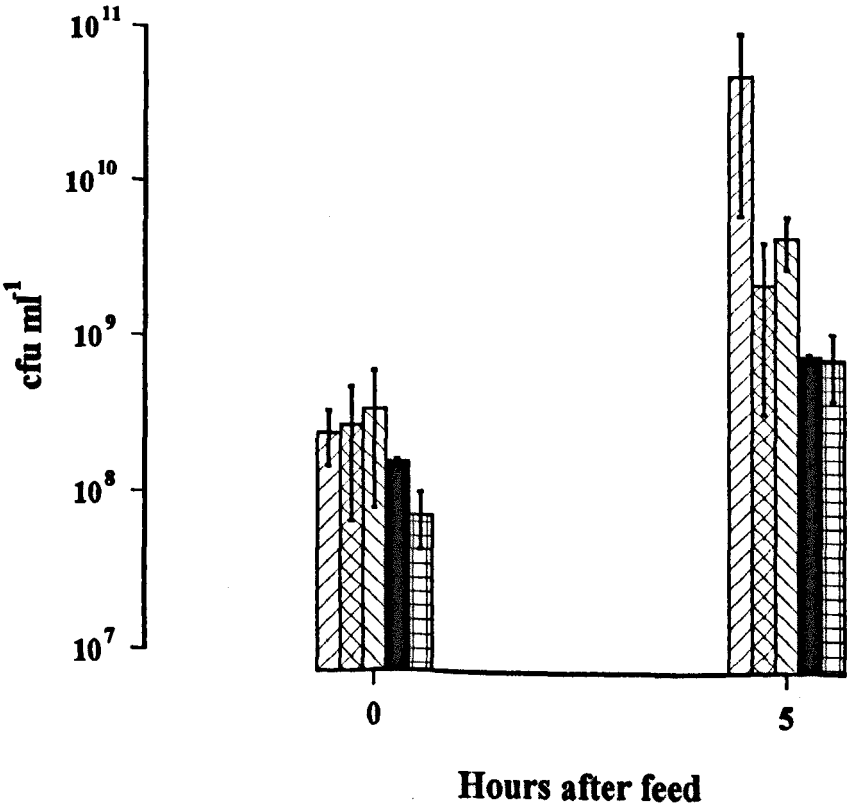

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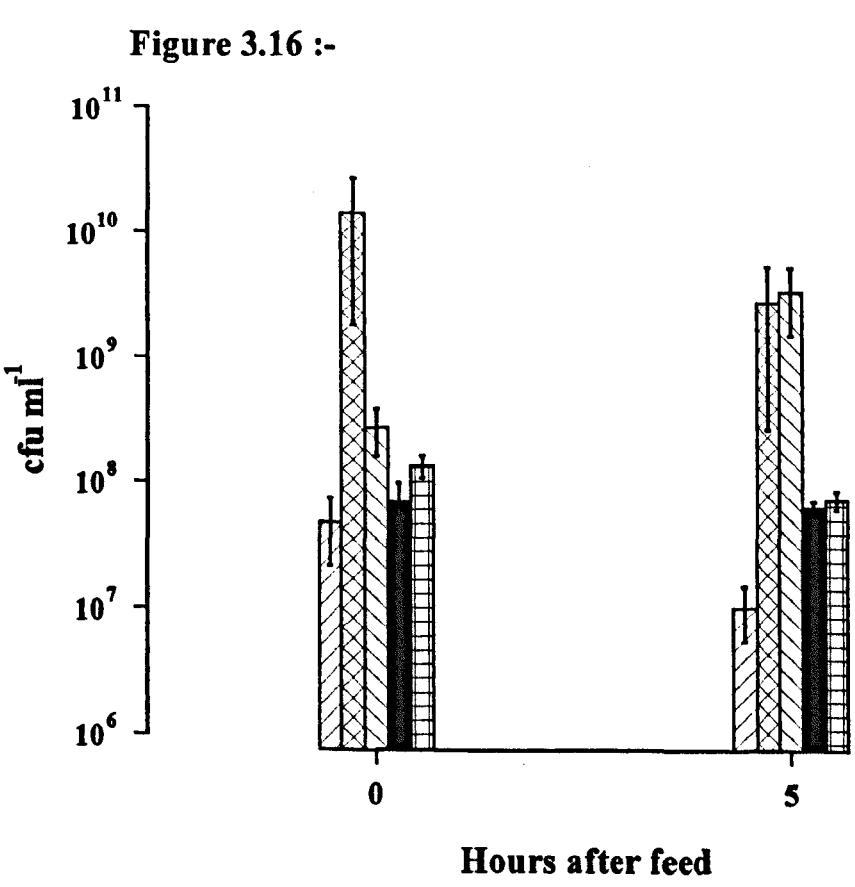


Figure 3.2 :-
Production of succinate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

Figure 3.21 :-
Production of lactate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

Figure 3.22 :-
Production of acetate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

Figure 3.23 :-
Production of propionate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

Figure 3.24 :-
Production of butyrate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

Figure 3.25 :-
Production of valerate (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

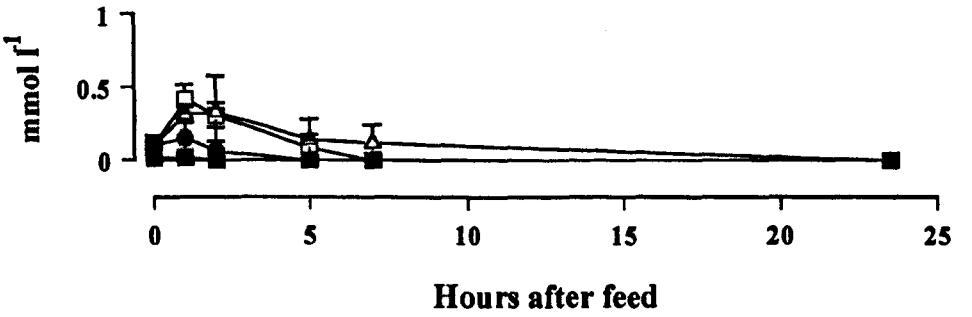
Figure 3.26 :-
Production of isoacids (isobutyrate and isovalerate) (mmol l^{-1}) from the single-stage fermentation system at various time intervals postfeed, when different starches were added to the media.

For all above figures the following key applies :-

●	Waxy maize
■	High amylose maize
▲	Maize
□	Tapioca
Δ	potato

Data represent the mean ± SEM of triplicate determinations.

Figure 3.2 :-



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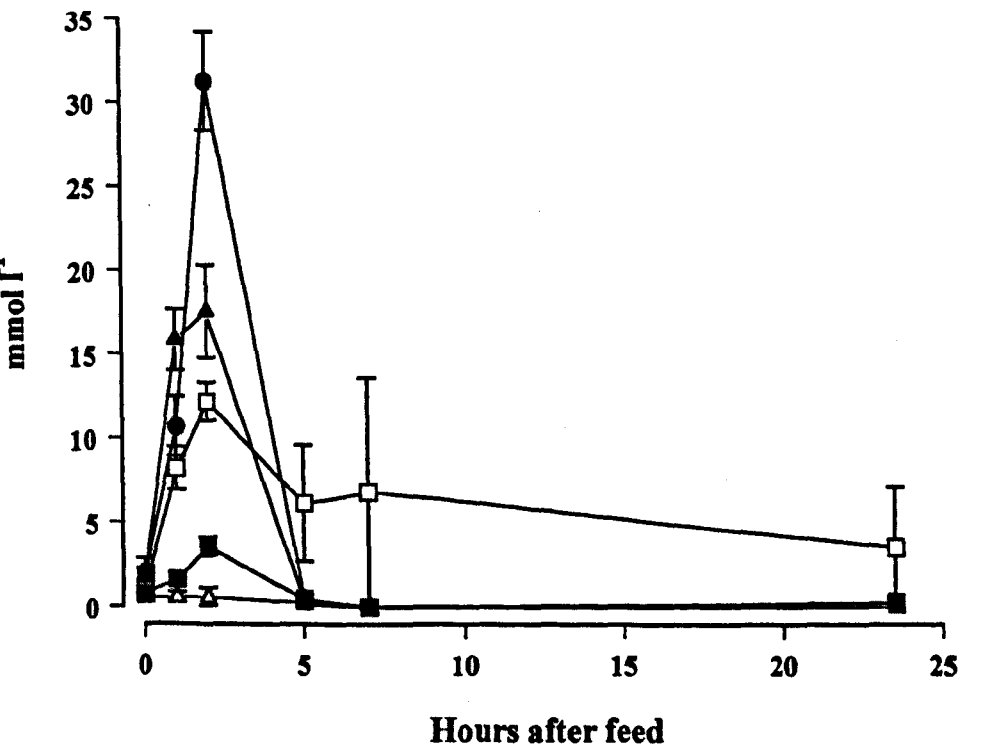

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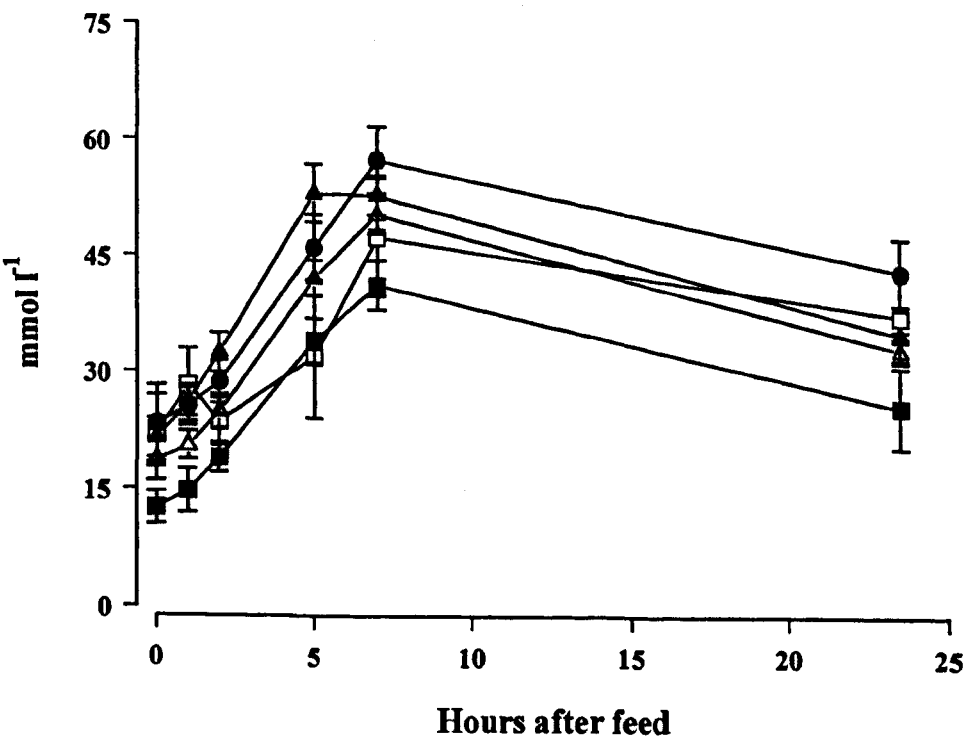

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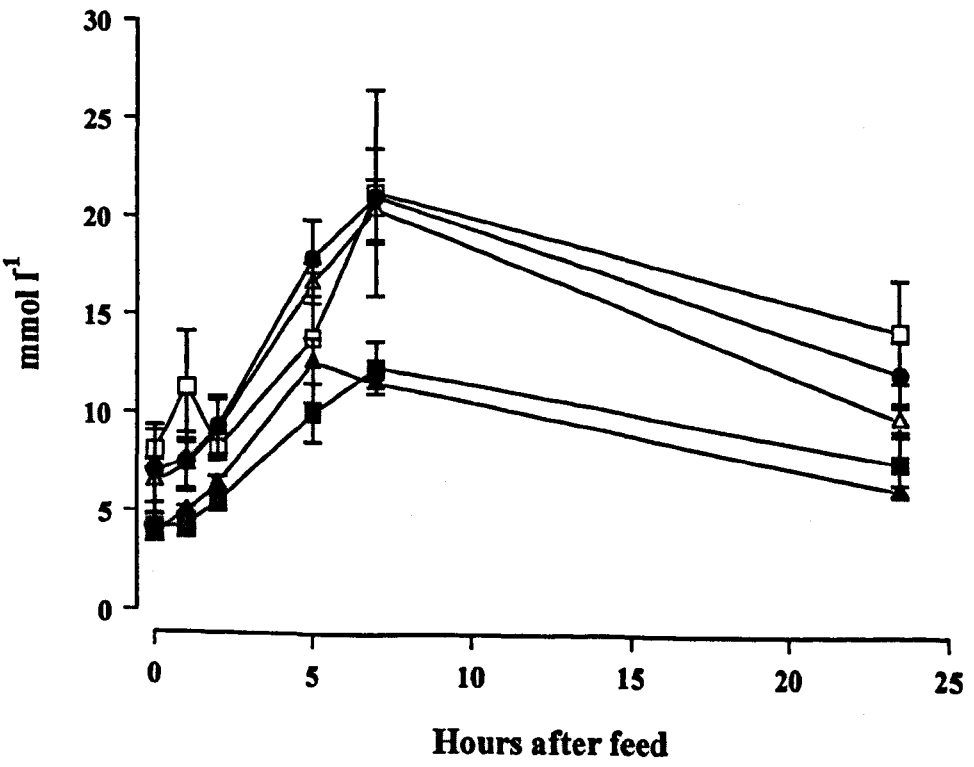

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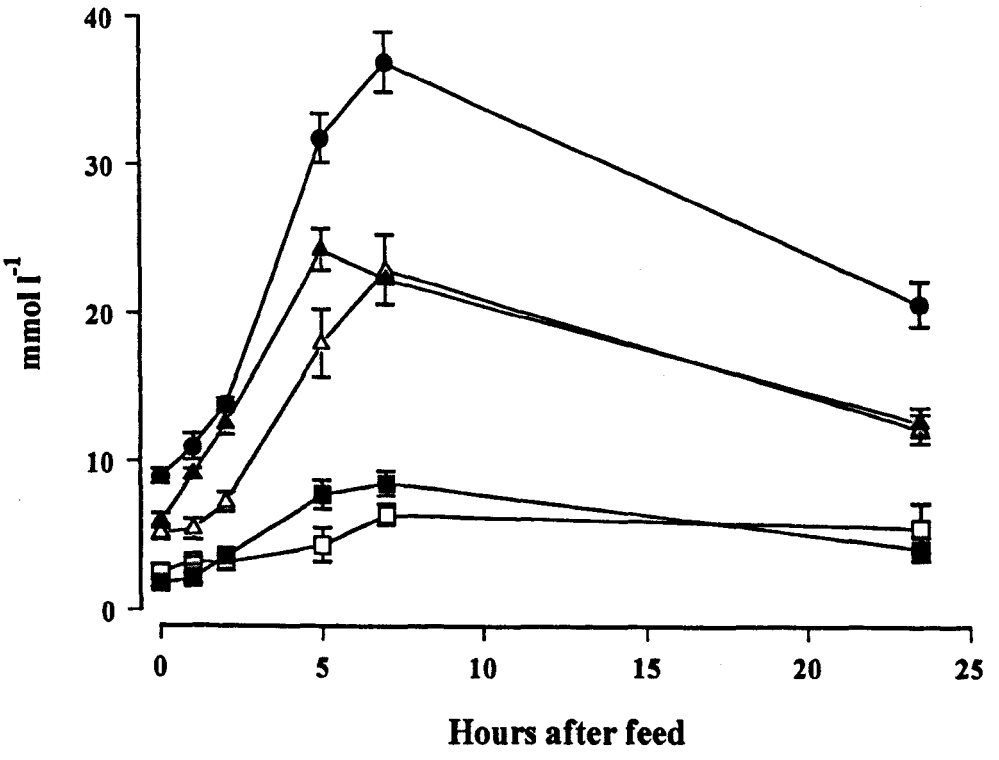

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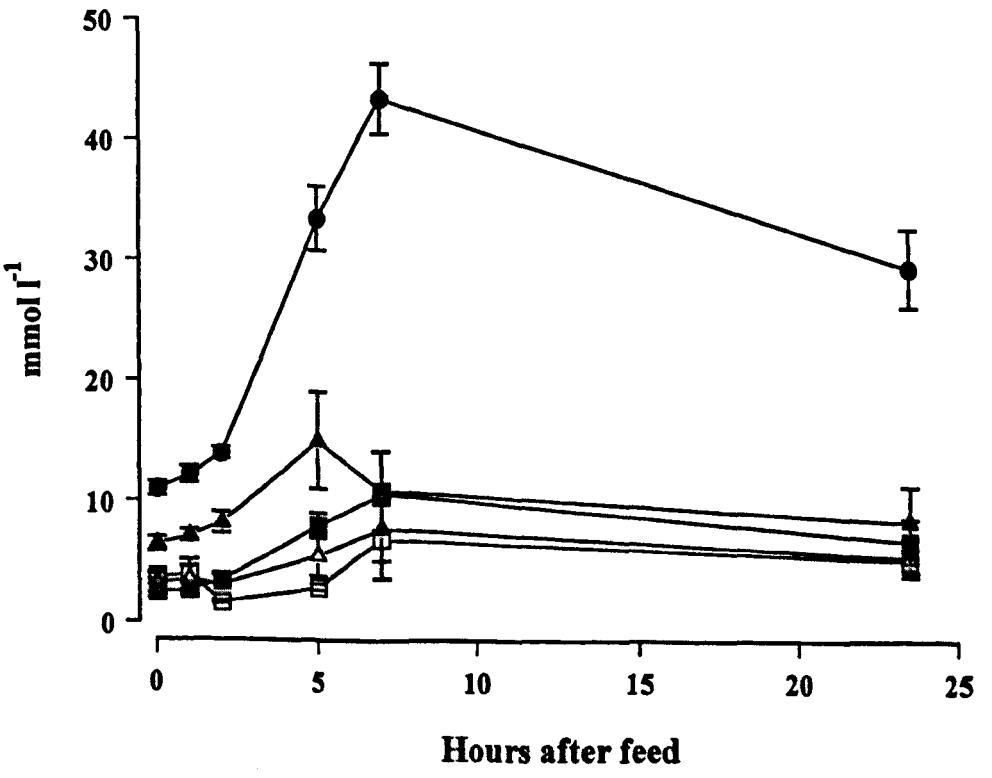

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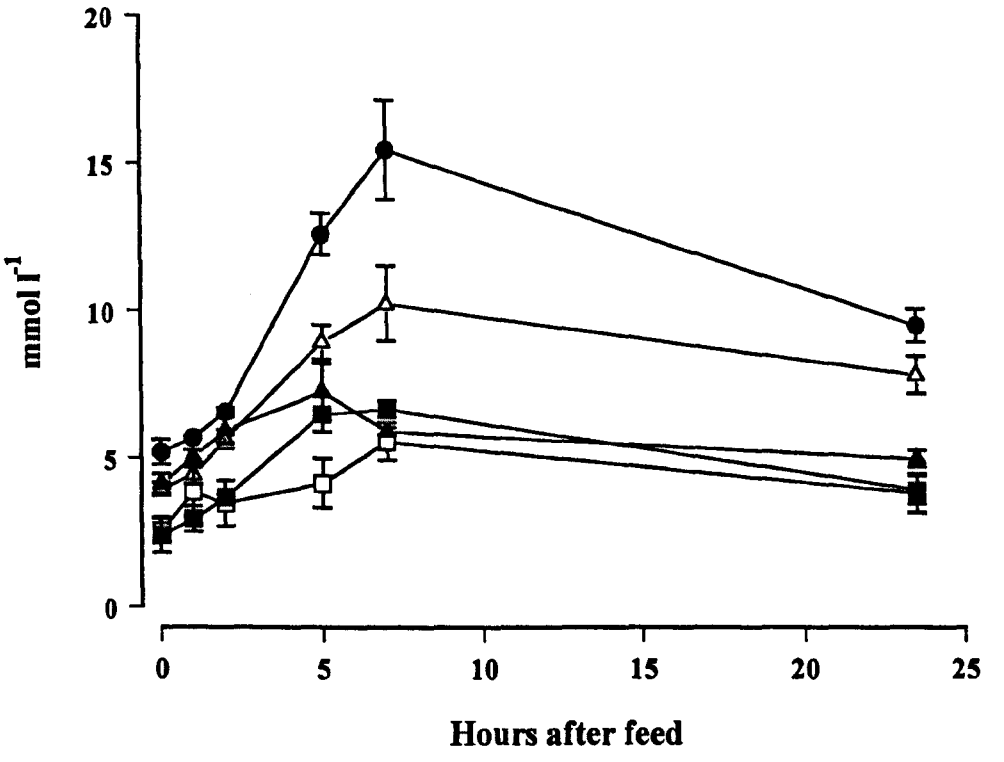


Figure 3.3 :-
Correlations between butyrate production (mmol l^{-1}) and the reported blue value of Waxy maize, Maize and HAM respectively, at 5 and 7 hours postfeed.

Figure 3.31 :-
Correlations between total SCFA production (mmol l^{-1}) and the reported blue value of Waxy maize, Maize and HAM respectively, at 5 and 7 hours postfeed.

Figure 3.32 :-
Correlations between lactate production (mmol l^{-1}) and the reported blue value of Waxy maize, Maize and HAM respctively, at 2 hours postfeed.

Figure 3.33 :-
Correlations between total SCFA production (mmol l^{-1}) and the reported starch damage of HAM, Maize and Waxy maize respectively, at 5 and 7 hours postfeed.

Figure 3.34 :-
Correlations between lactate production (mmol l^{-1}) and the reported starch damage of HAM, Maize and Waxy maize respectively, at 2 hours postfeed.

Figure 3.35 :-
Correlations between isoacid (isobutyrate and isovalerate) production (mmol l^{-1}) and the reported starch damage of HAM, Maize and Waxy maize respectively, at 5 and 7 hours postfeed.

For figures 3.3, 3.31, 3.33 and 3.35 the following key applies :-

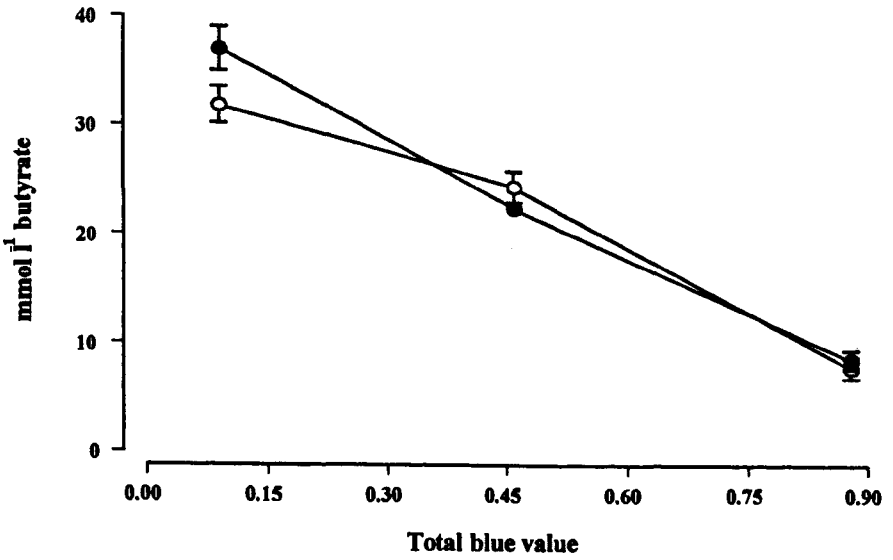
○	5 h postfeed
●	7 h postfeed

For figures 3.32 and 3.34 the following key applies :-

○	2 h postfeed

For all figures data represent the mean ± SEM of triplicate determinations.

Figure 3.3 :-



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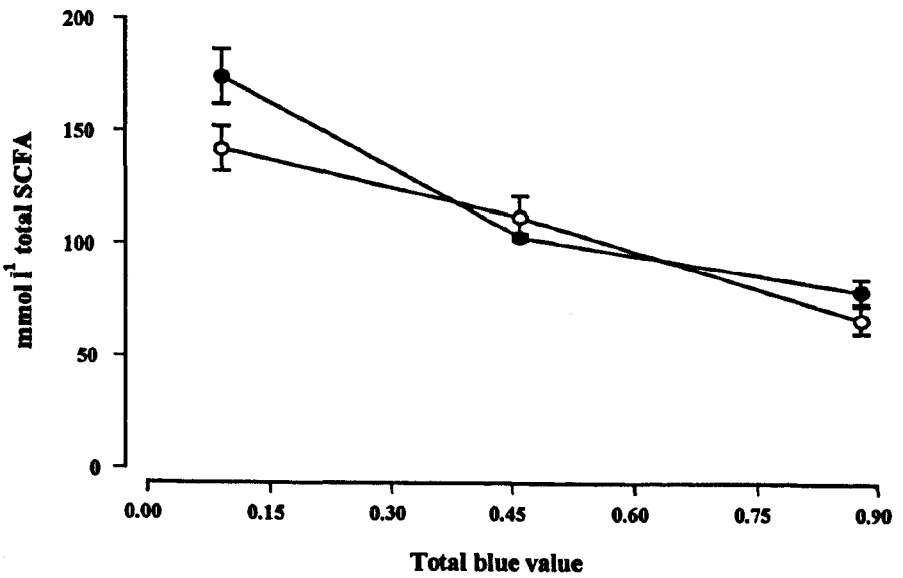

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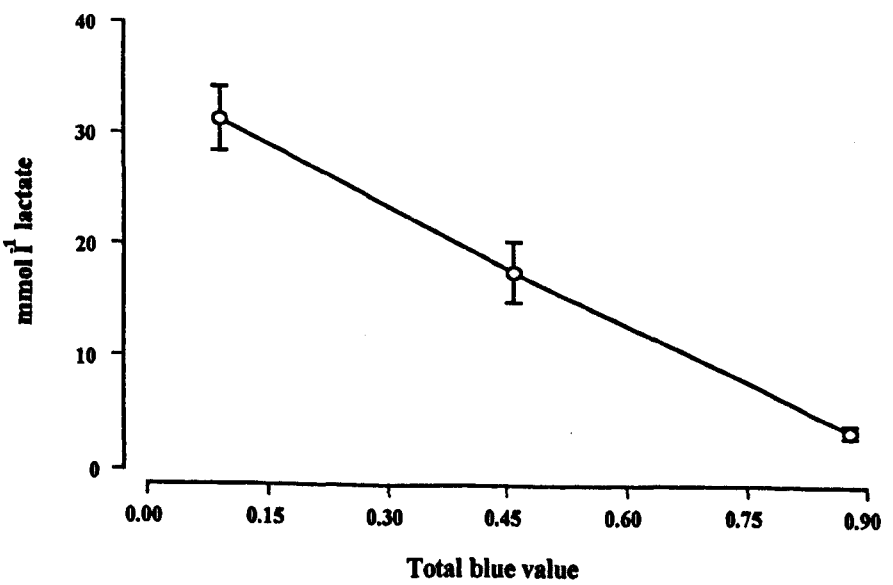

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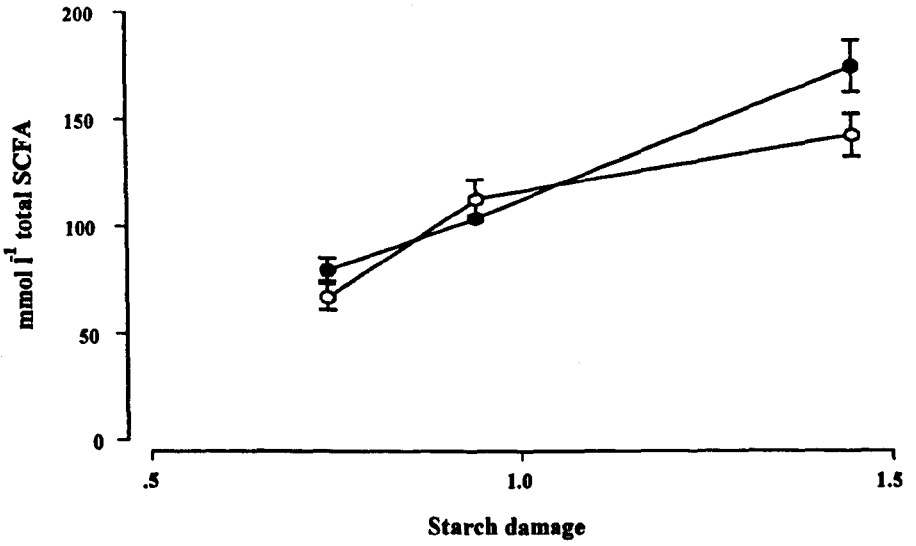

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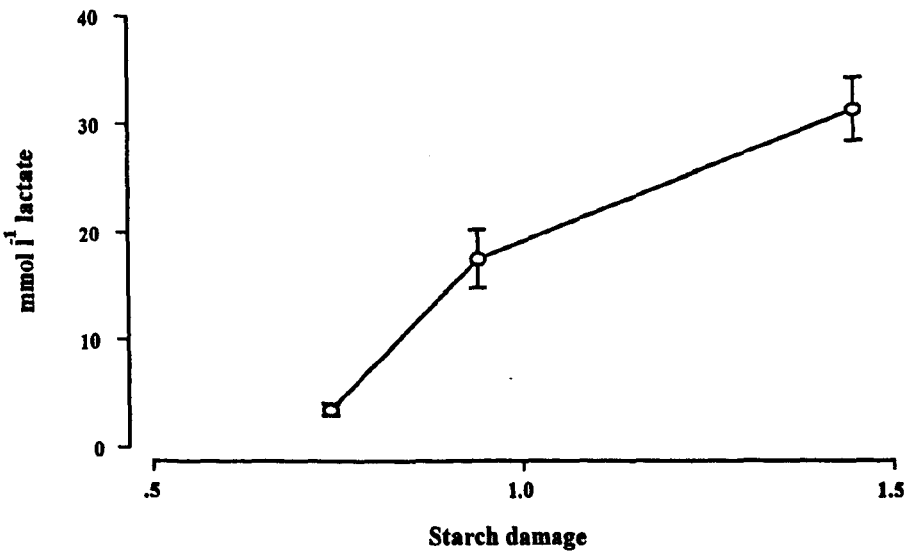

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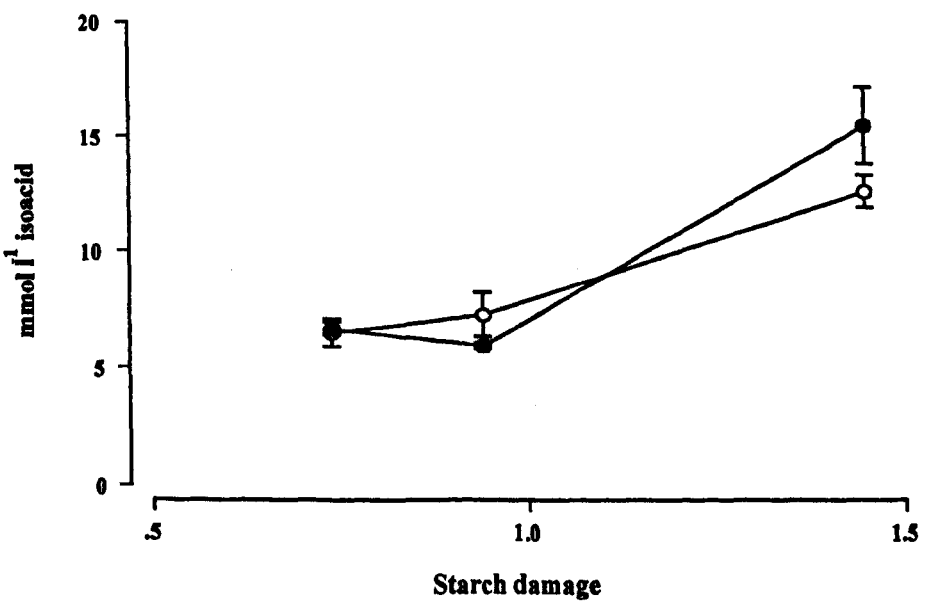


Figure 3.4 :-
KOH usage (ml) in the single-stage fermentation system over 4 days when native (untreated) waxy maize starch was added to the media.

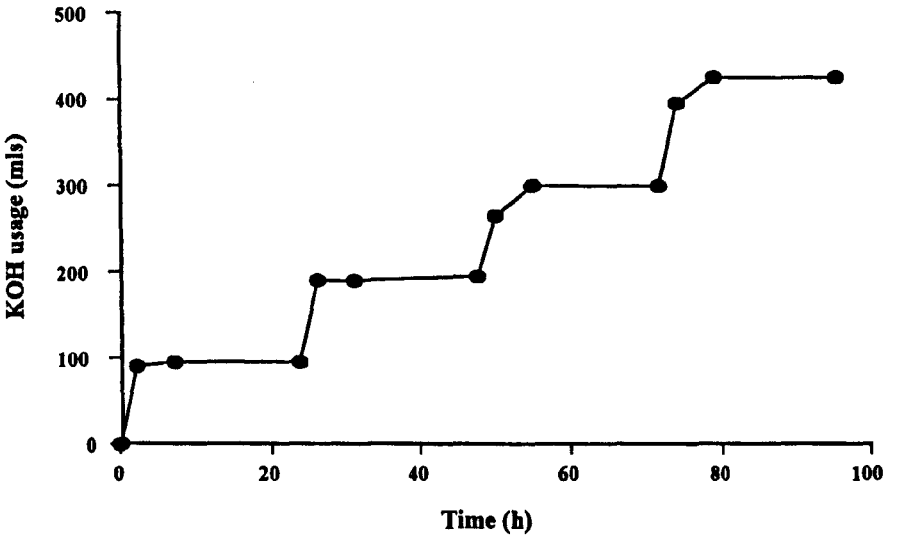
Figure 3.41 :-
KOH usage (ml) in the single-stage fermentation system over 4 days when native (untreated) high amylose maize was added to the media.

Figure 3.42 :-
KOH usage (ml) in the single-stage fermentation system over 4 days when native (untreated) maize starch was added to the media.

Figure 3.43 :-
KOH usage (ml) in the single-stage fermentation system over 4 days when native (untreated) tapioca starch was added to the media.

Figure 3.44 :-
KOH usage (ml) in the single-stage fermentation system over 4 days when native (untreated) potato starch was added to the media.

Figure 3.4 :-



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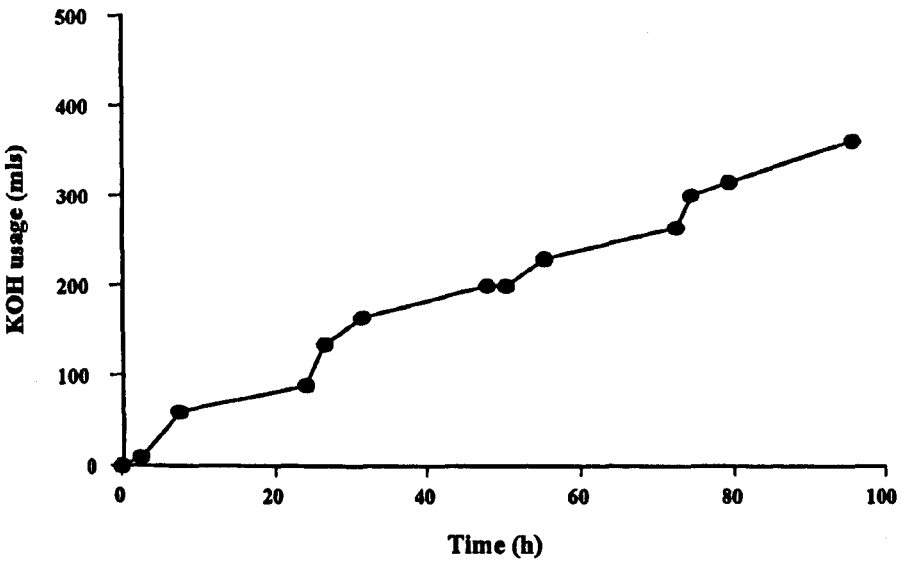


Figure 3.42 :-

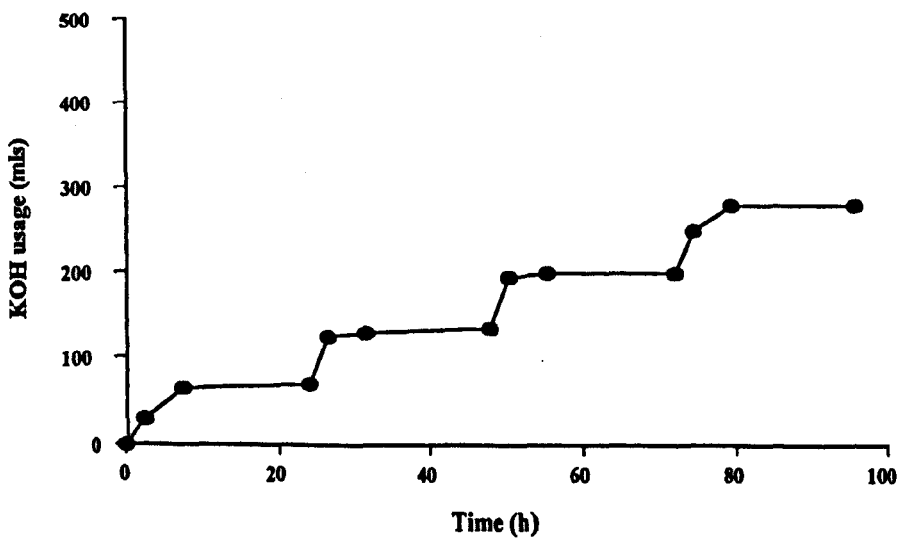


Figure 3.43 :-

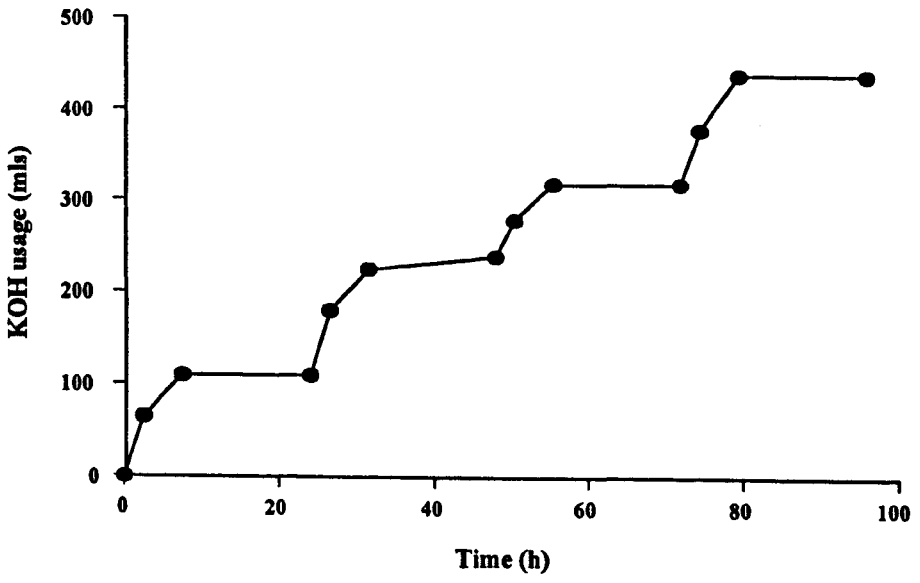


Figure 3.44 :-

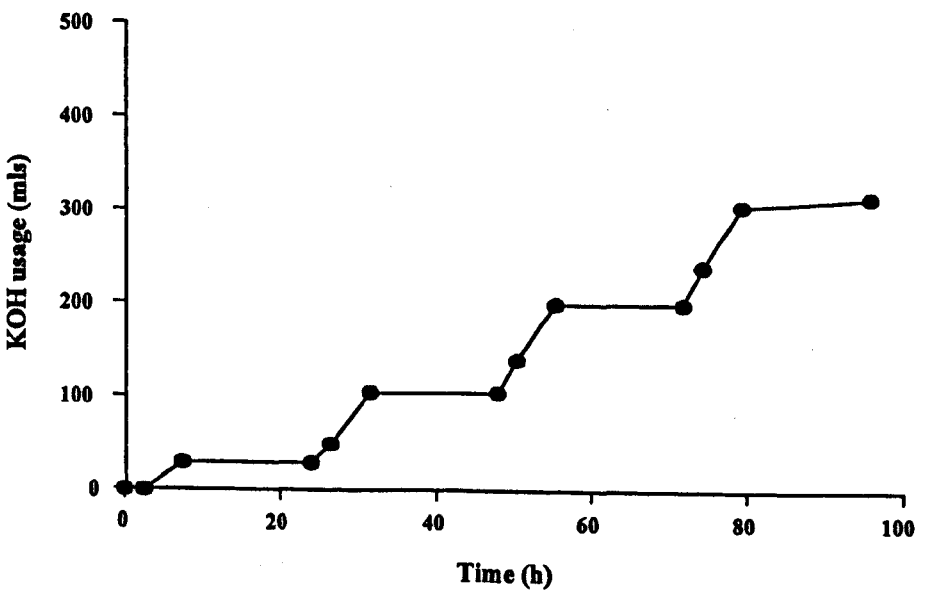


Table 3.1 :-

Correlation co-efficients (r^2) calculated using SCFA data and three parameters measured from the starch granules

	Parameter compared		
SCFA compared :-	**Total blue**	**Starch damage**	**Surface + true starch lipids**
Acetate - 5h postfeed	0.429	0.175	ND
Acetate - 7h postfeed	0.957	0.777	ND
Propionate - 5h postfeed	0.949	0.997	ND
Propionate - 7h postfeed	0.649	0.882	ND
Butyrate - 5h postfeed	0.969	0.803	0.857
Butyrate - 7h postfeed	0.997	0.948	0.975
Isoacids* - 5h postfeed	0.817	0.975	0.947
Isoacids* - 7h postfeed	0.651	0.884	0.832
Total SCFA - 5h postfeed	0.993	0.872	0.916
Total SCFA - 7h postfeed	0.905	0.999	0.989
Lactate - 2h postfeed	0.999	0.938	ND

Data determined from that obtained in the maize, waxy maize and high amylose maize samples only and is based on averages from three consecutive sampling days. *Isoacids = the sum of isovalerate and isobutyrate detected in the samples; total blue value, starch damage and surface lipid estimation was carried out by Dr Andrew Lynn at S.A.C. Auchincruive; ND = Not determined.

Table 3.2 :-

Rate of starch fermentation for the first 2.5 hours after feeding measured by KOH usage (mls h^{-1})

	Starch Type				
Sample day	**Waxy maize**	**High amylose maize**	**Maize**	**Tapioca**	**Potato**
Day 1	40	4	12	26	0
Day 2	38	18	22	28	8
Day 3	28	0	24	16	14
Day 4	38	17.5	20	24	16

Data determined using gradient measurements from graphs where KOH usage (mls) was plotted against time (h). KOH used was at 0.5M concentration.

Table 3.3 :-

Lactobacillus : coliform ratio determined from bacterial counts obtained from the single-stage fermenter at time zero and 5h postfeed.

	Starch Type				
Time interval	**Waxy maize**	**High amylose maize**	**Maize**	**Tapioca**	**Potato**
zero (0h)	42.1	1.2	0.9	3.1	0.9
5h postfeed	84.3	10.6	8.1	16.4	3.2

Data represents means of triplicate determinations

3.5 Discussion

Samples were taken from the single-stage fermentation system prior to feeding and five hours after feeding pancreatin-digested starches, and analysed for counts of various bacterial species. Samples were not examined for bacterial counts after the 5h sampling and so it is not known whether or not the bacterial counts continued to change after this sampling period. The five starches which were added to the fermenter vessel had been subjected to treatment with acid and porcine pancreatin, which was carried out to simulate the conditions encountered as the starch passes from the stomach and through the small intestine, leaving a fraction comparable to that which enters the monogastric colon. The starches had not been subjected to any form of processing treatment such as heating and cooling, and therefore should not have been either gelatinised or retrograded, prior to digestion with pancreatin.

The five starches which were added to the single-stage fermentation system appeared to exert varying effects on the seven bacterial groups studied. The largest variations were observed with the *Bacteroides* spp., where each starch had a different effect on the numbers of this species. Increases in counts were observed at the 5h postfeed sampling with the total anaerobes, aerotolerant and total *Lactobacillus* spp., and the *Bifidobacterium* spp., where the largest increases were observed. These results suggest that the majority of the starches studied were available to these bacterial groups for fermentation once they had been digested by pancreatin. The potato starch appeared to enhance the increase in numbers of the total anaerobic population, whereas the waxy maize starch appeared to enhance the numbers of the *Bifidobacterium* population. These findings indicate that not all the bacterial species studied fermented the same starches, even when they were in the native (untreated) form. This may be indicative of the reactions of these bacterial species in the monogastric colon, when these starches are encountered, as this particular model of the colon has been shown to be a good simulation of the proximal region of the monogastric porcine colon (Khaddour *et al.* 1998). The *Bifidobacterium* spp. are amongst the chief anaerobic species in the monogastric gut, comprising as much as 25% of the faecal bacteria (Macfarlane & Macfarlane, 1997). Therefore, if the findings that the *Bifidobacterium* spp. are stimulated by the waxy maize starch (i.e. high in amylopectin) can be extrapolated to the gut, this may be significant. The pancreatin-digested starches did not

increase the numbers of the coliforms up to 5h postfeeding. If this is also indicative of events in the monogastric colon this would be desirable, as members of the coliform group such as *E.coli*, are known to exert certain effects on the gut which may be considered as harmful (Fuller & Gibson, 1997). Therefore, large increases in the numbers of this particular bacterial group may be a problem in the gut. The bacterial data also show that with each of the starches studied, the numbers of *Lactobacillus* spp. increased at the 5h postfeed sampling compared to that at the time zero sampling. The highest increase was observed with the HAM starch, and the highest proportion of *Lactobacillus* spp. were observed with the waxy maize starch. These findings show that the type of starch used can affect the population of *Lactobacillus* spp., with the HAM and waxy maize starches appearing to have an enhanced effect. The *Lactobacillus* population are important in the monogastric gut as they play a part in inhibiting the growth of pathogenic micro-organisms from exogenous sources (Fuller & Gibson, 1997). Certain species of lactobacilli have also been shown to suppress organisms which may be involved in the production of pre-carcinogens (Rafter, 1995). However, the numbers of these organisms are "quantitatively less important" (Wang & Gibson, 1993), therefore any dietary component which succeeds in increasing the numbers of Lactobacilli can be considered important.

The majority of the SCFA production was observed between the first 5 and 7h postfeed, which correlated with the time of most KOH usage i.e. highest level of fermentation and hence acid production. The increase in SCFA production also appeared to correlate with the increase in bacterial numbers, in most cases. Within the SCFA a similar pattern of production was observed, with an increase in production over the first 7h postfeed, followed mostly by a decrease in the level of production or a maintained level from the 7 to 24h postfeed sampling. The exceptions to this were observed with the productions of succinate and lactate, where rapid productions within 1 and 2h postfeed were observed, which then appeared to decrease just as rapidly. These patterns of SCFA production were observed by Macfarlane & Macfarlane (1993) when mixed populations of gut bacteria were grown on starch, in pH-controlled batch culture. In this particular system, samples were taken for 48 hours. They observed the stabilisation phase (levelling out) for each SCFA to be between 24 and 48 hours, and not prior to 24 hours as in the present

experiment. However, some levelling out was observed before 24 hours in the Macfarlane & Macfarlane (1993) system. The starches studied in the present experiment appeared to be fermented more quickly than those studied by Macfarlane & Macfarlane (1993), as noted by the major production of SCFA no later than 7h postfeed. These differences may be explained by the treatment with pancreatin received by the starches in the present experiment, prior to feeding. This treatment may have allowed the bacteria easier access and hence a faster fermentation, either by stripping away material which is more difficult for the bacteria to break through, or by producing pits or holes in the starch granules which can be invaded by the bacteria. The continuous fermenter system employed in the present experiment allowed the washing out of spent media and bacterial cells which would not have occurred in the batch culture system of Macfarlane & Macfarlane (1993). This may have had a bearing on the results. High productions of butyrate were observed by Macfarlane & Macfarlane (1993) with their starch, as was observed in the present experiment. Large proportions of butyrate have been reported by others when starch was used as the carbohydrate source for human faecal bacteria (Englyst *et al.* 1987; Scheppach *et al.* 1988; Macfarlane & Macfarlane, 1993). A similar pattern of lactate production was observed in the present experiment as compared to that from Macfarlane & Macfarlane (1993) i.e. a rapid initial production followed by a decrease, almost as rapidly. However, in the Macfarlane & Macfarlane (1993) model, the decrease in lactate was found to occur more slowly. This pattern of lactate production will be due to the lactate being produced quickly and then converted to SCFA products by the bacterial population present (Macfarlane & Macfarlane, 1993). This means that the lactate will still be produced throughout the sampling period, but it will not be detected at high levels due to it being further metabolised quickly. This explanation can also be applied to the pattern of succinate production, as succinate like lactate, is used as a hydrogen sink and is also further metabolised to propionate (Wolin & Miller, 1983; Cummings & Macfarlane, 1991; Macfarlane & Macfarlane, 1993). The overall amounts of the specific SCFA produced in the single-stage fermentation system i.e. acetate, propionate and butyrate, were also in a similar range to those found by Macfarlane & Macfarlane (1993), although it is not possible to make specific comparisons as the source of the starch which was studied in their system was not specified.

The "blue value" was a measurement carried out on the five starches studied to estimate the quantity of amylose in the starch, with a higher value indicating a higher level of amylose. The blue values were compared with the data from specific SCFA to determine if the level of amylose present in the starch was a factor in the bacterial breakdown and the SCFA resulting from this. For the purposes of this experiment, any r^2 value of 0.8 or above was considered indicative of a positive correlation. The levels of SCFA at the time of maximum production i.e. 5 and 7h postfeed for butyrate and total SCFA, and 2h postfeed for lactate, were used for the comparisons. Only the blue values from the maize, waxy maize and HAM starches were used, as these starches are all extracted from the same botanical source (maize), and hence the complication of comparing cereal starches with root starches was avoided. With each of the blue value correlation graphs, as the level of amylose increased, the amount of the specific SCFA produced decreased. The best correlation was observed with the lactate production, where a doubling of the blue value led to a reduction of the lactate concentration by half. This result was supported by a correlation co-efficient (r^2) of 0.999. This suggests that the production of lactate may be dependent on the amylopectin portion of the starch i.e. the high amylopectin starches, such as waxy maize, may enhance the production of lactate. This may be expected as the production of lactate by the bacteria present was rapid, suggesting that the source of this production must have been readily available and easy to degrade. The outside of starch granules have been shown to be covered with protruding chains of amylopectin (as well as amylose) (Lineback, 1984) which may be the site of initial α-amylase attack (Stark & Yin, 1986). If this is correct, the porcine pancreatin may have contributed to the initial breakdown of the starch at this point and may not have completely degraded the amylopectin on the surface. This could leave an initial readily available source for bacterial fermentation. This may be possible as the starches were in the native form and so the protruding branches will still be on the surface. A more likely explanation is that the porcine pancreatin has opened the starch granules sufficiently to allow bacterial access, where amylose and amylopectin are available in varying amounts, and starches with higher amounts of amylopectin may be fermented more quickly. This extends the theory that starches containing too much amylose may be more difficult for the bacteria to ferment.

The production of butyrate was also negatively correlated to the level of amylose present in the starch, with a correlation co-efficient (r^2) of 0.997 at the 7h postfeed sampling. This suggests that the production of butyrate is strongly influenced by the level of amylose present in the starch. This can also be observed in the SCFA data graphs, where the highest amounts of butyrate were obtained from starches such as waxy maize which contain virtually no amylose. The correlation between blue value and total SCFA showed a negative correlation. These findings from the butyrate and total SCFA correlations suggest that if starches entering the gut contain very high levels of amylose, not only will the bacteria not be able to degrade them, but the production of butyrate (which is one of the major SCFA in the monogastric colon) and total SCFA will be decreased. The production of isoacids (isobutyrate and isovalerate) did not appear to correlate with the amount of amylose in the starch. This is as expected due to the isoacids being produced from the breakdown of proteins in the gut and not starch. Hence, the level of amylose in the starch should not have an effect on isoacid production (Macfarlane *et al.* 1992b). The production of acetate and propionate appeared to show changes in the correlations with the blue value when the 5 and 7h postfeed correlations were examined. The production of acetate did not appear to be influenced by the amylose content of the starch at the 5h postfeed sampling ($r^2 = 0.429$), but then did appear to correlate at the 7h postfeed sampling ($r^2 = 0.957$). This change may be due to the production of acetate in the early stages of the experiment arising from another source, such as from lactate or from acetogenesis (Wolin & Miller, 1993; Lojoie *et al.* 1988; Bernalier *et al.* 1996). Any production of acetate arising from the starch source at this time may have been overshadowed by that arising from these other sources. However, as this source started to deplete, the acetate production from the starch will be a main source of production and so it is likely that the amylose content will have a direct effect on this. With the production of propionate, the reverse was observed, with the amylose content having a direct effect on production at the 5h postfeed sampling, but not at the 7h postfeed sampling. This may be due to the initial production of propionate arising from the starch source while the carbohydrate is readily available, but then as this source starts to deplete, the production of propionate would arise from the bacterial metabolism of accumulated succinate. Therefore, the amylose content of the starch would not have a direct effect on propionate production at this time.

Starch damage is a physical process carried out on the starch granules, where they are subjected to enzymatic degradation and then examined under a microscope. A score is then given depending on the amount of starch damage which has occurred, with a higher score indicating that the starch is potentially more susceptible to enzyme attack. The correlation graphs between starch damage and specific SCFA indicate that as the score for starch damage increases the SCFA production (and hence fermentation) increases. The waxy maize starch appeared to have the highest starch damage score, and also appeared to result in the highest levels of SCFA production. This suggests that as this high amylopectin starch is more accessible to enzyme attack, it may also be more accessible to bacterial breakdown. The best correlations were observed with propionate at the 5h postfeed sampling and the total SCFA production at the 7h postfeed sampling. This result with the propionate reinforces the probability of the initial propionate production arising from the starch and not the conversion of succinate, which appears to occur later. These results also suggest that access to the starch is a major factor in its bacterial breakdown, particularly in the case of propionate and total SCFA production. The production of acetate did not appear to correlate with starch damage at either of the sampling times. As this is the major SCFA produced from the bacterial breakdown of starch, the results suggest that there may be a sufficient alternative supply of metabolites for the production of acetate so that bacterial access to the starch is not a major factor. The production of isoacids appeared to correlate with the degree of starch damage. A source of protein for degradation will be that associated with the starch components, and hence access into the starch granules may affect the production of isoacids in this way. The bacterial species which degrade proteins as an alternative energy source may not obtain glucose from the breakdown of starch as the major starch-degrading species may obtain this glucose first. This may mean that these other bacterial groups will switch to the breakdown of proteins to obtain their energy.

The correlations between surface lipids and specific SCFA show that the best correlations were obtained with the production of total SCFA. When the data were plotted (graph not shown) they showed that as the measurement of surface lipid increased, the production of SCFA decreased. This suggests that the lipids which are on the surface of the granules, and those associated with the major components, are major factors in determining

the amount of starch which is fermented by the bacteria. The total SCFA production appears to be affected by the amylose content, the starch damage score and the amount of lipids present, suggesting that all of the above are significant factors affecting the overall breakdown of starch by the bacterial population.

The cumulative KOH usage by the single-stage fermentation system was used as an indication of the extent of the fermentation taking place. The KOH usage was monitored over the four days of each fermenter run to determine whether or not there was a change in the breakdown pattern. The measurements of KOH usage were taken at the same times each day and so are comparable for each starch. The KOH usage graph for each starch appeared to show a different rate and extent of breakdown due to the differences in the requirements for KOH. The "step-pattern" of KOH usage for four of the five starches indicated that the majority of SCFA production (and KOH usage) was observed within the first 7h postfeeding. Between the 7 and 24h postfeed samplings, little or no KOH was added to the fermenter system. This indicated that most acid production was observed within the first 7 hours and little fermentation was carried out after this time. The KOH usage for the maize starch appeared to be low each day, over the four day sampling period, and also resulted in the lowest cumulative KOH usage over the sampling period. This may be due to the pancreatin treatment removing the fractions of the starch more easily degraded by the bacteria, leaving material which takes longer to degrade. The actual amount of KOH added to the fermenter per day with this starch appeared stable, at around 20ml. The tapioca starch appeared to require large amounts of KOH each day, and also appeared to require large amounts of KOH cumulatively, over the four day period. This suggests that this starch may be degraded to a greater extent by the bacteria, than the other starches, resulting in a higher production of SCFA. This may be due to the tapioca starch containing smaller granules, which may be easier to degrade by the bacteria. With the potato starch, the initial usage of KOH over the first two days was low, in the first 7h postfeed, but this appeared to increase over the next few days. This starch contains larger granules than the tapioca which may take longer for the bacteria to breakdown. However, the KOH usage for each day appeared to increase as both the graphs and the data in Table 3.2 shows, indicating that the bacteria may be adapting to the degradation of this particular

starch. The KOH usage for the HAM starch appears to de different from the pattern produced by the other starches, with the usage per day being low throughout the sampling period. Unlike the other four starches, the HAM starch appeared to show a continuation of fermentation between the 7 and 24h postfeed samplings. Table 3.2 shows that the actual rate of KOH usage for HAM starch was amongst the slowest observed out of the five starches. These results indicate that this starch which contains a higher than average amylose content is fermented more slowly and to a lesser extent than the other starches, particularly those which are lower in amylose such as waxy maize. The graphs of KOH usage for waxy maize starch show that for the first two days of sampling, this starch resulted in the fastest rate of KOH usage, with the majority of this usage being in the first 2h postfeed. This appeared to alter slightly over the remaining two days, with the KOH usage for each day increasing slightly. This particular starch appeared to show the highest usage of KOH each day, as shown in Table 3.2. The cumulative KOH usage for this particular starch also appeared to be amongst the highest for all the starches tested. The waxy maize starch appears to be fermented quickly by the bacteria present, which results in the production of high amounts of SCFA. This may result from this starch containing all amylopectin with virtually no amylose fraction, which may be more readily available to the bacteria due to pancreatin making it more accessible.

The findings from this chapter show that in a mixed population of colonic bacteria, starches are degraded to different extents and at different rates. High levels of SCFA are produced, with butyrate being a major product from starch breakdown. Certain species of bacteria also appeared to be enhanced by different starches, such as *Bifidobacterium* spp. being enhanced by waxy maize starch. The previous chapter suggested that amylose/amylopectin ratio was a big factor in influencing the amount of starch reaching the colon. This chapter indicates that other factors, such as starch damage and the lipids present not only influences the amount of starch reaching the colon, but also influences the way the starch is fermented by the bacteria and the products resulting from this. The results also appear to indicate that out of the native starches tested, the waxy maize starch appears to be degraded more easily and quickly, producing high levels of SCFA (particularly butyrate). This particular starch also appears to enhance beneficial species of bacteria, such

as *Bifidobacterium* and *Lactobacillus* spp., and at the same time reducing the levels of the coliform group, which contain potential pathogenic micro-organisms. These findings also suggest that the high levels of amylose present in some starches may reduce the bacterial fermentation of them. All these findings appear to be significant, especially as they can be extrapolated to the monogastric colon. These findings are also significant as the single-stage fermentation system employed has been shown to be a representative model of the colon.

CHAPTER 4

The effect of retrogradation and amylose/amylopectin content on starch fermentation - Study of a monogastric animal model.

4.1 Introduction

Amongst the most common methods employed for the study of the intestinal flora have been those which use *in vitro* techniques, such as the fermentation systems described previously. However, the best methods for studying the digestion of various dietary components are those which use animal systems. Those usually employed are laboratory animals such as conventional rats, especially for the study of resistant starches (Livesey, 1990; Gee *et al.* 1991; Tovar *et al.* 1992; Faulks *et al.* 1992; Asp *et al.* 1992; Berggren *et al.* 1993; Granfeldt *et al.* 1993). Various problems can be associated with these types of animal studies, especially when findings from gut microflora studies are extrapolated to humans (Rumney & Rowland, 1992). These problems are usually associated with the numbers and types of micro-organisms present in the rat systems, and the reactions of the animals to the dietary components. To try and reduce some of these problems, rat systems have been employed which contain gut microflora from human origin in place of their own indigenous intestinal flora (Roland *et al.* 1995; Djouzi & Andrieux, 1997). Gnotobiotic animals have also been employed, either with germ-free intestines or containing an intestinal flora of which all species are known (Coates *et al.* 1988).

One of the best models for human nutrition in terms of similarities in digestive function, is the pig (Miller & Ullrey, 1987). It has been suggested that the pig may be an adequate model to study the effects of dietary components on the microflora of the intestine (Allison, 1989), but Moore *et al.* (1987) concluded that the microflora of the porcine intestine was different from that of humans. Despite these findings, the pig appears to be the closest model of human intestinal fermentation which exists today.

The previous chapters have focussed on the use of *in vitro* techniques to study the bacterial breakdown of native and retrograded starches. The findings from the previous chapters suggest that micro-organisms from the intestinal population may degrade the amylopectin fraction of the starch more easily, and to a greater extent, than the amylose fraction. The amylopectin fraction of starch also appears to enhance certain bacterial groups within an *in vitro* environment. This chapter extends the previous work, by using the pig as a model for the breakdown of native and retrograded starches in monogastrics,

such as humans. The ability to extend the region of carbohydrate fermentation along the length of the colon with these starches was also examined. In addition, the potential to enhance particular groups of bacteria and hence the potential to benefit intestinal health was also examined.

4.2 Materials

4.2.1 Preparation of culture media :-

(1) The following media were prepared as per manufacturer's instructions:

MacConkey agar no. 3
Slanetz & Bartley medium
de Man, Rogosa, Sharpe (MRS) agar
Reinforced Clostridial agar (RCA)
Maximum recovery diluent (MRD)
Plate count agar

(II) Columbia blood agar + MUG supplement (CBA+MUG)

Prepared as detailed in **Chapter 3, section 3.2.1 (II)**, but with the addition of one vial of MUG supplement prior to pouring.

(III) Wilkins-Chalgren blood agar (WCBA)

Prepared as detailed in **Chapter 3, section 3.2.1 (III)**.

(IV) G-N agar (for the growth of Gram-negative anaerobes)

Wilkins-Chalgren anaerobe agar was prepared as per manufacturer's instructions. Prior to pouring the plates, 5% (v/v) defibrinated sheep blood and one vial of G-N supplement were added.

(V) N-S agar (for the growth of non-sporing anaerobes)

Wilkins-Chalgren anaerobe agar was prepared as per manufacturer's instructions. Prior to pouring the plates, 5% (v/v) defibrinated sheep blood and one vial of N-S supplement were added.

(VI) Starch agar

Plate count agar was prepared as per manufacturer's instructions. Potato starch (1% w/v) was added to the agar as a suspension in distilled water, mixed, and the mixture autoclaved at 121°C for 20 minutes.

(VII) Rifampicin agar (RIF)

Prepared as follows :-

Brain-heart infusion agar	23.5g
Distilled water	450ml
Rifampicin (5 mg/ml)	5ml
Defibrinated horse blood	50ml

The Brain-heart infusion agar was dissolved in distilled water and sterilised by autoclaving at 121°C for 20 min. The rifampicin and horse blood were added aseptically prior to pouring the plates (Drasar and Roberts, 1991).

(VIII) Kanamycin vancomycin agar (KV)

Prepared as detailed in **Chapter 3, section 3.2.1 (V).**

(IX) Bifidobacterium agar (BIF)

Prepared as detailed in **Chapter 3, section 3.2.1 (IV).**

4.2.2 Chemicals used :-

4.2.2.1 Half-strength Grams iodine

Prepared as detailed in **Chapter 3, section 3.2.4.3.**

4.2.3 Preparation of starches :-

4.2.3.1 Starches used

Native maize
Native waxy maize
Retrograded maize
Retrograded waxy maize
Retrograded mixture
Retrograded potato

The native forms of the feed grade starches (maize and waxy maize) were added to the animal diets without prior treatment, at a concentration of 50% (w/w). The retrograded maize, waxy maize and potato starches were prepared by mixing feed grade starches with distilled water (10% w/v), autoclaving at 121°C for 20 minutes, and then cooling for 3 days at 4°C. The retrograded starch mixture was prepared by mixing feed grade maize and waxy maize starches (50% v/v) with distilled water (10% w/v). The mixture was autoclaved and stored at 4°C as above. Excess water was removed and the retrograded starch gels chopped prior to inclusion in the animal diets.

4.2.4 Preparation of animal diets :-

A semi-synthetic diet was prepared which contained the following ingredients:

	Formulation (% w/w)
Starch*	50
White fish meal	10
Dried milk powder	10
Casein	10
Soyabean oil	8
Cane sugar	4.55
Solka Floc	3.50
Molasses	2
Dicalcium phosphate	1.20
Salt	0.50
Sowvite 12 (2.5)	0.25
Vitamin E	0.02

The starch component in each diet was replaced with one of the six starch preparations detailed above.

The suppliers of the media and chemicals used is detailed in **Appendix 1**

4.2.5 Analyses carried out on animal diets :-

4.2.5.1 Determination of water content

The water content of each of the animal diets containing one of six starches, was measured by drying portions of each diet (10g) to constant weight at 74°C in a drying oven. These were determined as :-

	Water content (%)
Retrograded maize	38.45
Retrograded potato	41.50
Native maize	12.15
Retrograded waxy maize	43.08
Retrograded mixture	38.45
Native waxy maize	8.07

4.2.5.2 Determination of diet constituents

The six feeds were also analysed by the SAC Analytical unit, Aberdeen to determine the exact constituents. Measurements are expressed as g kg^{-1} dry matter in each case.

	Starch type					
Determination	**RM**	**RP**	**NM**	**RWM**	**RMIX**	**NWM**
Dry matter	624.00	600.60	885.80	619.10	622.10	902.90
Crude protein	344.20	324.20	222.00	311.40	309.30	211.40
Amylase NDF	107.50	112.40	58.40	111.40	116.30	59.00
Fat AEE	181.50	169.00	130.80	169.30	169.60	119.40
Digestible energy	19.30	18.70	18.80	18.70	18.70	18.50
Ash	68.00	72.90	50.20	72.50	69.50	50.30
Starch	289.40	267.90	526.80	329.80	278.80	589.90

RM = Retrograded maize, RP = Retrograded potato, NM = Native maize, RWM = Retrograded waxy maize, RMIX = Retrograded mixture NWM = Native waxy maize.
NDF = Neutral detergent fibre, Fat AEE = Determined by acidified ether extract.

4.3 Methods

4.3.1 Experimental animals and diets

Thirty six newly weaned piglets (Landrace x Large white) from Tillycorthie farm, of approximately 10kg, were split into six groups. Each group of six pigs was housed in a separate pen and fed the semi-synthetic diet detailed, containing one of the six starch forms. The diets were fed *ad libitum* and the piglets had free access to drinking water. For three days prior to inclusion on the diets, the piglets were fed on a normal piglet feed. The diets were fed for a period of three weeks with the amount fed and the amount refused recorded each day. The amount of feed added to the troughs was increased as and when the growth of the animals dictated. Feed samples were analysed for retrograded starch, on dry matter, by the method of Englyst *et al.* (1992).

4.3.2. Analyses of faecal material

Faecal samples were taken from all piglets prior to the start of feeding and at various intervals during the feeding of the diets. The samples were collected at the following times :-

Sample name	Time taken
Baseline	Immediately before start of diet
Sample 1	48h after start of diet
Sample 2	4 days after start of diet
Sample 3	7 days after start of diet
Sample 4	11 days after start of diet
Sample 5	14 days after start of diet
Sample 6	18 days after start of diet

The piglets were weighed at the same time as the faecal samples were collected.
The faecal samples were used for the following analyses :-

4.3.2.1 Bacterial analyses

Approximately 1g of each sample was added to MRD and mixed until dispersed. This was diluted to 10^{-8} in MRD and plated out either on the bench, or in an anaerobic cabinet where

appropriate, using a modification of the Miles & Misra (1938) method. All incubations were carried out at 39°C, and the agar plates incubated as follows :-

	Conditions		
	Bacterial species	**Incubation time**	**Gas phase**
Slanetz & Bartley	*Enterococcus* spp.	24h	aerobic
CBA + MUG	Total aerobes	48h	aerobic
CBA + MUG	*Escherichia coli*	48h	aerobic
Starch agar	Starch degraders	48h	aerobic
Starch agar	Starch degraders	48h	anaerobic
G-N agar	Gram -ve anaerobes	48h	anaerobic
N-S agar	Non-sporing anaer.	48h	anaerobic
RCA	*Clostridium* spp.	48h	anaerobic
RIF	*Clostridium*, *Eubacterium* & *Fusobacterium* spp.	4 days	anaerobic

Samples were also plated onto MacConkey, MRS (aerotolerant and total), Wilkins-Chalgren blood agar, Bifidobacterium agar and Kanamycin-vancomycin agar, using a modification of the Miles & Misra (1938) method. The plates were incubated under the conditions previously described in **Chapter 3, Section 3.3.3.1.** Counts of *E. coli* were obtained by examining plates for fluorescence under a UV lamp at 365nm. Starch-degrading bacterial species were enumerated by flooding the plates with half-strength Gram's iodine after incubation, and examining colonies for zones of clearing. (Macfarlane & Englyst, 1986; Dettori-Campus *et al.* 1992).

4.3.2.2 SCFA analysis

Approximately 1g of each faecal sample was added to MRD and mixed until dissolved. Samples were analysed for SCFA using the method described in **Chapter 2, Section 2.3.3.**

4.3.2.3 Determination of α-Amylase content

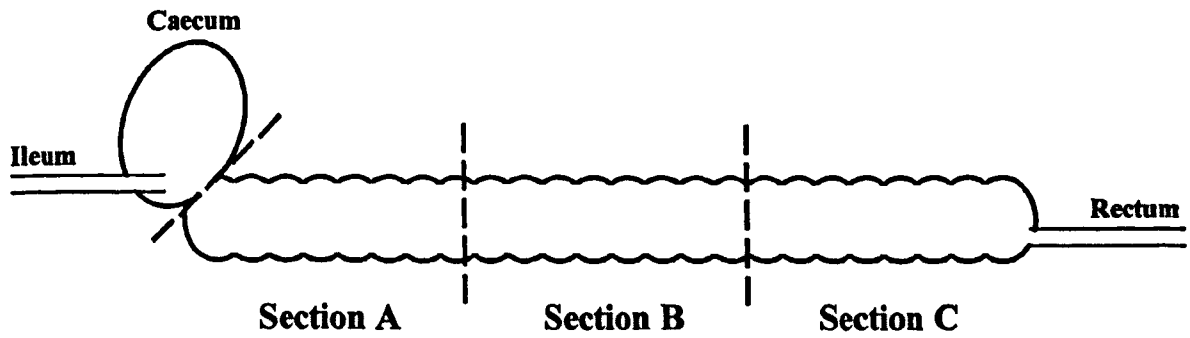
Faecal samples were added to distilled water (10% w/v) and spun for ten minutes at 7000 x *g*. The level of α-amylase present (U/L) was determined as detailed in the Amylase 10 kit.

4.3.2.4 Determination of total starch

Samples of faecal material were analysed for total starch by the Rowett Research Institute, Aberdeen, using the method of Aman & Hesselman (1984).

4.3.3 Analysis of colonic material

After three weeks on the diets, three piglets from each pen were selected and humanely killed by terminal anaesthesia using sodium pentobarbitone (Euthatal). Once pronounced dead, each piglet was dissected and the intestines removed. The small intestine was separated off at the ileo-caecal valve and discarded. The caecum was removed, weighed and also discarded. The remaining colon was equally sectioned into three, as detailed below, and the contents from each section removed and used for analysis (Sections A, B and C refer to the proximal, mid and distal colon respectively) :-



4.3.3.1 Bacterial analysis

Bacterial analysis was carried out on the colonic material from each section in the same manner as for the faecal material, as detailed in **Section 4.3.2.1.**

4.3.3.2 SCFA analysis

SCFA analysis was carried out on the colonic material from each section in the same manner as for the faecal material, as detailed in **Section 4.3.2.2.**

4.3.3.3 Determination of α-Amylase

α-Amylase analysis was carried out on the colonic material from each section in the same manner as for the faecal material, as detailed in **Section 4.3.2.3.**

4.3.3.4 Determination of total starch

Total starch analysis was carried out on the colonic material from each section in the same manner as for the faecal material, as detailed in **Section 4.3.2.4.**

4.3.3.5 Determination of skatole

Skatole analysis was carried out on the colonic material from each section using the HPLC method of Hansen-Møller (1992).

4.3.4 Statistical analysis

Statistical analysis was carried out on triplicate data using one-way analysis of variance (ANOVA), where comparisons were made between each data set within a column. This was achieved by use of the MINITAB statistical package (Ryan *et al.* 1985). Mean and standard deviations were also carried out on the data using the same MINITAB package, and are detailed in **Appendix 4.**

4.4 Results

Figure 4.1 (a)- 4.19 show the counts of various bacterial species isolated from the faeces of animals fed on diets containing one of six different starches, over a period of 18 days. For all the aerobic bacterial species isolated, apart from the *Enterococcus* and aerobic starch-degrading species, a peak in numbers after 7 days was observed with the diets containing retrograded waxy maize, retrograded mixture and native waxy maize starches.

Figures 4.1 (a) and 4.1 (b) show the counts of coliforms and *E. coli* respectively, isolated from the faecal samples over the 18 day sampling period. These two bacterial groups showed a similar pattern in the counts observed. The counts obtained for both groups varied for the first 7 days (sample 3) with each starch, and then levelled out to approximately 10^8 cfu g^{-1}. The highest counts of both bacterial groups prior to the 7 day sampling were obtained with the retrograded maize starch, and from the native waxy maize starch at the 7 day sampling. After 7 days on the diets containing retrograded waxy maize starch, the counts of the coliforms obtained were significantly higher ($P<0.05$) than those obtained prior to feeding (baseline) and also significantly higher ($P<0.05$) than those obtained with the retrograded potato starch at the same sampling time. After 18 days on the diets, the *E. coli* and coliforms counts obtained with the retrograded maize starch were significantly lower ($P<0.05$) than the counts obtained prior to feeding.

Figures 4.11 (a) and 4.11 (b) show the total aerobic and total anaerobic counts respectively, obtained from the faecal samples. The two groups of bacteria showed different patterns of counts throughout the sampling period. The total aerobic species appeared to show a larger variation in counts than the total anaerobic species throughout. The highest counts of total aerobic species were obtained with the native waxy maize starch after 7 days, and were recorded at approximately 10^{11} cfu g^{-1}. With the total aerobic counts, the retrograded waxy maize, native waxy maize and retrograded mixture starches increased in numbers after 7 days on the diets, whereas the other three starches showed a decrease in numbers at this sampling time. However, with the total anaerobic bacterial species, all starches showed the highest counts after 7 days on the diets apart from the retrograded mixture starch which showed the highest counts after only 4 days of feeding this starch. The highest counts obtained for the total anaerobic species were not as high as the peak counts for the total aerobic species. The highest counts of the total anaerobic

species after 7 days of feeding were observed with the retrograded maize starch, which was significantly higher ($P<0.05$) than the counts obtained with the retrograded waxy maize, native waxy maize and retrograded mixture starches. After 18 days of feeding, the total aerobic counts levelled out to approximately 10^8 cfu g^{-1}, and the counts of the total anaerobic species levelled out at approximately 10^9 cfu g^{-1}. With the total anaerobic bacterial species, the counts obtained with the native waxy maize starch after 7 days were significantly higher ($P<0.01$) than the counts obtained prior to feeding. With the total aerobic species, the counts obtained with the retrograded maize starch after 18 days of feeding were significantly lower ($P<0.01$) than the counts obtained prior to feeding.

Figures 4.12 (a) and 4.12 (b) show the counts of aerotolerant and total *Lactobacillus* spp. respectively, obtained from the faecal samples over the 18 day sampling period. With these two bacterial groups a similar pattern of counts was observed with each starch, where a large variation in the counts with each starch was observed throughout the sampling period. The highest counts of both bacterial species were observed with the retrograded waxy maize, native waxy maize and retrograded mixture starches after 7 days of feeding. The counts obtained with the retrograded maize starch for both bacterial groups appeared to be maintained at approximately the same level throughout the sampling period, more so with the anaerobic *Lactobacillus* group. Apart from the peak in numbers after 7 days on the diets, the retrograded waxy maize starch resulted in the lowest counts of both bacterial groups. After two weeks on the diets, the counts observed from the native maize starch (both aerotolerant and total lactobacilli) were significantly higher ($P<0.05$ aerotolerant; $P<0.01$ total) than the counts obtained with the retrograded waxy maize, native waxy maize and retrograded mixture starches.

Figures 4.13 (a) and 4.13 (b) show an estimation of the aerobic and anaerobic starch-degrading bacterial species respectively, obtained from the faecal samples. The counts obtained for the anaerobic starch-degrading species were higher and showed a greater variation than the counts obtained for the aerobic species. The two bacterial groups appeared to produce very different patterns of counts throughout the sampling period. The counts of aerobic starch-degrading species appeared to have a base level of approximately $5x10^2$ cfu g^{-1}, with each starch giving rise to a peak in numbers at a different sampling time, followed by a return to the base level. No significant differences were observed in the

counts of starch-degrading bacteria from any of the six starches at any of the sampling times.

Figures 4.14 (a) and 4.14 (b) show the counts of the Gram-negative and non-sporing anaerobes respectively, obtained from the faecal samples over an 18 day period. The counts obtained for these two bacterial groups were closer together for each of the starches than was observed with the aerobic bacterial species, and tended to be in the region of 10^8-10^9 cfu g^{-1}. With both bacterial groups, the counts obtained with the retrograded maize starch after 7 days of feeding, were significantly higher ($P<0.05$) than the counts obtained with the retrograded mixture starch at the same sampling time. However, the counts of both bacterial groups obtained with the retrograded maize and retrograded mixture starches were significantly lower ($P<0.05$) after 18 days on the diets, compared to the counts obtained prior to feeding.

Figure 4.15 shows the counts of *Enterococcus* spp. obtained from the faecal samples over the 18 day sampling period. The counts obtained for this bacterial species varied greatly over the 18 day sampling period, and did not follow the pattern observed for the other aerobic bacterial species studied. This bacterial population did not stabilise over the 18 day sampling period. All starches except the native waxy maize and retrograded mixture starches gave counts after 18 days which were lower than those obtained prior to feeding, although this was not significant. After 14 days, the *Enterococcus* counts obtained with the native maize starch were significantly higher ($P<0.05$) than the counts obtained from all other starches at this sampling time, except the retrograded maize starch.

Figure 4.16 shows the counts of *Clostridium* spp. which were isolated from faecal samples over the 18 day sampling period. The counts of *Clostridium* species obtained with the native and retrograded maize starches appeared to follow a similar trend over the sampling period. The counts obtained with the retrograded mixture diet i.e. composed of both maize and waxy maize, followed a trend which was between that obtained with the retrograded maize and retrograded waxy maize starches. After 7 days on the diets, the counts obtained with the retrograded maize starch were significantly higher ($P<0.01$; $P<0.05$ retrograded mixture) than the counts obtained from the other starches studied, apart from the native maize starch. The counts obtained with the retrograded waxy maize starch were the lowest throughout, and appeared to be stable at 10^8 cfu g^{-1}.

Figure 4.17 shows the counts of *Bifidobacterium* species obtained from the faecal samples over the 18 day period. The highest counts of *Bifidobacterium* species throughout the sampling period were obtained with the retrograded maize starch, which was significantly higher ($P<0.05$) than the counts obtained with the native maize starch after 14 days of feeding. The counts obtained with the native waxy maize and retrograded mixture diets followed a similar trend throughout the 18 day sampling period. The lowest counts of *Bifidobacterium* species throughout were from the animals fed the retrograded waxy maize starch, although the counts with this particular starch increased steadily over the 18 day sampling period.

Figure 4.18 shows the counts of *Eubacterium*, *Fusobacterium* and *Clostridium* species detected on rifampicin agar from the faecal samples over 18 days. The counts obtained with the native maize, retrograded waxy maize and retrograded potato starches appeared to be mostly stable throughout the 18 day sampling period. The counts obtained with the native waxy maize and retrograded mixture starches decreased after 18 days compared to the counts obtained prior to feeding. The counts obtained with the retrograded mixture starch after 14 days of feeding were significantly lower ($P<0.05$) than the counts obtained with the retrograded waxy maize starch.

Figure 4.19 shows the counts of *Bacteroides* species isolated from the faecal samples over 18 days. With all six starches studied, a similar trend was observed throughout the sampling period, with a peak in numbers after approximately 7 days (2 days for the retrograded mixture starch) followed by a decrease in numbers over the rest of the sampling period. The counts obtained after 7 days with the retrograded maize starch were significantly higher than the counts obtained with the retrograded waxy maize and retrograded mixture starches, at the same sampling time. The retrograded maize and retrograded mixture starches showed a drop in the mean counts of *Bacteroides* species of between 1-2 orders of magnitude after 18 days of feeding, when compared with the counts prior to feeding. However, this decrease was not significant, which may be due to a large variation in the actual counts of this bacterial species obtained. The counts obtained with the retrograded maize starch after 7 days of feeding were significantly higher than those obtained with the same starch 4 days later.

Figures 4.2 (a) - 4.29 show the counts of various bacterial species obtained from three sections of colon, removed from animals which had been fed on diets containing one of six different starches for 21 days. Sections A, B and C refer to the proximal, mid and distal colon sections respectively. The bacterial counts along the length of the colon from the pigs fed the retrograded maize starch, were either very similar in numbers or followed a trend similar to that obtained from the native maize-fed animals, in the majority of cases. To a lesser extent, the native and retrograded waxy maize starches followed similar trends in bacterial counts along the length of the colon.

The graphs show that with the samples from the animals fed the retrograded maize starch, the counts of the various bacterial species studied remained fairly stable along the length of the colon. In the majority of cases, the counts obtained with the retrograded maize starch followed a similar trend to that observed with the native maize starch. The largest difference in the counts obtained with the retrograded maize starch was observed with the aerobic starch-degrading species (Figure 4.23 (a)). Here a non-significant drop in the mean counts was observed in the mid colon, followed by an increase in counts in the distal colon to the same level as detected in the proximal colon. The mean counts of *Clostridium* species obtained with the retrograded maize starch (Figure 4.26) also increased in the distal colon section compared to the mid section of the colon. Again however, this increase was not significant.

The animals fed the retrograded potato starch showed a consistently low count of aerobic starch-degrading species along the length of the colon, at approximately $5x10^2$ cfu g^{-1} (Figure 4.23 (a)). This count was the lowest obtained for the aerobic starch-degrading species, and was significantly lower ($P<0.05$) than the counts obtained with the retrograded mixture starch in the distal region of the colon. With the retrograded potato starch, the counts of the majority of the bacterial species studied were lower in the mid section of the colon than those observed in either the proximal or distal sections. However, the counts of *Enterococcus* species obtained with the retrograded potato starch increased slightly in the mid colon compared to the other two colon sections (Figure 4.25), and the counts of *Bacteroides* species decreased throughout the length of the colon in the animals fed the retrograded potato starch (Figure 4.29). The counts of *Bacteroides* species in the mid colon section obtained with the retrograded potato starch, were significantly higher

($P<0.01$; $P<0.05$ native maize) than the counts obtained from any of the other starches in this section of the colon. The counts of the various species of anaerobic bacteria studied were highest throughout the length of the colon with the retrograded potato starch, as were the counts of *E. coli* (Figure 4.2 (b)).

In the samples from the animals fed the native maize, the counts of the bacterial species were either at a fairly consistent level or decreased in numbers, along the length of the colon. The lowest counts of *Enterococcus* species and the highest counts of *Clostridium* species (Figure 4.26) were detected in the mid colon section of the animals fed the native maize starch. The highest mean count from the animals fed the native maize starch was observed with the aerobic starch-degrading species in the distal colon (Figure 4.23 (a)). This resulted in a count of approximately 10^7 cfu g^{-1} in the distal colon section, but this value was not significantly greater than the numbers found in the mid colon section..

The samples from the animals fed the retrograded waxy maize showed an increase between the proximal and mid sections of the colon with the coliform, *Enterococcus*, and the aerotolerant and total *Lactobacillus* counts, but these numbers returned to the same level in the distal section, as observed in the proximal section. The *E. coli* counts were unchanged along the length of the colon with the animals fed this starch (Figure 4.2 (b)). The rest of the bacterial species studied were fairly stable along the length of the colon with the retrograded waxy maize starch except for the aerobic starch-degrading species which exhibited a non-significant decrease in the mid-colon section as compared to the proximal colon, and the anaerobic starch-degrading bacteria which exhibited a non-significant increase between the mid and distal colon. The counts of the aerobic starch-degrading species returned to the same level in the distal colon as recorded in the proximal colon section (Figure 4.23 (a)). The highest counts of both aerotolerant and total *Lactobacillus* species in the mid section of the colon, and the highest counts of *Bifidobacterium* species in the distal section of the colon, were obtained with the retrograded waxy maize starch. The coliform count from the animals fed the retrograded waxy maize starch was the lowest obtained in the distal section of the colon.

The lowest coliform counts were detected in the proximal and mid sections of the colon from the animals fed the retrograded mixture starch, but in the distal section of the colon, the counts increased by approximately three orders of magnitude to give a final count

of 10^8 cfu g^{-1}. The *E. coli* counts with the retrograded mixture starch followed the same trend as the coliform bacteria, with the increase between the mid and distal sections being only one order of magnitude (Figure 4.2 (b)). The counts of *Enterococcus* species with the retrograded mixture starch decreased in numbers along the length of the colon, but were at least 1 order of magnitude higher than the highest counts observed with the other five starches (Figure 4.25). The *Enterococcus* counts with this diet did not appear to follow the same trend as the counts observed with the other starches studied. The counts of both the aerobic and anaerobic starch-degrading species were consistently low but constant throughout the length of the colon with this starch (Figure 4.23 (a) and 4.23 (b)). The lowest counts along the length of the colon were detected with the retrograded mixture starch for the majority of the bacterial species studied. The exception to this was with the aerotolerant *Lactobacillus* species, where the highest counts in the proximal section were detected with this starch, but the counts decreased greatly along the length of the colon so that in the distal section of the colon, this starch resulted in the lowest aerotolerant *Lactobacillus* counts (Figure 4.22 (a)). The counts of aerotolerant *Lactobacillus* species in the distal colon from the retrograded mixture starch samples were significantly lower ($P<0.05$) than the counts from the retrograded waxy maize and retrograded maize starches, in the same colon section. The counts on the rifampicin agar and those of the *Bacteroides* species obtained with the retrograded mixture and native waxy maize starches followed a similar trend along the length of the colon (Figures 4.28 and 4.29 respectively).

The colon samples taken from the pigs fed the native waxy maize starch showed the highest counts of coliform bacteria in the mid section of the colon, which decreased in the distal colon to the same counts observed in the proximal colon (Figure 4.2 (a)). The counts of both aerotolerant and total *Lactobacillus* species decreased along the length of the colon with this starch, more so with the total population (Figures 4.22 (a) and 4.22 (b)). The counts of *E. coli* and the total aerobes (Figures 4.2 (b) and 4.21 (a)) were fairly stable along the length of the colon with this starch, as were the majority of the other bacterial species studied. A non-significant increase in the mean count of the total anaerobic species was observed between the mid and distal colon sections with the native waxy maize samples (Figure 4.21 (b)). An increase in the counts of *Clostridium* species was also observed between the same two sampling sites (Figure 4.26). With the native waxy maize starch the

counts of the total anaerobic species were generally low compared to the other five starches.

Figures 4.3-4.32 show the proportions of acetate : propionate : butyrate detected in the three colon sections taken from each of the dissected animals. In all sections of the colons, the production of the three SCFA were detected as acetate > propionate >butyrate. The proportions of acetate : propionate : butyrate were detected between 60 : 20 : 20 and 70 : 20 : 10 respectively. The proportions of butyric acid increased from the proximal to distal colon in the samples obtained from the pigs which had been fed on the retrograded potato and retrograded maize starches (Figures 4.3 and 4.32). The proportion of propionic acid in the samples obtained from the pigs fed on the retrograded mixture starch increased from the proximal to distal colon by approximately 10%, at the expense of the acetic acid. The samples from the pigs fed on the retrograded waxy maize starch showed an increase in the proportion of acetate along the length of the colon. The proportions of acetic acid appeared to decrease and the proportions of propionic acid appeared to increase along the length of the colon, with the native waxy maize starch (Figures 4.3-4.32).

The levels of α-amylase detected from the faecal samples of the animals fed diets containing one of six different starches over an 18 day period are recorded in Figures 4.4 - 4.43. From the animals fed on five of the starches, the levels detected prior to the start of the feeding trial i.e Sample B, were below 200 U/L, and the levels detected from all six starches prior to slaughter were approximately 200 U/L.

Figure 4.4 shows the α-amylase activity from the diets containing the retrograded and native maize starches. These two starches showed a peak level of activity at samples 1 and 2 respectively, followed by a decrease in activity back to base level. With the retrograded maize starch, this peak in numbers after two days of feeding was significantly higher ($P<0.05$) than the level recorded at the same sampling time with the native maize starch. The level of α-amylase detected from the retrograded maize starch at sampling no 5, was significantly lower ($P<0.01$) than the level detected from the same animals prior to feeding (sample B). The α-amylase levels did not reach higher than approximately 350 U/L for both these starches.

The highest α-amylase levels (approximately 600 U/L) were detected in the samples from the animals fed the native waxy maize starch after 4 days of feeding, but after this samping time the levels decreased, to result in approximately 100 U/L after 11 days on the diets (Figure 4.41). The samples from the animals fed the retrograded waxy maize starch (Figure 4.41) peaked at sample 5, i.e after 14 days of feeding. Prior to this sampling, the levels of α-amylase detected with this starch were very low, reaching levels of approximately 100 U/L.

The α-amylase levels detected from the retrograded mixture starch samples (Figure 4.42) were the lowest found throughout. The trend of α-amylase activity mirrored that found with the samples from the animals fed the retrograded potato starch, apart from the levels detected in the baseline samples.

The animals fed the retrograded potato starch contained levels of α-amylase in the faecal material prior to feeding, which were at least twice that detected from the faecal material of the animals fed the other starches (200 U/L). The levels observed in the samples from the retrograded potato-fed animals decreased over the sampling period with a small rise in amylase activity to 300 U/L, after the animals had been on the diet for 11 days (Figure 4.43).

Figure 4.5 shows the levels of α-amylase detected in the proximal, mid and distal colon sections of the animals at slaughter. The only samples which showed an increase in the levels of α-amylase activity (approximately 300 U/L) along the length of the colon, were those obtained from the animals fed the diet containing the retrograded maize starch. The other starches showed a decrease in α-amylase activity along the length of the colon from the caecum to the rectum end. The largest decrease (450 U/L) was observed in the samples from the pigs fed the native maize starch and was observed between the proximal and mid sections of the colon. The samples from the animals fed the retrograded waxy maize starch showed the lowest levels of α-amylase activity along the length of the colon (approximately 100 U/L). The levels of α-amylase detected from the native waxy maize starch samples, were also low along the length of the colon, with the levels of α-amylase detected in the distal section of the colon being significantly lower ($P<0.01$) than the levels detected with the retrograded mixture starch. The levels of α-amylase detected in the distal colon from

the animals fed the retrograded potato starch were significantly lower ($P<0.05$) than the levels detected in the proximal colon, from the same animals. The levels of α-amylase detected in the distal colon with the retrograded maize starch, were significantly higher ($P<0.05$) than the levels detected with the retrograded waxy maize and the native waxy maize starches.

Table 4.1 shows the proportions of SCFA levels detected in the faecal samples of the animals fed on diets containing one of six different starches over an 18 day period. The proportions of acetate : propionate : butyrate changed slightly over the sampling period, but were generally in the ranges of 60 : 20 : 20 or 70 : 20 : 10 respectively. After 7 days of feeding the starches (sample 3), the proportions of acetate decreased and butyrate increased with the retrograded maize and retrograded mixture starches; the proportions of butyric acid increased with the native waxy maize starch, and the proportions of all three SCFA remained the same with the retrograded potato starch.

The largest changes in the proportions of the three SCFA were detected between the first (B) and last samplings (sample 6), where the proportions of acetic acid decreased between 1 and 22% and the butyrate increased between 1 and 16%, depending on the starch. The isoacid values are the sum of the isobutyric and isovaleric levels detected in the samples. The lowest values were detected in the samples 14 days after the start of feeding (sample 5) with the majority of starches, except the retrograded mixture and native waxy maize starches, where the latter showed an isoacid level which was almost the highest detected for this starch throughout the 18 days. The highest overall isoacid production was also detected in the samples from the pigs fed the native waxy maize diet, 18 days after the start of feeding (at sampling no 6, prior to slaughter), which was 20% of the total SCFA produced. The levels of isoacids detected varied between the seven sampling times and with each starch. The most constant levels of isoacids detected over the 18 days was found in the samples from the pigs fed the diet containing the retrograded potato starch.

Table 4.2 shows the levels of the various SCFA detected from the colon sections taken from animals fed on diets containing one of six different starches over a period of 18 days. The

levels of total SCFA production decreased between the proximal and mid-colon sections in the samples from the pigs fed on most of the diets, except from those fed on the retrograded potato diet, where an increase was observed between these two sampling sites. Three of the diets i.e. retrograded maize, retrograded waxy maize and retrograded mixture, showed increased levels of total SCFA production between the mid and distal colon. The native maize starch showed a significant decrease ($P<0.05$) in the level of total SCFA production of approximately 50mM between the mid and distal sections of the colon. The levels of total SCFA detected in the distal section of the colon from the animals fed the retrograded potato and retrograded waxy maize starches, were significantly higher ($P<0.05$) than the levels detected from the animals fed the native maize starch. In general, the total SCFA levels detected in the samples decreased from the caecum end to the rectum end of the colon.

The percentage of butyrate detected in the samples varied along the length of the colon depending on the type of starch added to the diet. The percentage increased throughout the length of the colon with the retrograded maize, retrograded potato and native maize diets. The samples from the animals fed the native waxy maize starch showed levels of butyrate which were almost constant along the colon length, and the retrograded mixture starch showed levels which decreased along the colon length. The retrograded waxy maize starch showed a decrease in the butyrate level from the proximal to mid colon, but a slight rise in the percentage of butyrate between the mid and distal colon. The level of butyrate detected in the mid colon with the retrograded maize starch was significantly higher ($P<0.05$) than that detected from the native maize, retrograded waxy maize and native waxy maize starches.

The percentages of isoacids (isobutyrate and isovalerate) detected in the colon sections also varied depending on the starch fed in the diet. An increase was observed along the colon length with the retrograded mixture, retrograded potato and retrograded maize starches, with the percentage from the retrograded maize starch almost doubling in the distal section, compared with that detected in the proximal section. (Table 4.2). With the samples taken from the pigs fed the diet containing the retrograded waxy maize starch, a large decrease from 14% to 5% isoacids was observed between the proximal and mid colon sections. In the distal region of the colon, the percentage of isoacids detected from the

retrograded maize starch was significantly higher ($P<0.05$) than that detected from the retrograded waxy maize starch. In general, an increase was observed in the isoacid levels detected from the caecum to the rectum end of the colon samples.

Table 4.3 shows the feed conversion ratio (FCR) calculated at weekly intervals, for the animals on each of the six diets. The FCR for the diets containing the retrograded and native maize starches increased over the three week period with the native maize starch showing the highest ratio of 0.95 at the end of week three. With the native and retrograded waxy maize diets, the FCR was different after the first week of feeding with the native waxy maize diet giving a higher ratio. However, over the next two weeks the two starches showed FCR which were very similar. The FCR from the retrograded potato diet started off high at week one at 0.60, but did not increase until week three where it reached 0.80. The FCR for the retrograded mixture starch appeared to show a value similar to that for the retrograded waxy maize diet at weeks one and two, but showed an FCR closer to that observed with the retrograded maize diet at week three.

Table 4.4 shows the Lactobacillus : coliform ratios calculated from the bacterial counts obtained from the colon sections taken from the animals fed on diets containing one of six different starches. The samples from the retrograded waxy maize-fed animals had the highest calculated ratios throughout the length of the colon, although the ratios were not significantly higher than those from the animals fed the other starches. The ratios obtained with the counts from the animals fed the retrograded mixture starch, were high in the proximal section of the colon, but then decreased along the two remaining sections of the colon. With the retrograded maize starch, the ratios in the proximal and mid sections of the colon were low, but they increased in the distal section of the colon. The ratio obtained in the mid section of the colon with the retrograded potato starch was approximately twice that detected in the proximal colon, but this decreased by a large amount in the distal section of the colon. The lowest ratios of Lactobacillus : coliforms were obtained in the distal sections of the colon, and were calculated from the samples containing the native starches i.e. native maize and native waxy maize. The ratios calculated using the data from the animals fed the native maize starch were very low along the whole length of the colon.

Table 4.5 shows the levels of skatole detected in the colon sections from the animals fed one of six different starches. The highest levels of skatole detected along the length of the colon were detected in the samples from the animals fed the native waxy maize starch. The lowest levels of skatole detected throughout the colon were detected in the samples from the retrograded mixture-fed animals. The highest level of skatole measured in the distal section of the colon was detected in the samples from the native maize starch. The levels of skatole detected appeared to decrease along the length of the colon with the retrograded maize starch. With the retrograded waxy maize starch, the levels of skatole appeared to increase slightly along the length of the colon, with the levels detected in the distal section being similar to those detected with the retrograded maize starch at this section.

Figure 4.1 (a) and 4.1 (b) :-
Counts of coliforms and *E. coli* species (cfu g^{-1}) respectively, obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figures 4.11 (a) and (b) :-
Counts of total aerobic and total anaerobic bacteria (cfu g^{-1}) respectively, obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figures 4.12 (a) and (b) :-
Counts of aerotolerant and total *Lactobacillus* species (cfu g^{-1}) respectively, obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figures 4.13 (a) and (b) :-
Counts of aerobic and anaerobic starch-degrading species (cfu g^{-1}) respectively, obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figures 4.14 (a) and (b) :-
Counts of Gram-negative anaerobes and non-sporing anaerobes (cfu g^{-1}) respectively, obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figure 4.15 :-
Counts of *Enterococcus* species (cfu g^{-1}) obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figure 4.16 :-
Counts of *Clostridium* species (cfu g^{-1}) obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figure 4.17 :-
Counts of *Bifidobacterium* species (cfu g^{-1}) obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

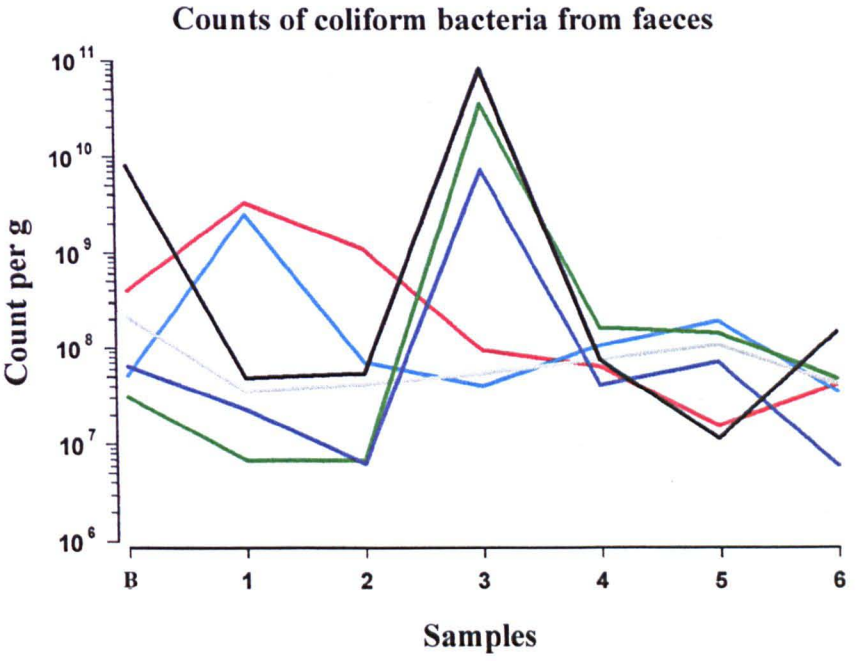
Figure 4.18 :-
Counts on Rifampicin agar (cfu g^{-1}) obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Figure 4.19 :-
Counts of *Bacteroides* species (cfu g^{-1}) obtained from faecal samples taken at various intervals from pigs fed a diet containing one of six starches.

Data represents mean of triplicate determinations. For all figures the following key applies :-

_____	Retrograded maize	_____	Retrograded potato
_____	Native maize	_____	Retrograded waxy maize
_____	Retrograded mixture	_____	Native waxy maize

Figure 4.1 (a) :-



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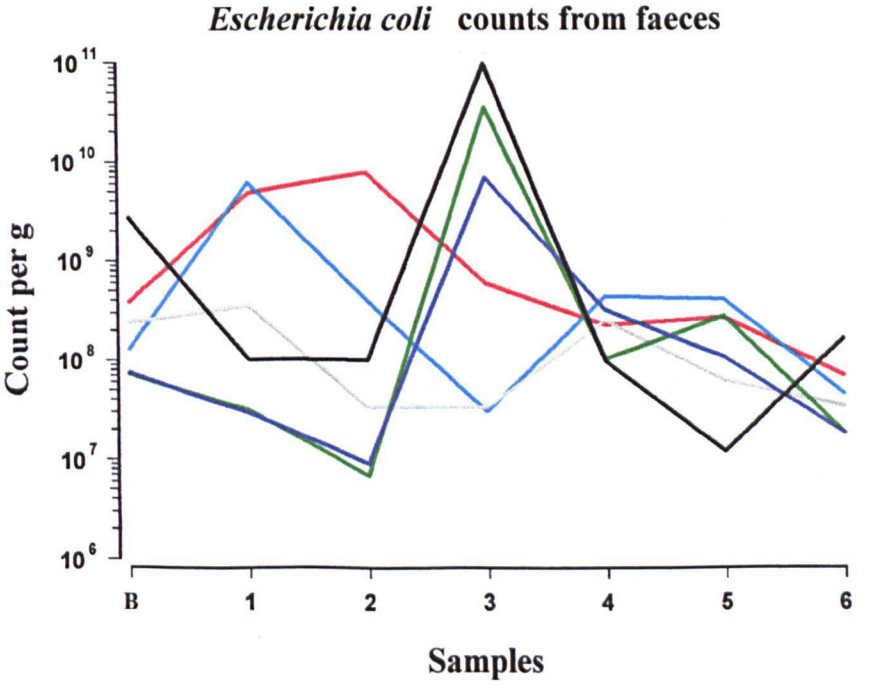


Figure 4.11 (a) :-

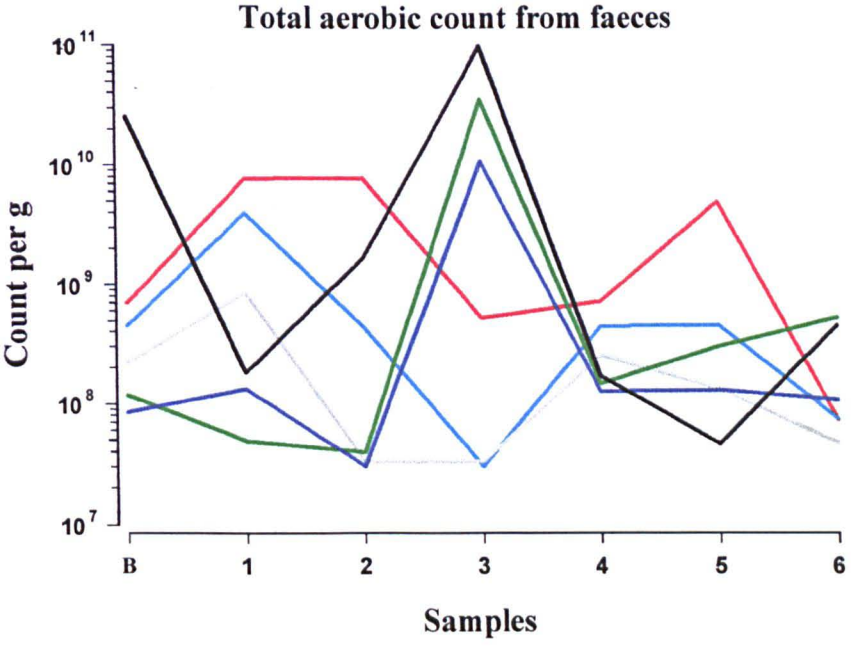
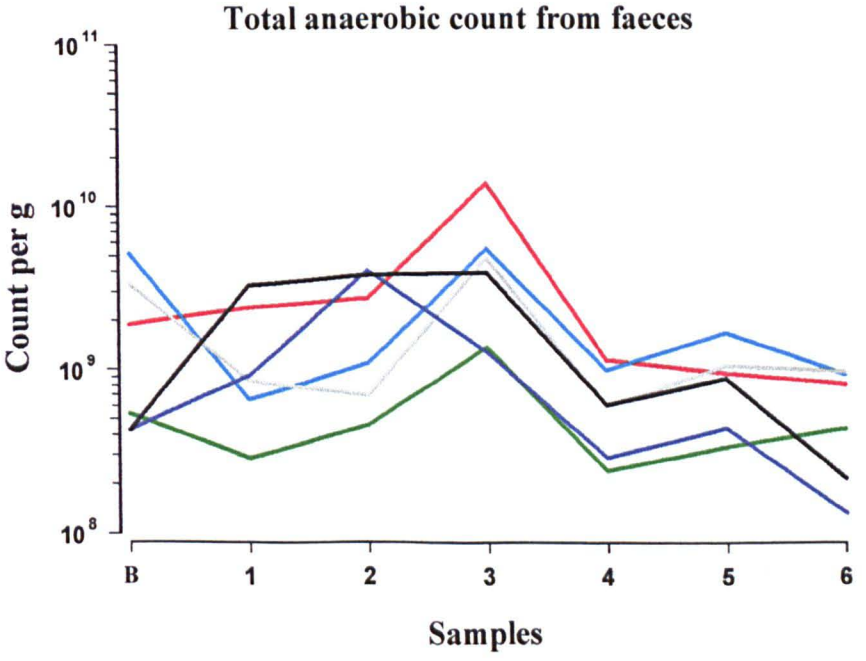


Figure 4.11 (b) :-



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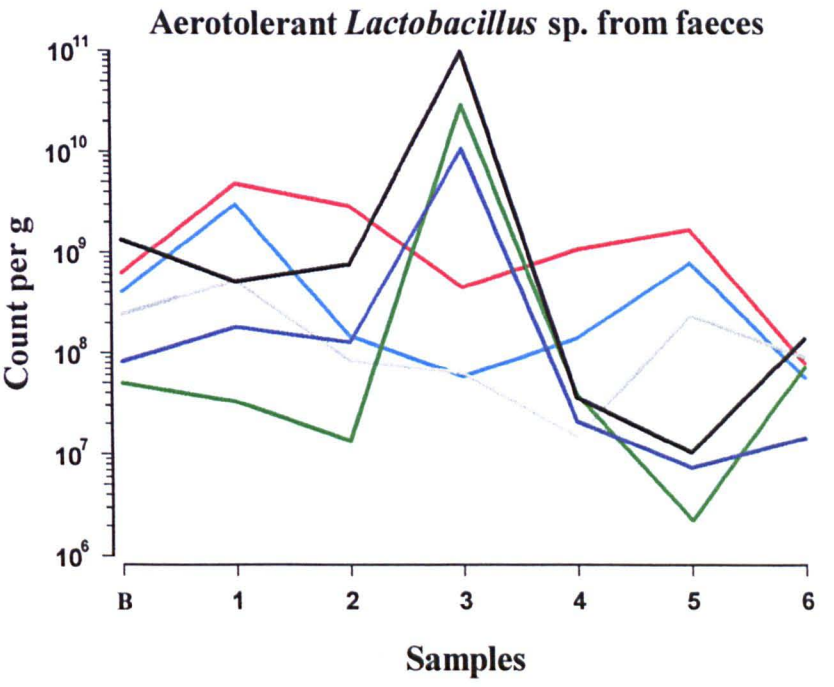

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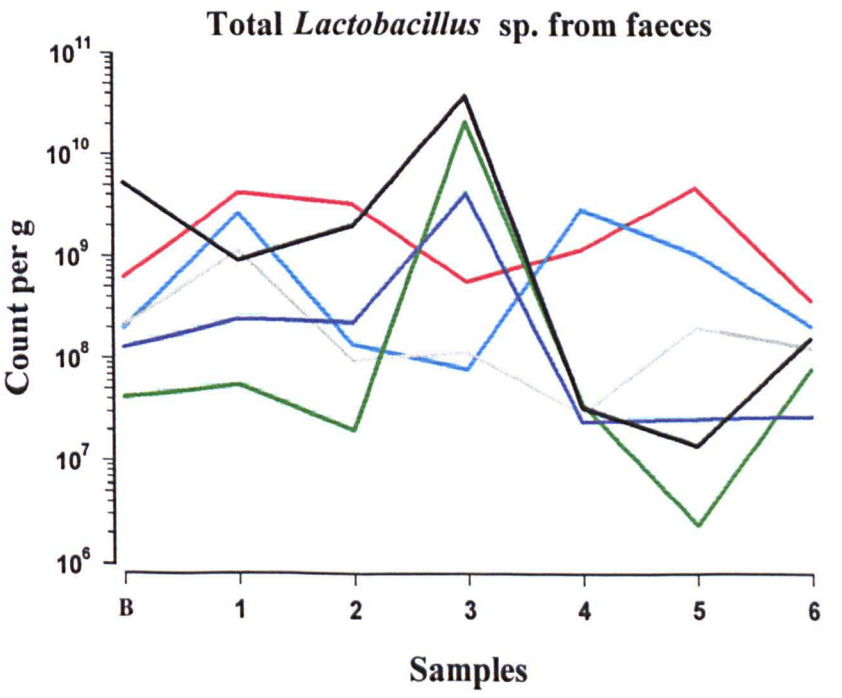

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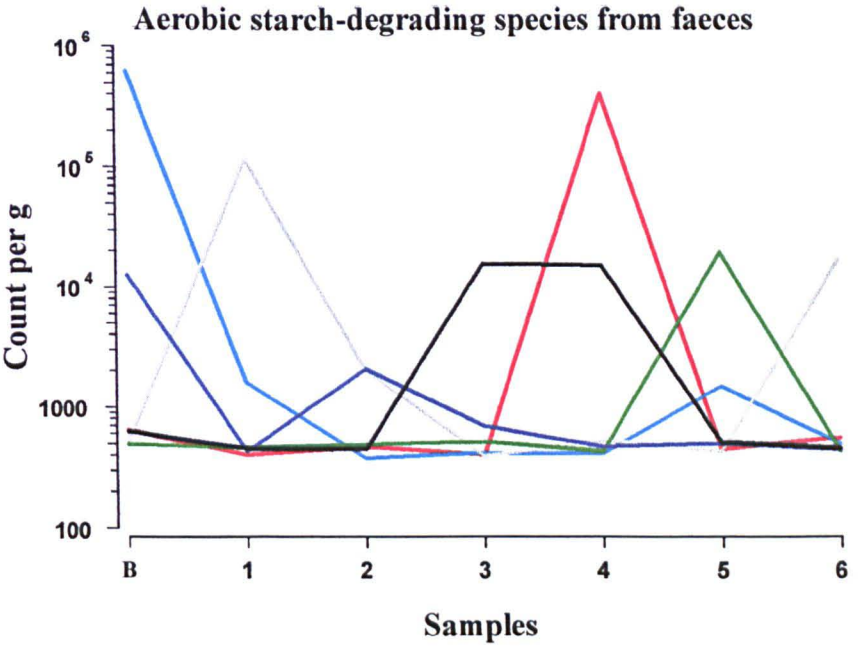


Figure 4.13 (b) :-

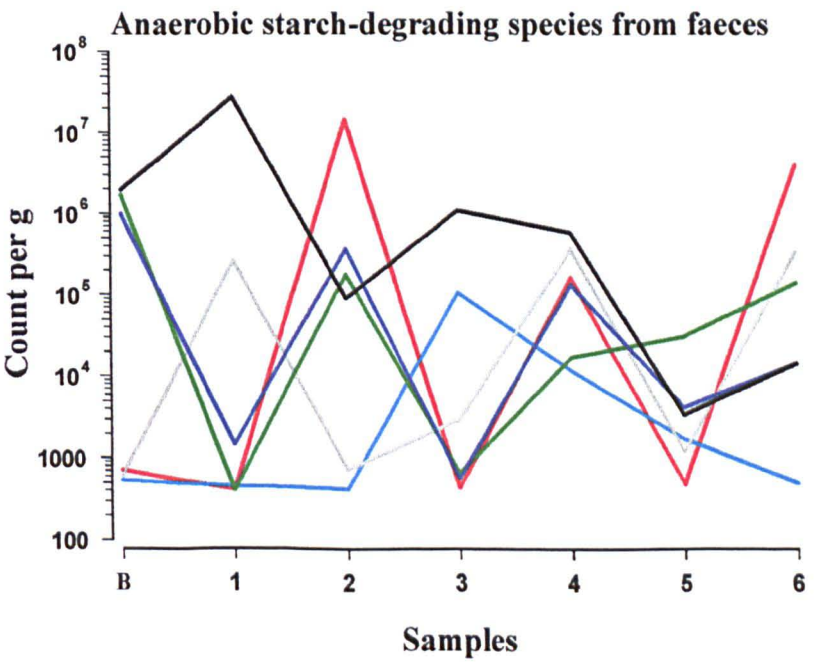


Figure 4.14 (a) :-

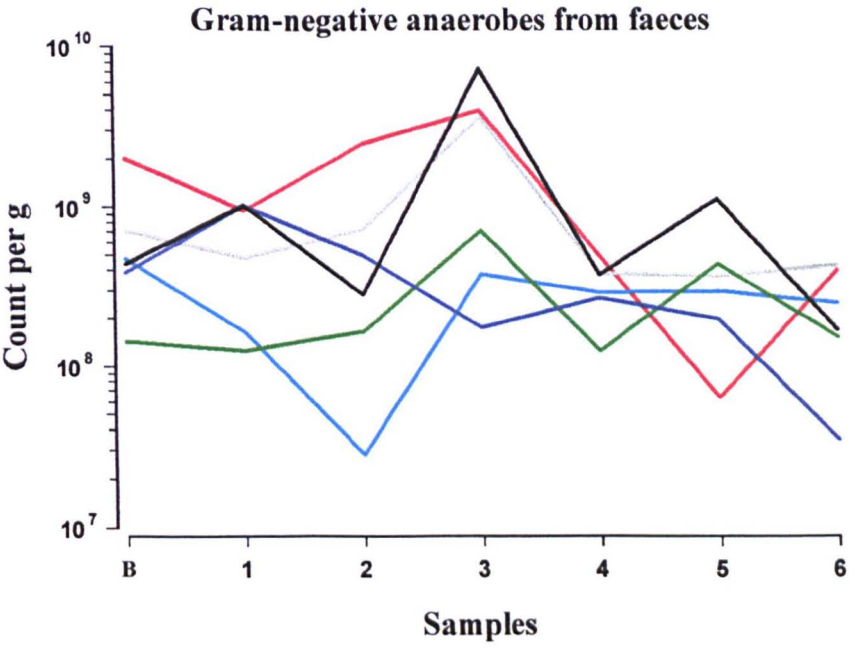
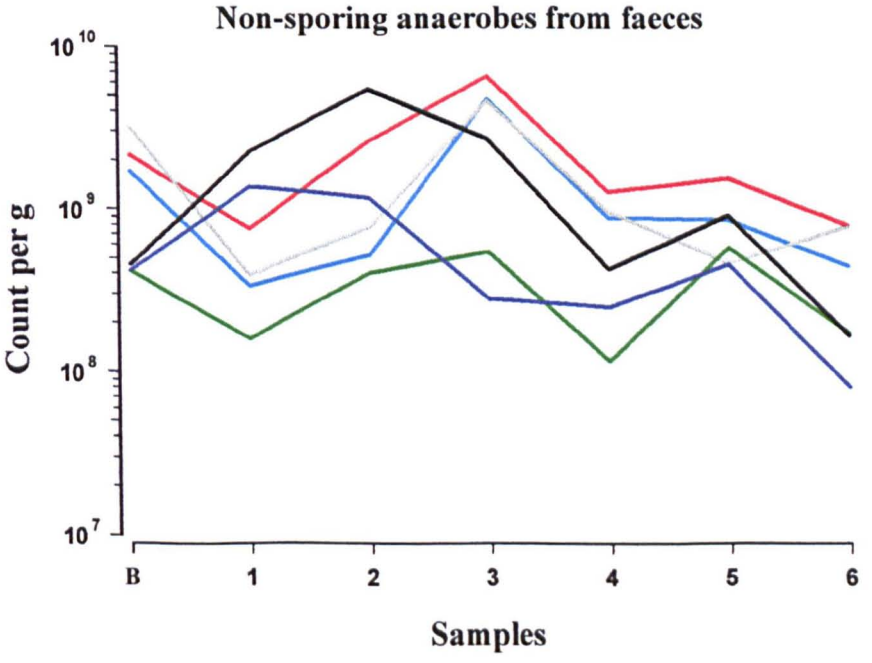


Figure 4.14 (b) :-



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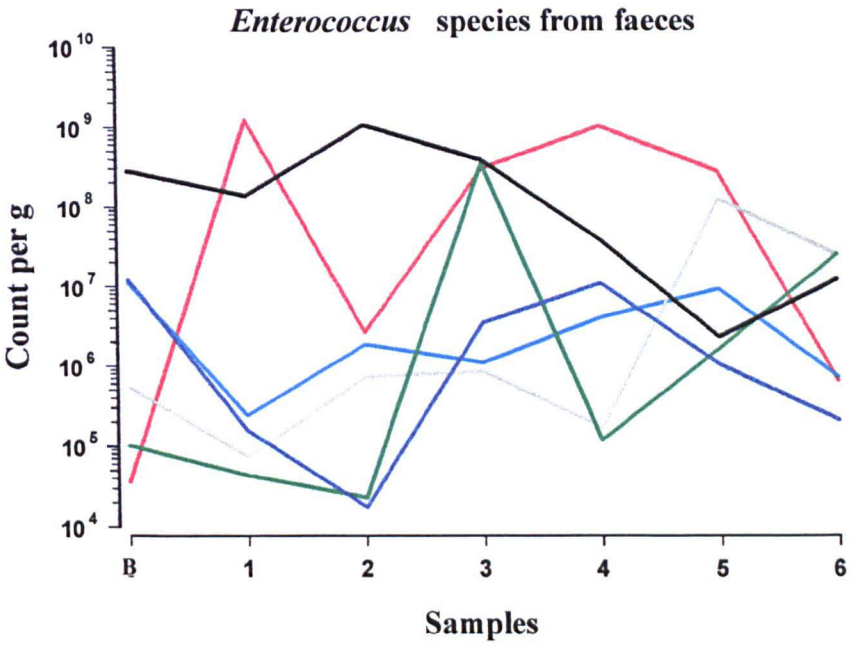


Figure 4.16 :-

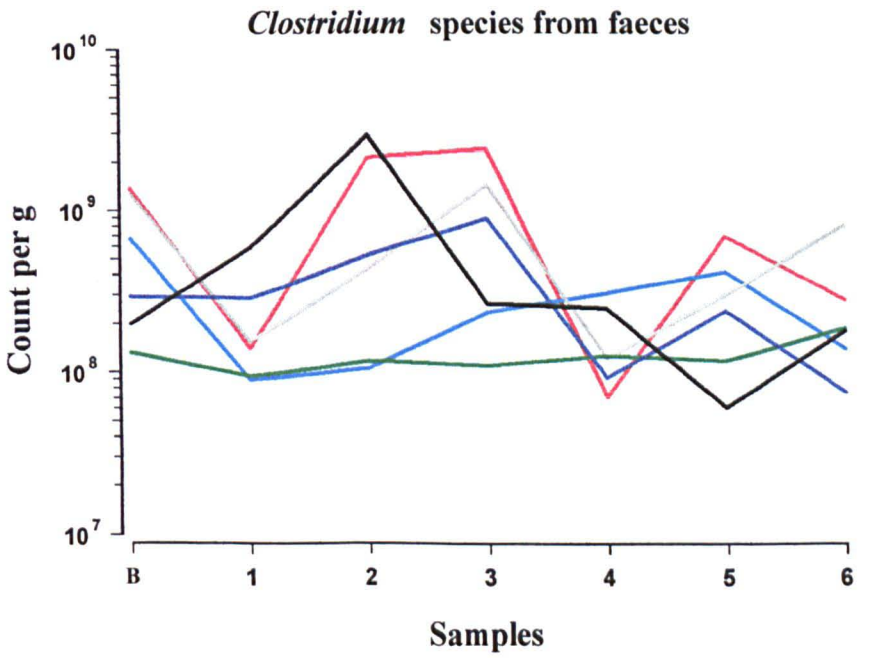


Figure 4.17 :-

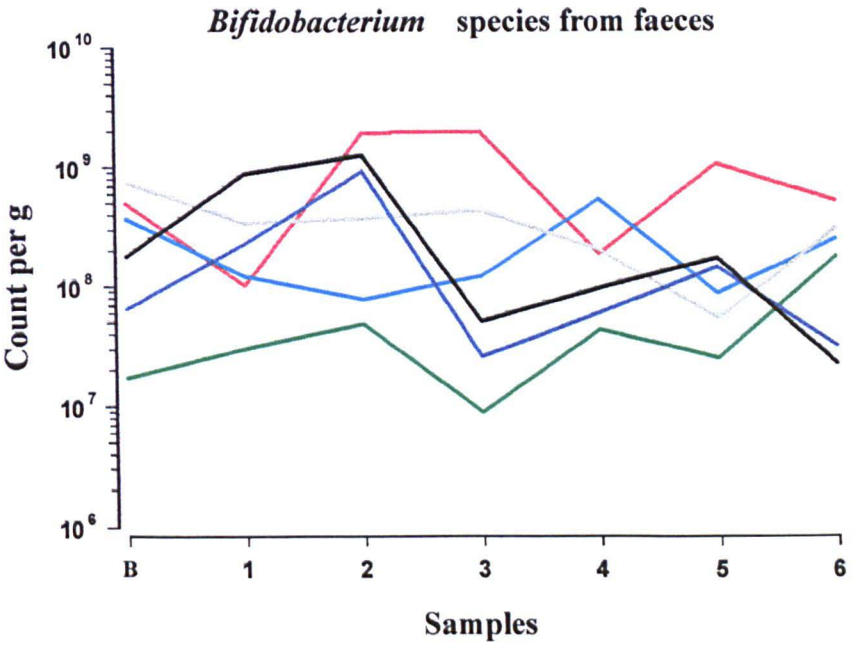


Figure 4.18 :-

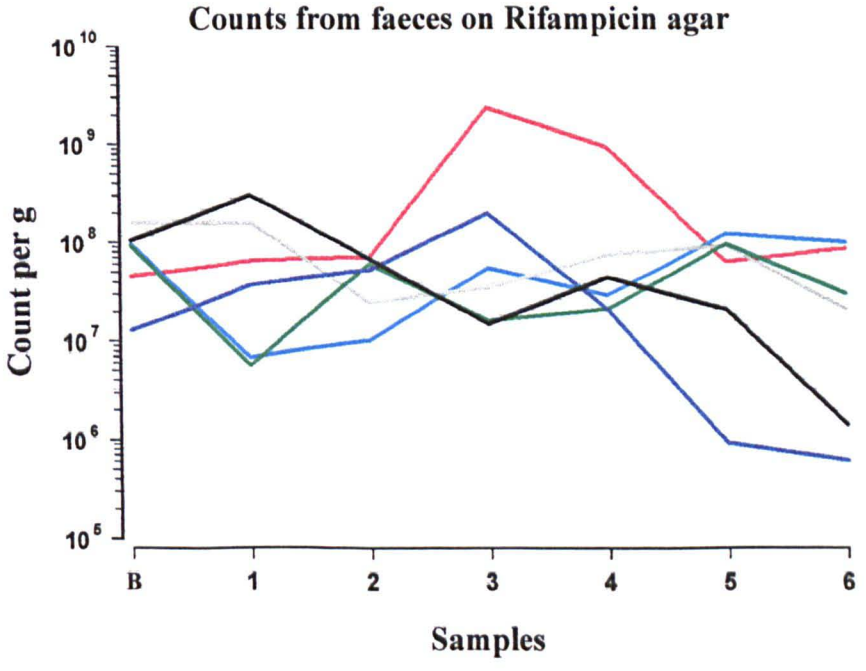


Figure 4.19 :-

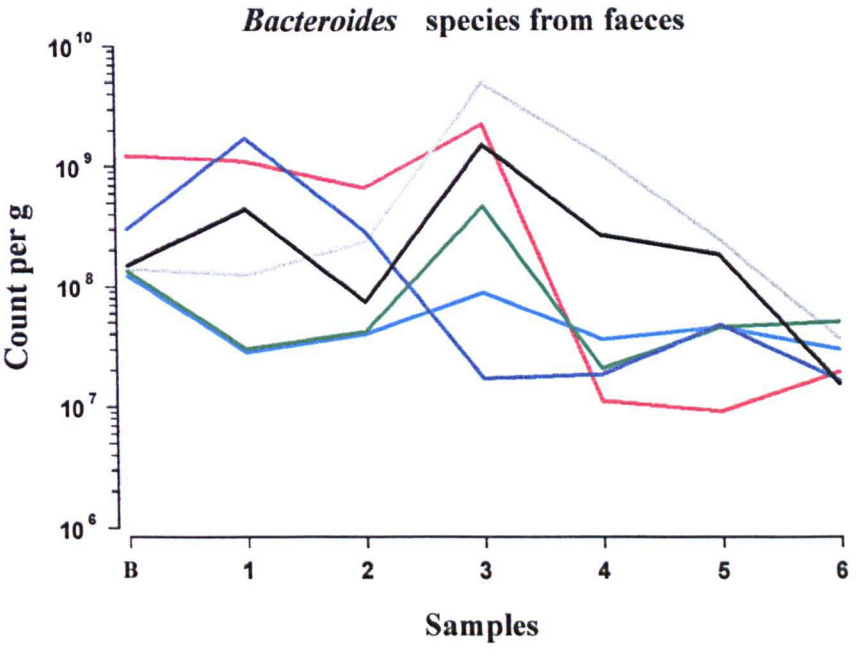

Bacteroides species from faeces
Count per g
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
B
1
2
3
4
5
6
Samples

Figure 4.2 (a) and (b) :-
Counts of coliforms and *E. coli* species (cfu g^{-1}) respectively, obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.21 (a) and (b) :-
Counts of total aerobic and total anaerobic species (cfu g^{-1}) respectively, obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figures 4.22 (a) and (b) :-
Counts of aerotolerant and total *Lactobacillus* species (cfu g^{-1}) respectively, obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figures 4.23 (a) and (b) :-
Counts of aerobic and anaerobic starch-degrading species (cfu g^{-1}) respectively, obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figures 4.24 (a) and (b) :-
Counts of Gram-negative anaerobes and non-sporing anaerobes (cfu g^{-1}) respectively, obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.25 :-
Counts of *Enterococcus* species (cfu g^{-1}) obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.26 :-
Counts of *Clostridium* species (cfu g^{-1}) obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.27 :-
Counts of *Bifidobacterium* species (cfu g^{-1}) obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.28 :-
Counts on Rifampicin agar (cfu g^{-1}) obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Figure 4.29 :-
Counts of *Bacteroides* species (cfu g^{-1}) obtained from three colon sections, taken from pigs fed a diet containing one of six starches.

Data represents mean of triplicate determinations. For all figures the following key applies :-

———	Retrograded maize	———	Retrograded potato
———	Native maize	———	Retrograded waxy maize
———	Retrograded mixture	———	Native waxy maize

Figure 4.2 (a) :-

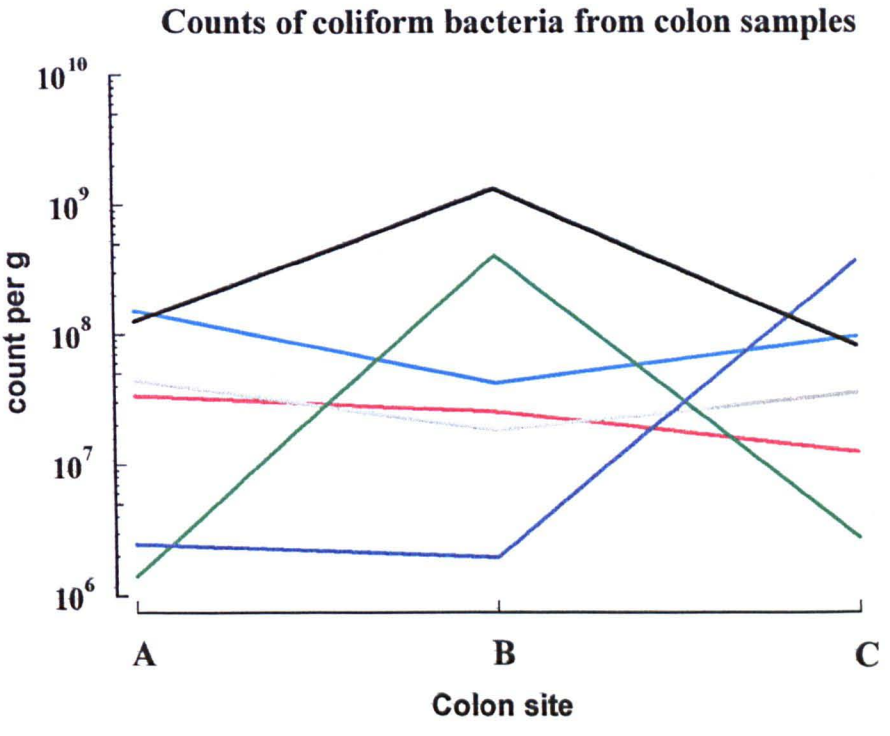


Figure 4.2 (b) :-

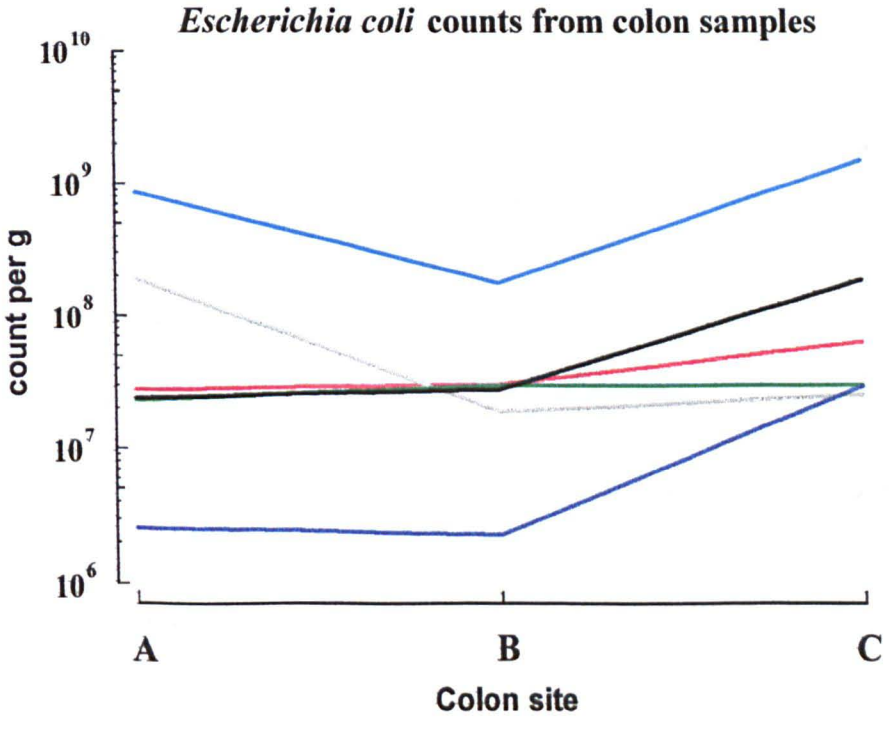


Figure 4.21 (a) :-

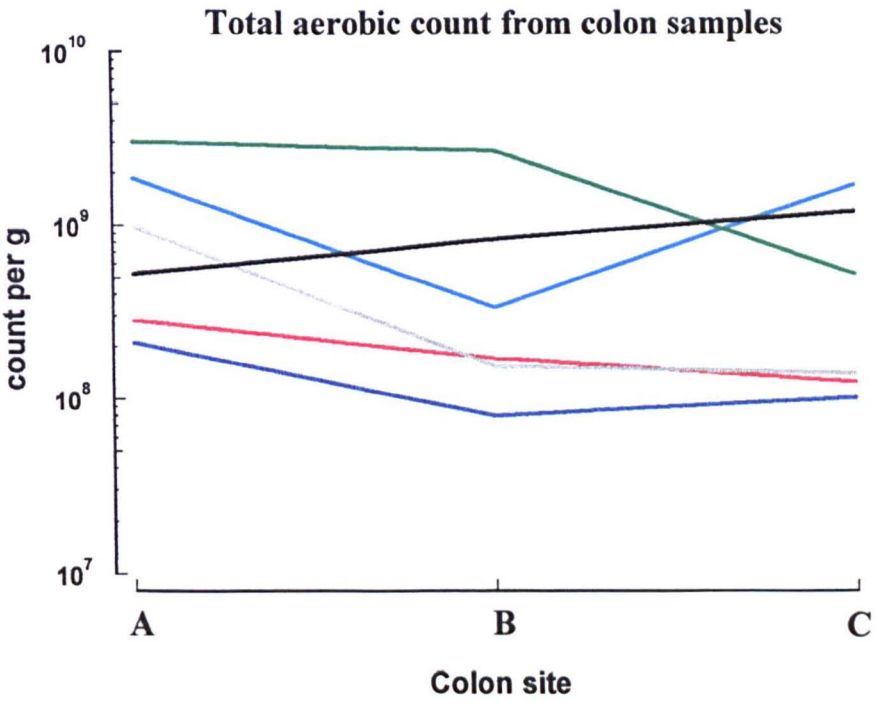


Figure 4.21 (b) :-

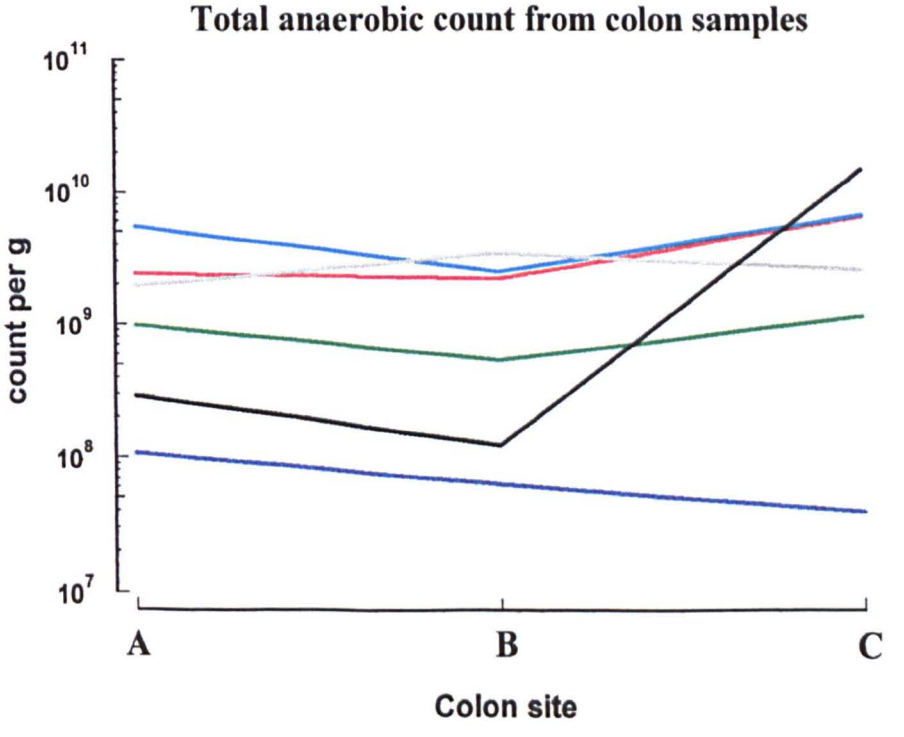


Figure 4.22 (a) :-

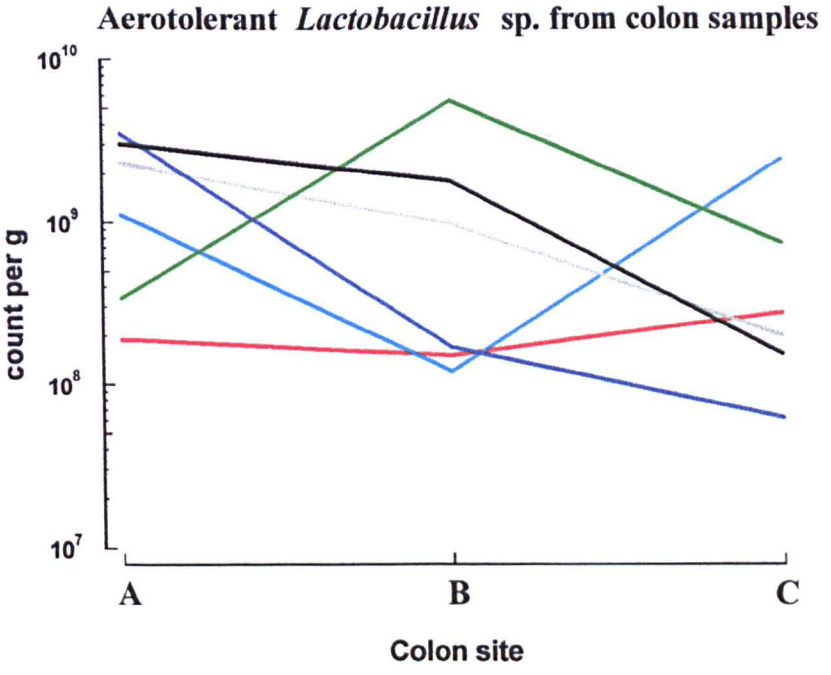


Figure 4.22 (b) :-

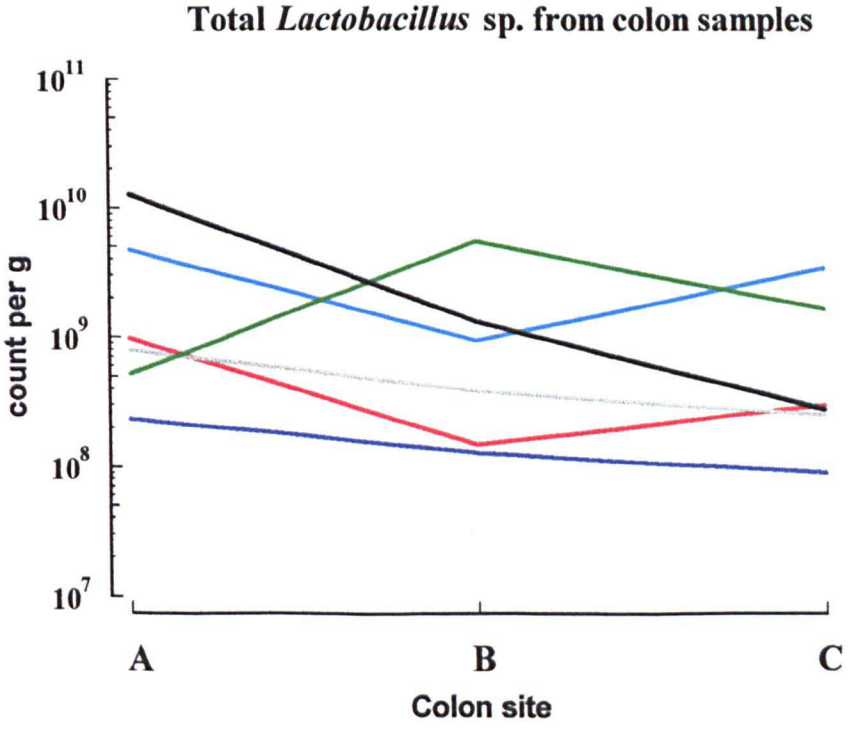


Figure 4.23 (a) :-

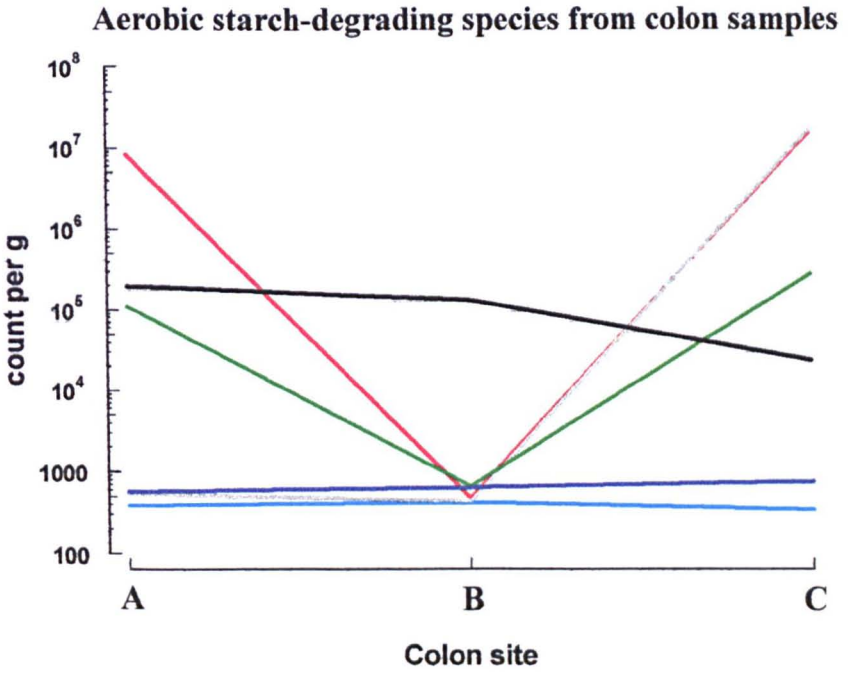


Figure 4.23 (b) :-

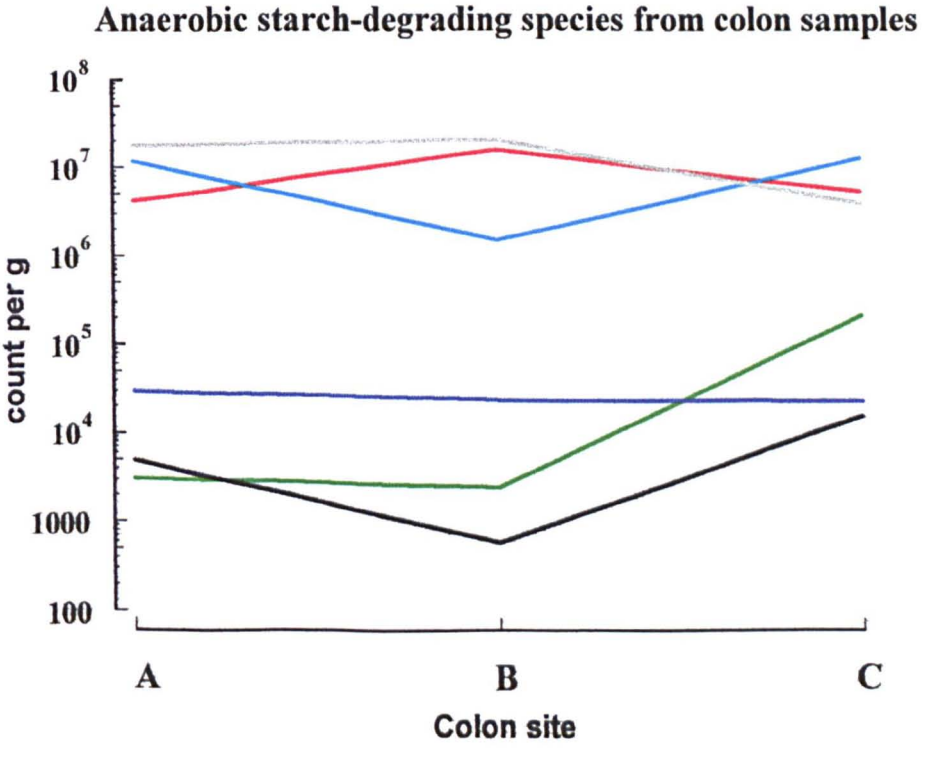


Figure 4.24 (a) :-

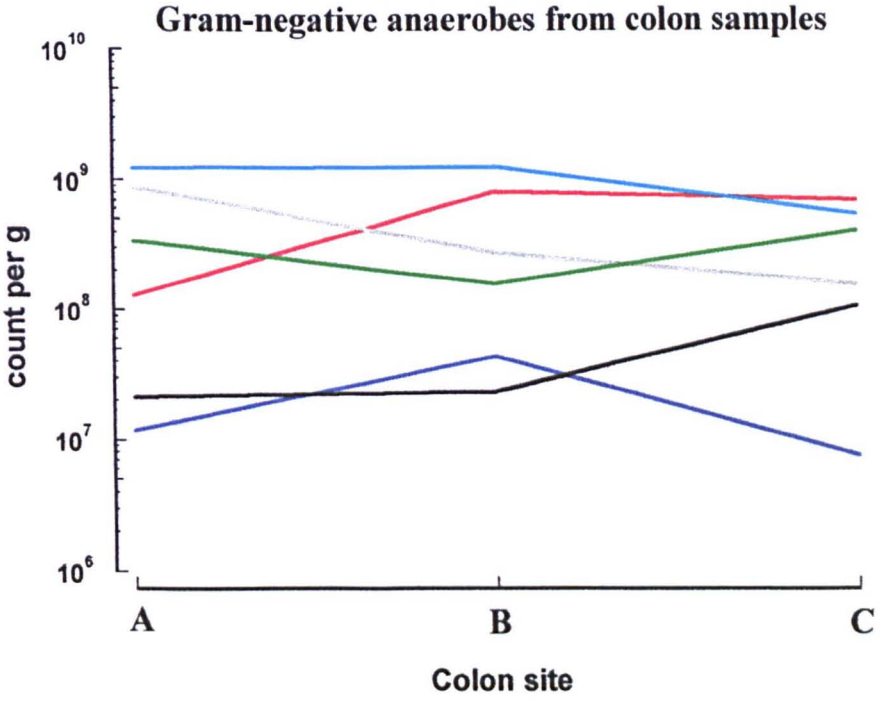


Figure 4.24 (b) :-

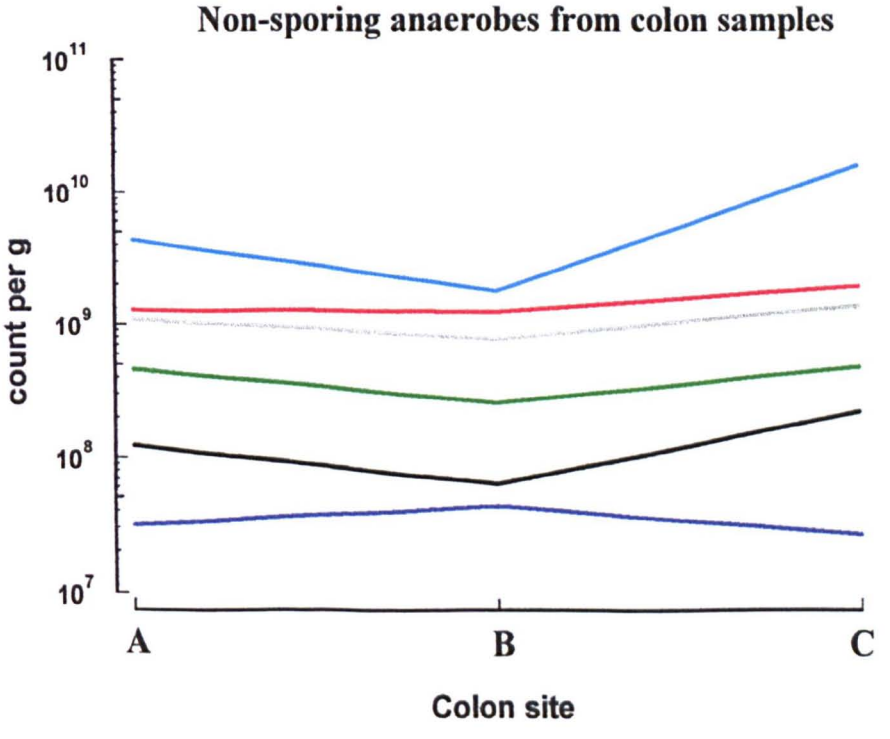


Figure 4.25 :-

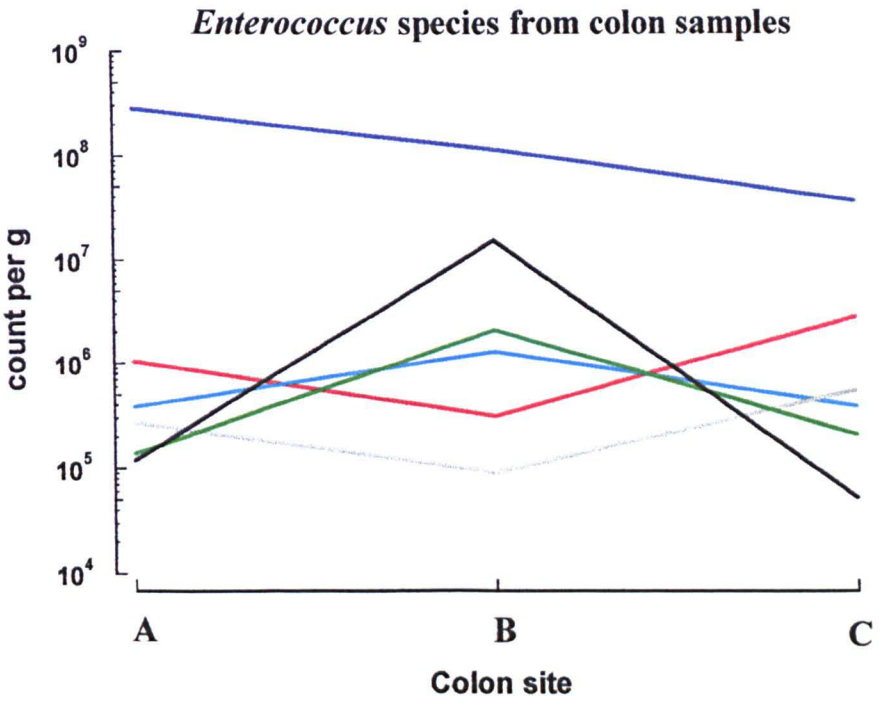


Figure 4.26 :-

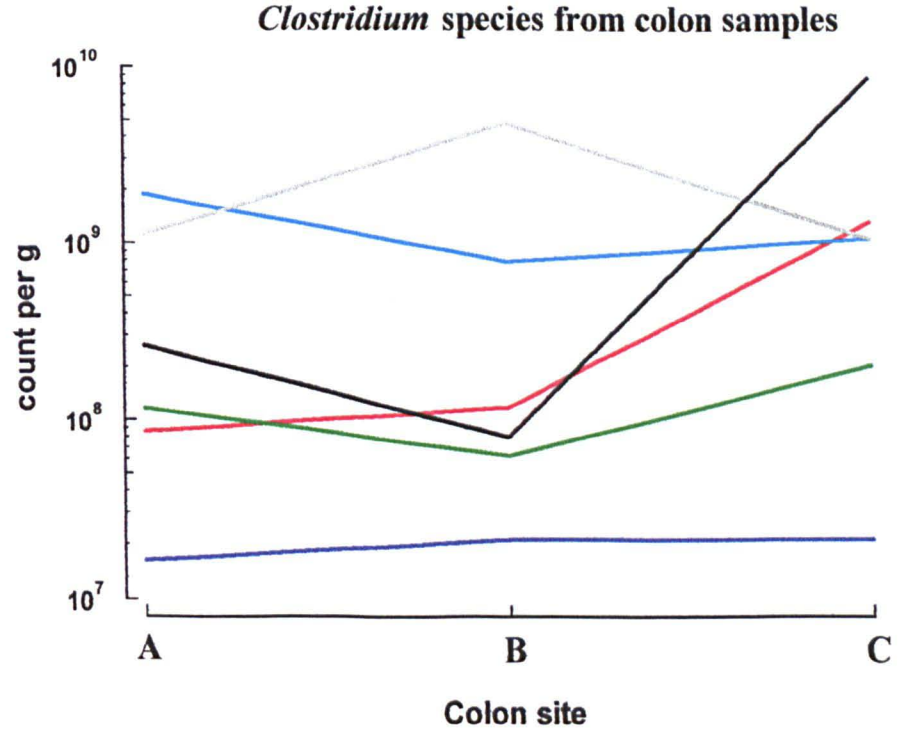


Figure 4.27 :-

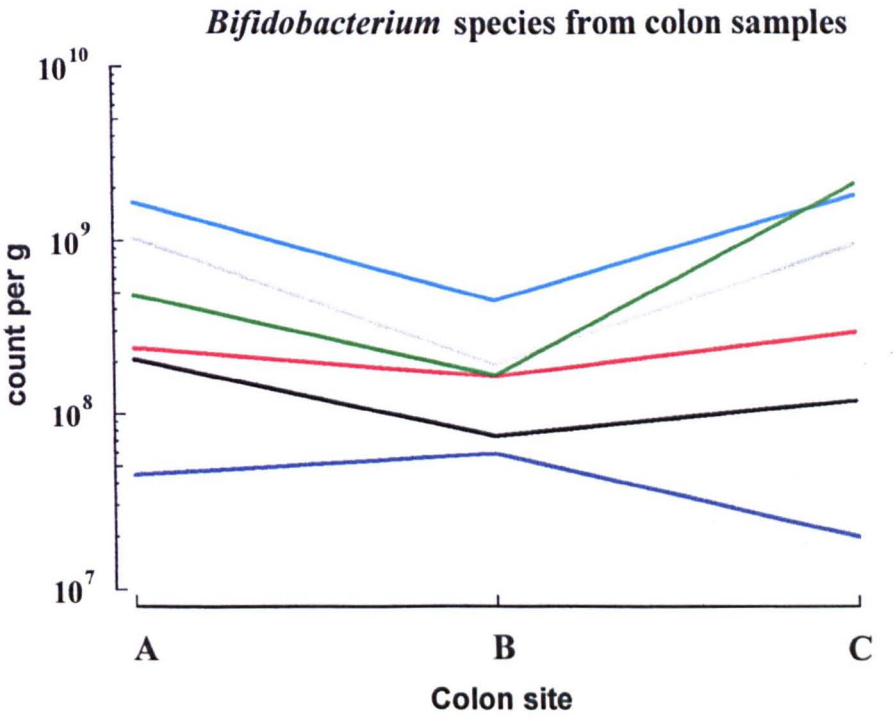


Figure 4.28 :-

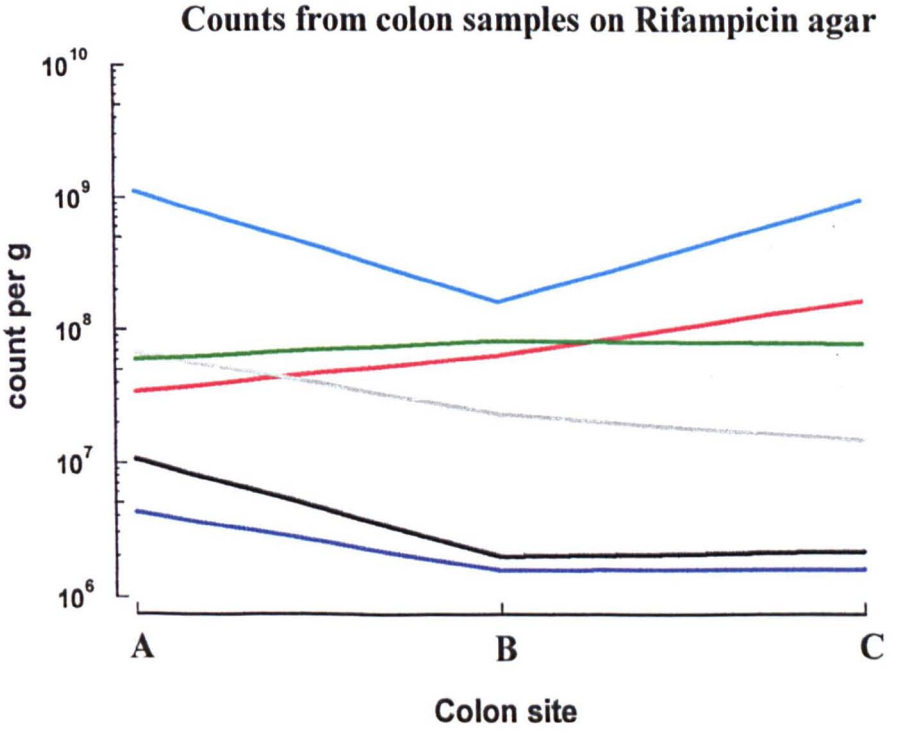


Figure 4.29 :-

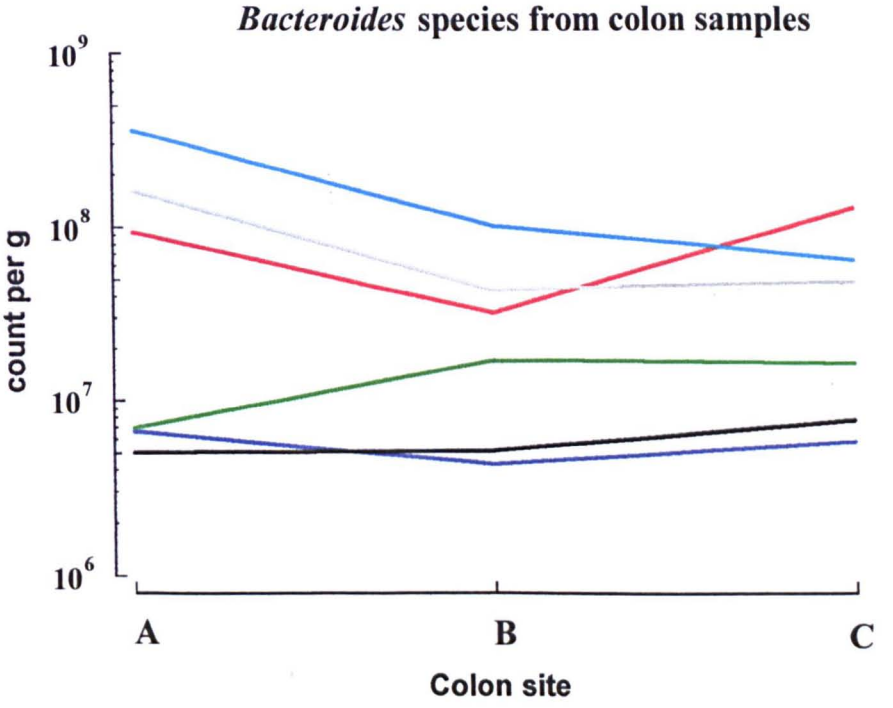
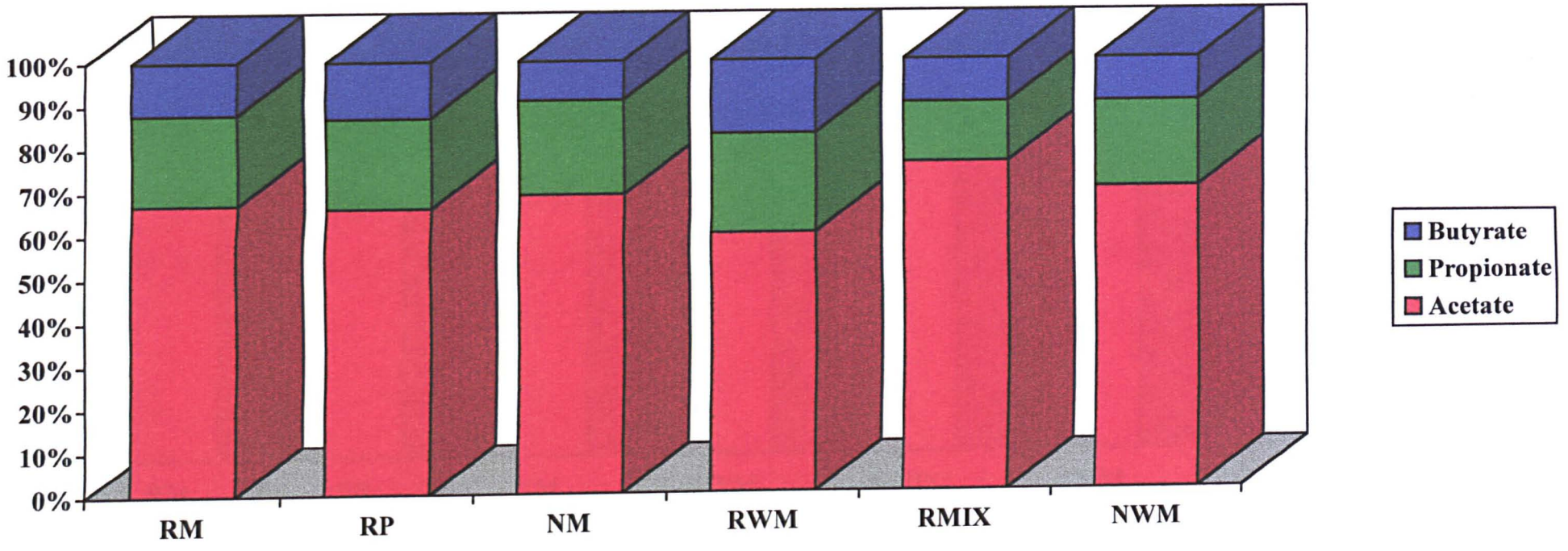
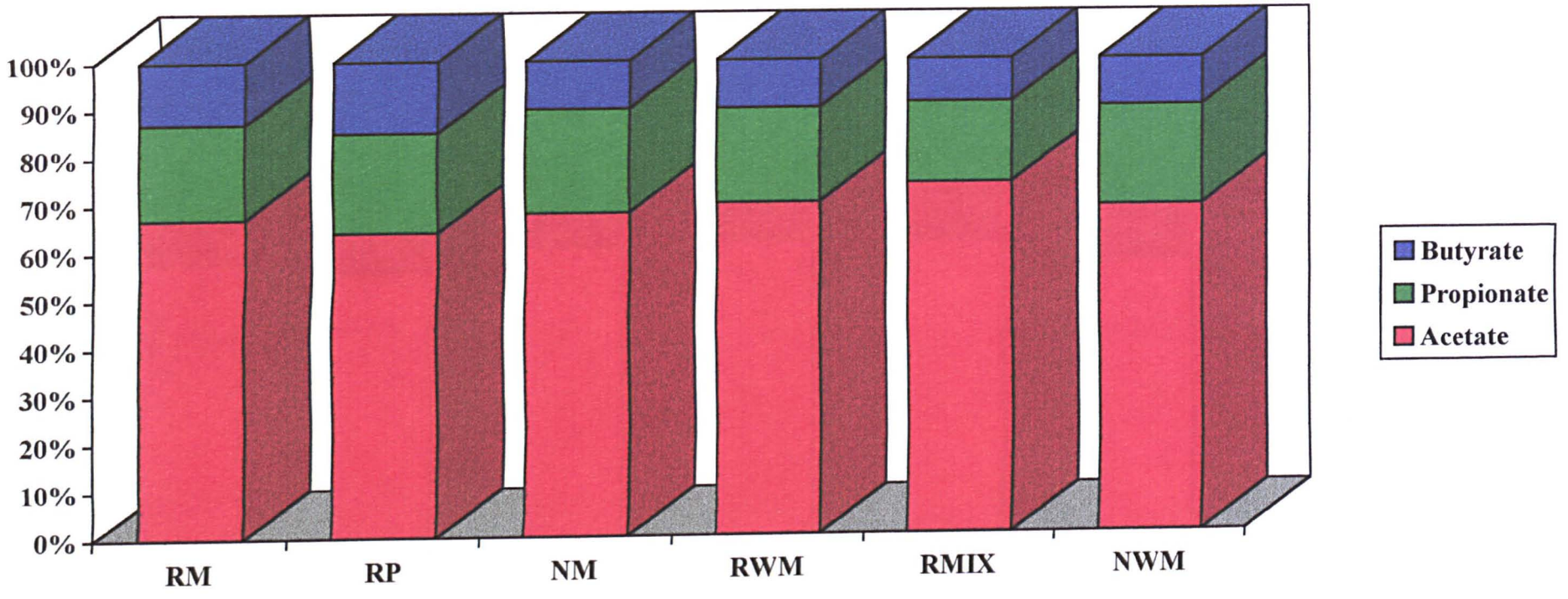

Bacteroides species from colon samples
10^9
10^8
10^7
10^6
count per g
A
B
C
Colon site

Figure 4.3 :-
Proportions of acetate : propionate : butyrate determined from SCFA data from the porcine colon - proximal.



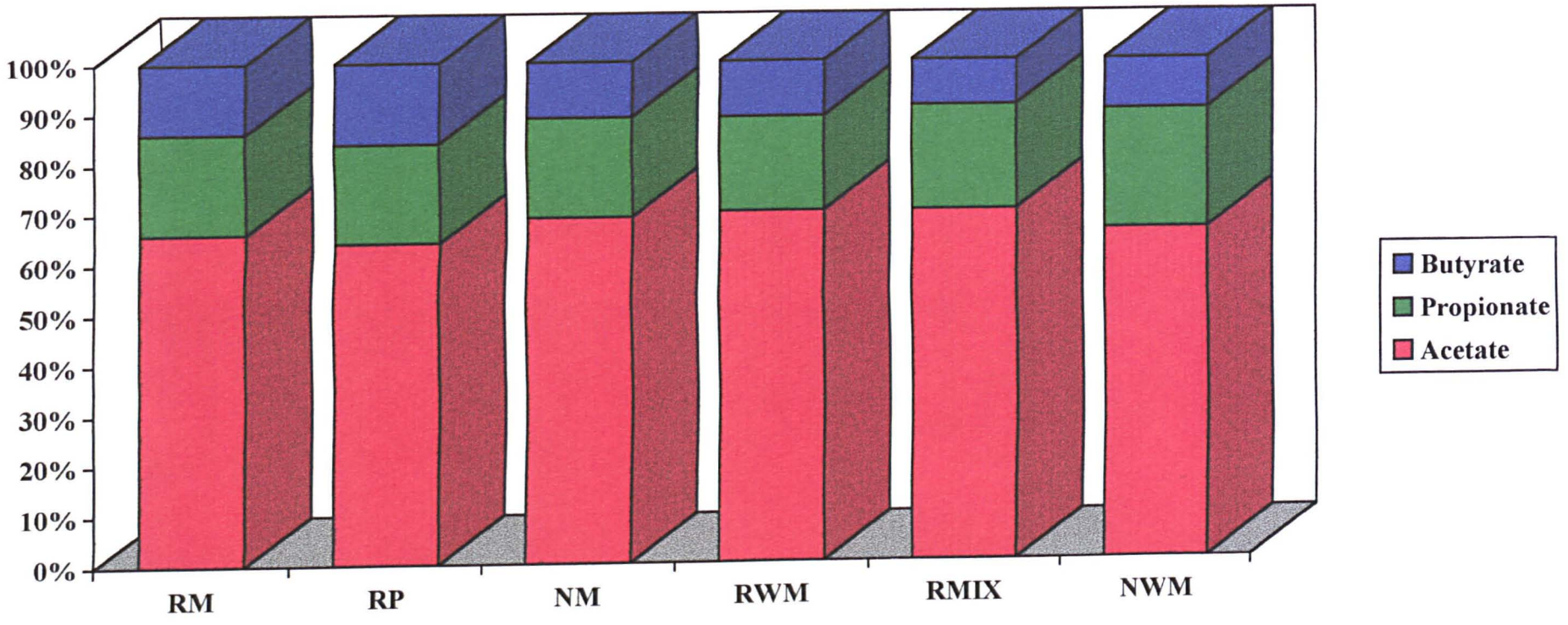
Data represent the mean of samples from three pigs on each diet.
RM = Retrograded maize; RP = Retrograded potato; NM = Native maize; RWM = Retrograded waxy maize; RMIX = Retrograded mixture; NWM = Native waxy maize

Figure 4.31 :-
Proportions of acetate : propionate : butyrate determined from SCFA data from the porcine colon - mid.



Data represent the mean of samples from three pigs on each diet.
RM = Retrograded maize; RP = Retrograded potato; NM = Native maize; RWM = Retrograded waxy maize; RMIX = Retrograded mixture; NWM = Native waxy maize

Figure 4.32 :-
Proportions of acetate : propionate : butyrate determined from SCFA data from the porcine colon - distal.



Data represent the mean of samples from three pigs on each diet.
RM = Retrograded maize; RP = Retrograded potato; NM = Retrograded maize; RWM = Retrograded waxy maize; RMIX = retrograded mixture; NWM = Native waxy maize

Figure 4.4 :-
Amylase activity (U/L) determined from faecal samples over a three week period from pigs fed on a diet containing maize starch (native or retrograded).

Figure 4.41 :-
Amylase activity (U/L) determined from faecal samples over a three week period from pigs fed on a diet containing waxy maize starch (native or retrograded).

Figure 4.42 :-
Amylase activity (U/L) determined from faecal samples over a three week period from pigs fed on a diet containing a retrograded starch mixture.

Figure 4.43 :-
Amylase activity (U/L) determined from faecal samples over a three week period from pigs fed on a diet containing retrograded potato starch.

For all above figures the following key applies :-

Retrograded maize
Native maize
Retrograded waxy maize
Native waxy maize
Retrograded mixture
Retrograded potato

Figure 4.5 :-
Amylase activities (U/L) determined from the proximal, mid and distal colon of pigs which had been fed on diets containing different starches, at slaughter. Data represents the mean of samples from three pigs on each diet, two samples from each pig.

The following key applies :-

Retrograded maize
Retrograded potato
Native maize
Retrograded waxy maize
Retrograded mixture
Native waxy maize

Figure 4.4 :-

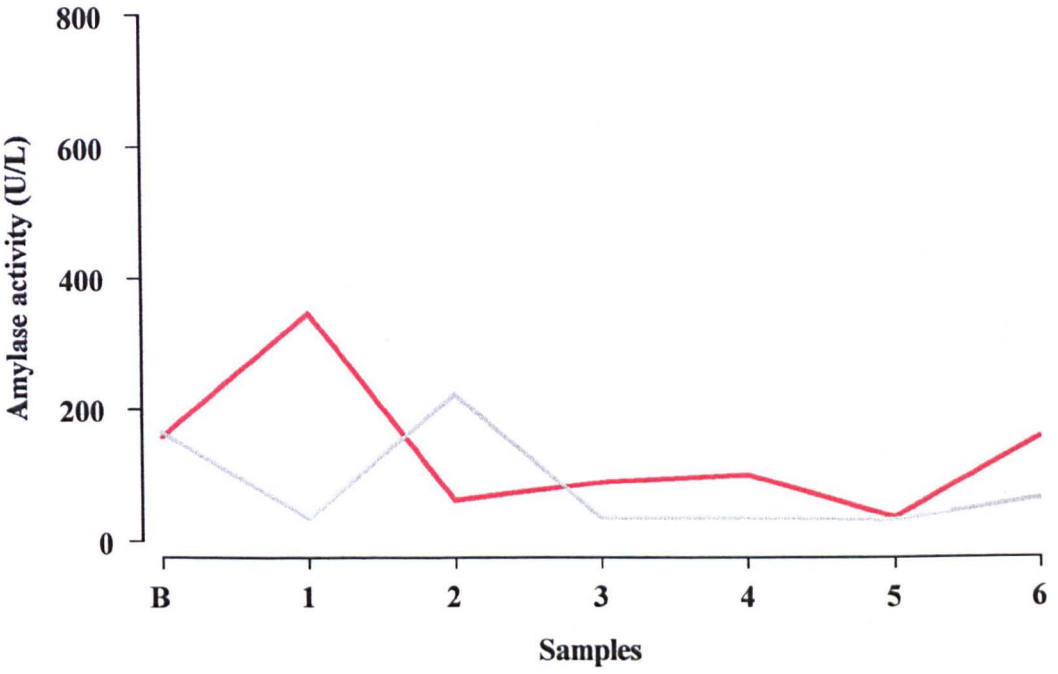


Figure 4.41 :-

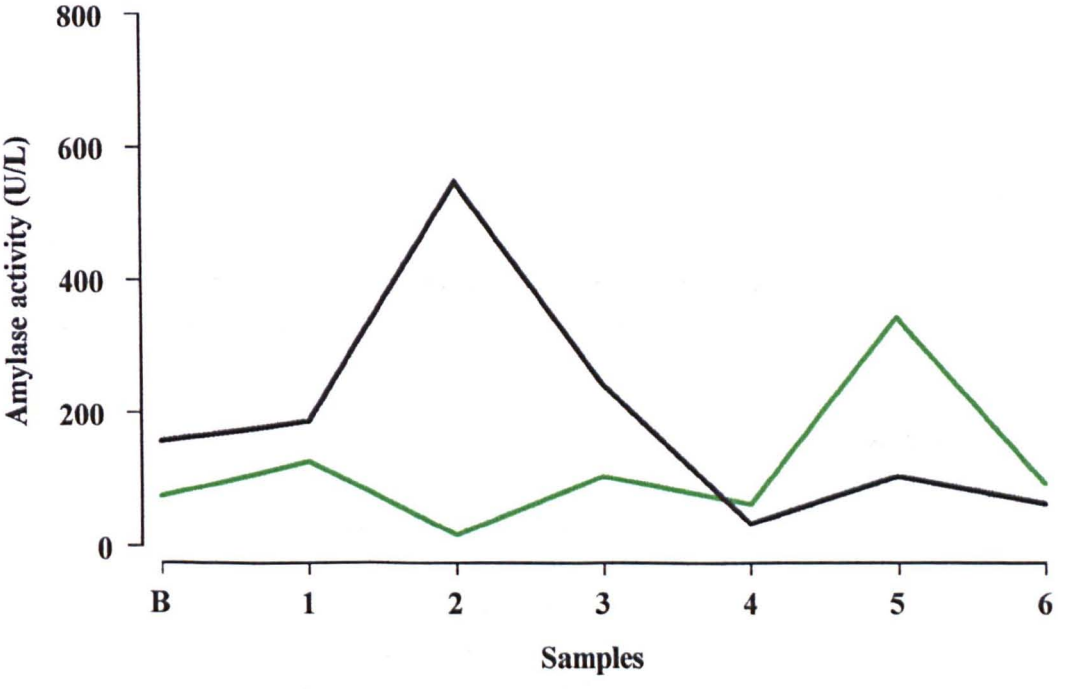


Figure 4.42 :-

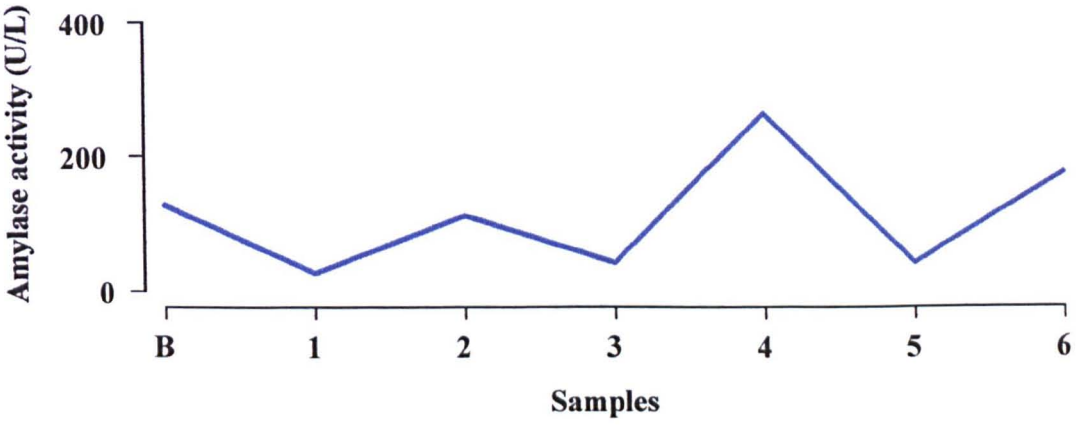


Figure 4.43 :-

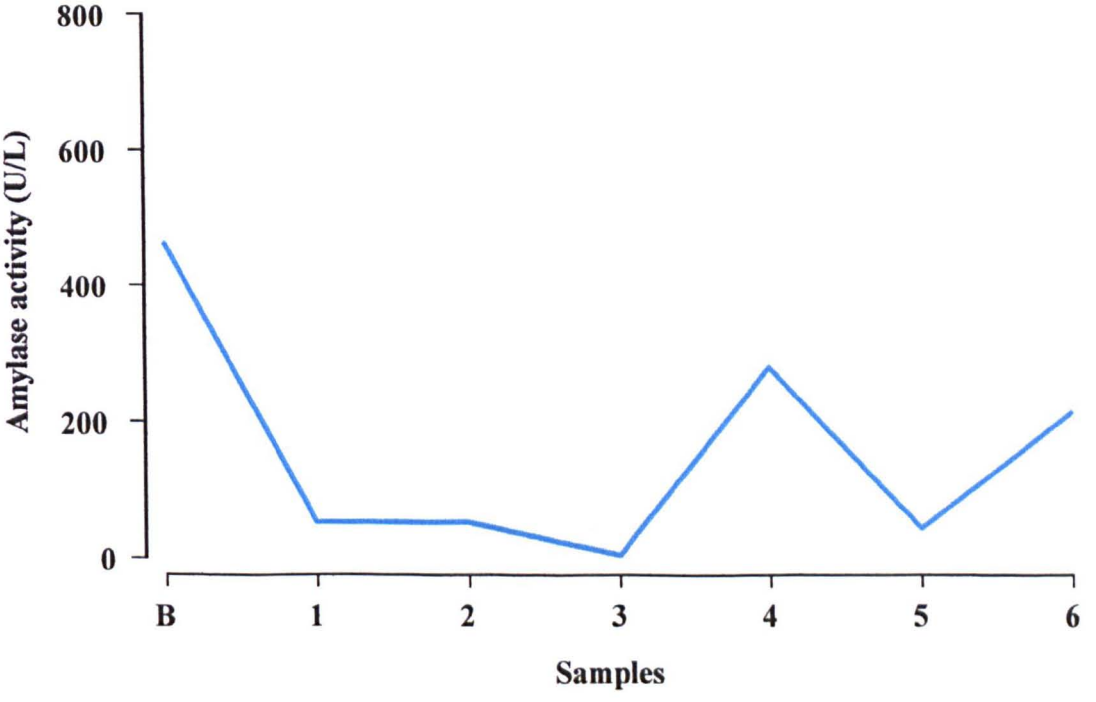


Figure 4.5 :-

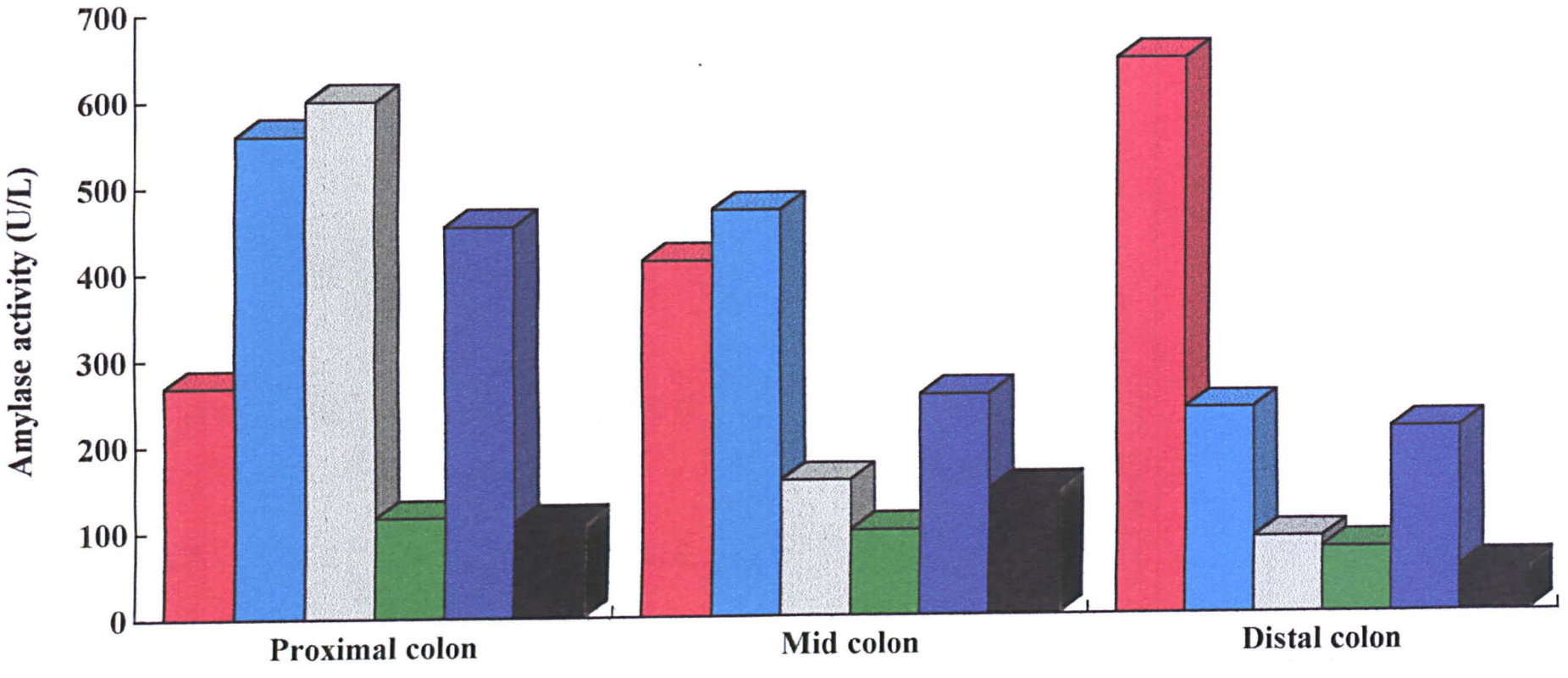

Amylase activity (U/L)
700
600
500
400
300
200
100
0
Proximal colon
Mid colon
Distal colon

Table 4.1 :-

SCFA data determined from faecal samples over a three week period from pigs fed on diets containing different starches.

Baseline	Short-Chain Fatty Acids			
Starch type :-	**Acetate : Propionate : Butyrate**			**Isoacids* (%)**
Retrograded maize	80	15	5	14.88
Retrograded potato	63	22	15	13.48
Native maize	71	20	9	11.39
Retrograded waxy maize	72	19	9	8.46
Retrograded mixture	70	24	6	16.06
Native waxy maize	78	22	0	13.52

Sample 1	Short-Chain Fatty Acids			
Starch type :-	**Acetate : Propionate : Butyrate**			**Isoacids* (%)**
Retrograded maize	69	22	9	21.89
Retrograded potato	67	19	14	13.38
Native maize	71	21	8	13.14
Retrograded waxy maize	53	36	11	15.04
Retrograded mixture	76	16	8	11.81
Native waxy maize	72	24	4	11.33

Sample 2	Short-Chain Fatty Acids			
Starch type :-	**Acetate : Propionate : Butyrate**			**Isoacids* (%)**
Retrograded maize	71	16	13	16.73
Retrograded potato	65	21	14	14.86
Native maize	73	21	6	20.43
Retrograded waxy maize	66	19	15	12.47
Retrograded mixture	71	15	14	9.46
Native waxy maize	70	16	14	10.54

Sample 3	Short-Chain Fatty Acids			
Starch type :-	**Acetate : Propionate : Butyrate**			**Isoacids* (%)**
Retrograded maize	68	19	13	10.92
Retrograded potato	63	22	15	11.98
Native maize	75	18	7	16.20
Retrograded waxy maize	72	21	7	13.44
Retrograded mixture	67	22	11	18.12
Native waxy maize	79	13	8	13.65

Table 4.1 continued :-

Sample 4	Short-Chain Fatty Acids			
Starch type :-	Acetate : Propionate : Butyrate			Isoacids*(%)
Retrograded maize	71	20	9	8.82
Retrograded potato	62	23	15	11.34
Native maize	73	16	11	12.56
Retrograded waxy maize	65	26	9	13.70
Retrograded mixture	76	16	8	14.52
Native waxy maize	77	13	10	12.75

Sample 5	Short-Chain Fatty Acids			
Starch type :-	Acetate : Propionate : Butyrate			Isoacids* (%)
Retrograded maize	72	17	11	7.31
Retrograded potato	62	27	11	9.13
Native maize	77	12	11	7.25
Retrograded waxy maize	78	18	4	8.45
Retrograded mixture	76	17	7	10.30
Native waxy maize	81	13	6	17.92

Sample 6	Short-Chain Fatty Acids			
Starch type :-	Acetate : Propionate : Butyrate			Isoacids* (%)
Retrograded maize	58	21	21	11.53
Retrograded potato	59	24	17	11.87
Native maize	66	19	15	14.13
Retrograded waxy maize	69	22	9	12.91
Retrograded mixture	69	24	7	8.78
Native waxy maize	73	16	11	20.67

Data represent the mean of multiple determinations for each diet. Acetate : propionate : butyrate = the proportion of each detected in the samples; * = The sum of isovaleric and isobutyric acids detected in each sample.

Table 4.2 :-

SCFA data determined from three sites along the colon of pigs fed on diets containing different starches.

Proximal colon	SCFA production		
Starch type :-	Total (mM)	Butyrate (%)	Isoacids* (%)
Retrograded maize	131.03	10.74	5.82^{ab}
Retrograded potato	133.49	11.98	5.59^{a}
Native maize	119.11	7.45	9.27^{b}
Retrograded waxy maize	182.90	12.21	14.03^{ab}
Retrograded mixture	133.17	9.06	5.11^{ab}
Native waxy maize	146.68	8.38	8.21^{ab}

Mid colon	SCFA production		
Starch type :-	Total (mM)	Butyrate (%)	Isoacids* (%)
Retrograded maize	82.19	11.23^{ac}	7.59
Retrograded potato	168.36	12.89^{a}	7.64
Native maize	111.76	8.65^{b}	8.59
Retrograded waxy maize	102.10	9.11^{b}	4.90
Retrograded mixture	92.11	8.33^{bc}	6.45
Native waxy maize	143.72	8.44^{b}	11.34

Distal colon	SCFA production		
Starch type :-	Total (mM)	Butyrate (%)	Isoacids* (%)
Retrograded maize	95.90^{abc}	11.40^{ab}	10.57^{a}
Retrograded potato	87.54^{ac}	13.59^{a}	8.37^{ab}
Native maize	62.86^{b}	8.96^{b}	14.00^{ab}
Retrograded waxy maize	110.66^{c}	9.88^{ab}	5.96^{b}
Retrograded mixture	124.27^{abc}	7.59^{b}	7.92^{ab}
Native waxy maize	91.23^{abc}	8.41^{ab}	10.08^{ab}

Data represent the mean of triplicate determinations for each diet. Values within a column bearing different superscript letters are significantly different ($P<0.05$), columns not bearing letters are not significantly different; * = The sum of isovaleric and isobutyric acids detected in each sample; Percentages are expressed as a percentage of the total SCFA production.

Table 4.3 :-

Feed conversion ratio* from the animals fed on diets containing one of six different starches.

	Time course		
Starch type :-	**Week 1**	**Week 2**	**Week 3**
Retrograded maize	0.45	0.67	0.75
Retrograded potato	0.60	0.57	0.80
Native maize	0.36	0.71	0.95
Retrograded waxy maize	0.53	0.74	0.65
Retrograded mixture	0.64	0.79	0.80
Native waxy maize	0.80	0.70	0.67

* calculated as :-
the total weight gain (Kg) from each pen per week / total feed consumption (Kg) per pen per week. The data has been calculated from the feed wet weight

Table 4.4 :-

Lactobacillus : coliform ratios determined from three colon sections taken from animals fed on a diet containing one of six different starches.

	Colon section		
Starch Type :-	**Proximal**	**Mid**	**Distal**
Retrograded maize	87	17	127
Retrograded potato	297	677	162
Native maize	12	38	8
Retrograded waxy maize	1809	2141	1820
Retrograded mixture	840	203	121
Native waxy maize	297	2	5

Data represents mean of triplicate determinations.

Table 4.5 :-

Measurement of skatole (mg kg^{-1}) determined from three colon sections taken from animals fed on a diet containing one of six different starches.

	Colon section		
Starch Type :-	**Proximal**	**Mid**	**Distal**
Retrograded maize	50	43	40
Retrograded potato	37	50	33
Native maize	42	39	97
Retrograded waxy maize	27	40	43
Retrograded mixture	26	19	26
Native waxy maize	80	83	83

Data represents mean of triplicate determinations.

Table 4.6 :-

Measurements of starch and resistant starch (% dry matter) determined from each of the six diets, by the Rowett Research Institute.

	Measurement of starch and resistant starch	
Starch Type :-	**% starch in diets**	**% of starch resistant**
Retrograded maize	32.45	1.08
Retrograded potato	31.58	0.37
Native maize	48.59	0.28
Retrograded waxy maize	38.49	0.04
Retrograded mixture	39.11	0.70
Native waxy maize	52.31	0.02

Table 4.7 :-

Measurement of starch detected (%) in faecal samples (dry matter) from the animals fed one of six different starches, over a period of 18 days.

	Sampling number						
Starch :-	**B**	**1**	**2**	**3**	**4**	**5**	**6**
RM	0.24	0.11	0.16	0.23	0.16	0.14	0.11
RP	0.15	0.16	0.13	0.10	0.12	<0.10	0.14
NM	0.19	0.14	0.10	0.14	0.11	0.10	0.03
RWM	0.12	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
RMIX	0.10	0.16	0.11	<0.10	<0.10	0.07	0.03
NWM	0.12	<0.10	0.07	0.07	0.03	0	0.03

Data represents mean of multiple determinations.
RM = retrograded maize; RP = retrograded potato; NM = native maize; RWM = retrograded waxy maize; RMIX = retrograded mixture; NWM = native waxy maize. B = baseline.

Table 4.8 :-

Measurement of starch detected (%) in samples from three colon sections, taken from animals fed one of six different starches.

	Colon section		
Starch Type :-	**Proximal**	**Mid**	**Distal**
Retrograded maize	0.41	0.30	0.14
Retrograded potato	0.97	0.35	0.15
Native maize	0.11	0.11	0.13
Retrograded waxy maize	0.12	0.13	0.15
Retrograded mixture	0.38	0.37	0.13
Native waxy maize	0.12	0.07	0.03

Data represents mean of triplicate determinations.

4.5 Discussion

Faecal samples were taken from the pigs at various intervals during the feeding of diets containing one of six different starches. These starches were added at a concentration of 50% (wet weight), which is higher than in an average animal diet, in order to heighten any effects which may have existed due to the nature and type of the starch included. Analysis of the same bacterial species over the three weeks the diets were fed should give a good indication of the effect these starches can have on the bacterial population resident in the colon. However, the faecal samples will only give an indication of the effects on the flora present in the later part of the colon (Finegold & Sutter, 1978). The counts of the various bacterial species which were isolated from the faecal samples of the animals, varied greatly between animals fed the same starch. This led to large standard errors with some of the bacterial species studied, which is a hazard when studies are carried out using living animals. Therefore, the discussion will focus mostly on the mean values represented, and the trends observed with the bacterial species studied, rather than the actual numbers detected.

The faecal samples taken from the animals during the three weeks contained similar numbers of aerobic and anaerobic bacteria, although the aerobic counts were recorded at slightly higher numbers. The highest counts recorded for the aerobic species were 10^{11} cfu g^{-1} wet weight, and the highest for the anaerobic species were recorded at 10^{10} g^{-1} wet weight. These results are contrary to the observations from human faecal material where the anaerobic population has been reported to outnumber the aerobic population (Simon & Gorbach, 1981). Hillman *et al.* (1993) found that the gut of newly weaned piglets contains oxygen at an average concentration of 70 μmol l^{-1}. This concentration will be higher than that found in the adult pig, since as observed by Hillman *et al.* (1996), dissolved oxygen in the monogastric intestine is a function of surface area : volume ratio. Researchers have shown that the gut environment becomes more anaerobic as the animal becomes older, and the bacterial species found in the gut will be subject to change (Maczulak *et al.* 1989; Hillman, 1998). This higher concentration of oxygen in the newly weaned piglet may account for the higher numbers of total aerobes detected. The numbers of aerobic species detected in the faecal samples prior to feeding the diets, were very different, sometimes

significantly different. This is expected when dealing with animal material as there will be a natural variation in the numbers and species of bacteria between animals.

Previous studies examining the faecal flora of adult humans have reported numbers of total counts as at least 10^{11}cfu g^{-1} (quoted from both wet and dry weight faeces) (Finegold *et al.* 1983; Wang *et al.* 1996; Savage, 1983; Simon & Gorbach, 1981). These reported counts from human faecal studies are similar to those obtained from studies on both faecal and large intestinal contents from adult pigs, where counts of 10^{10}-10^{11} cfu g^{-1} were detected (Salanitro *et al.* 1977; Russell, 1979). The average counts isolated from the porcine faeces over the three week sampling period was 10^{8} cfu g^{-1} wet weight. As the majority of the bacterial species isolated in this study were facultative, the results obtained relate more closely to the counts of facultative anaerobes (10^{8}-10^{9}) reported by Finegold *et al.* (1983), which had been isolated from the faecal material of Japanese-Americans, using the roll-tube method of Moore & Holdeman (1974). Comparison of the counts obtained in this study with those obtained from the faecal material of other monogastric sources such as humans, can be very difficult as factors such as diet, stress, environment and age can affect the numbers and types of bacterial species isolated (Holdeman *et al.* 1976; Finegold & Sutter, 1978; Mallett & Rowland, 1988). As the animals used in this study were newly weaned, the gut flora is unlikely to be fully developed, which may also cause discrepancies when comparisons between other studies are made. Another factor which could affect the numbers of the bacterial species studied is the media which were used for their isolation. The types of media employed may have allowed the growth of more than one bacterial species and would not have supported the growth of just one single species. This makes it difficult to obtain the exact enumeration of one particular bacterial species, and hence the counts obtained will only be an estimation on that particular agar. The "total" counts reported will also be estimates as the media used will not support the growth of all the aerobic or anaerobic species which are known to inhabit the colon. Some of the media used will also allow the growth of a single genus on more than one agar, e.g. *Clostridium* species will be isolated on both reinforced Clostridial agar (RCA) and Rifampicin agar (RIF), which would give an inaccurate total count if the numbers from each agar were simply added together. All these factors may result in lower counts being obtained from the animals in this study, compared to those published in previous studies. The extrapolation of bacterial

data from one species to another is never without its problems, as Moore *et al.* (1987) stated "the flora of pigs was grossly different from that of humans" at the species level. However, for this study the exact numbers and species of bacteria isolated are not wholly important as this study sets out to examine the effects of starch type (source, treatment and content) on the bacterial flora which is already established. The counts and species obtained in this study however, were sufficiently comparable to those already found in studies employing human and porcine faecal material as were the principles of bacterial activity, to allow extrapolation of this porcine study to other monogastric animals such as humans.

With the majority of the aerobic species studied, the counts isolated with the retrograded waxy maize, native waxy maize and retrograded mixture starches showed a peak in the numbers after 7 days on the diets, to approximately 10^{10}-10^{11} cfu g^{-1}. This increase in bacterial numbers will be due to an increase in the amount of available carbohydrate entering the large intestine which will increase bacterial growth. When pigs are weaned, the solid food entering the gut may cause damage to the epithelium due to its abrasive nature. This damage will result in an increase in the supply of blood to the affected area (Hillman *et al.* 1996) to aid the healing process, which will then increase the supply of oxygen to this area (Hillman *et al.* 1996). If this theory is true, this may account for the growth of the aerobic bacterial species observed with the starches containing higher levels of amylopectin, as these starches may simply be more abrasive on the gut wall than the other starches studied. This increase in the aerobic bacterial species may be due to a theory other than the abrasive nature of this starch on the gut wall. It has been proposed that bacteria are able to utilise the amylopectin part of the starch more easily than the amylose portion (Reid *et al.* 1996), and hence the aerobic species studied may have increased further in numbers due to the utilisation of amylopectin. The amylopectin portion of starch is less affected by the heating-cooling process (Filer, 1988) and so would naturally be more accessible to bacteria. However, once starches have been autoclaved and cooled, the amylopectin portion of an average starch (typical in amylose and amylopectin quantities) such as maize and potato, may be mixed in with the amylose portion and so would not be readily available to the bacteria. The more amylose that a starch contains, the greater this effect and the less accessible the amylopectin may be. However, this effect will not be seen with waxy maize starch as very little or no amylose is found in this starch, and so

theoretically the starch should be more readily available for bacterial fermentation. It has been discovered recently that when pigs are newly weaned the villi of the small intestine shorten and the crypts of the villi increase. The maximum change in the physiology of the small intestine occurs from 3-7 days post-weaning, with the recovery usually being complete between 10-15 days post-weaning (Anon, 1998). This reduction in villus height is associated with a decrease in absorption from the small intestine, which will increase the available carbohydrate entering the large intestine and will in turn promote bacterial growth (Anon, 1988). This phenomenon has also been encountered by other researchers (Pluske *et al.* 1996). As the starches which showed an increase in counts contained more amylopectin, it supports the previous data suggesting that this portion of the starch may be more easily fermented by the bacteria present, and also suggests that the effects observed may be related to the amylopectin portion of the starch. After the first seven days on each diet, the numbers of aerobic bacterial species from the faecal samples stabilised, indicating that the increase in available carbohydrate entering the colon could not support a continuing rise in bacterial numbers, and so the bacterial population stabilised at a level which would be supported easily by this available carbohydrate. In general, the anaerobic species studied from the faecal samples showed a similar trend to the aerobic species over the 18 days (increase in numbers followed by stabilisation), but the peak in numbers after seven days on the diets was more pronounced with the aerobic population. This suggests that the aerobic bacterial population may be more easily manipulated by dietary components than the anaerobic populations.

The strictly anaerobic species studied such as the *Bifidobacterium* spp., *Bacteroides* spp., and counts on rifampicin agar (*Clostridium* spp., *Eubacterium* spp. and *Fusobacterium* spp.), did not follow the trend outlined above. The numbers of these bacterial species appeared to be closer together throughout the 18 day sampling period, with no apparent stabilising at the end. The findings from the aerobic, facultative and strictly anaerobic bacterial populations suggest that the aerobes are more easily affected by changes in the diet than the anaerobic population. An increase in the numbers of this bacterial group may be easier to detect in the older animal, as they have more scope to increase as they are reported to be between 10^2-10^4 cfu g^{-1} lower than the anaerobic bacterial group. A vast increase in

the numbers of the anaerobic bacterial species would need to take place before a change was noticed, as this group already exists in the older animal at very large numbers in the gut. The periods of large variations in the bacterial counts suggests that the bacterial population as a whole takes approximately seven days to adjust to a new diet. One factor which could also affect the species and numbers of the gut bacteria studied initially, could be the stress the animals may have experienced due to the removal from the sow, the change in their environment and the change of the diet from a milk-based diet to one containing solid food. After the peak in numbers observed around day seven, the bacterial populations studied settled back to numbers similar to those detected from the animals prior to the start of the diet. This is in accordance with published findings which state that diet variation leads to relatively minor effects on the faecal microflora (Simon & Gorbach, 1980).

The most numerous bacterial species which inhabit the colon have been stated as belonging to the Genera *Bacteroides*, *Fusobacterium*, *Eubacterium* and *Bifidobacterium* (Gorbach & Goldin, 1990; Moore, 1978). These bacterial groups are also known to be among the most numerous starch-degrading species isolated from human faeces (Macfarlane & Englyst, 1986). This would mean that the anaerobic starch-degrading bacteria present in faeces, would be detected at higher numbers than the aerobic starch-degrading bacteria. The results obtained in this study match those of previous findings, with the anaerobic species being detected in higher numbers. The starch-degrading population from the porcine faeces was estimated using a modification of a simple plate method described by Macfarlane and Englyst (1986). From this method, Macfarlane & Englyst reported counts of anaerobic starch-degrading bacteria from faeces at 10^{10}-10^{12} cfu g^{-1}. The numbers of anaerobic starch-degrading bacteria isolated from the study with porcine faeces yielded counts no higher than 10^{7} cfu g^{-1}. The reason for the lower numbers from the porcine study may be due to the differences in the isolation and growth conditions employed in this study. In this study, a non-selective plate count agar (PCA) with added starch was employed instead of the PY medium used by Macfarlane & Englyst (1986). The incubation of the plates in this study was also limited to two days instead of five as reported in the Macfarlane & Englyst study (1986), which might not be long enough to isolate the dominant anaerobic starch degrading species described above, as they are slow growing.

The retrograded mixture diet is composed of a mixture of 50% waxy maize and 50% maize which was then retrograded to obtain a starch with an amylopectin content between that of the waxy maize starch and the maize starch. In theory this starch should give bacterial counts which were intermediate between those of the waxy maize and maize starch. The graphs of the various bacterial species isolated from the faecal samples, show that in most cases this did apply. However, in the case of the *Clostridium* spp., the counts for the retrograded mixture diet followed a similar trend to that of the retrograded waxy maize diet for the first seven days of feeding and then followed a trend similar to that for the retrograded maize diet for the remaining eleven days of feeding. This finding suggests that the *Clostridium* spp. probably ferments the amylopectin portion of the mixture initially, possibly as it is more readily available, and then adapts to the fermentation of the maize starch in the mixture. The *Bifidobacterium* counts obtained with the native waxy maize and retrograded mixture diets followed a similar trend throughout. This suggests that the *Bifidobacterium* species isolated from the pigs may be utilising the waxy maize part of the starch mixture.

To ensure the gut of man and animals remains healthy, the correct balance of the gut microflora is necessary. This includes a low or stable population of the coliform group (particularly in the proximal colon) and a high population of lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* species. The lactic acid group maintain a low pH in the gut which is usually low enough to prevent a large increase in the coliform population, which prefer a higher pH. Low numbers of *Bacteroides* species are also preferred even though this species are major starch degraders in the large intestine, as high numbers of this group have been implicated in the risk of colon cancer. A dietary component which promotes a beneficial bacterial population and maintains low levels of the coliform population, or one which gives rise to a stable bacterial population, is the ideal. In addition to this, a continued starch fermentation and a reduced protein fermentation in the distal sections of the colon are also required to maintain a healthy gut. To detect which starches were depleted in this region, and hence allowed protein fermentation to take place, the levels of isoacids were measured, as these SCFA come from the degradation of amino acids and therefore do not result from the fermentation of carbohydrates.

The counts of the beneficial species described appeared to remain at fairly high levels throughout with each of the starches, and the coliforms and *Bacteroides* spp. did not appear to give elevated counts over the 18 day period. The counts of the *Lactobacillus* population remained stable with the retrograded maize starch and the numbers of coliforms also decreased over the 18 day sampling period with this starch. The highest counts of *Bifidobacterium* species and *Bacteroides* species were also obtained with this starch. The retrograded maize starch samples also appear to show the highest proportions of butyric acid and one of the lowest levels of isoacids, after 18 days on the diets. With the retrograded waxy maize starch, the numbers of *Lactobacillus* species reached a peak after seven days on the diets but gave fairly low counts throughout. The counts of *Bacteroides* species with this starch were also consistently low over the 18 day sampling period. However, the counts of *Lactobacillus* spp. with the retrograded and native waxy maize starches appeared to increase after the 14 day sampling (Sample 5). The counts of *Bifidobacterium* species also appeared to increase over the 18 day sampling period with the retrograded waxy maize starch. The levels of SCFA detected from the native and retrograded waxy maize samples, show that the retrograded waxy maize starch shows high levels of propionic acid throughout, and low levels of isoacids throughout. The levels of isoacids and acetic acid detected from the native waxy maize starch were the highest detected after 18 days on the diets. These results indicate that feeding the retrograded starches, in particular the retrograded maize starch, appeared to provide the most desired bacterial populations. The beneficial species appeared to be adapting over the period of time to the retrograded waxy maize starch. However, it must be noted that these feeding studies were carried out over a three week period only and so any long term effects were not examined.

The graphs of the amylase activity (U/L) from the faecal samples over the eighteen day period show that each of the six starches studied gave rise to different trends in α-amylase detected. The amounts of α-amylase determined in the faecal material did not appear to reach a stable level, but instead fluctuated over the eighteen days. It appeared that each of the six starches gave rise to levels of amylase which peaked after a different number of days on that particular starch. These peak levels of α-amylase should correspond to a peak in

the numbers of starch-degrading bacteria, as the α-amylase activity in the faecal matter can only have come from bacterial activity and not the animal itself. However, this was found not to be the case, and could be due to the problems with the isolation methods described previously, which would not have isolated the most dominant species of starch-degrading bacteria. As the starch-degrading bacteria consist of all bacteria able to break down starch and no single species was monitored, it is difficult to determine the trigger for the release of α-amylase. It may be the presence of starch, or it may be the more likely factor of the absence of sugars such as maltose and glucose. As the amylase in the faecal samples was shown to peak in amount at different sampling times depending on the starch, this suggests that the starch in the faecal material is still being degraded by the bacteria and that the bacteria adapted to each of the different starches at a different time during the feeding course. However, the highest level of α-amylase in the faecal samples was detected with the native waxy maize starch.

The examination of the bacteria directly from the sites of the monogastric colon provides exact information of the types and numbers of bacteria present. This data could only be obtained once from each animal as the animals were slaughtered to obtain the results. The large intestines of the animals at slaughter were 1.5m long on average, which gives a massive area for bacteria to accumulate. The human large intestine can be split into three key areas- the proximal, transverse (mid) and distal colon. Each of these sites contains different numbers and possibly species of bacteria, and each receives components of the diet in a different form and at different rates (Gibson *et al.* 1993). As the physiology of the porcine colon is very similar to that of the human (Miller & Ullrey, 1987), this led to the colons removed from the animals being split into three sections to obtain data which would be comparable with other studies. In this study, the caecum was also removed from the rest of the large intestine. This is known to be the site of most starch fermentation in the pig, but is almost redundant in humans. Therefore, the inclusion of this data would have been misleading for extrapolation to other monogastric species.

With the majority of the bacterial species from the six diets, excessive variability in the counts rendered differences in the mean values detected in the mid sections of the colons. The reasons for these effects however are unclear, as this section of the

monogastric colon has a pH and dietary flow rate intermediate between that of the proximal and distal colons.

The counts obtained with the retrograded maize starch appeared to be fairly constant along the length of the colon, apart from the *Clostridium* species. The counts of the aerobic starch-degrading species and *Bacteroides* species were the highest in the distal colon with this starch. The counts of the *Lactobacillus* species detected in the animals fed the retrograded maize starch appeared to be low along the length of the colon. The majority of the bacterial species obtained with the retrograded maize starch, in particular the anaerobic starch degrading species, appeared to follow a similar trend to those produced with the native maize starch. However, the trend obtained for the aerobic starch-degrading species with the retrograded maize starch showed counts which were the highest in the proximal and mid sections of the colon, and then followed a trend similar to the native maize starch between the mid and distal sections of the colon. The Lactobacillus : coliform ratio obtained with the retrograded maize starch was low in the first two-thirds of the colon, but then increased in the distal section of the colon. This starch also showed levels of total SCFA which increased between the mid and distal sections, and levels of skatole which were high in the proximal section of the colon, but were lowered in the mid and distal sections of the colon. The production of butyrate also increased along the length of the colon with the retrograded form of the starch. High counts of *Clostridium* and starch-degrading species, and low levels of *Lactobacillus* species were detected along the length of the colon with the native maize starch. The native maize also showed a low Lactobacillus : coliform ratio along the colon length. This form of the starch also showed a large reduction in the production of total SCFA to give the lowest levels detected in the distal colon, and an increase in the production of isoacids and skatole along the length of the colon. These results show that the retrograded maize starch appears to be more effective in maintaining the levels of the majority of the beneficial species (*Lactobacillus* and *Bifidobacterium* spp.) throughout the colon, but also gives high counts of *Bacteroides* species. This form of the starch also appears to be degraded along the whole length of the colon as indicated by the SCFA data, whereas the native form of the starch does not. The native form of the starch appears to be depleted in the early stages of the colon which then results in the degradation

of proteins by the bacteria present, as indicated by the highest levels of isoacids in the distal colon.

With the retrograded waxy maize starch, the majority of the counts remained stable along the length of the colon, apart from the *Lactobacillus* species and the starch degrading species, which showed a higher count in section C than that detected in section A. The coliform counts obtained with this starch were the lowest detected in the proximal and distal colon, and the counts of *Bifidobacterium* species were the highest detected in the distal section of the colon. The counts of *E. coli* remained unchanged along the length of the colon with this starch. The retrograded form of this starch also gave the highest Lactobacillus : coliform ratio throughout the length of the colon. This starch also resulted in a level of total SCFA which remained high along the length of the colon, and the lowest level of isoacids in the distal section of the colon. The native form of the waxy maize starch showed an increase in the total anaerobic population and in the *Clostridium* species between the mid and distal sections of the colon. The majority of the counts were low with this form of the starch, including the *Lactobacillus* species. The Lactobacillus : coliform ratio was very low along the length of the colon with the native form of the starch. The levels of total SCFA were fairly high along the length of the colon, as were the levels of skatole and isoacids with this form of the starch. These results suggest that the retrograded form of the waxy maize starch is more effective in maintaining the beneficial population along the length of the colon, and appeared to promote the growth of the *Bifidobacterium* species. The retrograded form also appeared to be fermented by the bacterial species along the whole length of the colon, with the necessity for protein breakdown being low. The native form of the waxy maize starch appeared to promote the growth of species such as *Clostridium* in the mid section of the colon, and also appeared to be fermented along the length of the colon, with the majority of this starch being fermented in the first two-thirds of the colon. The early depletion of the starch led to an increased breakdown of proteins in the distal section of the colon.

With the retrograded mixture starch the counts of the majority of the bacterial species studied, including the *Lactobacillus* and *Bifidobacterium* species, were the lowest throughout the length of the colon. The counts of *Bacteroides* species and those isolated on rifampicin agar followed the same trend along the length of the colon as with the native

waxy maize starch. This suggests that these bacterial species may be utilising the waxy maize portion of the mixture starch. With the retrograded mixture starch, low counts of the coliforms and *E. coli* were obtained in the proximal section of the colon, but this increased between the mid and distal sections. This increase in the coliform group may not be a problem in itself, but it does suggest that the pH has increased sufficiently in the last third of the colon to allow this group to proliferate. This may partly be due to the low levels of *Lactobacillus* and *Bifidobacterium* species, in the distal section of the colon. The coliform group contains pathogens such as *Salmonella* species, which could become established in the colon if this diet was fed to the animals. These results with the retrograded mixture starch were matched by a high Lactobacillus : coliform ratio in the proximal section of the colon, which decreased drastically in the mid and distal sections. This starch gave the highest level of total SCFA in the distal section of the colon (which had increased from that detected in the mid colon), increasing isoacid levels along the colon, and low levels of skatole. This suggests that although the starch is obviously fermented along the length of the colon, it is being fermented by bacterial groups which may be harmful to the large intestine in large numbers, and not the beneficial population.

The bar graph showing the amylase activity (U/L) from three different sites along the porcine colon shows that in most cases the amount of amylase detected dropped from section A through to section C, where it was detected at the lowest level. The only diet which showed the opposite effect was the retrograded maize diet. The factor for α-amylase production is not clear but it is probable that the level of α-amylase will increase as the levels of available sugars decreases, in order to stimulate the breakdown of the available polysaccharide. This theory may match the findings with the retrograded maize starch as there are still high levels of total SCFA detected in the distal section of the colon, and hence substrate is still available to be degraded.

The feed conversion ratio (FCR) was calculated for each of the six diets to determine how efficiently the starches were utilised. A value of 1.00 is designated as the most efficiently used by the animal. The ratios with some of the starches were low at the start, but as the length of time on the diets increased, the FCR increased. The only diet where this did not

occur was the native waxy maize diet, where the ratio actually decreased over the time period. At week three of the feeding trial the ratios for the retrograded starches were lower than for the native ones (apart from the waxy maize starch which will be less affected by the retrograding procedure). This suggests that the animals being fed the retrograded starches were making less efficient use of the starches compared to those on the native diet and that more of the retrograded diet was available to the microflora. This indicates that the procedure used to create resistant starch has actually resulted in the formation of such starch, and that the starch is not merely in a gelatinized state which would have been more available to the animal.

The levels of resistant starch detected in the faecal and colon samples were low with each starch, indicating that the resistant starch is being rapidly fermented by the bacteria present, and that it is not in a state which is also resistant to the bacterial population present. The levels of resistant starch measured in each of the diets were higher than the levels detected in the faecal material, indicating that a proportion of the starch has been retrograded by the procedure. The levels of resistant starch present in the colon samples from the animals fed the retrograded maize starch decreased along the length of the colon, and those from the retrograded waxy maize starch samples were constant along the length of the colon, with the highest level detected with this starch in the distal section of the colon. The levels of resistant starch detected from the animals fed the native waxy maize starch were very low, particularly in the mid and distal sections of the colon. These findings show that residues of all the starches were detected along the length of the colon, but higher levels of the retrograded starches were detected. The fact that very low levels of resistant starch were detected in these samples indicates that the majority of starch fermentation may be occuring in the caecum, and hence the effects observed on the bacterial population and the resulting fermenation products in the colon, are occurring with very low levels of starch.

A fraction of the starch included in the diet will be resistant to the enzymes present in the small intestine, and will enter the colon where it will be fermented by the bacteria present. The depletion of this starch will result in the bacterial species present switching to the breakdown of proteins for energy. The lack of starch will result in a decrease in the

numbers of *Lactobacillus* and *Bifidobacterium* spp. and an increase in pH. This will not only encourage the growth of coliforms (possibly *Salmonella* spp.), but will also increase the production of ammonia, amines and toxic phenols. If the starch reaches the colon in a form and at a concentration which allowed its fermentation to continue along the length of the colon, such adverse effects could be avoided.

From the faecal and colon data obtained from the animals, it appears that retrograding the starches prior to inclusion in the diet will give these desired effects. The native starches appeared to deplete too quickly and so did not give desired effects any further along the colon. The retrograded maize starch showed good results both from the faecal and the colon data, and maintained the majority of the species along the length of the colon. However, this starch also promoted the growth of the bacterial species which may be harmful to the gut in large numbers, and maintained only low levels of the beneficial groups, namely the *Lactobacillus* and *Bifidobacterium* species. The retrograded waxy maize starch did not appear to perform as well as the retrograded maize starch, in relation to the levels of butyrate and isoacids, but increasing levels of the beneficial groups and low levels of *Bacteroides* species were detected with this form of starch. The counts of *Lactobacillus* species also considerably outnumber those from the coliform group along the length of the colon with this particular starch. The feed conversion ratios for these two starches show that the animals grew fairly well on these starches (but not as well as with the native forms), with the values for the retrograded maize starch increasing over the three weeks and the values with the retrograded waxy maize starch maintaining relatively good growth.

It appears that retrogradation is a major factor in ensuring the starch source persists further along the length of the monogastric large intestine. It also appears that high levels of amylopectin, such as those found in the waxy maize starch, are a further factor in ensuring the starch reaches the distal portion of the large intestine. High levels of amylopectin also appear to promote the growth of certain beneficial species in the colon. All these effects may also be achieved with low levels of resistant starch. Therefore, to obtain the most beneficial effects for the gut, retrograding a starch containing high levels of amylopectin i.e. the waxy maize starch, appears best.

CONCLUSIONS AND FUTURE WORK

Conclusions

This thesis set out to examine the bacterial fermentation of resistant (retrograded) starches in the monogastric colon, with emphasis on the potential health effects which may be gained with these starches. In particular, the potential to extend the carbohydrate fermenting region of the colon with these starches was assessed.

Experimental procedures were carried out to examine the breakdown of these starches by pure cultures of known starch-degrading bacterial species, in mixed cultures using a simulation of the porcine colon, and in an actual monogastric animal model. The findings from the above experiments were very similar throughout, and were as follows :-

- Each bacterial group studied used a different botanical starch form more efficiently, indicating that many forms of starch present in the diet will be fermented, which will limit the competition between starch-degrading species in the monogastric gut.

- Treating the starch prior to adding as a substrate for bacterial fermentation will alter the way in which the starch is fermented, both in terms of how much of the starch is fermented, and the levels of SCFA resulting from this fermentation.

- The levels of amylose and amylopectin present within a starch will affect the change in the starch brought about by retrogradation or digestion with pancreatin. This will then have an effect on the resulting bacterial fermentation.

- Butyric acid was found to be a major product from the breakdown of the starch forms tested. The production of butyrate appeared to be correlated with the level of amylose within the starch, with less butyrate produced as the level of amylose increases.

- The degree of estimated starch damage and the lipids which may be associated with the starch are also factors in the bacterial breakdown of the starches.

- The starches tested were fermented at different rates, with the waxy maize starches being fermented fastest, and the high amylose maize starch showing the slowest fermentation.

- The levels of amylose found in starches such as high amylose maize i.e. ~52 %, may be too high and consequently bacterial fermentation is reduced once the starch has been retrograded.

- Certain starch forms were found to enhance specific bacterial groups, such as waxy maize starch which enhanced the *Bifidobacterium* and *Lactobacillus* populations *in vitro*. Bacterial groups which may be harmful to the gut in large numbers were also reduced in numbers with this starch. These findings were also encountered in the porcine colon. This suggests that there may be scope for manipulation of the gut flora by inclusion of this type of starch in the monogastric diet.

- The starches which had been retrograded prior to inclusion in the porcine diets were fermented further along the colon than the starches which were untreated, and consequently reduced the bacterial breakdown of proteins.

- In all cases, the waxy maize starch enhanced the desired bacterial populations, reduced the levels of potential pathogens, extended the fermentation region along the length of the colon, reduced the breakdown of proteins, was readily fermentable by the bacteria, and resulted in high levels of SCFA production along the length of the colon. This finding is contrary to the theory that, as it is the amylose portion which retrogrades, this will be the contributing factor towards the properties of these starches.

Future work

This thesis has made the proposal that starches which contain higher than average levels of amylopectin i.e. waxy maize, may be beneficial to the health of the monogastric colon, and may be able to enhance the growth and fermentation of the microflora present. Based on the experimental work which was carried out within this thesis, several areas of further work have come to light.

The data from the animal experiment showed that after the three weeks on the diets, some of the bacterial species studied did not appear to have stabilised. It would have been interesting to have continued the studies to determine whether or not these species did stabilise, or whether further changes in counts could be observed. It would also have been relevant to continue the study for a longer period of time to determine whether or not the beneficial populations which had been enhanced by the waxy maize starch were maintained as the animal grew older. It would also have been relevant to determine whether or not these bacterial species could be maintained at these elevated levels with starches other than waxy maize, or whether that starch needs to be included in the diet continuously to maintain the beneficial population.

The data from the pure culture experiments involving *Cl. butyricum* showed that some of the treated starches reduced fermentative activity by this organism. It was not certain however, if the pancreatin was removing the more fermentable parts of the starches, or whether the pancreatin was interfering with the breakdown of the starches, possibly by binding to certain sites on the starch granules. Research should continue in this area to determine the role of pancreatin in the fermentation of resistant starches and to determine if these problems are also encountered in the gut environment.

The actual role of certain species of the gut flora in the fermentation of resistant starches in the gut does not appear to be entirely clear. It is apparent that the non-starch-degrading bacterial species present in the gut benefit from the breakdown of the starches present, but it is not certain how they are able to benefit. They are obviously in a position to benefit, but how? Is there a potential biofilm scenario which operates, with the non-starch degrading species binding to the starch-degrading species so that they are in the best place to obtain breakdown products such as sugars. These interactions may not as yet be fully understood, and may need to be examined further.

The fermentation of native and retrograded starches by both pure and mixed bacterial cultures was carried out, and the gas and SCFA produced measured, as an indication of the rate and extent of these fermentations. However, it is not clear what proportion of these starches were either fermented by the bacteria or removed during pancreatin digestion. Therefore, measurements of the residual starch fractions should have been made after these events to determine the level and structure of the remaining starch.

This thesis has examined the effects of resistant starches in the diet on the microflora present, focussing mainly on retrograded starches. However, more foods appear to contain modified starches which have mostly been chemically modified, and as yet, there appears to be little knowledge available on the effect these starches have on the gut microflora. Are they degraded by the bacteria present in either the small or large intestine, and if so, what are the resulting products? Are they different from the products from the breakdown of retrograded starches due to the nature of the modification. These starch forms should be assessed further.

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APPENDICES

Appendix 1

List of media and chemical sources

Culture media - agars and broths

Brain-heart infusion broth	Oxoid CM370
Brain-heart infusion agar	Oxoid CM375
Columbia agar base	Oxoid CM331
MacConkey agar no. 3	Oxoid CM115
de Man, Rogosa, Sharpe (MRS) agar	Oxoid CM361
MRS broth	Oxoid CM359
Nutrient broth	Oxoid CM1
Plate count agar	Oxoid CM325
Reinforced Clostridial agar (RCA)	Oxoid CM151
Slanetz & Bartley medium	Oxoid CM377
Wilkins-Chalgren anaerobe agar	Oxoid CM619

Culture media supplements

Amylopectin	Sigma A-7780
Arabinogalactan	Sigma A-9029
Bile salts	Oxoid L55
Casein	Sigma C-3400
Cooked meat granules	Lab M - Lab 24
Fructose	Fisons F/1952/53
Glucose	Sigma G-5767
G-N supplement	Oxoid SR108B
Haemin (bovine)	Sigma H-2250
Horse blood (defibrinated)	Oxoid SR50
Kanamycin solution (10 mg/ml)	Sigma K-0129
Maltose	Fisons M/1450/48
MUG supplement	Oxoid BR071E
Nalidixic acid	Sigma N-8878
N-S supplement	Oxoid SR107B
Pectin	Sigma P-2157
Peptone water	Sigma P-8388
Potato starch	Sigma S-2630
Rifampicin	Sigma R-3501
Sheep blood (defibrinated)	Oxoid SR51
Tomato juice (sterile)	Oxoid SR032C
Vancomycin hydrochloride	Sigma V-2002
Vitamin K_1	Sigma V-3501
Xylan	Sigma X-0627
Yeast extract powder	Fisons Y/0200/48

Diluents

Maximum recovery diluent (MRD)	Oxoid CM733
Ringers	Oxoid BR52

Animal Trial diets

Cane sugar	Rowett Research
Casein	Rowett Research
Dicalcium phosphate	Rowett Research
Dried milk powder	Harbro

Maize starch (cornflour)	Cerestar GL03401
Molasses	Rowett Research
Potato starch	Cerestar
Salt	Rowett Research
Solka Floc	Harbro
Sowvite 12 (2.5)	Harbro
Soyabean oil	Rowett Research
Vitamin E	Rowett Research
Waxy maize starch (snowflake)	Cerestar 06439
White fish meal	Harbro

Commercial kits

Amylase 10 kit	Sigma 577-10

Chemicals

Antifoam A	Sigma A-5758
Biotin	Sigma B-4501
Calcium chloride	Sigma C-1016
Calcium chloride dihydrate	Sigma C-3881
$CoCl_2.6H_20$	Sigma C-2644
$CuCl_2.2H_20$	BDH 278344Y
EDTA	Sigma E-4884
L-cysteine hydrochloride	Sigma C-7880
2-ethyl n-butyric acid	Sigma 10,995-9
Ferric (III) chloride	Sigma F-7134
Ferrous sulphate heptahydrate	Sigma F-7002
H_3BO_3	Sigma B-5270
Hydrochloric acid	BDH 285073U
Iodine	Fisons 1/0450
Magnessium chloride hexahydrate	Sigma M-0250
Magnessium sulphate heptahydrate	Sigma M-1880
$MnCl_2.4H_20$	Sigma M-3634
Menadione	Sigma M-5625
Metaphosphoric acid	Fisons M/3851/53
$Na_2Mo0_4.2H_20$	Sigma S-6646
$NiCl_2.6H_20$	Fisons
Nicotinamide	Sigma N-3376
Pantothenate	Sigma P-5710
Para-aminobenzoic acid	Sigma A-9878
Phytone peptone	Becton Dickinson 81-1906-0
Polyoxyethylene-sorbitan monooleate (Tween 80)	Sigma P-1754
Porcine pancreatin	Sigma P-1500
di-potassium hydrogen orthophosphate	Fisons P/5240
Potassium hydroxide	Fisons P/5600
Potassium iodide	Sigma P-8256
Sodium chloride	Sigma S-9625
Sodium formaldehyde sulphoxylate	Fisons S/4040/53
Sodium hydrogen carbonate	BDH 10247
Sulphuric acid	BDH 102760B

Thiamine	Sigma T-4625
Trypticase peptone	Becton Dickinson 81-1921-0
Vitamin B_{12}	Sigma V-2876
Zinc sulphate heptahydrate	BDH 30621

Bacterial cultures

Bifidobacterium suis	20211 DSM
Clostridium butyricum	NCIMB 7423
Fusobacterium sp.	NCIMB 12177

Starches used

Amylopectin	Sigma A-7780
Maize	National starch 116688
Waxy maize	National starch 116686
High amylose maize	BDH
Tapioca	National starch 116687
Potato	National starch 116689
Wheat	BDH 30265
Amylose azure (potato)	Sigma A-3508
Amylose (pure-potato)	Sigma A-0512
Amylopectin azure (potato)	Sigma A-4640
Amylopectin (pure-potato)	Sigma A-8515

Appendix 2

Data from Chapter 2

Appendix 2.1 :-

Gas production (ml) detected during 48 hour incubation from pure cultures of *Cl. butyricum* grown in cooked meat media containing a different amount of starch under various treatments.

Maize 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	12.33 ± 1.30	11.67 ± 0.67	8.00 ± 1.00	10.33 ± 1.67	14.67 ± 0.33	3.33 ± 0.33
24 hours	3	13.07 ± 1.41	14.17 ± 0.93	9.67 ± 1.13	12.83 ± 1.83	16.67 ± 0.33	4.27 ± 0.37
42 hours	3	13.27 ± 1.41	14.33 ± 1.00	10.60 ± 1.20	13.97 ± 1.89	17.40 ± 0.40	4.53 ± 0.33
48 hours	3	13.73 ± 1.44	14.57 ± 1.04	10.87 ± 1.18	14.37 ± 1.89	17.80 ± 0.40	4.73 ± 0.33

W. maize 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	10.67 ± 0.33	11.67 ± 0.33	9.33 ± 1.20	6.17 ± 0.17	11.00 ± 0.50	14.33 ± 3.18
24 hours	3	11.40 ± 0.20	13.83 ± 0.44	11.00 ± 1.31	9.03 ± 0.23	12.67 ± 0.83	16.47 ± 3.56
42 hours	3	11.50 ± 0.10	14.07 ± 0.41	11.73 ± 1.37	10.23 ± 0.23	13.33 ± 0.87	16.67 ± 3.55
48 hours	3	11.73 ± 0.09	14.30 ± 0.38	11.90 ± 1.35	10.63 ± 0.23	13.73 ± 0.87	16.93 ± 3.53

HAM - 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	8.67 ± 0.33	10.17 ± 0.44	6.33 ± 0.17	4.67 ± 0.33	6.33 ± 0.33	5.33 ± 0.33
24 hours	3	9.50 ± 0.35	12.33 ± 0.33	7.53 ± 0.17	7.87 ± 0.44	7.33 ± 0.33	6.73 ± 0.44
42 hours	3	9.77 ± 0.38	12.53 ± 0.33	8.13 ± 0.17	9.13 ± 0.47	7.93 ± 0.33	6.73 ± 0.44
48 hours	3	10.17 ± 0.38	12.73 ± 0.33	8.33 ± 0.17	9.53 ± 0.47	8.27 ± 0.37	6.97 ± 0.47

Appendix 2.1 continued :-

Amylo. 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	10.33 ± 0.17	12.00 ± 0	12.83 ± 3.68	9.67 ± 0.67	19.33 ± 0.33	10.83 ± 2.09
24 hours	3	10.73 ± 0.17	13.20 ± 0	18.03 ± 5.83	11.93 ± 0.64	22.67 ± 0.33	12.77 ± 2.62
42 hours	3	11.20 ± 0.20	14.67 ± 0.03	19.50 ± 6.41	13.07 ± 0.67	23.60 ± 0.31	12.90 ± 2.55
48 hours	3	11.57 ± 0.23	15.17 ± 0.09	19.73 ± 6.53	13.47 ± 0.67	24.00 ± 0.31	13.17 ± 2.52

Potato 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	9.50 ± 0.29	14.67 ± 0.33	7.83 ± 0.17	8.67 ± 0.33	21.33 ± 0.88	3.00 ± 0
24 hours	3	16.33 ± 0.33	17.47 ± 0.07	9.17 ± 0.03	11.40 ± 0.76	24.67 ± 0.33	3.87 ± 0.07
42 hours	3	17.33 ± 0.33	19.33 ± 0.13	9.97 ± 0.03	12.93 ± 0.44	25.67 ± 0.47	3.93 ± 0.07
48 hours	3	17.57 ± 0.35	20.13 ± 0.24	10.27 ± 0.03	13.40 ± 0.40	26.07 ± 0.47	4.13 ± 0.07

Tapioca 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	11.00 ± 0	11.83 ± 0.17	6.00 ± 0.58	7.00 ± 0.58	9.00 ± 0	5.00 ± 0
24 hours	3	11.40 ± 0	13.10 ± 0.21	7.40 ± 0.58	9.40 ± 0.42	10.00 ± 0	6.33 ± 0.07
42 hours	3	11.83 ± 0.03	14.57 ± 0.26	8.20 ± 0.58	10.33 ± 0.47	10.80 ± 0.12	6.93 ± 0.07
48 hours	3	12.20 ± 0.06	15.00 ± 0.23	8.20 ± 0.58	10.53 ± 0.47	11.00 ± 0.12	6.93 ± 0.07

Wheat 0.5%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	10.17 ± 0.44	11.17 ± 0.60	4.67 ± 0.33	15.33 ± 1.76	8.00 ± 0	4.00 ± 0
24 hours	3	12.17 ± 0.44	12.67 ± 0.54	5.67 ± 0.33	18.53 ± 2.12	9.33 ± 0.17	5.00 ± 0
42 hours	3	12.37 ± 0.44	14.07 ± 0.45	6.13 ± 0.37	19.53 ± 2.12	10.13 ± 0.17	5.00 ± 0
48 hours	3	12.53 ± 0.41	14.67 ± 0.48	6.13 ± 0.37	19.93 ± 2.12	10.53 ± 0.17	5.20 ± 0

Appendix 2.1 continued :-

Maize 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	9.33 ± 0.33	15.00 ± 0.58	11.00 ± 1.00	17.67 ± 0.33	22.33 ± 0.33	5.67 ± 0.33
24 hours	3	17.50 ± 1.32	18.73 ± 0.55	13.20 ± 1.01	21.60 ± 0.72	26.33 ± 0.33	6.93 ± 0.29
42 hours	3	19.83 ± 2.20	20.73 ± 0.55	13.93 ± 0.94	23.27 ± 1.07	29.07 ± 0.29	7.47 ± 0.24
48 hours	3	20.10 ± 2.32	21.47 ± 0.48	14.13 ± 0.94	23.67 ± 1.07	29.53 ± 0.24	7.67 ± 0.24

W. maize 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	8.33 ± 0.33	20.17 ± 1.09	15.17 ± 1.88	16.00 ± 2.00	19.33 ± 0.33	21.00 ± 0
24 hours	3	17.00 ± 0	21.90 ± 1.05	19.70 ± 4.51	19.80 ± 2.30	21.33 ± 0.33	26.33 ± 0.88
42 hours	3	20.17 ± 1.33	24.03 ± 1.02	21.17 ± 5.16	21.33 ± 2.37	22.13 ± 0.33	27.20 ± 1.33
48 hours	3	20.53 ± 1.37	24.43 ± 1.02	21.37 ± 5.36	21.80 ± 2.30	22.53 ± 0.33	27.60 ± 1.33

HAM 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	7.33 ± 0.33	12.33 ± 0.88	9.33 ± 0.33	10.33 ± 0.17	11.83 ± 0.44	7.00 ± 0
24 hours	3	13.00 ± 0	14.93 ± 0.66	10.97 ± 0.32	13.27 ± 0.22	13.83 ± 0.44	8.40 ± 0
42 hours	3	14.33 ± 0.17	16.60 ± 0.61	11.37 ± 0.52	14.73 ± 0.20	14.83 ± 0.44	8.60 ± 0
48 hours	3	14.60 ± 0.20	17.23 ± 0.59	11.67 ± 0.52	15.33 ± 0.20	15.23 ± 0.44	8.87 ± 0.03

Amylo. 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	15.17 ± 0.44	20.00 ± 0.58	16.17 ± 0.73	20.33 ± 2.33	18.33 ± 0.33	19.33 ± 0.88
24 hours	3	20.17 ± 0.44	22.13 ± 0.33	21.50 ± 4.00	23.67 ± 2.77	20.33 ± 0.33	26.27 ± 0.47
42 hours	3	22.63 ± 0.23	23.07 ± 0.37	23.70 ± 5.50	25.53 ± 2.98	21.07 ± 0.37	27.60 ± 0.94
48 hours	3	23.50 ± 0.32	23.37 ± 0.37	24.23 ± 5.73	25.93 ± 2.98	21.47 ± 0.37	28.07 ± 1.01

Appendix 2.1 continued :-

Potato 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	9.50 ± 1.04	17.67 ± 0.17	13.67 ± 0.33	15.33 ± 1.20	24.33 ± 0.33	6.00 ± 0
24 hours	3	18.33 ± 1.20	22.53 ± 0.26	15.20 ± 0.31	18.73 ± 1.69	27.67 ± 0.33	7.20 ± 0
42 hours	3	23.17 ± 0.60	24.80 ± 0.25	15.93 ± 0.18	20.27 ± 1.80	29.33 ± 0.37	7.40 ± 0
48 hours	3	23.67 ± 0.52	25.67 ± 0.65	16.17 ± 0.09	20.80 ± 1.74	29.87 ± 0.33	7.60 ± 0

Tapioca 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	17.67 ± 0.33	18.83 ± 0.73	8.67 ± 0.33	19.00 ± 2.52	21.83 ± 0.73	8.67 ± 0.67
24 hours	3	21.33 ± 0.33	23.73 ± 0.63	9.87 ± 0.24	23.07 ± 2.68	23.83 ± 0.73	10.67 ± 0.67
42 hours	3	24.13 ± 0.58	25.60 ± 0.23	10.33 ± 0.18	25.40 ± 2.41	24.77 ± 0.67	11.40 ± 0.60
48 hours	3	25.20 ± 0.69	26.00 ± 0.15	10.33 ± 0.18	25.87 ± 2.34	24.97 ± 0.67	11.60 ± 0.60

Wheat 1%		Starch treatment					
Sample time	n	Native starch	Native + pancreatin	Retrograded starch	Retrograded + pancreatin	Native panc. dig.	Retro. panc. dig.
18 hours	3	13.50 ± 1.04	16.33 ± 3.18	11.33 ± 0.73	23.83 ± 0.73	14.00 ± 0	6.67 ± 0.17
24 hours	3	17.50 ± 1.23	20.00 ± 3.76	12.67 ± 0.72	27.83 ± 0.91	16.00 ± 0	8.07 ± 0.17
42 hours	3	18.10 ± 1.35	21.73 ± 3.70	13.47 ± 0.72	30.03 ± 1.07	16.80 ± 0	8.40 ± 0.10
48 hours	3	18.43 ± 1.41	22.23 ± 3.65	13.47 ± 0.72	30.50 ± 1.13	17.20 ± 0	8.80 ± 0.10

Data represent mean ± SEM of triplicate determinations. W. maize = waxy maize starch; HAM = high amylose maize starch; Amylo. = amylopectin.

Appendix 2.2 :-

Gas production (ml) and bacterial counts (cfu ml^{-1}) detected from pure cultures of *Bifidobacterium suis* which had been incubated in TPY media containing various amounts of starch which had undergone various treatments.

Gas production (ml)		Starch treatment			
Starch source - 0.25%	**n**	**Native**	**Retrograded**	**Native pancreatin digested**	**Retrograded pancreatin digested**
Maize	3	0.20 ± 0	1.03 ± 0.07	1.20 ± 0.10	1.00 ± 0
Waxy maize	3	0.50 ± 0.12	0.93 ± 0.07	1.07 ± 0.07	1.07 ± 0.07
High amylose maize	3	0.27 ± 0.09	1.30 ± 0	1.23 ± 0.03	0.50 ± 0

Gas production (ml)		Starch treatment			
Starch source - 0.5%	**n**	**Native**	**Retrograded**	**Native pancreatin digested**	**Retrograded pancreatin digested**
Maize	3	0.83 ± 0.07	1.40 ± 0	1.00 ± 0	0.83 ± 0.13
Waxy maize	3	1.53 ± 0.07	1.87 ± 0.07	0.40 ± 0	1.20 ± 0.12
High amylose maize	3	1.27 ± 0.07	1.57 ± 0.13	1.60 ± 0	0.70 ± 0.23

Gas production (ml)		Starch treatment			
Starch source - 0.75%	**n**	**Native**	**Retrograded**	**Native pancreatin digested**	**Retrograded pancreatin digested**
Maize	3	0.60 ± 0.12	1.83 ± 0.03	1.37 ± 0.09	1.10 ± 0.10
Waxy maize	3	0.67 ± 0.03	2.03 ± 0.12	1.00 ± 0	1.00 ± 0.12
High amylose maize	3	0.67 ± 0.07	1.07 ± 0.07	1.13 ± 0.07	0.70 ± 0.06

Bacterial counts (cfu ml^{-1})		Starch treatment			
Starch source - 0.5%	**n**	**Native**	**Retrograded**	**Native pancreatin digested**	**Retrograded pancreatin digested**
Maize	3	5.7e7 ± 3.2e7	1.3e8 ± 9.4e7	8.3e7 ± 5.9e7	6.7e8 ± 3.7e8
Waxy maize	3	9.1e6 ± 3.7e6	5.3e6 ± 2.7e6	4.1e6 ± 5.9e5	1.8e8 ± 7.5e7
High amylose maize	3	2.3e10 ± 8.5e9	7.4e8 ± 4.3e7	5.6e8 ± 1.6e8	6.0e8 ± 3.0e8

Data represent average ± SEM of triplicate determinations.

Appendix 2.3 :-

Gas production (ml) and bacterial counts (cfu ml^{-1}) detected from pure cultures of *Fusobacterium sp.* which had been incubated in TPY media containing various amounts of starch which had undergone various treatments.

Gas production (ml)		Starch treatment			
Starch source - 0.25%	n	Native	Retrograded	Native pancreatin digested	Retrograded pancreatin digested
Maize	3	4.53 ± 0.33	4.50 ± 0	5.67 ± 0.38	4.60 ± 0.12
Waxy maize	3	5.47 ± 0.17	5.73 ± 0.18	5.47 ± 0.07	4.60 ± 0.12
High amylose maize	3	4.10 ± 0	4.47 ± 0.03	4.60 ± 0.12	4.37 ± 0.07

Gas production (ml)		Starch treatment			
Starch source - 0.5%	n	Native	Retrograded	Native pancreatin digested	Retrograded pancreatin digested
Maize	3	3.93 ± 0.17	2.13 ± 0.18	5.67 ± 0.07	3.43 ± 0.13
Waxy maize	3	4.37 ± 0.17	2.90 ± 0.15	4.93 ± 0.07	4.93 ± 0.13
High amylose maize	3	3.70 ± 0	1.83 ± 0.87	4.73 ± 0.07	4.17 ± 0.07

Gas production (ml)		Starch treatment			
Starch source - 0.75%	n	Native	Retrograded	Native pancreatin digested	Retrograded pancreatin digested
Maize	3	5.57 ± 0.17	5.37 ± 0.09	6.37 ± 0.19	5.97 ± 0.09
Waxy maize	3	5.30 ± 0	6.43 ± 0.03	5.93 ± 0.18	6.47 ± 0.12
High amylose maize	3	5.17 ± 0.17	4.37 ± 0.17	5.17 ± 0.03	6.07 ± 0.23

Bacterial counts (cfu ml^{-1})		Starch treatment			
Starch source - 0.5%	n	Native	Retrograded	Native pancreatin digested	Retrograded pancreatin digested
Maize	3	1.9e7 ± 1.5e7	4.3e7 ± 1.6e7	8.3e7 ± 1.9e7	2.1e9 ± 1.1e9
Waxy maize	3	3.1e7 ± 8.0e6	3.2e7 ± 1.6e7	1.3e8 ± 4.5e7	5.7e8 ± 1.8e8
High amylose maize	3	1.6e7 ± 4.7e6	1.5e7 ± 6.4e6	4.1e6 ± 5.2e5	4.8e7 ± 1.2e7

Data represent average ± SEM of triplicate determinations.

Appendix 2.4 :-

SCFA levels (mM) measured from pure cultures of *Cl. butyricum* which were grown in Hungate tubes containing cooked meat media and either 0.5 or 1% starch, after 48 hours incubation at 39°C.

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Maize	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	8.17 ± 1.77	9.71 ± 0.64	37.19 ± 3.73	15.87 ± 4.34	8.08 ± 0.29	53.16 ± 6.36
N+P	3	10.84 ± 0.98	8.32 ± 0.11	33.11 ± 2.52	19.76 ± 1.11	8.32 ± 0.46	45.34 ± 1.09
R	3	4.89 ± 0.64	3.26 ± 0.05	23.10 ± 3.24	8.22 ± 1.05	2.98 ± 0.22	30.73 ± 1.46
R+P	3	14.91 ± 2.59	4.25 ± 0.31	37.60 ± 4.11	29.71 ± 3.66	4.29 ± 0.28	69.98 ± 7.26
NPD	3	19.26 ± 0.50	4.57 ± 0.07	47.00 ± 1.06	37.41 ± 1.00	4.08 ± 0.07	70.71 ± 2.11
RPD	3	5.94 ± 0.06	5.84 ± 0.10	13.12 ± 0.21	7.37 ± 0.05	4.98 ± 0.27	19.47 ± 0.29

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Waxy maize	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	6.34 ± 0.13	8.69 ± 0.21	33.59 ± 0.96	17.17 ± 0.71	9.46 ± 1.41	58.66 ± 2.07
N+P	3	14.19 ± 0.57	8.55 ± 1.41	29.98 ± 2.24	23.99 ± 0.67	8.44 ± 0.51	51.96 ± 1.91
R	3	7.00 ± 1.13	3.89 ± 0.03	29.97 ± 3.89	13.45 ± 6.08	2.62 ± 0.27	38.61 ± 8.70
R+P	3	12.10 ± 0.33	5.50 ± 0.18	31.80 ± 0.77	26.41 ± 3.38	4.05 ± 0.19	60.81 ± 7.08
NPD	3	12.99 ± 1.08	4.34 ± 0.17	33.36 ± 0.39	23.04 ± 0.44	3.76 ± 0.48	49.11 ± 0.89
RPD	3	17.63 ± 3.89	4.56 ± 0.52	45.47 ± 8.58	30.18 ± 2.35	3.87 ± 0.17	72.15 ± 4.88

Appendix 2.4 continued :-

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
HAM	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	5.48 ± 0.41	10.48 ± 0.23	30.06 ± 1.00	7.38 ± 0.12	7.05 ± 0.31	34.61 ± 0.79
N+P	3	9.77 ± 0.62	9.94 ± 0.18	27.59 ± 0.25	13.39 ± 2.10	8.03 ± 0.86	33.85 ± 3.85
R	3	4.99 ± 0.18	4.24 ± 0.28	19.76 ± 0.50	6.85 ± 0.17	3.89 ± 0.36	29.91 ± 1.37
R+P	3	10.85 ± 1.61	5.29 0.43	31.07 ± 4.27	19.15 ± 1.31	4.99 ± 0.30	48.69 ± 3.45
NPD	3	7.65 0.80	5.23 ± 0.15	21.69 ± 1.79	15.25 ± 0.65	4.69 ± 0.34	38.76 ± 1.00
RPD	3	8.00 ± 0.29	5.96 ± 0.17	18.84 ± 1.20	9.79 ± 0.10	5.70 ± 0.20	23.96 ± 0.31

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Amylopectin	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	11.75 ± 0.24	3.97 ± 0.06	31.35 ± 0.88	18.61 ± 2.40	5.57 ± 0.17	46.96 ± 2.87
N+P	3	11.20 ± 0.34	4.30 ± 0.28	32.98 ± 1.10	21.46 ± 0.95	7.43 ± 0.58	48.83 ± 2.07
R	3	13.90 ± 5.00	2.94 ± 0.43	37.70 ± 10.40	18.50 ± 5.66	2.63 ± 0.60	47.09 ± 7.85
R+P	3	15.67 ± 2.40	4.92 ± 0.18	43.16 ± 3.37	37.07 ± 6.85	3.52 ± 0.36	78.50 ± 10.70
NPD	3	24.87 ± 1.05	3.52 ± 0.02	55.17 ± 2.81	21.72 ± 0.96	3.83 ± 0.19	47.60 ± 2.47
RPD	3	13.17 ± 2.54	4.68 ± 0.36	35.58 ± 5.13	33.60 ± 0.64	3.44 ± 0.23	76.86 ± 1.70

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Potato	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	8.50 ± 0.74	9.08 ± 1.82	40.96 ± 3.77	22.24 ± 1.25	6.34 ± 0.83	52.59 ± 3.15
N+P	3	15.61 ± 0.36	12.52 ± 1.84	45.40 ± 3.05	44.61 ± 2.71	4.10 ± 0.17	88.88 ± 1.45
R	3	4.60 ± 0.58	3.40 ± 0.09	23.57 ± 0.60	8.43 ± 0.82	3.27 ± 0.07	36.17 ± 0.82
R+P	3	14.72 ± 1.67	6.42 ± 0.50	49.72 ± 1.93	33.77 ± 3.72	4.23 ± 0.53	76.51 ± 7.34
NPD	3	28.70 ± 1.21	3.66 ± 0.17	60.49 ± 2.24	41.98 ± 3.06	3.27 ± 0.58	72.44 ± 5.36
RPD	3	6.27 ± 0.28	5.90 ± 0.07	14.22 ± 0.48	7.89 ± 0.20	5.55 ± 0.32	20.66 ± 0.81

Appendix 2.4 continued :-

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Tapioca	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	11.65 ± 0.53	3.60 ± 0.24	32.20 ± 0.84	22.61 ± 0.84	5.35 ± 0.12	47.96 ± 3.58
N+P	3	11.85 ± 0.79	4.24 ± 0.15	34.20 ± 0.54	26.26 ± 1.93	6.85 ± 0.12	55.23 ± 2.04
R	3	3.24 ± 1.01	3.97 ± 0.33	20.83 ± 3.45	6.38 ± 0.31	3.60 ± 0.07	13.94 ± 0.81
R+P	3	10.22 ± 1.30	5.23 ± 0.72	34.63 ± 1.18	29.41 ± 3.42	4.08 ± 0.27	72.54 ± 4.62
NPD	3	10.54 ± 0.68	4.91 ± 0.12	30.00 ± 0.48	25.19 ± 0.64	3.26 ± 0.07	52.03 ± 1.15
RPD	3	7.71 ± 1.03	5.37 ± 0.36	17.58 ± 0.74	10.99 ± 0.64	4.90 ± 0.21	28.63 ± 1.32

		SCFA (mM) - 0.5% starch			SCFA (mM) - 1% starch		
Wheat	**n**	**Acetate**	**Propionate**	**Butyrate**	**Acetate**	**Propionate**	**Butyrate**
N	3	10.84 ± 0.26	3.92 ± 0.18	29.65 ± 0.91	11.39 ± 0.74	7.17 ± 0.44	40.30 ± 2.23
N+P	3	10.47 ± 0.90	4.71 ± 0.46	34.91 ± 2.66	18.37 ± 4.10	8.22 ± 0.59	49.40 ± 6.87
R	3	3.44 ± 0.15	3.85 ± 0.25	13.00 ± 0.56	7.00 ± 0.39	3.28 ± 0.15	29.00 ± 0.89
R+P	3	25.46 ± 3.94	3.91 ± 0.16	59.54 ± 5.39	39.45 ± 3.57	3.67 ± 0.17	83.10 ± 3.09
NPD	3	8.31 ± 0.27	4.82 ± 0.30	26.88 ± 0.94	15.80 ± 0.95	4.20 ± 0.16	40.76 ± 2.11
RPD	3	6.23 ± 0.22	5.77 ± 0.10	13.73 ± 0.69	7.72 ± 0.14	5.68 ± 0.18	22.91 ± 0.52

Data represent mean ± SEM of triplicate data. HAM = High amylose maize; N = native (untreated) starch; N+P = Native starch with added porcine pancreatin ; R = Retrograded starch; R+P = Retrograded starch with added porcine pancreatin; NPD = Native pancreatin-digested starch; RPD = Retrograded pancreatin-digested starch.

Appendix 2.5 :-

Final pH values recorded after 48 hours incubation of pure cultures of *Cl. butyricum* in nutrient broth containing cooked meat and starches (0.5 and 1%) which had undergone various treatments.

	Type of starch added to media (0.5%)					
Starch source :	**Native**	**Native + panc.**	**Retrograded**	**Retrograded + panc.**	**Native panc. dig.**	**Retro. panc. dig.**
Maize	5.10 ± 0.06	4.80 ± 0	5.67 ± 0.03	5.20 ± 0.10	5.00 ± 0	5.83 ± 0.03
Waxy maize	5.20 ± 0	4.73 ± 0.07	5.53 ± 0.09	5.37 ± 0.03	5.20 ± 0	5.10 ± 0.15
High am. maize	5.43 ± 0.03	5.03 ± 0.03	5.83 ± 0.03	5.40 ± 0.06	5.57 ± 0.09	5.80 ± 0.06
Amylopectin	5.00 ± 0	5.07 ± 0.03	5.17 ± 0.27	5.20 ± 0.06	4.90 ± 0	5.20 ± 0.12
Potato	5.00 ± 0.06	5.10 ± 0.15	5.67 ± 0.03	5.23 ± 0.03	4.80 ± 0.06	5.90 ± 0
Tapioca	4.80 ± 0.06	4.97 ± 0.07	5.23 ± 0.26	5.40 ± 0	5.40 ± 0	5.70 ± 0
Wheat	4.93 ± 0.03	5.13 ± 0.12	5.87 ± 0.03	4.87 ± 0.09	5.47 ± 0.03	5.90 ± 0

	Type of starch added to media (1%)					
Starch source :	**Native**	**Native + panc.**	**Retrograded**	**Retrograded + panc.**	**Native panc. dig.**	**Retro. panc. dig.**
Maize	4.83 ± 0.07	4.60 ± 0	5.33 ± 0.12	4.97 ± 0.03	4.77 ± 0.03	5.70 ± 0.06
Waxy maize	4.87 ± 0.07	4.43 ± 0.03	5.17 ± 0.20	4.87 ± 0.07	4.90 ± 0	4.83 ± 0.09
High am. maize	5.03 ± 0.03	4.77 ± 0.12	5.50 ± 0.06	5.17 ± 0.03	5.13 ± 0.03	5.63 ± 0.03
Amylopectin	4.70 ± 0.07	4.37 ± 0.07	4.97 ± 0.14	4.73 ± 0.09	4.97 ± 0.03	4.80 ± 0
Potato	4.67 ± 0.07	4.73 ± 0.03	5.40 ± 0.06	4.87 ± 0.03	4.73 ± 0.03	5.73 ± 0.03
Tapioca	4.60 ± 0	4.27 ± 0.03	4.40 ± 0	4.80 ± 0	4.83 ± 0.03	5.40 ± 0.06
Wheat	5.00 ± 0.06	4.60 ± 0.15	5.37 ± 0.07	4.77 ± 0.07	5.07 ± 0.03	5.73 ± 0.03

Data represent average ± SEM of triplicate determinations. high am. maize = high amylose maize; Native + panc. = native starch with pancreatin added; retrograded + panc. = retrograded starch with pancreatin added; native panc. dig. = native pancreatin-digested starch; retro. panc. dig. = retrograded pancreatin-digested starch.

Appendix 2.6 :-
Colourimeter readings at 600nm taken at various time intervals of *Cl. butyricum* starch breakdown using blue amylose and amylopectin.

	Time of reading (hours)					
	0	1	2	3	4	24
***Cl. butyricum* + amylose azure + white amylop.**	0.59 ± 0.01	0.97 ± 0	1.15 ± 0.09	1.26 ± 0.08	1.32 ± 0.11	1.37 ± 0.12
***Cl. butyricum* + amylop. azure + white amylose**	0.06 ± 0.12	0.09 ± 0.01	0.12 ± 0.01	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.01
Filtrate + amylose azure + white amylop.	0.61 ± 0.01	0.90 ± 0.03	1.07 ± 0.09	1.13 ± 0.12	1.20 ± 0.14	1.36 ± 0.18
Filtrate + amylop. azure + white amylose	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
***Cl. butyricum* + amylose azure**	0.58 ± 0.04	0.87 ± 0.06	1.03 ± 0.06	1.07 ± 0.07	1.17 ± 0.08	1.23 ± 0.10
***Cl. butyricum* + amylop. azure**	0.08 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.15 ± 0.02	0.17 ± 0.01	0.16 ± 0.01
Filtrate + amylose azure	0.62 ± 0.03	1.02 ± 0.11	1.09 ± 0.16	1.14 ± 0.20	1.20 ± 0.22	1.33 ± 0.28
Filtrate + amylop. azure	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01

Data represents mean ± SEM of triplicate determinations. amylop. = amylopectin

Appendix 2.7 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.25% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	1.02 ± 0.03	13.17 ± 0.22	27.06 ± 0.28	7.92 ± 0.16	0.38 ± 0.14	0.60 ± 0.06	0.18 ± 0.03
R	3	0.90 ± 0.01	8.05 ± 0.07	15.24 ± 0.15	7.62 ± 0.05	0.25 ± 0	0.47 ± 0.08	0.21 ± 0.04
NPD	3	0.89 ± 0.05	12.74 ± 0.55	22.25 ± 0.78	7.08 ± 0.29	0.25 ± 0.01	0.48 ± 0.05	0.14 ± 0.07
RPD	3	0.85 ± 0.02	8.78 ± 0.01	14.40 ± 0.05	6.88 ± 0.14	0.19 ± 0.01	0.36 ± 0.04	0.17 ± 0.02
Waxy maize :-								
N	3	1.10 ± 0.01	14.15 ± 0.09	31.73 ± 0.18	7.97 ± 0.14	0.65 ± 0	0.51 ± 0	0.04 ± 0.04
R	3	1.12 ± 0.04	14.37 ± 0.75	31.50 ± 1.75	7.96 ± 0.20	0.71 ± 0.06	0.46 ± 0.03	0.13 ± 0.07
NPD	3	0.83 ± 0.04	11.90 ± 0.67	19.79 ± 1.27	6.57 ± 0.26	0.21 ± 0.03	0.38 ± 0.01	0.08 ± 0.05
RPD	3	0.92 ± 0.01	10.39 ± 0.08	16.89 ± 0.32	7.34 ± 0.05	0.19 ± 0.01	0.31 ± 0.16	0.09 ± 0.04
HAM :-								
N	3	0.96 ± 0.02	10.03 ± 0.12	19.42 ± 0.21	7.77 ± 0.14	0.37 ± 0.01	0.44 ± 0.02	0.13 ± 0.08
R	3	1.01 ± 0.01	9.21 ± 0.19	16.35 ± 0.34	8.25 ± 0.08	0.26 ± 0.01	0.42 ± 0.01	0.16 ± 0.02
NPD	3	0.86 ± 0.03	9.51 ± 0.28	16.00 ± 0.56	7.07 ± 0.22	0.26 ± 0.02	0.43 ± 0.02	0.19 ± 0.05
RPD	3	0.89 ± 0	9.32 ± 0.14	15.52 ± 0.18	7.06 ± 0.04	0.21 ± 0.02	0.43 ± 0.01	0.16 ± 0.04

Appendix 2.71 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.5% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	0.99 ± 011	12.66 ± 2.45	27.32 ± 5.34	7.69 ± 0.76	0.70 ± 0.17	0.34 ± 0.02	0.13 ± 0.01
R	3	0.96 ± 0.03	10.70 ± 1.05	22.95 ± 2.63	7.66 ± 0.07	0.65 ± 0.05	0.27 ± 0.01	0.14 ± 0.01
NPD	3	1.11 ± 0.01	19.70 ± 0.42	34.33 ± 0.45	8.02 ± 0.17	0.38 ± 0.01	0.26 ± 0.01	0.06 ± 0.06
RPD	3	1.01 ± 0.02	12.26 ± 1.14	18.00 ± 0.63	8.21 ± 0.09	0.26 ± 0.03	0.10 ± 0.01	0.14 ± 0.07
Waxy maize :-								
N	3	1.14 ± 0.02	15.97 ± 0.92	37.13 ± 1.88	8.10 ± 0.11	0.89 ± 0.02	0.36 ± 0.02	0.16 ± 0.01
R	3	1.20 ± 0.01	20.36 ± 0.10	40.44 ± 3.26	8.06 ± 0.08	0.49 ± 0.22	0.37 ± 0.01	0.03 ± 0.03
NPD	3	1.08 ± 0.02	19.74 ± 0.59	35.36 ± 1.00	7.54 ± 0.12	0.37 ± 0.02	0.26 ± 0.01	0.11 ± 0.06
RPD	3	1.03 ± 0.02	11.96 ± 0.19	19.73 ± 0.18	7.85 ± 0.31	0.22 ± 0.02	0.13 ± 0.02	0.04 ± 0.04
HAM :-								
N	3	0.92 ± 0.04	8.84 ± 0.43	19.27 ± 0.94	7.69 ± 0.28	0.62 ± 0.03	0.30 ± 0.01	0.10 ± 0.05
R	3	0.93 ± 0.04	9.68 ± 1.33	20.50 ± 3.12	7.60 ± 0.05	0.45 ± 0.15	0.25 ± 0.01	0.08 ± 0.04
NPD	3	0.97 ± 0.01	12.01 ± 0.19	21.19 ± 0.26	7.88 ± 0.08	0.44 ± 0.01	0.22 ± 0	0.05 ± 0.05
RPD	3	0.96 ± 0.16	16.12 ± 1.82	21.84 ± 3.06	8.63 ± 0.44	0.26 ± 0.03	0.43 ± 0.18	0.18 ± 0.09

Appendix 2.72 :-

SCFA levels (mM) measured from pure cultures of *B. suis* grown in Hungate tubes containing TPY media and 0.75% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	1.10 ± 0.09	22.47 ± 1.62	42.39 ± 3.16	7.77 ± 0.56	0.55 ± 0.10	0.46 ± 0.05	0.18 ± 0.01
R	3	1.01 ± 0.03	13.22 ± 0.57	24.94 ± 1.17	8.06 ± 0.36	0.38 ± 0.02	0.54 ± 0.08	0.15 ± 0.08
NPD	3	1.17 ± 0.05	25.97 ± 1.19	42.94 ± 2.66	7.67 ± 0.31	0.24 ± 0	0.58 ± 0.03	0.20 ± 0.02
RPD	3	1.00 ± 0	13.47 ± 0.08	22.18 ± 0.09	7.64 ± 0.02	0.21 ± 0	0.48 ± 0.01	0.19 ± 0.04
Waxy maize :-								
N	3	1.35 ± 0.08	31.21 ± 4.47	58.86 ± 7.31	8.89 ± 0.45	0.71 ± 0.03	0.66 ± 0.06	0.24 ± 0.01
R	3	1.35 ± 0.10	33.09 ± 3.59	54.82 ± 5.97	9.02 ± 0.52	0.33 ± 0.06	0.62 ± 0.07	0.22 ± 0.03
NPD	3	1.31 ± 0.08	29.75 ± 2.12	46.46 ± 2.42	8.77 ± 0.29	0.26 ± 0.03	0.60 ± 0.04	0.23 ± 0.03
RPD	3	1.00 ± 0.01	17.66 ± 0.21	28.49 ± 0.39	7.38 ± 0.10	0.21 ± 0	0.46 ± 0.01	0.10 ± 0.06
H.A.M :-								
N	3	0.95 ± 0.03	11.80 ± 0.66	23.54 ± 1.59	7.49 ± 0.27	0.52 ± 0.03	0.49 ± 0.02	0.19 ± 0.04
R	3	0.79 ± 0.01	9.30 ± 0.15	16.48 ± 0.27	6.64 ± 0.15	0.28 ± 0.02	0.37 ± 0.01	0.12 ± 0.01
NPD	3	0.97 ± 0.05	13.20 ± 0.70	23.35 ± 1.27	7.53 ± 0.39	0.23 ± 0.01	0.48 ± 0.02	0.13 ± 0.01
RPD	3	0.95 ± 0.02	13.25 ± 0.34	22.21 ± 0.44	7.16 ± 0.17	0.27 ± 0.02	0.51 ± 0.02	0.14 ± 0.08

HAM = high amylose maize; N = native; R = retrograded; NPD = native pancreatin digested; RPD = retrograded pancreatin digested starch.

Appendix 2.8 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.25% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	0.13 ± 0.01	0.03 ± 0.03	26.02 ± 1.07	10.47 ± 0.12	2.04 ± 0.40	7.69 ± 0.09	0.54 ± 0.09
R	3	0.02 ± 0.02	0.08 ± 0.08	26.40 ± 0.63	9.50 ± 0.17	1.03 ± 0.47	8.03 ± 0.18	0.35 ± 0.21
NPD	3	0.25 ± 0.02	0.08 ± 0.08	24.27 ± 0.91	11.57 ± 0.14	2.29 ± 0.08	6.38 ± 0.53	0.55 ± 0.07
RPD	3	0.08 ± 0.01	0.12 ± 0.12	22.96 ± 2.72	10.28 ± 0.72	1.07 ± 0.02	7.25 ± 0.42	0.34 ± 0.09
Waxy maize :-								
N	3	0.05 ± 0.02	0	26.62 ± 0.63	9.72 ± 0.14	2.01 ± 0.95	7.42 ± 0.05	0.38 ± 0.11
R	3	0.14 ± 0.03	0	27.23 ± 0.65	9.52 ± 0.04	2.56 ± 0.31	7.10 ± 0.18	0.69 ± 0.13
NPD	3	0.23 ± 0.01	0	22.11 ± 0.22	17.67 ± 0.46	2.15 ± 0.02	3.40 ± 0.16	0.56 ± 0
RPD	3	0.12 ± 0.01	0	22.92 ± 0.44	11.59 ± 0.48	1.34 ± 0.04	7.24 ± 0.27	0.29 ± 0.02
HAM :-								
N	3	0.05 ± 0.05	0.03 ± 0.03	23.87 ± 1.37	9.86 ± 0.23	1.89 ± 0.45	7.59 ± 0.11	0.54 ± 0.08
R	3	0.05 ± 0	0.02 ± 0.02	24.41 ± 0.64	8.88 ± 0.04	1.18 ± 0.03	7.92 ± 0.04	0.38 ± 0.02
NPD	3	0.12 ± 0.01	0	20.73 ± 0.60	9.74 ± 0.35	1.11 ± 0.03	6.80 ± 0.12	0.28 ± 0.02
RPD	3	0.07 ± 0.01	0	21.77 ± 0.93	10.31 ± 0.50	1.30 ± 0.06	7.26 ± 0.33	0.27 ± 0.04

Appendix 2.81 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.5% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	1.50 ± 012	0	10.82 ± 0.68	19.14 ± 1.20	1.13 ± 0.06	0	0.25 ± 0.13
R	3	0.01 ± 0	0.06 ± 0	0.12 ± 0	0.15 ± 0	0.05 ± 0.01	0	0
NPD	3	1.94 ± 0.03	0.18 ± 0	13.08 ± 0.21	13.89 ± 0.25	0.15 ± 0.01	0.19 ± 0	0.65 ± 0.06
RPD	3	1.64 ± 0.05	0.16 ± 0.02	9.61 ± 0.12	11.96 ± 0.19	3.35 ± 0.01	0.15 ± 0	0.42 ± 0.02
Waxy maize :-								
N	3	0.02 ± 0	0	0.19 ± 0.02	0.33 ± 0.03	0.03 ± 0	0	0.01 ± 0
R	3	0.44 ± 0.01	3.94 ± 0.10	6.13 ± 0.28	6.83 ± 0.24	2.70 ± 0.08	0	0
NPD	3	0.11 ± 0.02	0.23 ± 0.06	28.56 ± 0.32	16.67 ± 0.19	3.29 ± 0.11	6.51 ± 0.10	0.36 ± 0.14
RPD	3	1.38 ± 0.03	0.46 ± 0.18	9.70 ± 0.10	10.60 ± 0.17	3.30 ± 0.54	0.14 ± 0.01	0.27 ± 0.02
HAM :-								
N	3	0.03 ± 0	0	0.20 ± 0.01	0.36 ± 0.01	0.03 ± 0	0	0.01 ± 0
R	3	0.67 ± 0.12	4.50 ± 0.06	5.23 ± 0.30	7.37 ± 0.28	0.33 ± 0.20	0.08 ± 0.04	0.06 ± 0.03
NPD	3	2.00 ± 0.04	0.31 ± 0.11	21.45 ± 0.53	15.40 ± 0.25	0.17 ± 0	0.19 ± 0	0.59 ± 0.03
RPD	3	1.53 ± 0.01	0.25 ± 0.07	10.38 ± 0.08	11.05 ± 0.04	2.96 ± 1.27	0.14 ± 0.01	0.36 ± 0.02

Appendix 2.82 :-

SCFA levels (mM) measured from pure cultures of *Fusobacterium sp.* grown in Hungate tubes containing TPY media and 0.75% starch

		SCFA (mM)						
Maize :-	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**
N	3	0.13 ± 0	0.03 ± 0.03	27.35 ± 0.20	9.93 ± 0.09	3.30 ± 0.02	7.30 ± 0.05	0.61 ± 0.02
R	3	0.20 ± 0.02	0.07 ± 0.07	26.17 ± 0.62	9.65 ± 0.28	2.42 ± 0.10	7.33 ± 0.11	0.68 ± 0.08
NPD	3	0.37 ± 0.01	0.25 ± 0.06	20.80 ± 0.49	22.85 ± 0.52	1.73 ± 0.11	1.31 ± 0.14	0.57 ± 0.11
RPD	3	0.20 ± 0.02	0	26.67 ± 1.50	14.70 ± 0.68	1.07 ± 0.47	7.28 ± 0.40	0.27 ± 0.06
Waxy maize :-								
N	3	0.15 ± 0.04	0.02 ± 0.02	27.27 ± 0.13	9.87 ± 0.21	2.87 ± 0.71	7.38 ± 0.11	0.38 ± 0.19
R	3	0.16 ± 0	0	27.39 ± 0.25	9.81 ± 0.07	2.21 ± 0.82	7.55 ± 0.16	0.58 ± 0.07
NPD	3	0.38 ± 0.02	0.37 ± 0.07	22.20 ± 0.99	25.16 ± 1.10	1.86 ± 0.07	1.57 ± 0.10	0.45 ± 0.02
RPD	3	0.22 ± 0.01	0.15 ± 0.01	24.24 ± 1.45	21.83 ± 1.09	1.82 ± 0.08	3.64 ± 0.31	0.45 ± 0.05
HAM :-								
N	3	0.07 ± 0.01	0	26.90 ± 0.59	10.98 ± 0.22	1.95 ± 0.02	8.17 ± 0.18	0.53 ± 0.02
R	3	0.11 ± 0.02	0.03 ± 0.03	21.02 ± 1.03	9.08 ± 0.44	0.72 ± 0.31	7.09 ± 0.39	0.23 ± 0.06
NPD	3	0.15 ± 0.01	0.03 ± 0.03	21.59 ± 0.91	10.08 ± 0.57	1.44 ± 0.05	6.70 ± 0.33	0.47 ± 0.07
RPD	3	0.13 ± 0.01	0.03 ± 0.03	23.13 ± 0.65	12.88 ± 0.30	1.50 ± 0.03	6.36 ± 0.14	0.29 ± 0.04

HAM = high amylose maize; N = native; R = retrograded; NPD = native pancreatin digested; RPD = retrograded pancreatin digested.

Appendix 3

Data from Chapter 3

Appendix 3.1 :-

Bacterial counts (cfu ml^{-1}) detected in samples from a single-stage fermentation system which had been fed media containing different untreated starches.

		Bacterial species enumerated (cfu ml^{-1}) - Time zero						
Starch type	**n**	**Coliforms**	***Lactobacillus* spp. (aero)**	***Lactobacillus* spp. (total)**	**Total aerobic count**	**Total anaerobic count**	***Bifidobacterium* spp.**	***Bacteroides* spp.**
Native waxy maize	3	2.65E7 ± 1.26E7	1.70E9 ± 1.40E9	4.33E8 ± 1.36E8	4.00E9 ± 6.43E8	2.43E9 ± 1.22E9	2.44E8 ± 9.54E7	4.85E7 ± 2.65E7
Native HA maize	3	1.93E7 ± 2.96E6	1.90E7 ± 8.02E6	1.90E7 ± 1.16E7	4.10E8 ± 1.36E8	6.50E10 ± 3.33E10	2.73E8 ± 2.06E8	1.46E10 ± 1.27E10
Native maize	3	9.40E7 ± 2.12E7	9.83E7 ± 1.30E7	7.67E7 ± 1.76E7	5.27E8 ± 8.19E7	1.22E11 ± 2.49E10	3.47E8 ± 2.67E8	2.77E8 ± 1.13E8
Native tapioca	3	6.00E7 ± 1.55E7	1.80E8 ± 3.51E7	1.53E8 ± 1.76E7	1.41E9 ± 9.05E8	4.93E8 ± 1.59E8	1.60E8 ± 5.77E6	7.07E7 ± 2.93E7
Native potato	3	1.40E8 ± 3.00E7	1.03E8 ± 9.33E6	1.02E8 ± 4.29E7	9.33E8 ± 2.89E8	2.93E8 ± 2.96E7	7.23E7 ± 2.88E7	1.37E8 ± 2.73E7

		Bacterial species enumerated (cfu ml^{-1}) - 5h postfeed						
Starch type	**n**	**Coliforms**	***Lactobacillus* spp. (aero)**	***Lactobacillus* spp. (total)**	**Total aerobic count**	**Total anaerobic count**	***Bifidobacterium* spp.**	***Bacteroides* spp.**
Native waxy maize	3	2.70E7 ± 1.05E7	1.67E9 ± 9.22E8	1.51E9 ± 4.88E8	3.33E9 ± 2.09E9	4.04E10 ± 3.98E10	5.11E10 ± 4.46E10	1.05E7 ± 4.87E6
Native H.A maize	3	1.08E7 ± 3.11E6	9.17E7 ± 2.03E6	1.02E8 ± 8.33E6	4.00E8 ± 1.76E8	5.79E10 ± 2.94E10	2.29E9 ± 1.96E9	2.92E9 ± 2.64E9
Native maize	3	7.40E7 ± 1.30E7	6.20E8 ± 1.92E8	6.77E8 ± 3.65E8	1.70E9 ± 1.25E9	1.23E11 ± 1.20E10	4.57E9 ± 1.69E9	3.53E9 ± 1.95E9
Native tapioca	3	4.40E7 ± 1.43E7	6.00E8 ± 5.29E7	5.90E8 ± 2.08E7	1.02E9 ± 2.47E8	2.50E9 ± 7.77E8	7.80E8 ± 3.06E7	6.50E7 ± 6.81E6
Native potato	3	8.40E7 ± 1.70E7	2.63E8 ± 1.18E8	2.05E8 ± 7.57E7	5.00E8 ± 5.00E7	2.05E10 ± 1.97E10	7.52E8 ± 3.44E8	7.50E7 ± 1.25E7

Data represent mean ± SEM of triplicate data. HA maize = high amylose maize. Coliforms were enumerated on MacConkey agar; *Lactobacillus* spp. were enumerated on MRS agar; Total aerobic count was enumerated on Columbia blood agar; Total anaerobic count was enumerated on Wilkins-Chalgren blood agar; *Bifidobacterium* spp. were enumerated on Bifidobacterium agar; *Bacteroides* spp. were enumerated on Kanamycin-Vancomycin agar.

Appendix 3.2 :-
SCFA levels (mM) detected in samples from a single-stage fermentation system when fed media containing different starches.

Pre-feed (oh)		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	0.10 ± 0.07	2.04 ± 0.85	23.26 ± 4.94	7.06 ± 2.30	2.37 ± 0.16	8.97 ± 0.51	2.83 ± 0.26	10.98 ± 0.57
Native H A maize	3	0.01 ± 0.01	0.72 ± 0.24	12.56 ± 2.09	4.18 ± 0.66	1.16 ± 0.35	1.85 ± 0.30	1.25 ± 0.24	2.50 ± 0.50
Native maize	3	ND	1.96 ± 0.34	21.42 ± 2.48	3.90 ± 0.44	2.22 ± 0.09	5.97 ± 0.51	1.92 ± 0.25	6.40 ± 0.60
Native tapioca	3	0.10 ± 0.01	1.12 ± 0.33	22.30 ± 4.54	8.09 ± 0.96	1.09 ± 0.12	2.56 ± 0.48	1.48 ± 0.31	3.70 ± 0.25
Native potato	3	0.10 ± 0.03	0.53 ± 0.13	18.62 ± 2.58	6.47 ± 1.10	1.65 ± 0.24	5.19 ± 0.46	2.31 ± 0.03	3.24 ± 0.82

Post-feed 1 hour		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	0.15 ± 0.12	10.71 ± 1.75	25.59 ± 2.60	7.54 ± 1.40	2.49 ± 0.08	3.29 ± 0.56	3.19 ± 0.12	12.16 ± 0.73
Native H A maize	3	0.02 ± 0.02	1.67 ± 0.33	14.72 ± 2.79	4.29 ± 0.59	1.53 ± 0.20	2.17 ± 0.35	1.43 ± 0.24	2.62 ± 0.51
Native maize	3	ND	15.86 ± 1.80	26.02 ± 1.81	5.03 ± 0.21	2.81 ± 0.05	9.12 ± 0.35	2.22 ± 0.20	7.11 ± 0.47
Native tapioca	3	0.42 ± 0.10	8.23 ± 1.29	28.15 ± 4.74	11.30 ± 2.84	1.75 ± 0.48	3.30 ± 0.26	2.11 ± 0.37	3.84 ± 1.26
Native potato	3	0.31 ± 0.06	0.56 ± 0.32	20.44 ± 1.81	7.32 ± 1.32	1.55 ± 0.16	5.42 ± 0.71	2.90 ± 0.22	3.50 ± 1.04

Post-feed 2 hours		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	0.06 ± 0.06	31.31 ± 2.91	28.63 ± 2.71	9.34 ± 1.47	2.99 ± 0.26	13.82 ± 0.42	3.57 ± 0.12	13.94 ± 0.49
Native H A maize	3	ND	3.56 ± 0.52	18.94 ± 1.91	5.53 ± 0.52	1.73 ± 0.20	3.67 ± 0.16	1.96 ± 0.11	3.41 ± 0.37
Native maize	3	ND	17.56 ± 2.73	32.42 ± 2.56	6.38 ± 0.38	3.46 ± 0.22	12.52 ± 0.74	2.47 ± 0.32	8.20 ± 0.88
Native tapioca	3	0.31 ± 0.08	12.17 ± 1.12	23.52 ± 3.04	8.23 ± 0.65	1.83 ± 0.44	3.20 ± 0.45	1.65 ± 0.32	1.63 ± 0.14
Native potato	3	0.33 ± 0.25	0.58 ± 0.52	24.79 ± 2.21	9.21 ± 1.43	2.04 ± 0.12	7.25 ± 0.62	3.59 ± 0.24	3.06 ± 0.87

Appendix 3.2 continued :-

Post-feed 5 hours		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	ND	0.49 ± 0.03	46.01 ± 4.23	17.93 ± 1.95	6.43 ± 0.85	31.75 ± 1.67	6.15 ± 0.30	33.44 ± 2.68
Native H A maize	3	ND	0.46 ± 0.23	33.95 ± 2.86	10.04 ± 1.49	2.84 ± 0.50	7.75 ± 0.94	3.63 ± 0.11	7.98 ± 1.05
Native maize	3	ND	0.17 ± 0.09	52.94 ± 3.77	12.67 ± 1.15	4.00 ± 0.57	24.26 ± 1.39	3.26 ± 0.40	15.00 ± 4.01
Native tapioca	3	0.09 ± 0.09	6.18 ± 3.46	31.88 ± 7.93	13.86 ± 3.31	2.38 ± 0.63	4.38 ± 1.13	1.78 ± 0.21	2.86 ± 0.25
Native potato	3	0.14 ± 0.14	0.26 ± 0.26	42.02 ± 2.26	16.65 ± 1.02	3.36 ± 0.21	17.60 ± 1.95	5.56 ± 0.42	5.48 ± 1.70

Post-feed 7 hours		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	ND	ND	57.31 ± 4.28	21.09 ± 2.42	7.53 ± 1.36	36.93 ± 2.03	7.91 ± 0.43	43.46 ± 2.93
Native H A maize	3	ND	ND	41.18 ± 3.13	12.36 ± 1.32	2.72 ± 0.27	8.50 ± 0.84	3.95 ± 0.08	10.61 ± 0.82
Native maize	3	ND	ND	52.73 ± 2.56	11.58 ± 0.21	3.01 ± 0.13	22.31 ± 0.09	2.89 ± 0.04	10.89 ± 3.23
Native tapioca	3	ND	6.81 ± 6.78	47.35 ± 7.62	21.28 ± 5.27	3.30 ± 0.26	6.43 ± 0.71	2.26 ± 0.38	6.86 ± 3.26
Native potato	3	0.12 ± 0.12	0.08 ± 0.08	50.21 ± 2.48	20.42 ± 1.54	3.74 ± 0.41	22.95 ± 2.33	6.50 ± 0.87	7.74 ± 2.71

Post-feed 24 hours		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Native waxy maize	3	ND	0.25 ± 0.25	43.14 ± 4.26	12.19 ± 1.66	3.43 ± 0.36	20.74 ± 1.53	6.11 ± 0.36	29.32 ± 3.25
Native H A maize	3	ND	0.31 ± 0.31	25.60 ± 5.22	7.64 ± 1.61	1.56 ± 0.04	4.20 ± 0.80	2.39 ± 0.52	6.75 ± 1.83
Native maize	3	ND	ND	35.06 ± 0.54	6.25 ± 0.30	2.20 ± 0.23	12.74 ± 0.98	2.80 ± 0.16	8.35 ± 2.87
Native tapioca	3	ND	3.54 ± 3.54	37.38 ± 5.99	14.33 ± 2.60	2.00 ± 0.67	5.59 ± 1.65	1.84 ± 0.06	5.11 ± 0.90
Native potato	3	ND	ND	32.99 ± 1.25	9.87 ± 0.84	3.29 ± 0.56	12.27 ± 0.98	4.55 ± 0.24	5.39 ± 1.55

Data represent the mean ± SEM of multiple determinations; **n** = Number of samples tested; ND = Not detected; H A maize = High amylose maize

Appendix 3.3 :-

Amount of KOH (mls) needed in the single-stage fermenter to stabilise the pH at 6.0 over a period of 4 days, when different starches were incorporated into the media.

Waxy maize :-	Time (h)	Cummulative KOH (ml)
Day 1	0	0
Day 1	2.25	90
Day 1	7.25	95
Day 2	23.75	95
Day 2	26.25	190
Day 2	31.25	190
Day 3	47.75	195
Day 3	50.25	265
Day 3	55.25	300
Day 4	71.75	300
Day 4	74.25	395
Day 4	79.25	425
Day 5	95.75	425

High amylose maize :-	Time (h)	Cummulative KOH (ml)
Day 1	0	0
Day 1	2.50	10
Day 1	7.50	60
Day 2	24.00	90
Day 2	26.50	135
Day 2	31.50	165
Day 3	48.00	200
Day 3	50.50	200
Day 3	55.50	230
Day 4	72.50	265
Day 4	74.50	300
Day 4	79.50	315
Day 5	96.00	360

Maize	Time (h)	Cummulative KOH (ml)
Day 1	0	0
Day 1	2.50	30
Day 1	7.50	65
Day 2	24.00	70
Day 2	26.50	125
Day 2	31.50	130
Day 3	48.00	135
Day 3	50.50	195
Day 3	55.50	200
Day 4	72.00	200
Day 4	74.50	250
Day 4	79.50	280
Day 5	96.00	280

Appendix 3.3 continued :-

Tapioca	Time (h)	Cummulative KOH (ml)
Day 1	0	0
Day 1	2.50	65
Day 1	7.50	110
Day 2	24.00	110
Day 2	26.50	180
Day 2	31.50	225
Day 3	48.00	240
Day 3	50.50	280
Day 3	55.50	320
Day 4	72.00	320
Day 4	74.50	380
Day 4	79.50	440
Day 5	96.00	440

Potato	Time (h)	Cummulative KOH (ml)
Day 1	0	0
Day 1	2.50	0
Day 1	7.50	30
Day 2	24.00	30
Day 2	26.50	50
Day 2	31.50	105
Day 3	48.00	105
Day 3	50.50	140
Day 3	55.50	200
Day 4	72.00	200
Day 4	74.50	240
Day 4	79.50	305
Day 5	96.00	315

Data represent a single measurement taken at approximately the same time during the four day fermenter run.

Appendix 3.4 :-
Lactobacillus : coliform ratio determined from bacteria; counts obtained from the single-stage fermenter at time zero and 5h postfeed.

	Sampling time (h)	
Starch Type :-	**Time zero samples (0h)**	**5h postfeed samples**
Waxy maize - day 2	100.0	77.8
Waxy maize - day 3	10.9	15.0
Waxy maize - day 4	15.5	160.0
High amylose maize - day 2	2.8	13.4
High amylose maize - day 3	0.4	11.8
High amylose maize - day 4	0.3	6.5
Maize - day 2	1.0	3.7
Maize - day 3	0.6	6.7
Maize - day 4	1.0	14.0
Tapioca - day 2	5.3	24.0
Tapioca - day 3	1.8	17.7
Tapioca - day 4	2.2	7.6
Potato - day 2	1.6	6.7
Potato - day 3	0.9	1.9
Potato - day 4	0.2	0.9

Appendix 4

Data from Chapter 4

Appendix 4.1 :-

Bacterial counts (cfu g^{-1}) obtained from faecal samples over a period of three weeks from pigs fed on diets containing different starches.

Coliform bacteria (on MacConkey agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	4.00E8 ± 8.47E7	6	5.23E7 ± 1.61E7	3	2.11E8 ± 9.39E7	4	3.11E7 ± 1.23E7	4	6.47E7 ± 4.29E7	4	8.11E9 ± 8.03E9
Sample 1	4	3.36E9 ± 2.41E9	4	2.56E9 ± 2.55E9	4	3.50E7 ± 1.67E7	4	6.85E6 ± 3.85E6	4	2.30E7 ± 1.88E7	3	4.91E7 ± 2.29E7
Sample 2	4	1.12E9 ± 8.82E8	4	7.82E7 ± 2.92E7	4	4.26E7 ± 3.45E7	3	6.98E6 ± 5.37E6	3	6.30E6 ± 3.23E6	3	5.56E7 ± 3.96E7
Sample 3	3	9.69E7 ± 8.11E7	4	4.12E7 ± 1.50E7	3	5.56E7 ± 2.45E7	3	3.65E10 ± 1.69E10	3	7.63E9 ± 5.80E9	3	8.41E10 ± 4.85E10
Sample 4	3	6.49E7 ± 2.42E7	4	1.08E8 ± 3.68E7	3	7.67E7 ± 7.51E7	3	1.67E8 ± 1.45E8	3	4.17E7 ± 1.07E7	3	7.64E7 ± 2.48E7
Sample 5	2	1.56E7 ± 1.42E7	4	1.94E8 ± 1.38E8	4	1.09E8 ± 8.45E7	3	1.47E8 ± 6.49E7	3	7.30E7 ± 6.41E7	3	1.16E7 ± 6.48E6
Sample 6	3	4.21E7 ± 1.85E7	3	3.59E7 ± 3.08E7	3	4.33E7 ± 1.42E7	3	4.92E7 ± 4.35E7	3	6.09E6 ± 1.78E6	3	1.51E8 ± 1.21E8

***Enterococcus* species (on S&B agar)**

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	3.68E4 ± 7.57E3	6	1.12E7 ± 1.11E7	3	5.53E5 ± 4.94E5	4	1.02E5 ± 3.93E4	4	1.24E7 ± 9.49E6	4	2.81E8 ± 2.80E8
Sample 1	4	1.24E9 ± 1.24E9	4	2.45E5 ± 1.98E5	4	7.61E4 ± 5.02E4	4	4.39E4 ± 2.17E4	4	1.58E5 ± 8.22E4	3	1.42E8 ± 1.42E8
Sample 2	4	2.75E6 ± 1.84E6	4	1.93E6 ± 1.43E6	4	7.61E5 ± 4.34E5	3	2.34E4 ± 8.80E3	3	1.77E4 ± 3.74E3	3	1.10E9 ± 7.78E8
Sample 3	3	3.08E8 ± 2.32E8	4	1.13E6 ± 6.56E5	3	8.84E5 ± 8.73E5	3	3.64E8 ± 1.72E8	3	3.55E6 ± 1.53E6	3	3.99E8 ± 3.75E8
Sample 4	3	1.06E9 ± 7.59E8	4	4.11E6 ± 3.80E6	3	1.76E5 ± 1.65E5	3	1.20E5 ± 6.30E4	3	1.13E7 ± 1.06E7	3	3.94E7 ± 1.53E7
Sample 5	2	2.80E8 ± 2.71E8	4	9.40E6 ± 9.07E6	4	1.28E8 ± 4.07E7	3	1.69E6 ± 1.32E6	3	1.07E6 ± 9.03E5	3	2.34E6 ± 1.31E6
Sample 6	3	6.62E5 ± 2.27E5	3	7.50E5 ± 2.50E5	3	2.59E7 ± 2.45E7	3	2.64E7 ± 2.62E7	3	2.15E5 ± 1.38E5	3	1.27E7 ± 1.24E7

Appendix 4.1 continued :-

Aerotolerant *Lactobacillus* species (on MRS agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	6.25E8 ± 2.88E8	6	4.04E8 ± 2.03E8	3	2.41E8 ± 1.16E8	4	5.02E7 ± 3.04E7	4	8.31E7 ± 3.58E7	4	1.34E9 ± 1.25E9
Sample 1	4	4.90E9 ± 2.38E9	4	3.06E9 ± 2.88E9	4	5.34E8 ± 3.31E8	4	3.34E7 ± 1.25E7	4	1.82E8 ± 6.26E7	3	5.15E8 ± 3.62E8
Sample 2	4	2.95E9 ± 1.28E9	4	1.50E8 ± 2.83E7	4	8.57E7 ± 3.65E7	3	1.36E7 ± 4.06E6	3	1.29E8 ± 6.26E7	3	7.74E8 ± 5.39E8
Sample 3	3	4.57E8 ± 3.82E8	4	5.90E7 ± 1.45E7	3	6.42E7 ± 3.05E7	3	2.95E10 ± 1.42E10	3	1.09E10 ± 8.33E9	3	9.95E10 ± 6.59E10
Sample 4	3	1.07E9 ± 6.06E8	4	1.42E8 ± 5.33E7	3	1.52E7 ± 7.81E6	3	3.93E7 ± 3.21E7	3	2.11E7 ± 1.13E7	3	3.60E7 ± 2.02E7
Sample 5	2	1.71E9 ± 1.26E9	4	8.03E8 ± 4.18E8	4	2.37E8 ± 5.20E7	3	2.29E6 ± 1.06E6	3	7.42E6 ± 5.02E6	3	1.05E7 ± 8.28E6
Sample 6	3	8.00E7 ± 2.29E7	3	5.75E7 ± 3.38E7	3	9.08E7 ± 2.28E7	3	7.15E7 ± 2.34E7	3	1.46E7 ± 4.76E6	3	1.39E8 ± 1.06E8

Total *Lactobacillus* species (on MRS agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	6.27E8 ± 4.33E7	6	2.02E8 ± 7.92E7	3	2.13E8 ± 9.69E7	4	4.20E7 ± 1.48E7	4	1.29E8 ± 8.72E7	4	5.30E9 ± 5.04E9
Sample 1	4	4.28E9 ± 1.80E9	4	2.72E9 ± 2.12E9	4	1.17E9 ± 5.57E8	4	5.54E7 ± 3.35E7	4	2.46E8 ± 9.53E7	3	9.28E8 ± 6.05E8
Sample 2	4	3.26E9 ± 4.20E8	4	1.37E8 ± 2.15E7	4	9.63E7 ± 2.69E7	3	2.00E7 ± 8.26E6	3	2.24E8 ± 1.64E8	3	2.02E9 ± 1.48E9
Sample 3	3	5.75E8 ± 4.73E8	4	8.01E7 ± 2.62E7	3	1.19E8 ± 4.00E7	3	2.12E10 ± 1.20E10	3	4.19E9 ± 3.42E9	3	3.80E10 ± 2.14E10
Sample 4	3	1.18E9 ± 6.75E8	4	2.92E9 ± 2.73E9	3	2.97E7 ± 1.66E7	3	3.65E7 ± 3.33E7	3	2.45E7 ± 1.32E7	3	3.38E7 ± 1.61E7
Sample 5	2	4.70E9 ± 4.45E9	4	1.06E9 ± 8.72E8	4	2.07E8 ± 2.21E7	3	2.40E6 ± 1.06E6	3	2.61E7 ± 1.77E7	3	1.42E7 ± 1.13E7
Sample 6	3	3.71E8 ± 2.84E8	3	2.08E8 ± 9.82E7	3	1.25E8 ± 0	3	7.89E7 ± 1.93E7	3	2.75E7 ± 1.18E7	3	1.58E8 ± 8.05E7

Appendix 4.1 continued :-

Total aerobic count (on CBA agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	7.04E8 ± 1.04E8	6	4.54E8 ± 2.35E8	3	2.21E8 ± 1.04E8	4	1.19E8 ± 5.41E7	4	8.53E7 ± 4.23E7	4	2.54E10 ± 1.50E10
Sample 1	4	7.58E9 ± 3.36E9	4	3.95E9 ± 3.82E9	4	8.57E8 ± 3.48E8	4	4.92E7 ± 2.83E7	4	1.33E8 ± 4.85E7	3	1.82E8 ± 7.82E7
Sample 2	4	7.79E9 ± 5.78E9	4	4.37E8 ± 2.65E8	4	3.35E7 ± 2.33E7	3	3.98E7 ± 1.86E7	3	3.05E7 ± 1.15E7	3	1.67E9 ± 8.28E8
Sample 3	3	5.24E8 ± 3.59E8	4	3.04E7 ± 9.06E6	3	3.35E7 ± 1.58E7	3	3.52E10 ± 1.93E10	3	1.06E10 ± 8.46E9	3	9.72E10 ± 6.63E10
Sample 4	3	7.24E8 ± 3.38E8	4	4.45E8 ± 3.69E8	3	2.56E8 ± 2.51E8	3	1.46E8 ± 9.77E7	3	1.27E8 ± 7.54E7	3	1.70E8 ± 5.88E7
Sample 5	2	4.90E9 ± 4.69E9	4	4.58E8 ± 2.62E8	4	1.33E8 ± 5.78E7	3	3.02E8 ± 1.36E8	3	1.30E8 ± 1.08E8	3	4.64E7 ± 1.15E7
Sample 6	3	7.31E7 ± 3.05E7	3	7.50E7 ± 2.89E7	3	4.67E7 ± 1.20E7	3	5.18E8 ± 4.36E8	3	1.06E8 ± 4.17E7	3	4.49E8 ± 3.21E8

***Escherichia coli* (on CBA+MUG agar)**

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	3.89E8 ± 8.18E7	6	1.32E8 ± 4.96E7	3	2.45E8 ± 1.12E8	4	7.35E7 ± 2.88E7	4	7.56E7 ± 4.52E7	4	2.73E9 ± 2.49E9
Sample 1	4	4.88E9 ± 2.25E9	4	6.24E9 ± 6.15E9	4	3.61E8 ± 2.46E8	4	3.22E7 ± 2.64E7	4	3.01E7 ± 2.41E7	3	1.04E8 ± 4.97E7
Sample 2	4	7.79E9 ± 5.78E9	4	4.01E8 ± 2.75E8	4	3.35E7 ± 2.33E7	3	6.87E6 ± 5.44E6	3	9.10E6 ± 6.71E6	3	1.01E8 ± 7.96E7
Sample 3	3	5.93E8 ± 4.68E8	4	3.04E7 ± 9.06E6	3	3.35E7 ± 1.58E7	3	3.52E10 ± 1.93E10	3	6.96E9 ± 4.81E9	3	9.72E10 ± 6.63E10
Sample 4	3	2.29E8 ± 1.67E8	4	4.45E8 ± 3.69E8	3	2.54E8 ± 2.51E8	3	1.03E8 ± 6.25E7	3	3.25E8 ± 3.01E8	3	9.85E7 ± 3.95E7
Sample 5	2	2.70E8 ± 1.54E8	4	4.11E8 ± 2.78E8	4	6.30E7 ± 4.40E7	3	2.78E8 ± 1.26E8	3	1.06E8 ± 9.09E7	3	1.21E7 ± 4.83E6
Sample 6	3	6.85E7 ± 3.46E7	3	4.58E7 ± 1.82E7	3	3.33E7 ± 1.86E7	3	1.83E7 ± 1.40E7	3	1.84E7 ± 1.41E7	3	1.61E8 ± 1.38E8

Appendix 4.1 continued :-

Aerobic starch degraders (on starch agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	6.49E2 ± 0	6	6.34E5 ± 6.29E5	3	5.38E2 ± 6.47E1	4	5.02E2 ± 7.29E1	4	1.26E4 ± 1.21E4	4	6.36E2 ± 1.32E2
Sample 1	4	4.01E2 ± 8.08E1	4	1.60E3 ± 1.10E3	4	1.17E5 ± 1.15E5	4	4.61E2 ± 2.40E1	4	4.36E2 ± 1.25E1	3	4.55E2 ± 1.09E2
Sample 2	4	4.69E2 ± 2.36E1	4	3.75E2 ± 3.94E1	4	2.02E3 ± 1.17E3	3	4.89E2 ± 5.46E1	3	2.09E3 ± 1.77E3	3	4.52E2 ± 7.46E1
Sample 3	3	4.01E2 ± 4.91E1	4	4.18E2 ± 5.11E1	3	3.91E2 ± 3.70E1	3	5.15E2 ± 2.70E1	3	6.93E2 ± 1.25E2	3	1.59E4 ± 1.54E4
Sample 4	3	4.12E5 ± 2.06E5	4	4.12E2 ± 4.43E1	3	5.27E2 ± 1.13E1	3	4.27E2 ± 4.16E1	3	4.66E2 ± 4.07E1	3	1.53E4 ± 1.48E4
Sample 5	2	4.46E2 ± 2.15E1	4	1.48E3 ± 1.04E3	4	4.30E2 ± 4.79E1	3	1.96E4 ± 1.90E4	3	4.98E2 ± 9.91E1	3	5.11E2 ± 1.21E1
Sample 6	3	5.60E2 ± 3.61E1	3	5.00E2 ± 0	3	1.70E4 ± 1.65E4	3	4.36E2 ± 1.30E1	3	4.43E2 ± 1.36E1	3	4.56E2 ± 1.07E1

Anaerobic starch degraders (on starch agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	7.14E2 ± 0	6	5.40E2 ± 1.18E2	3	6.26E2 ± 5.91E1	4	1.74E6 ± 1.54E6	4	1.01E6 ± 8.40E5	4	1.98E6 ± 1.51E6
Sample 1	4	4.18E2 ± 3.68E1	4	4.59E2 ± 2.92E1	4	2.68E5 ± 1.55E5	4	4.07E2 ± 8.14E1	4	1.51E3 ± 1.15E3	3	2.79E7 ± 2.58E7
Sample 2	4	1.44E7 ± 1.44E7	4	4.26E2 ± 5.43E1	4	7.34E2 ± 1.12E2	3	1.79E5 ± 1.76E5	3	3.67E5 ± 1.68E5	3	9.24E4 ± 9.18E4
Sample 3	3	4.43E2 ± 2.77E1	4	1.07E5 ± 1.03E5	3	2.99E3 ± 1.26E3	3	6.68E2 ± 8.15E1	3	5.76E2 ± 4.15E1	3	1.11E6 ± 1.11E6
Sample 4	3	1.67E5 ± 1.62E5	4	1.20E4 ± 1.06E4	3	3.69E5 ± 1.91E5	3	1.76E4 ± 1.70E4	3	1.36E5 ± 9.29E4	3	5.86E5 ± 6.20E4
Sample 5	2	4.90E2 ± 1.19E2	4	1.75E3 ± 1.29E3	4	1.21E3 ± 6.93E2	3	3.16E4 ± 2.74E4	3	4.23E3 ± 2.07E3	3	3.40E3 ± 1.44E3
Sample 6	3	3.94E6 ± 1.98E6	3	5.00E2 ± 0	3	3.35E5 ± 1.65E5	3	1.42E5 ± 1.39E5	3	1.43E4 ± 1.39E4	3	1.44E4 ± 1.40E4

Appendix 4.1 continued :-

Total anaerobic count (on WCBA agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	1.90E9 ± 2.37E8	6	5.19E9 ± 3.61E9	3	3.34E9 ± 7.95E8	4	5.42E8 ± 1.46E8	4	4.29E8 ± 1.15E8	4	4.29E8 ± 1.27E8
Sample 1	4	2.44E9 ± 1.34E9	4	6.65E8 ± 2.93E8	4	8.63E8 ± 4.98E8	4	2.90E8 ± 7.52E7	4	9.29E8 ± 1.18E8	3	3.32E9 ± 1.35E9
Sample 2	4	2.78E9 ± 4.30E8	4	1.10E9 ± 4.74E8	4	7.03E8 ± 1.55E8	3	4.65E8 ± 5.60E7	3	4.12E9 ± 3.34E9	3	3.87E9 ± 1.85E9
Sample 3	3	1.40E10 ± 2.98E9	4	5.55E9 ± 5.09E9	3	4.91E9 ± 1.66E9	3	1.39E9 ± 4.76E8	3	1.30E9 ± 5.30E8	3	3.99E9 ± 4.15E8
Sample 4	3	1.16E9 ± 5.60E8	4	1.00E9 ± 1.77E8	3	6.05E8 ± 1.33E8	3	2.42E8 ± 7.60E7	3	2.92E8 ± 5.52E7	3	6.11E8 ± 1.25E8
Sample 5	2	9.55E8 ± 3.25E8	4	1.70E9 ± 7.65E8	4	1.06E9 ± 4.34E8	3	3.38E8 ± 6.87E7	3	4.41E8 ± 9.59E7	3	8.89E8 ± 3.72E8
Sample 6	3	8.16E8 ± 5.46E8	3	9.42E8 ± 2.95E8	3	9.75E8 ± 1.66E8	3	4.38E8 ± 3.01E8	3	1.34E8 ± 1.96E7	3	2.18E8 ± 4.09E7

***Clostridium* species (on RCA agar)**

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	1.37E9 ± 3.90E8	6	6.70E8 ± 3.38E8	3	1.28E9 ± 5.57E8	4	1.33E8 ± 4.22E7	4	2.94E8 ± 1.11E8	4	2.00E8 ± 9.61E7
Sample 1	4	1.44E8 ± 7.79E7	4	9.09E7 ± 5.57E7	4	1.57E8 ± 5.37E7	4	9.53E7 ± 4.23E7	4	2.93E8 ± 2.91E7	3	5.95E8 ±1.19E8
Sample 2	4	2.15E9 ± 1.18E9	4	1.07E8 ± 1.72E7	4	4.43E8 ± 7.67E7	3	1.19E8 ± 3.73E7	3	5.34E8 ± 1.72E8	3	2.96E9 ± 1.81E9
Sample 3	3	2.45E9 ± 2.51E8	4	2.36E8 ± 5.73E7	3	1.46E9 ± 6.29E8	3	1.11E8 ± 2.14E7	3	9.04E8 ± 2.54E8	3	2.69E8 ± 4.32E7
Sample 4	3	7.14E7 ± 1.33E7	4	3.12E8 ± 2.85E7	3	1.23E8 ± 5.93E7	3	1.28E8 ± 2.71E7	3	9.37E7 ± 2.84E7	3	2.51E8 ± 1.22E8
Sample 5	2	7.00E8 ± 3.10E8	4	4.24E8 ± 1.74E8	4	3.02E8 ± 6.51E7	3	1.19E8 ± 2.63E7	3	2.41E8 ± 1.36E8	3	6.08E7 ± 1.23E7
Sample 6	3	2.82E8 ± 4.14E7	3	1.42E8 ± 2.20E7	3	8.17E8 ± 3.71E8	3	1.89E8 ± 1.55E8	3	7.57E7 ± 4.83E7	3	1.82E8 ± 9.67E7

Appendix 4.1 continued :-

Gram-negative anaerobes (on GN agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	2.02E9 ± 4.30E8	6	4.76E8 ± 1.39E8	3	7.12E8 ± 3.51E8	4	1.43E8 ± 3.81E7	4	3.91E8 ± 1.01E8	4	4.41E8 ± 1.22E8
Sample 1	4	9.56E8 ± 4.40E8	4	1.65E8 ± 3.83E7	4	4.80E8 ± 2.26E8	4	1.25E8 ± 3.66E7	4	1.02E9 ± 4.54E8	3	1.03E9 ± 8.49E7
Sample 2	4	2.49E9 ± 7.02E8	4	2.84E7 ± 1.74E7	4	7.36E8 ± 2.39E8	3	1.68E8 ± 4.01E7	3	5.04E8 ± 1.58E8	3	2.85E8 ± 1.04E8
Sample 3	3	4.04E9 ± 1.19E9	4	3.85E8 ± 9.52E7	3	3.72E9 ± 1.68E9	3	7.18E8 ± 1.64E8	3	1.77E8 ± 5.96E7	3	7.29E9 ± 5.19E9
Sample 4	3	4.95E8 ± 1.51E8	4	2.92E8 ± 6.02E7	3	3.87E8 ± 8.06E7	3	1.25E8 ± 1.60E7	3	2.70E8 ± 9.86E7	3	3.78E8 ± 5.89E7
Sample 5	2	6.42E7 ± 2.72E7	4	2.96E8 ± 5.13E7	4	3.68E8 ± 1.12E8	3	4.40E8 ± 1.91E8	3	1.98E8 ± 9.32E7	3	1.12E9 ± 6.60E8
Sample 6	3	4.03E8 ± 1.24E8	3	2.50E8 ± 1.42E8	3	4.33E8 ± 8.82E7	3	1.54E8 ± 9.40E7	3	3.51E7 ± 8.92E6	3	1.70E8 ± 5.28E7

Non-sporing anaerobes (on NS agar)

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	2.14E9 ± 4.13E8	6	1.72E9 ± 6.75E8	3	3.20E9 ± 1.54E9	4	4.16E8 ± 1.00E8	4	4.19E8 ± 6.02E7	4	4.60E8 ± 1.66E8
Sample 1	4	7.53E8 ± 4.36E8	4	3.34E8 ± 7.70E7	4	3.88E8 ± 4.80E7	4	1.59E8 ± 3.98E7	4	1.36E9 ± 4.44E8	3	2.23E9 ± 5.31E8
Sample 2	4	2.60E9 ± 8.77E8	4	5.17E8 ± 3.01E8	4	7.68E8 ± 1.71E8	3	4.01E8 ± 1.07E7	3	1.16E9 ± 2.40E8	3	5.38E9 ± 3.22E9
Sample 3	3	6.44E9 ± 1.83E9	4	4.71E9 ± 2.45E9	3	4.54E9 ± 2.05E9	3	5.42E8 ± 2.04E8	3	2.80E8 ± 1.07E8	3	2.66E9 ± 7.46E8
Sample 4	3	1.27E9 ± 3.42E8	4	8.76E8 ± 4.39E8	3	9.51E8 ± 1.35E8	3	1.16E8 ± 5.78E7	3	2.49E8 ± 1.16E8	3	4.24E8 ± 4.85E7
Sample 5	2	1.53E9 ± 6.02E8	4	8.45E8 ± 3.34E8	4	4.61E8 ± 9.49E7	3	5.77E8 ± 2.65E8	3	4.55E8 ± 1.69E8	3	9.01E8 ± 3.09E8
Sample 6	3	7.78E8 ± 1.97E8	3	4.42E8 ± 1.02E8	3	7.58E8 ± 1.08E8	3	1.70E8 ± 4.10E7	3	8.01E7 ± 5.51E7	3	1.65E8 ± 2.08E7

Appendix 4.1 continued :-

***Bifidobacterium* species (on BIF agar)**

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	5.00E8 ± 2.38E8	6	3.73E8 ± 9.94E7	3	7.57E8 ± 2.05E8	4	1.76E7 ± 8.17E6	4	6.59E7 ± 2.62E7	4	1.81E8 ± 1.27E8
Sample 1	4	1.04E8 ± 6.14E7	4	1.24E8 ± 3.82E7	4	3.49E8 ± 5.35E7	4	3.07E7 ± 1.12E7	4	2.31E8 ± 8.33E7	3	8.92E8 ± 1.29E8
Sample 2	4	1.96E9 ± 8.80E8	4	8.03E7 ± 2.50E7	4	3.83E8 ± 1.53E8	3	4.98E7 ± 2.69E7	3	9.52E8 ± 3.85E8	3	1.28E9 ± 7.38E8
Sample 3	3	2.03E9 ± 1.11E9	4	1.26E8 ± 1.11E8	3	4.48E8 ± 1.51E8	3	9.07E6 ± 1.73E6	3	2.67E7 ± 3.91E6	3	5.18E7 ± 1.72E7
Sample 4	3	1.93E8 ± 8.47E7	4	5.51E8 ± 2.02E8	3	2.07E8 ± 1.11E8	3	4.43E7 ± 3.89E7	3	6.12E7 ± 2.42E7	3	9.91E7 ± 4.66E7
Sample 5	2	1.08E9 ± 5.66E8	4	8.92E7 ± 1.50E7	4	5.58E7 ± 1.02E7	3	2.54E7 ± 1.22E7	3	1.49E8 ± 1.47E8	3	1.76E8 ± 1.38E8
Sample 6	3	5.32E8 ± 1.12E8	3	2.58E8 ± 5.83E7	3	3.12E8 ± 1.33E8	3	1.84E8 ± 1.58E8	3	3.24E7 ± 1.68E7	3	2.30E7 ± 1.71E7

Counts on Rifampicin (RIF) agar

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	4.64E7 ± 8.23E6	6	9.64E7 ± 4.09E7	3	1.60E8 ± 1.06E8	4	9.26E7 ± 3.81E7	4	1.31E7 ± 7.83E6	4	1.07E8 ± 3.80E7
Sample 1	4	6.62E7 ± 4.00E7	4	6.94E6 ± 3.32E6	4	1.62E8 ± 4.50E7	4	5.76E6 ± 2.73E6	4	3.78E7 ± 3.67E7	3	3.05E8 ± 1.30E8
Sample 2	4	7.11E7 ± 3.10E7	4	1.04E7 ± 2.87E6	4	2.49E7 ± 6.19E6	3	6.10E7 ± 2.70E7	3	5.31E7 ± 1.87E7	3	6.85E7 ± 4.11E7
Sample 3	3	2.34E9 ± 1.23E9	4	5.60E7 ± 5.07E7	3	3.58E7 ± 1.33E7	3	1.64E7 ± 9.83E6	3	2.00E8 ± 8.37E7	3	1.51E7 ± 9.84E5
Sample 4	3	9.45E8 ± 3.46E8	4	2.96E7 ± 1.28E7	3	7.55E7 ± 5.84E7	3	2.12E7 ± 1.39E7	3	2.12E7 ± 1.09E7	3	4.50E7 ± 2.59E7
Sample 5	2	6.38E7 ± 5.82E7	4	1.24E8 ± 6.70E7	4	9.23E7 ± 8.20E7	3	9.79E7 ± 3.25E7	3	9.27E5 ± 1.56E5	3	2.09E7 ± 8.24E6
Sample 6	3	8.73E7 ± 5.04E7	3	1.01E8 ± 3.95E7	3	2.08E7 ± 3.63E6	3	3.03E7 ± 1.39E7	3	6.19E5 ± 4.94E5	3	1.44E6 ± 7.45E5

Appendix 4.1 continued :-

***Bacteroides* species (on KV agar)**

	Starch type											
	Retrograded maize		Retrograded potato		Native maize		Retrograded waxy maize		Retrograded mixture		Native waxy maize	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	1.26E9 ± 4.63E8	6	1.25E8 ± 2.95E7	3	1.41E8 ± 8.29E7	4	1.37E8 ± 4.63E7	4	3.06E8 ± 1.33E8	4	1.52E8 ± 4.78E7
Sample 1	4	1.12E9 ± 5.27E8	4	2.89E7 ± 7.13E6	4	1.28E8 ± 1.59E7	4	3.06E7 ± 8.99E6	4	1.75E9 ± 6.31E8	3	4.46E8 ± 8.14E7
Sample 2	4	6.88E8 ± 2.97E8	4	4.06E7 ± 1.02E7	4	2.47E8 ± 4.31E7	3	4.30E7 ± 4.10E6	3	3.00E8 ± 1.30E8	3	7.59E7 ± 2.22E7
Sample 3	3	2.33E9 ± 2.68E8	4	9.10E7 ± 3.91E7	3	5.03E9 ± 3.26E9	3	4.84E8 ± 1.15E8	3	1.74E7 ± 6.21E6	3	1.58E9 ± 9.04E8
Sample 4	3	1.13E7 ± 4.09E6	4	3.70E7 ± 1.69E7	3	1.28E9 ± 7.29E8	3	2.10E7 ± 6.88E6	3	1.88E7 ± 8.34E6	3	2.74E8 ± 5.24E7
Sample 5	2	9.21E6 ± 5.50E4	4	4.65E7 ± 1.83E7	4	2.52E8 ± 9.42E7	3	4.67E7 ± 5.09E6	3	4.87E7 ± 2.75E7	3	1.89E8 ± 1.24E8
Sample 6	3	1.97E7 ± 5.27E6	3	3.08E7 ± 5.83E6	3	3.75E7 ± 1.01E7	3	5.21E7 ± 4.70E7	3	1.68E7 ± 9.32E6	3	1.59E7 ± 4.17E6

Data represents the mean ± SEM of multiple determinations; **n** = Number of samples tested; E = 10^X

Appendix 4.2 :
Bacterial counts (cfu g^{-1}) obtained from three sites along the colon of pigs fed on diets containing different starches.

Coliform bacteria (on MacConkey agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	3.37E7 ± 2.69E7	1.55E8 ± 1.32E8	4.45E7 ± 2.68E7	1.42E6 ± 7.40E5	2.52E6 ± 1.14E6	1.27E8 ± 9.74E7
Section B	2.55E7 ± 1.40E7	4.25E7 ± 3.41E7	1.82E7 ± 9.68E6	4.18E8 ± 4.16E8	1.96E6 ± 1.12E6	1.35E9 ± 1.25E9
Section C	1.25E7 ± 1.04E7	9.72E7 ± 6.04E7	3.53E7 ± 7.95E6	2.79E6 ± 1.69E6	3.70E8 ± 3.70E8	8.29E7 ± 5.74E7

***Enterococcus* species (on S&B agar)**

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	1.04E6 ± 5.85E5	3.86E5 ± 7.88E4	2.74E5 ± 1.42E5	1.40E5 ± 3.68E4	2.80E8 ± 2.60E8	1.20E5 ± 5.44E4
Section B	3.17E5 ± 1.58E5	1.31E6 ± 1.13E6	9.15E4 ± 3.96E4	2.11E6 ± 1.86E6	1.18E8 ± 5.95E7	1.56E7 ± 1.53E7
Section C	2.85E6 ± 2.74E6	3.90E5 ± 1.68E5	5.55E5 ± 2.07E5	2.11E5 ± 5.30E4	3.75E7 ± 2.95E7	5.39E4 ± 2.67E4

Aerotolerant *Lactobacillus* species (on MRS agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	1.88E8 ± 6.65E7	1.12E9 ± 4.82E8	2.32E9 ± 1.09E9	3.42E8 ± 1.02E8	3.52E9 ± 3.34E9	3.04E9 ± 2.98E9
Section B	1.52E8 ± 5.08E7	1.22E8 ± 2.20E7	9.95E8 ± 3.58E8	5.64E9 ± 5.08E9	1.72E8 ± 8.71E7	1.82E9 ± 1.78E9
Section C	2.81E8 ± 6.82E7	2.48E9 ± 2.31E9	2.03E8 ± 1.07E8	7.56E8 ± 1.85E8	6.25E7 ± 2.93E7	1.56E8 ± 1.47E8

Appendix 4.2 continued :-

Total *Lactobacillus* species (on MRS agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	9.75E8 ± 5.61E8	4.74E9 ± 3.89E9	7.87E8 ± 6.11E8	5.22E8 ± 1.42E8	2.34E8 ± 1.97E8	1.27E10 ± 1.24E10
Section B	1.47E8 ± 5.48E7	9.33E8 ± 5.89E8	3.83E8 ± 7.12E7	5.42E9 ± 3.62E9	1.27E8 ± 9.46E7	1.31E9 ± 1.08E9
Section C	2.96E8 ± 9.10E7	3.38E9 ± 3.17E9	2.47E8 ± 2.66E7	1.62E9 ± 4.16E8	8.91E7 ± 8.04E7	2.76E8 ± 1.33E8

Total aerobic count (on CBA agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	2.82E8 ± 1.39E8	1.87E9 ± 1.35E9	9.83E8 ± 8.84E8	3.03E9 ± 1.48E9	2.10E8 ± 1.95E8	5.26E8 ± 3.51E8
Section B	1.72E8 ± 1.02E8	3.42E8 ± 1.29E8	1.55E8 ± 6.80E7	2.72E9 ± 2.19E9	8.04E7 ± 5.00E7	8.41E8 ± 7.37E8
Section C	1.26E8 ± 5.27E7	1.74E9 ± 1.49E9	1.41E8 ± 7.86E7	5.29E8 ± 4.46E8	1.02E8 ± 5.91E7	1.22E9 ± 1.20E9

***Escherichia coli* (on CBA+MUG agar)**

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	2.79E7 ± 1.74E7	8.62E8 ± 8.19E8	1.90E8 ± 1.55E8	2.30E7 ± 1.15E7	2.56E6 ± 2.29E6	2.42E7 ± 2.36E7
Section B	3.08E7 ± 1.68E7	1.79E8 ± 1.06E8	1.90E7 ± 1.14E7	3.01E7 ± 2.66E7	2.30E6 ± 1.63E6	2.82E7 ± 2.76E7
Section C	6.35E7 ± 3.34E7	1.49E9 ± 1.34E9	2.54E7 ± 4.35E6	3.03E7 ± 2.66E7	2.96E7 ± 2.69E7	1.86E8 ± 1.85E8

Appendix 4.2 continued :-

Aerobic starch degraders (on starch agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	8.33E6 ± 8.33E6	3.86E2 ± 1.73E1	5.37E2 ± 1.87E1	1.11E5 ± 1.11E5	5.71E2 ± 7.13E1	1.97E5 ± 1.53E5
Section B	4.72E2 ± 2.77E1	4.14E2 ± 4.38E1	4.29E2 ± 1.23E1	6.67E2 ± 1.12E2	6.32E2 ± 4.57E1	1.34E5 ± 1.26E5
Section C	1.57E7 ± 8.82E6	3.36E2 ± 4.18E1	1.67E7 ± 1.67E7	2.78E5 ± 2.77E5	7.41E2 ± 9.23E1	2.42E4 ± 2.36E4

Anaerobic starch degraders (on starch agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	4.25E6 ± 4.14E5	1.19E7 ± 1.19E7	1.82E7 ± 1.59E7	3.11E3 ± 1.69E3	3.00E4 ± 2.67E4	4.93E3 ± 2.21E3
Section B	1.61E7 ± 9.80E6	1.51E6 ± 1.51E6	2.08E7 ± 1.74E7	2.42E3 ± 1.92E3	2.34E4 ± 1.96E4	5.90E2 ± 6.41E1
Section C	5.32E6 ± 6.11E5	1.28E7 ± 1.28E7	3.89E6 ± 1.67E6	2.09E5 ± 2.08E5	2.29E4 ± 1.98E4	1.55E4 ± 1.49E4

Total anaerobic count (on WCBA agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	2.44E9 ± 1.13E9	5.51E9 ± 3.18E9	1.97E9 ± 7.62E8	9.95E8 ± 6.45E8	1.08E8 ± 5.68E7	2.91E8 ± 1.34E8
Section B	2.22E9 ± 1.95E9	2.51E9 ± 1.25E9	3.46E9 ± 2.34E9	5.41E8 ± 2.50E8	6.37E7 ± 1.51E7	1.25E8 ± 6.26E7
Section C	6.51E9 ± 6.14E9	6.71E9 ± 5.00E9	2.56E9 ± 1.19E9	1.16E9 ± 6.75E8	3.93E7 ± 9.34E6	1.47E10 ± 1.45E10

Appendix 4.2 continued :-

***Clostridium* species (on RCA agar)**

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	8.60E7 ± 5.52E7	1.90E9 ± 9.55E8	1.13E9 ± 9.37E8	1.16E8 ± 2.80E7	1.62E7 ± 1.14E7	2.62E8 ± 1.60E8
Section B	1.18E8 ± 5.71E7	7.74E8 ± 4.88E8	4.72E9 ± 4.59E9	6.28E7 ± 1.67E7	2.12E7 ± 1.80E7	8.04E7 ± 4.74E7
Section C	1.30E9 ± 1.22E9	1.05E9 ± 4.23E8	1.04E9 ± 7.32E8	2.02E8 ± 1.09E8	2.13E7 ± 1.97E7	8.53E9 ± 8.39E9

Gram-negative anaerobes (on G-N agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	1.28E8 ± 7.37E7	1.21E9 ± 5.43E8	8.75E8 ± 5.63E8	3.36E8 ± 1.29E8	1.17E7 ± 4.18E6	2.13E7 ± 3.65E6
Section B	8.14E8 ± 7.03E8	1.25E9 ± 8.79E8	2.73E8 ± 4.28E7	1.58E8 ± 8.75E7	4.31E7 ± 3.39E7	2.31E7 ± 9.19E6
Section C	6.85E8 ± 5.97E8	5.39E8 ± 1.56E8	1.57E8 ± 6.08E7	3.96E8 ± 1.26E8	7.57E6 ± 1.65E6	1.07E8 ± 8.73E7

Non-sporing anaerobes (on NS agar)

	Starch type					
	Retrograded maize	Retrograded potato	Native maize	Retrograded waxy maize	Retrograded mixture	Native waxy maize
Colon samples:						
Section A	1.30E9 ± 8.13E8	4.37E9 ± 1.69E9	1.11E9 ± 7.01E8	4.59E8 ± 1.07E8	3.13E7 ± 1.77E7	1.23E8 ± 5.16E7
Section B	1.26E9 ± 9.03E8	1.80E9 ± 4.77E8	7.85E8 ± 3.89E8	2.59E8 ± 6.58E7	4.41E7 ± 3.14E7	6.44E7 ± 8.92E6
Section C	1.95E9 ± 1.68E9	1.59E10 ± 1.07E10	1.40E9 ± 1.25E9	4.83E8 ± 5.39E7	2.66E7 ± 1.30E7	2.23E8 ± 1.70E8

Appendix 4.2 continued :-

***Bifidobacterium* species (on BIF agar)**

	Starch type					
	Retrograded maize	**Retrograded potato**	**Native maize**	**Retrograded waxy maize**	**Retrograded mixture**	**Native waxy maize**
Colon samples:						
Section A	2.39E8 ± 1.06E8	1.67E9 ± 9.46E8	1.04E9 ± 5.50E8	4.86E8 ± 1.86E8	4.47E7 ± 2.65E7	2.07E8 ± 8.32E7
Section B	1.65E8 ± 1.12E8	4.53E8 ± 6.38E7	1.97E8 ± 6.76E7	2.68E8 ± 5.48E7	5.92E7 ± 3.45E7	7.48E7 ± 3.21E7
Section C	2.97E8 ± 2.14E8	1.84E9 ± 1.67E9	9.61E8 ± 9.10E8	2.12E9 ± 1.75E9	2.01E7 ± 1.31E7	1.19E8 ± 7.88E7

Counts on Rifampicin (RIF) agar

	Starch type					
	Retrograded maize	**Retrograded potato**	**Native maize**	**Retrograded waxy maize**	**Retrograded mixture**	**Native waxy maize**
Colon samples:						
Section A	3.43E7 ± 9.95E6	1.11E9 ± 7.27E8	6.76E7 ± 5.38E7	6.04E7 ± 2.88E7	4.32E6 ± 4.09E6	1.07E7 ± 8.47E6
Section B	6.44E7 ± 5.13E7	1.61E8 ± 7.81E7	2.31E7 ± 6.54E6	8.28E7 ± 4.06E7	1.57E6 ± 1.02E6	2.01E6 ± 6.37E5
Section C	1.63E8 ± 1.38E8	9.35E8 ± 7.86E8	1.48E7 ± 9.33E5	7.79E7 ± 4.46E7	1.60E6 ± 1.34E6	2.19E6 ± 7.37E5

***Bacteroides* species (on KV agar)**

	Starch type					
	Retrograded maize	**Retrograded potato**	**Native maize**	**Retrograded waxy maize**	**Retrograded mixture**	**Native waxy maize**
Colon samples:						
Section A	9.43E7 ± 7.79E7	3.61E8 ± 1.91E8	1.63E8 ± 1.18E8	6.99E6 ± 1.80E6	6.67E6 ± 1.91E6	5.05E6 ± 1.02E6
Section B	3.28E7 ± 3.30E6	1.03E8 ± 1.47E7	4.42E7 ± 3.63E6	1.74E7 ± 6.23E6	4.34E6 ± 1.47E6	5.19E6 ± 9.77E5
Section C	1.30E8 ± 1.07E8	6.52E7 ± 4.24E7	4.91E7 ± 2.41E7	1.65E7 ± 5.34E6	5.81E6 ± 5.98E5	7.72E6 ± 3.66E6

Data represents the mean ± SEM of triplicate determinations. E = 10^x

Appendix 4.3 :-
SCFA levels (mM) detected in both faecal and colon samples from pigs fed on diets containing different starches.

Faecal samples- Baseline		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	ND	1.80 ± 0	41.90 ± 0	7.60 ± 0	6.20 ± 0	2.60 ± 0	3.50 ± 0	1.60 ± 0
Retrograded potato	6	1.78 ± 0.35	7.07 ± 2.62	84.10 ± 18.10	28.97 ± 7.07	9.28 ± 1.90	20.15 ± 7.56	13.15 ± 3.54	1.92 ± 0.65
Native maize	4	1.85 ± 0.88	4.30 ± 1.46	88.70 ± 25.00	24.23 ± 5.37	7.78 ± 1.12	11.07 ± 2.09	9.10 ± 2.49	1.20 ± 0.24
Retrograded waxy maize	4	0.12 ± 0.09	1.32 ± 0.17	40.20 ± 2.84	10.32 ± 1.15	1.20 ± 0.71	5.15 ± 0.65	4.35 ± 0.90	2.92 ± 1.24
Retrograded mixture	4	0.02 ± 0.02	0.38 ± 0.38	18.38 ± 5.78	6.15 ± 1.84	1.55 ± 0.51	1.60 ± 1.29	3.58 ± 0.97	0.28 ± 0.24
Native waxy maize	4	0.22 ± 0.05	0.68 ± 0.18	20.65 ± 3.78	5.75 ± 1.43	1.52 ± 0.45	0.02 ± 0.02	2.75 ± 1.05	ND

Faecal samples- Sample 1		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	4	0.85 ± 0.30	0.38 ± 0.34	29.58 ± 5.71	9.45 ± 2.93	5.77 ± 1.25	4.05 ± 2.72	6.82 ± 1.74	0.62 ± 0.39
Retrograded potato	4	0.52 ± 0.22	1.38 ± 0.38	50.00 ± 18.60	14.20 ± 4.74	4.40 ± 1.51	10.50 ± 4.83	8.02 ± 2.07	3.83 ± 1.33
Native maize	4	0.62 ± 0.24	1.30 ± 0.52	32.78 ± 5.86	9.45 ± 1.84	2.90 ± 0.50	3.80 ± 1.40	4.55 ± 0.95	1.30 ± 0.50
Retrograded waxy maize	4	0.35 ± 0.13	1.35 ± 0.48	22.62 ± 3.09	15.10 ± 9.99	5.22 ± 4.69	4.85 ± 4.19	2.75 ± 1.75	0.75 ± 0.65
Retrograded mixture	4	0.60 ± 0.21	1.20 ± 0.27	28.95 ± 4.18	5.92 ± 0.52	1.60 ± 0.73	3.22 ± 1.50	3.92 ± 1.27	1.32 ± 1.13
Native waxy maize	3	0.97 ± 0.32	3.17 ± 0.49	40.37 ± 9.05	13.27 ± 5.17	1.73 ± 0.37	2.23 ± 1.85	7.07 ± 2.77	8.87 ± 5.63

Faecal samples- Sample 2		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	4	0.28 ± 0.16	0.68 ± 0.35	25.35 ± 2.35	5.75 ± 0.68	2.58 ± 0.25	4.75 ± 1.20	5.25 ± 0.96	2.15 ± 0.25
Retrograded potato	4	0.92 ± 0.29	1.50 ± 0.38	40.58 ± 8.03	12.73 ± 2.36	3.42 ± 0.88	8.80 ± 1.24	8.45 ± 1.34	3.45 ± 0.67
Native maize	4	0.60 ± 0.40	0.85 ± 0.22	28.92 ± 4.64	8.07 ± 1.78	4.35 ± 1.29	2.52 ± 0.91	6.55 ± 1.16	1.50 ± 0.82
Retrograded waxy maize	3	0.77 ± 0.32	1.87 ± 1.13	45.50 ± 27.30	13.47 ± 7.84	2.83 ± 1.68	10.27 ± 6.72	8.07 ± 4.25	4.63 ± 3.05
Retrograded mixture	3	0.13 ± 0.09	1.00 ± 0.32	38.40 ± 11.00	8.23 ± 1.55	1.73 ± 0.43	7.67 ± 3.05	4.30 ± 1.22	2.30 ± 0.15
Native waxy maize	3	1.43 ± 0.70	7.70 ± 6.75	49.00 ± 18.60	11.63 ± 4.56	3.00 ± 1.32	9.87 ± 4.75	6.57 ± 3.00	1.63 ± 1.21

Appendix 4.3 continued :-

Faecal samples- Sample 3		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	0.67 ± 0.24	1.87 ± 1.27	47.80 ± 17.70	13.00 ± 5.95	3.13 ± 0.87	9.33 ± 3.54	6.30 ± 1.72	4.23 ± 1.15
Retrograded potato	4	0.12 ± 0.05	0.15 ± 0.06	21.65 ± 3.01	7.75 ± 0.88	1.38 ± 0.44	5.15 ± 0.85	3.78 ± 1.05	3.08 ± 0.63
Native maize	3	0.10 ± 0.06	0.13 ± 0.03	18.47 ± 3.14	4.40 ± 0.58	1.23 ± 0.38	1.80 ± 0.36	3.70 ± 0.96	0.60 ± 0.60
Retrograded waxy maize	3	0.27 ± 0.09	1.13 ± 0.24	33.90 ± 3.61	9.87 ± 1.88	2.83 ± 0.09	3.50 ± 0.26	5.10 ± 1.10	2.40 ± 0.57
Retrograded mixture	3	0.93 ± 0.43	1.13 ± 0.55	31.43 ± 5.00	10.07 ± 2.85	4.00 ± 1.86	5.07 ± 3.02	6.87 ± 3.05	0.47 ± 0.47
Native waxy maize	3	0.27 ± 0.14	0.83 ± 0.43	24.37 ± 5.51	3.97 ± 1.13	0.97 ± 0.65	2.57 ± 1.92	4.30 ± 1.51	1.33 ± 0.32

Faecal samples- Sample 4		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	0.17 ± 0.09	0.93 ± 0.41	28.47 ± 3.41	8.10 ± 1.17	2.13 ± 0.24	3.60 ± 0.67	1.87 ± 1.33	0.07 ± 0.03
Retrograded potato	4	0.68 ± 0.28	1.02 ± 0.32	43.70 ± 18.50	16.67 ± 6.86	3.03 ± 1.33	10.38 ± 5.53	6.47 ± 2.35	1.85 ± 1.66
Native maize	3	0.27 ± 0.27	0.17 ± 0.12	34.10 ± 14.30	7.40 ± 3.50	1.53 ± 0.44	5.33 ± 3.68	5.30 ± 0.98	0.27 ±0.27
Retrograded waxy maize	3	ND	0.30 ± 0.06	18.23 ± 2.96	7.37 ± 3.37	1.00 ± 0.53	2.60 ± 1.44	3.87 ± 1.52	2.17 ± 0.75
Retrograded mixture	3	ND	0.27 ± 0.07	20.63 ± 0.19	4.43 ± 0.96	0.67 ± 0.12	2.17 ± 0.77	4.17 ± 0.42	1.00 ± 0.35
Native waxy maize	3	0.53 ± 0.17	0.47 ± 0.20	32.87 ± 6.46	5.37 ± 1.66	1.93 ± 0.93	4.23 ± 2.23	4.93 ± 1.54	3.47 ± 1.88

Faecal samples- Sample 5		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	2	0.25 ± 0.05	1.00 ± 0.20	23.05 ± 0.85	5.55 ± 0.35	0.60 ± 0.50	3.65 ± 1.15	2.10 ± 0.60	0.75 ± 0.55
Retrograded potato	4	ND	0.30 ± 0.27	28.58 ± 6.58	12.13 ± 1.77	2.00 ± 1.03	5.18 ± 2.05	2.85 ± 0.67	2.10 ± 1.27
Native maize	4	ND	ND	27.23 ± 9.68	4.20 ± 0.57	0.88 ± 0.30	3.87 ± 1.65	2.08 ± 0.91	2.55 ± 1.23
Retrograded waxy maize	3	0.40 ± 0.20	0.73 ± 0.07	29.33 ± 6.27	6.53 ± 1.07	0.60 ± 0.30	1.50 ± 0.78	3.10 ± 1.70	1.60 ± 0.35
Retrograded mixture	3	0.30 ± 0.15	0.23 ± 0.19	45.37 ± 0.77	10.57 ± 1.48	1.97 ± 0.64	4.03 ± 1.23	5.40 ± 0.35	3.70 ± 0.32
Native waxy maize	3	0.17 ± 0.09	0.10 ± 0.06	30.33 ± 4.94	5.03 ± 3.21	3.13 ± 1.73	2.27 ± 1.00	5.53 ± 1.00	1.77 ± 0.89

Appendix 4.3 continued :-

Faecal samples- Sample 6		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	0.20 ± 0.12	0.33 ± 0.09	41.37 ± 8.18	14.73 ± 1.68	3.13 ± 0.48	15.33 ± 4.54	6.40 ± 1.01	1.13 ± 0.81
Retrograded potato	3	ND	ND	51.50 ± 12.80	21.33 ± 2.11	4.37 ± 0.41	14.83 ± 2.31	7.77 ± 1.87	2.47 ± 2.37
Native maize	3	0.07 ± 0.07	0.10 ± 0.06	54.00 ± 12.60	15.70 ± 3.10	3.03 ± 0.94	11.83 ± 1.46	10.87 ± 1.82	2.77 ± 1.11
Retrograded waxy maize	3	0.30 ± 0	0.17 ± 0.17	41.73 ± 3.77	13.53 ± 1.53	3.60 ± 0.40	5.40 ± 0.61	6.07 ± 0.20	4.13 ± 0.71
Retrograded mixture	3	0.40 ± 0.21	1.00 ± 0.65	62.17 ± 4.02	21.80 ± 7.42	2.93 ± 0.95	6.23 ± 1.05	6.00 ± 0.47	1.13 ± 1.08
Native waxy maize	3	0.50 ± 0.25	0.10 ± 0.10	50.20 ± 10.00	11.00 ± 3.64	6.27 ± 1.96	7.40 ± 6.20	12.67 ± 5.42	3.50 ± 3.40

Colon samples- Section A		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	1.20 ± 0.46	2.30 ± 0.74	76.10 ± 19.60	24.30 ± 6.91	3.90 ± 0.60	14.07 ± 2.75	3.73 ± 0.38	5.43 ± 1.37
Retrograded potato	3	0.83 ± 0.09	0.90 ± 0.61	78.90 ± 14.50	25.10 ± 5.64	3.83 ± 1.32	16.00 ± 2.15	3.63 ± 0.66	4.30 ± 2.11
Native maize	3	1.63 ± 0.27	1.17 ± 0.49	69.70 ± 15.10	22.10 ± 6.86	6.57 ± 0.48	8.87 ± 3.54	4.47 ± 2.27	4.60 ± 1.14
Retrograded waxy maize	3	1.33 ± 0.32	19.10 ± 17.30	77.87 ± 4.49	29.40 ± 7.05	10.27 ± 6.73	22.33 ± 9.46	15.40 ± 12.20	7.20 ± 6.23
Retrograded mixture	3	2.03 ± 0.64	1.00 ± 0.23	93.60 ± 20.00	17.57 ± 3.44	3.50 ± 1.25	12.07 ± 2.47	3.30 ± 1.72	0.10 ± 0.06
Native waxy maize	3	2.40 ± 0.68	1.57 ± 0.41	85.90 ± 14.50	24.47 ± 4.08	6.97 ± 1.89	12.30 ± 0.66	5.07 ± 1.62	8.00 ± 1.66

Colon samples- Section B		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	0.53 ± 0.07	1.23 ± 0.48	46.40 ± 9.63	14.23 ± 4.10	2.97 ± 1.12	9.23 ± 2.19	3.27 ± 1.57	4.33 ± 2..39
Retrograded potato	3	0.93 ± 0.09	1.33 ± 0.32	95.10 ± 26.70	31.60 ± 5.79	7.77 ± 2.07	21.70 ± 5.17	5.10 ± 1.07	4.83 ± 3.02
Native maize	3	1.13 ± 0.30	1.60 ± 0.74	66.93 ± 7.15	21.63 ± 2.37	4.90 ± 0.38	9.67 ± 1.97	4.70 ± 1.11	1.20 ± 1.20
Retrograded waxy maize	3	0.90 ± 0.25	1.10 ± 0.70	65.90 ± 11.90	18.37 ± 3.44	3.30 ± 0.26	9.30 ± 1.98	1.70 ± 0.20	1.53 ± 1.07
Retrograded mixture	3	2.00 ± 0.75	1.17 ± 0.50	61.17 ± 9.78	14.13 ± 2.36	3.27 ± 0.85	7.67 ± 2.11	2.67 ± 1.42	0.03 ± 0.03
Native waxy maize	3	2.43 ± 1.04	1.53 ± 0.66	80.30 ± 21.40	24.43 ± 6.33	9.63 ± 4.04	12.13 ± 3.84	6.67 ± 3.59	6.60 ± 2.06

Appendix 4.3 continued :-

Colon samples- Section C		Short-Chain Fatty Acids (mM)							
Starch type	**n**	**Succinate**	**Lactate**	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**
Retrograded maize	3	1.03 ± 0.09	1.80 ± 0.20	51.07 ± 4.52	15.93 ± 2.43	5.27 ± 0.81	10.93 ± 1.59	4.87 ± 0.95	5.00 ± 2.15
Retrograded potato	3	0.67 ± 0.12	1.37 ± 0.09	48.70 ± 2.39	15.60 ± 2.22	4.10 ± 0.93	11.90 ± 1.27	3.23 ± 0.66	1.97 ± 0.99
Native maize	3	0.87 ± 0.44	1.60 ± 0.59	34.73 ± 0.97	10.23 ± 2.22	4.33 ± 1.89	5.63 ± 0.90	4.47 ± 1.39	1.00 ± 0.46
Retrograded waxy maize	3	1.07 ± 0.20	1.33 ± 0.09	70.00 ± 2.25	18.73 ± 3.12	4.53 ± 1.27	10.93 ± 1.96	2.07 ± 1.33	2.00 ± 1.90
Retrograded mixture	3	1.60 ± 1.04	1.17 ± 0.57	76.80 ± 27.40	22.83 ± 2.60	7.37 ± 4.48	9.43 ± 3.11	2.47 ± 1.94	2.60 ± 1.30
Native waxy maize	3	1.73 ± 1.14	1.03 ± 0.93	49.20 ± 12.60	17.43 ± 2.79	5.50 ± 1.94	7.67 ± 2.91	3.70 ± 2.59	4.97 ± 3.41

Data represent the mean ± SEM of multiple determinations. **n** = Number of samples tested; ND = Not detected

Appendix 4.4 :-

α-amylase levels (U/L) detected in both faecal and colon samples from pigs fed on diets containing different starches.

	Starch type											
Samples	**Retrograded maize**		**Retrograded potato**		**Native maize**		**Retrograded waxy maize**		**Retrograded mixture**		**Native waxy maize**	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	157.62 ± 0	6	461.00 ± 160.00	3	163.40 ± 42.10	4	74.40 ± 23.00	4	127.00 ± 74.80	4	157.60 ± 65.10
Sample 1	4	346.00 ± 112.00	4	52.50 ± 41.70	4	35.00± 21.40	4	127.00 ± 110.00	4	26.30 ± 11.30	3	187.00 ± 126.00
Sample 2	4	61.30 ± 41.40	4	52.50 ± 31.20	4	223.00 ± 137.00	3	17.50 ± 17.50	3	110.90 ± 51.90	3	549.00 ± 203.00
Sample 3	3	87.60 ± 87.60	4	4.38 ± 4.38	3	35.00 ± 10.10	3	105.10 ± 35.00	3	40.90 ± 11.70	3	245.00 ± 245.00
Sample 4	3	99.20 ± 82.30	4	280.00 ± 245.00	3	35.00 ± 20.20	3	64.20 ± 21.00	3	263.00 ± 263.00	3	35.00 ± 10.10
Sample 5	2	35.00 ± 17.50	4	43.80 ± 11.30	4	30.60 ± 13.10	3	344.00 ± 240.00	3	40.90 ± 32.50	3	105.10 ± 96.50
Sample 6	3	157.60 ± 53.50	3	211.00 ± 166.00	3	64.20 ± 55.70	3	93.40 ± 30.90	3	175.10 ± 89.90	3	64.20 ± 30.90
Colon samples:												
Section A	3	269.00 ± 143.00	3	560.40 ± 96.50	3	601.00 ± 296.00	3	116.70 ± 49.90	3	455.00 ± 184.00	3	110.90 ± 67.30
Section B	3	414.00 ± 178.00	3	473.00 ± 168.00	3	157.60 ± 40.40	3	99.20 ± 47.80	3	257.00 ± 137.00	3	145.90 ± 60.90
Section C	3	648.00 ± 194.00	3	239.30 ± 58.40	3	87.60 ± 61.50	3	75.90 ± 23.30	3	216.00 ± 25.40	3	52.50 ± 10.10

Data represent the mean ± SEM of multiple determinations. **n** = Number of samples tested

Appendix 4.5 :-
Weights of animals (Kg) at certain intervals of the starch feeding trial.

	Time of animal weighing							
	Start weight (Kg)		End of week 1 (Kg)		End of week 2 (Kg)		End of week 3 (Kg)	
Starch type :-	n		n		n		n	
Retrograded maize	6	8.59 ± 0.44	6	9.26 ± 0.57	5	11.93 ± 0.79	5	15.85 ± 1.12
Retrograded potato	6	10.76 ± 0.46	6	12.31 ± 0.61	6	15.09 ± 0.89	6	20.28 ± 1.19
Native maize	6	11.16 ± 0.21	5	11.78 ± 0.54	5	14.15 ± 0.71	5	17.98 ± 0.89
Retrograded waxy maize	6	8.60 ± 0.50	6	9.90 ± 0.54	6	12.88 ± 0.70	6	17.02 ± 0.80
Retrograded mixture	6	9.33 ± 0.45	6	10.49 ± 0.36	6	13.14 ± 0.50	6	17.46 ± 0.87
Native waxy maize	6	10.04 ± 0.76	6	11.16 ± 0.78	6	13.51 ± 1.01	6	17.10 ± 1.38

n = Number of samples tested. Two of the diets contain only 5 samples as it was necessary to remove two animals due to diet unrelated poor growth.

Appendix 4.6 :-
Animal feed consumption (Kg) per week of diets containing different starches.

	Time interval		
Starch type :-	Week 1 (Kg)	Week 2 (Kg)	Week 3 (Kg)
Retrograded maize	1.29 ± 0.14	2.51 ± 0.15	3.74 ± 0.24
Retrograded potato	2.24 ± 0.15	4.21 ± 0.28	5.53 ± 0.18
Native maize	1.17 ± 0.11	2.38 ± 0.14	2.89 ± 0.08
Retrograded waxy maize	2.12 ± 0.11	3.47 ± 0.18	5.46 ± 0.23
Retrograded mixture	1.54 ± 0.14	2.87 ± 0.26	4.64 ± 0.20
Native waxy maize	1.19 ± 0.22	2.89 ± 0.25	4.61 ± 0.10

Data represents the mean ± SEM of seven daily feed consumption values.
Feed consumption was taken as the amount of feed added to each pen minus the amount refused, on a daily basis.

Appendix 4.7 :-
Lactobacillus : coliform ratio from colon samples obtained from pigs fed on diets containing different starches.

		Colon section		
Starch type :-	**n**	**Proximal colon**	**Mid colon**	**Distal colon**
Retrograded maize	3	87.4 ± 68.7	17.3 ± 12.2	126.6 ± 95.0
Retrograded potato	3	297.0 ± 268.0	677.0 ± 615.0	162.0 ± 137.0
Native maize	3	12.6 ± 4.5	37.8 ± 20.2	7.9 ± 2.1
Retrograded waxy maize	3	1809.0 ± 1587.0	2141.0 ± 1627.0	1820.0 ± 1182.0
Retrograded mixture	3	840.0 ± 830.0	203.0 ± 193.0	121.0 ± 116.0
Native waxy maize	3	297.0 ± 293.0	2.2 ± 0.7	4.8 ± 1.6

Data represents mean ± SEM of triplicate determinations.

Appendix 4.8 :-
Skatole measurements determined from colon material taken from pigs fed on diets containing different starches.

		Colon section		
Starch type :-	**n**	**Proximal colon**	**Mid colon**	**Distal colon**
Retrograded maize	3	50.0 ± 25.2	43.3 ± 18.6	40.0 ± 5.8
Retrograded potato	3	36.7 ± 8.8	50.0 ± 23.1	33.3 ± 6.7
Native maize	3	42.3 ± 18.6	39.0 ± 16.3	96.7 ± 53.6
Retrograded waxy maize	3	26.7 ± 8.8	40.0 ± 10.0	43.3 ± 14.5
Retrograded mixture	3	26.0 ± 9.4	18.7 ± 10.7	26.0 ± 12.5
Native waxy maize	3	80.0 ± 61.1	83.3 ± 60.1	83.3 ± 60.1

Data represents mean ± SEM of triplicate determinations, n = number of samples.

Appendix 4.9 :-

Measurement of starch detected (%) in samples from both faecal samples and colon samples from animals fed a diet containing one of six different starches

	Starch type											
Samples	**Retrograded maize**		**Retrograded potato**		**Native maize**		**Retrograded waxy maize**		**Retrograded mixture**		**Native waxy maize**	
Faecal samples:	**n**		**n**		**n**		**n**		**n**		**n**	
Baseline	3	0.24 ± 0	6	0.16 ± 0.02	3	0.19 ± 0.03	4	0.12 ± 0.02	4	0.10 ± 0	4	0.12 ± 0.02
Sample 1	4	0.11 ± 0	4	0.16 ± 0.02	4	0.14 ± 0.03	4	0.10 ± 0	4	0.16 ± 0.06	3	0.10 ± 0
Sample 2	4	0.16 ± 0.03	4	0.13 ± 0.01	4	0.10 ± 0	3	0.10 ± 0	3	0.10 ± 0	3	0.07 ± 0.07
Sample 3	3	0.23 ± 0.07	4	0.10 ± 0	3	0.14 ± 0.02	3	0.10 ± 0	3	0.10 ± 0	3	0.03 ± 0.03
Sample 4	3	0.16 ± 0.04	4	0.12 ± 0.01	3	0.11 ± 0.01	3	0.10 ± 0	3	0.10 ± 0	3	0.03 ± 0.03
Sample 5	2	0.14 ± 0.02	4	0.10 ± 0	4	0.10 ± 0	3	0.10 ± 0	3	0.07 ± 0.03	3	0 ± 0
Sample 6	3	0.11 ± 0.01	3	0.14 ± 0.09	3	0.03 ± 0.03	3	0.07 ± 0.03	3	0.03 ± 0.03	3	0.03 ± 0.03
Colon samples:												
Section A	3	0.41 ± 0.20	3	0.97 ± 0.47	3	0.11 ± 0.07	3	0.12 ± 0.02	3	0.38 ± 0.23	3	0.12 ± 0.07
Section B	3	0.30 ± 0.11	3	0.35 ± 0.14	3	0.11 ± 0.01	3	0.13 ± 0.02	3	0.37 ± 0.20	3	0.07 ± 0.03
Section C	3	0.14 ± 0.04	3	0.15 ± 0.11	3	0.13 ± 0.03	3	0.15 ± 0.02	3	0.13 ± 0.08	3	0.03 ± 0.03

Data represent mean ± SEM of multiple determinations. **n** = number of samples tested.

Publication record

Part of this work has been published:-

Refereed papers

Khaddour, R., Reid, C.-A. and Hillman, K. (1998) Maintenance *in vitro* of the microflora and fermentation patterns of the porcine intestine. *Pig News and Information* **19**, 111N-114N.

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Offered papers

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