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FERMENTATION CHARACTERISTICS OF SELECTED DIETARY SUBSTRATES: A HUMAN *IN VITRO* STUDY

By

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ABSTRACT

FERMENTATION CHARACTERISTICS OF SELECTED DIETARY SUBSTRATES: A HUMAN *IN VITRO* STUDY

By

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The objective of this study was to use batch fermentation methodology to assess the fermentability of several dietary substrates as measured by short chain fatty acid production, organic and dry matter disappearance, and water holding and potential water holding capacities. Specifically, molecular weight was examined as it relates to the potential fermentability of β-glucan. The substrates used were arabinogalactan, high viscosity β-glucan, medium viscosity β-glucan, low viscosity β-glucan, a mixture of inulin and fructooligosaccharide, a mixed fiber source, oat flour, oat groats, and Solka-Floc®. The production of total short chain fatty acids by fermentation with human fecal inocula ranged from 0.92 mmol per gram original organic matter (Solka-Floc®) to 5.30 mmol per gram original organic matter (high viscosity β-glucan). Substrate organic matter disappearance ranged from –1.79% (Solka-Floc®) to 83.84% (mixed fiber) and was positively correlated with short chain fatty acid production ($r = 0.77$, $p = 0.015$). Water holding capacity ranged from 3.79 grams water per gram fermented residue dry matter (oat groats) to 6.76 grams water per gram fermented residue dry matter (inulin/FOS). Potential water holding capacity was inversely correlated with organic matter disappearance ($r = -0.91$, $p = 0.001$). This data is suggestive of a relationship between increasing molecular weight and the fermentability of β-glucan, however further research is necessary to see if the existence of the effect can be confirmed.

I dedicate this thesis to my family: Mom, Dad, Kelly, and Alex.

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LIST OF ABBREVIATIONS

DMD ..... Dry Matter Disappearance

g ..... Gram

Mmol ..... Millimoles

OMD ..... Organic Matter Disappearance

PWHC ..... Potential Water Holding Capacity

SCFA ..... Short Chain Fatty Acid

WHC ..... Water Holding Capacity

INTRODUCTION

The process of digestion and utilization of nutrients in humans is complex and multifaceted. Certain fibers of plant origin escape normal digestion, as humans lack the necessary enzymes for their hydrolysis. These fibers enter the large bowel relatively unaltered and serve as substrates for fermentation by colonic microflora. The primary end products of this fermentation are short chain fatty acids (acetate, propionate, and butyrate), gases (CO_2, H_2, and CH_4), and microbial cells.

The objective of this research was to use *in vitro* batch fermentation methods to predict the organic matter disappearance (OMD), short chain fatty acid (SCFA) production, water holding capacity (WHC) and potential water holding capacity (PWHC) of fiber products potentially of interest for health promotion and disease prevention. The primary hypothesis to be tested in this study was that differences in the molecular weights of β-glucan-containing substrates do not affect the extent of fermentation as measured by the above indices. Other substrates were included in this study due to the funding corporation's proprietary interest in incorporating these substrates into processed cereal products.

Chapter 1

LITERATURE REVIEW

The wall of the digestive tract consists of four layers. From the innermost layer outward, they are the mucosa, submucosa, muscularis externa, and the serosa. The mucosa itself consists of three layers, the epithelium, the lamina propria, and the muscularis mucosa. The epithelial layer within the large bowel consists of crypts, which are approximately fifty cells deep and are the location of cellular growth (Potter, 1999). Proliferation of cells within the colon is very rapid, as they are replaced every four to eight days (Tappenden et al., 1998). Colonocytes begin their cell division in the lower 60% of the colonic crypts and, once mature, move towards the surface and become highly polarized. The health and proliferation of colonic cells requires a fuel source, of which glucose, short chain fatty acids and glutamine may serve. The brush borders of these colonocytes secrete enzymes, including hydrolases. The epithelium of the proximal colon absorbs chloride, sodium, and water and secretes potassium and bicarbonate (Scheppach, 1994).

The human colon contains about 222 grams of fecal matter distributed along the length of the bowel and is highly populated with bacterial flora (Cummings, 1997). It is estimated that 95% of the total cell count in the body consists of colonic bacteria, which is equivalent to 10^{12} bacterial cells per gram of dry feces. The species colonization is location-dependent within the intestine. The upper small bowel is dominated with facultative anaerobes and aerobes such as streptococci, staphylococci, and lactobacilli (Gibson et al., 1996). The bacterial population increases as the large bowel is reached, as

the pH is lower and transit time becomes longer (El Oufir et al, 1996). The predominant types of bacteria colonizing the lower gut include bacteroides, bifidobacteria, eubacteria, clostridia, lactobacilli, Gram-positive anaerobic cocci, coliforms, methanogens and dissimilatory sulphate-reducing bacteria (Gibson, 1998).

In general, intestinal bacteria do not contain mitochondria and do not use oxygen as a terminal electron acceptor for respiratory processes (Cummings, 1997). Therefore, the population of colonic microflora is maintained by anaerobic metabolism or the provision of an exogenous source of bacteria.

A probiotic is defined as a live microbial dietary supplement that transiently increases large bowel colonization of the selected bacteria. Bifidobacteria, lactic acid bacteria and saccharomyces are currently the most commonly supplemented. These bacteria are non-pathogenic, retain viability during storage, and survive passage into the large intestine of humans (Macfarlane and Cummings, 1999).

Prebiotics are non-digestible oligosaccharides that selectively stimulate growth and/or activity of existing colonic microflora (Macfarlane and Cummings, 1999). Supplemented prebiotics are meant to stimulate the growth of beneficial bacteria, which conversely, can competitively inhibit the growth of pathogenic bacteria. Prebiotic fibers are also utilized as a nutrition source by the bacteria in a process called fermentation, which results in the production of short chain fatty acids. Several food manufacturing companies are currently supplementing their products with prebiotics, such as fructooligosaccharide (FOS) and inulin.

The chief substrates for fermentation are non-digested dietary carbohydrates, with amino acids and endogenously produced carbohydrates and glycoproteins contributing to

a lesser degree. The rate of mucin production, an endogenous carbohydrate, appears to vary with the amount of fiber present in the diet, therefore the amount of host polysaccharide available for fermentation is dependent on diet (Salyers, 1990). Only mono- and oligosaccharides can enter the cytoplasm of a bacterial cell for fermentation. Therefore, the presence of an enzyme to break 1,4-β glucose or xylose linkages is required for the breakdown and subsequent fermentation of plant cell wall polysaccharides, such as cellulose or xylan (Soergel, 1982). Therefore, a synergy among bacteria may exist in the colon, as one bacterial strain may split a carbohydrate to small units or sugars and other species may then ferment the products to short chain fatty acids (Edwards, 1997).

The extent of carbohydrate fermentation, as well as the concentration and type of products produced is dependent on several factors. The synthesis and ratios of short chain fatty acids vary depending on the arrival rate and characteristics of the substrate, the transit time of the fecal matter, as well as the composition and total mass of the microflora present in the gut (Bourquin et al., 1993; El Oufir et al., 1996; McBurney, 1989; Soergel, 1982). Substrates vary in their degree of digestibility in many ways. High concentrations of lignin present in the cell-wall material can reduce potential digestibility. In addition, because the breakdown of fiber depends on the accessibility of the polymer to colonic microflora, an increasing degree of water solubility should increase colonic fermentation. It has been postulated that the types of sugars, the degree of cross linkages and the presence of side chains a substrate contains influences the proportion and total production of short chain fatty acids. For example, those substrates that contain rhamnose, arabinose, xylose, ribose, galacturonic and glucuronic acids tend to promote

the formation of propionate. Butyrate is formed in higher proportions from the fermentation of substrates containing sorbitol, galacturonic and glucuronic acids, and ribose to a lesser extent (Mortenson et al., 1988). Other factors affecting substrate digestibility include the amount of intake and the effects of food processing on chemical structure or availability (Cummings, 1982).

The major end products of bacterial fermentation of carbohydrate are organic acids, hydrogen and CO_2 (Cummings, 1997). The fermentation of proteins contributes additional short chain fatty acids, as well as branched chain fatty acids. Intermediates may be used as substrates for further fermentation: formic acid to CO_2 and H_2; succinate to propionate and H_2O; lactic acid to acetate, butyrate, propionate, and H_2; and methanogenic bacteria reduce H_2 to CO_2 and methane (Soergel, 1982; Gibson et al, 1996). Many of these compounds supply energy and have the ability to alter gut morphology or produce systemic effects.

Colonic fermentation can be studied in several ways. *In vivo* measurements of fermentation products have significant limitations. It is difficult to obtain human colonic contents, and those studies that have reported results in this fashion have utilized sudden death victims (Cummings et al., 1987). In addition, because short chain fatty acids are absorbed from the intestine and are metabolized by the liver and colonic epithelial cells, a peripheral blood measurement of short chain fatty acid concentrations would not be indicative of production. For the same reason, fecal concentrations of short chain fatty acids are also not an effective measurement of production.

Standardized *in vitro* batch fermentations have been widely used to simulate colonic activity and provides a rapid means of accurately quantifying estimates of fermentation of

specific sources of dietary fiber (Barry et al., 1995). A study examining the efficacy of using human fecal inocula for batch fermentations found a 24-hour system using at least three donors provides the most accurate estimates of colonic mechanisms (McBurney and Thompson, 1989).

Organic matter disappearance (OMD) also estimates substrate fermentability, as it approximates the loss of organic matter to digestion and fermentation. A study by Bourquin et al. (1996) reported the production of short chain fatty acids is directly correlated with organic matter disappearance.

The measurement of water holding capacity (WHC) is a representation of *in vivo* colonic water retention and is estimated by exerting an osmotic gradient through dialysis tubing containing the fermented substrates of interest. The value obtained from this method includes the water held by the substrates as well as the water holding capacity of the bacteria present in the fecal matter. WHC represents the amount of water in grams held by one gram of fermented residue dry matter. The measurement of water holding capacity is of interest in human health because it is an attempt to estimate the fecal bulking effects of the substrates of interest. Water holding is dependent on the manner in which water is held by the food matrix and by the way WHC is measured. Fermentation can alter the anatomy and physical properties of a fiber, therefore it is most accurate to quantify the WHC of the fermented residue (Robertson and Eastwood, 1981).

Potential water holding capacity (PWHC) is an extrapolation of the WHC estimate and represents the amount of water that would be excreted in the feces as a result of the ingestion of one gram of the substrate of interest. PWHC takes the extent of fermentation into account, and therefore is a measure of the relative WHC that equivalent amounts of

ingested fiber have in the colon (McBurney et al., 1985). PWHC is calculated by multiplying WHC for the specific substrate by the amount of dry matter remaining of that substrate following fermentation. PWHC is inversely related to *in vitro* OMD (Bourquin et al., 1996).

The *in vitro* fermentation procedure does not account for variances in subject transit time, therefore differences may exist in the comparison between these measurements and actual colonic production of short chain fatty acids, WHC and PWHC. Finally, organic matter disappearance will be slightly underestimated due to bacterial matter inclusion in the dry matter recovery estimates.

Rates of short chain fatty acid production in humans range from 300-400 mmol per day on the assumption of the daily ingestion of 32-42 grams carbohydrate (Cummings, 1994). It is estimated that 95 to 99% of the short chain fatty acids produced by bacterial fermentation are absorbed from the colonic lumen (Scheppach, 1994). The mechanism for absorption of short chain fatty acids from the colon is not completely understood, however certain aspects are understood through experimental evidence (See Appendix C for detail). First, the transport of short chain fatty acids through the colonic epithelium appears to be concentration dependent and independent of chain length. In addition, absorption of short chain fatty acids may be coupled with sodium absorption, probably via Na^+ - H^+ exchange. There is a luminal accumulation of HCO_3^- with SCFA absorption, which may be a byproduct of the CO_2 produced from the Krebs cycle. Finally, the rate of absorption of short chain fatty acids is thought to depend on the location of absorption in the colon (Soergel, 1982; Scheppach, 1994).

The short chain fatty acids produced by fermentation have a number of physiologic effects. Acetate, propionate and butyrate may influence epithelial cell replication, maturation, and death (Wijnands et al., 1999). Short chain fatty acids reduce colonic pH, thus causing precipitation of the bile acids present in the colon. This results in an inhibition of the formation of secondary bile acids, a product of bacterial metabolism of primary bile acids. High concentrations of secondary bile acids have been shown to increase colon cancer risk in high concentrations (Wijnands et al., 1999).

Short chain fatty acids are thought to influence homeostasis within the colon, as metabolic starvation causes a degeneration and inflammation of the colonic epithelium. This effect is abated when supplementation of butyrate is administered (Tappenden et al., 1997). Therefore, short chain fatty acids may prevent the TPN-induced atrophy of normal and resected intestine by increasing basolateral intestinal nutrient transport (Sagor et al., 1983; Tappenden et al., 1996; Tappenden et al., 1997). Specifically, short chain fatty acid exposure increases jejunal GLUT2 mRNA, ileal GLUT2 protein, and ileal proglucagon mRNA. GLUT2 is a basolateral glucose transporter (Tappenden et al., 1997). Proglucagon-derived peptides are associated with increased cellular proliferation during colonic adaptation (Bloom and Pollak, 1982; Rountree et al., 1992; Sagor et al., 1983; Reimer et al., 1996).

Each short chain fatty acid is thought to have several unique physiological functions throughout the body. The concentrations of acetate and propionate in the portal bloodstream is much higher than that of butyrate, which is suggestive of a selective use of butyrate as an oxidative fuel source for colonic epithelial cells. The affinity of the

colonocytes for butyrate as an energy source is higher in the distal than the proximal colon (Scheppach, 1994).

Acetate is thought to act as a minor energy source for body tissues and as a result, spares fatty acid oxidation in humans. The chemical structure for acetate is shown in Appendix B. Acetate has also been found to inhibit lypolysis and glycerol release in adipocytes (Cummings, 1988). Acetate may play a role in carbohydrate metabolism as well. Studies have shown that acetate functions to reduce serum free fatty acid concentrations (Wolever, 1988). Because free fatty acids compete with glucose for uptake within insulin sensitive tissues, fewer fatty acids present in the serum will lead to improved uptake of glucose into the cells.

A study of dog colon showed that acetate mediated an increase in mucosal blood flow. The same effect of acetate was found in surgical patients in a concentration-dependent manner (Tappenden et al., 1997). Finally, acetate is thought to increase colonic motility and may be involved in the chemical reflux barrier within the gastrointestinal tract (Scheppach, 1994).

There is little research on the physiological effects of propionate in humans. The chemical structure of propionate is shown in Appendix B. One study showed an increase in HDL cholesterol levels in those individuals who ingested 7.5 grams of sodium propionate per day as compared to the control group (Venter et al., 1990). Propionate is thought to mediate this reaction by inhibiting hepatic cholesterol synthesis. In addition, propionate is thought to act as a gluconeogenic agent. One study has shown that rectal infusion of propionate caused an increase in blood glucose, which is consistent with the action of a gluconeogenic agent (Wolever, 1988). However, the rate of propionate

infused (180 mmol) is higher than the concentration present in the colon, and therefore propionate is probably not a significant source of blood glucose in humans.

Butyrate is a linear four-carbon short chain fatty acid that is taken up by colonocytes where it is metabolized to CO_2 and ketones. Butyrate is considered the chief energy source for these cells. The chemical structure of butyrate is shown in Appendix B. It is estimated that butyrate supplies 70% of the fuel for colonic cellular growth and development (Hague et al., 1997). The importance of butyrate as a respiratory fuel source appears to increase from proximal to distal colon, while the importance of glutamine decreases (Roediger WE, 1982).

Butyrate has been found by many studies to induce growth of colonic mucosal cells. Butyrate-induced differentiation leads to the expression of a normal absorptive cell phenotype (Basson et al., 1998). However, butyrate affects cancer cells in the opposite manner. Butyrate has been shown to inhibit growth and induce apoptosis, or programmed cell death, and differentiation within malignant cells (Hague et al., 1997). The mechanisms responsible for these seemingly disparate effects are not fully understood. Cell number homeostasis in colonic epithelial cells is believed to be sustained by an equilibrium between cell proliferation and apoptosis. Apoptosis is a non-pathophysiologic type of cell death inherent in normal cell (Bedi et al., 1995). Specifically, Bcl-2, which inhibits apoptosis, is believed to counteract Bax, a protein that induces apoptosis of colonic cells within the crypt. One study found that the withdrawal of butyrate from the colon resulted in an increase in apoptosis and Bax concentration (Hass et al., 1997). Conversely, in normal cells, treatment with butyrate resulted in decreased apoptosis and decreased Bax concentration within the colonic epithelium

(Hague et al., 1997). Therefore, not only is butyrate the major energy source for the colonic mucosa, it also acts as a protective agent against cell death.

Butyrate is thought to inhibit DNA synthesis and hinder tumorogenic cell proliferation in the G1 phase of the cell cycle (Young, 1988). A study reported a significant decrease in Caco-2 cell proliferation as a result of treatment with 10mM butyrate (Basson et al., 1998). Acetate and propionate also inhibited cellular proliferation, however their effects were not as pronounced as butyrate. In addition to its effects of cancer cell proliferation, butyrate inhibited metastatic expansion and adhesion as well. Another study found that sodium butyrate inhibited the activity of SNB-19 and SNB-75 cancer cells (Gross et al., 1988).

The process by which butyrate induces cell death or reduced differentiation in malignant cell lines is not fully understood, however, several hypotheses have been suggested. First, butyrate is thought to effect cellular proliferation by the hyperacetylation of histones via the inhibition of histone deacetylase. Histones function to package DNA into nucleosomes. This action causes the loss of the positive charge on histone and subsequent weakening of the phosphate group within the DNA strand. This alteration induces a change in the chromatin structure and subsequent arrest of the cell cycle at the G1 stage, which limits cell proliferation (Young, 1988; Boffa et al., 1992). Hyperacetylation is also associated with increased transcriptional activity within areas of the cell which produces the activation of specific genes (Archer et al., 1998).

Histone hyperacetylation may be responsible for the induction of p21 *WAF1/Cip1*, a cell cycle inhibitor (Archer et al., 1998). p21 *WAF1/Cip1* inhibits certain G1 cyclin-dependent kinases by the suppression of the phosphorylation of retinoblastoma (RB)

protein (Nakano et al., 1997). There is some evidence that p21 *WAF1/Cip1* plays a role in the assembly and activation of kinase complexes as well. A tumor suppressor protein, p53, appears to mediate the activity of p21 *WAF1/Cip1* under certain conditions, however, butyrate affects p21 *WAF1/Cip1* action independently of p53 activation. Archer et al. found that p21 *WAF1/Cip1* is required for the growth inhibition that butyrate mediates (Archer et al., 1998; Nakano et al., 1997). Taken together, these data support the hypothesis that p21 *WAF1/Cip1* is a critical mediator of growth control exerted by butyrate.

There are several oncogenes that are responsible for regulating cellular growth and differentiation within the colon. C-myc is believed to control the production of a nuclear phosphoprotein that is involved in transcription of specific genes involved in cell growth control. C-fos and c-jun are responsible for activating DNA binding proteins that form the AP-1 transcription factor. AP-1 transcription factor may control genes involved in cellular growth and differentiation (Tappenden et al., 1998). Therefore these agents may control for cellular differentiation and growth, as well as apoptosis (Tappenden et al., 1998). Western blotting of Caco-2 lysates, a cancerous cell line, with antibodies to c-myc revealed that propionate, acetate, and butyrate downregulate c-myc 1 and 2 protein levels, with butyrate having the most significant effect (Basson et al., 1998; Velazquez et al., 1996).

Prebiotics effectively stimulate the production of short chain fatty acids, including butyrate, as measured by *in vitro* batch fermentation indices. Short chain fatty acids are thought to impart benefits not only to the health of the colon, but to systemic health as well. These above data provide a rational basis for the use of *in vitro* fermentation

techniques to investigate the fermentative capacity of the specific substrates used in this study.

Chapter 2

MATERIALS AND METHODS

Subjects

Two healthy adult females (age 25 and 28) and one healthy adult male (age 35) participated in this study. In addition, one male child of age eleven was included. All subjects were encouraged to ingest at least twenty-five grams of dietary fiber from varying sources per day. Subjects had not been on antibiotic therapy during the three months preceding the study.

Fermentation Media Preparation

The *in vitro* batch fermentation procedure was adapted from previously used methodology (Bourquin et al., 1996). Anaerobic dilution (Table 1) and fermentation media (Table 2) were made according to the following composition guidelines.

Table 1: Composition of anaerobic dilution medium (Bryant and Burkey, 1953)

Component	Concentration in medium
Mineral 1*	150 mL/L
Mineral 2 #	150 mL/L
Resazurin	0.0003 g/L
Cysteine hydrochloride	0.09 g/L
Sodium carbonate	0.32 g/L
Distilled water	Fill to 1L mark

* Composition: KH_2PO_4, 0.06 g/L.

Composition (g/L): KH_2PO_4, 0.06; $(NH_4)_2SO_4$, 0.12; NaCl, 0.05; $MgSO_4$, 0.18; $CaCl_2$, 0.01.

One liter of the anaerobic dilution medium was used. After the appropriate amounts of the above constituents were mixed, the solution was capped with cotton batting and autoclaved. Following the autoclave process, carbon dioxide was bubbled through the

solution for thirty minutes. The solution was then capped with a rubber stopper and stored at room temperature until use the same day.

Table 2: Composition of medium used for *in vitro* fermentation (Bourquin et al., 1996)

Component	Concentration in medium
Solution A *	330 mL/L
Solution B #	330 mL/L
Trace Mineral Solution °	10 mL/L
Water-soluble vitamin mix ♦	20 mL/L
Folate:biotin solution δ	5.0 mL/L
Riboflavin ¶	5.0 mL/L
Hemin solution §	2.5 mL/L
Short-chain fatty acid mix **	0.4 mL/L
Resazurin solution ##	1.0 mL/L
Distilled water	296 mL/L
Yeast extract	0.5 g/L
Trypticase	0.5 g/L
Na_2CO_3	4.0 g/L
Cysteine HCl·H_20	0.5 g/L

*Composition (g/L): NaCl, 5.4; KH_2PO_4, 2.7; $CaCl_2$·H2O, 0.16; $MgCl_2·6H_2O$, 0.12; $MnCl_2·4H_2O$, 0.06; $CoCl_2.6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4.
#Composition: KH_2PO_4, 2.7 g/L.
°Composition (mg/L): EDTA, 500; $FeSO_4·7H_2O$), 200; $ZnSO_4·7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3PO_4, 30; $CoCl_2·6H_2O$, 20; $CuCl_2·2H_2O$, 1; $NiCl_2·6H_2O$, 2; $Na_2MoO_4·2H_2O$, 3.
♦Composition (mg/L): thiamin·HCl, 100; pantothenic acid, 100; niacin, 100; pyridoxine, 100; *p*-aminobenzoic acid, 5; vitamin B-12, 0.25.
δComposition (mg/L): folic acid, 10; biotin, 2; NH_4CO_3, 100.
¶Composition: riboflavin, 10 mg/L in 5 mmol/L HEPES.
§Hemin, 500 mg/L in 10 mmol/L NaOH.
**250 mL/L each of *n*-valerate, isovalerate, isobutyrate, and DL-α-methylbutyrate.
##Resazurin, 1 g/L in distilled H_2O.

Three and one-half liters of fermentation media were needed for this study. All components but the water-soluble vitamins, Na_2CO_3 and cysteine hydrochloride were mixed and transferred to four one-liter bubble flasks. These flasks were individually heated with a Bunsen burner under a constant stream of CO_2 until they reached a boil. All flasks were capped with rubber stoppers and wired shut before sterilizing the medium

via autoclave. Just prior to use, the water-soluble vitamins were added via filter syringe. The other omitted components were added at this time as well.

Fermentation Methodology

Two sets of fermentations were performed simultaneously. The first fermentation was carried out in one hundred milliliter serum vials and was used to assess organic matter disappearance and short chain fatty acid production. The second set of fermentations was done in twenty-five milliliter Balch tubes and was used in the quantification of water holding capacity and potential water holding capacity. Each substrate was fermented in duplicate for each subject.

Substrates used in this study were arabinogalactan, low viscosity β-glucan, medium viscosity β-glucan, high viscosity β-glucan, mixed fiber, inulin/FOS, Solka-Floc®, oat flour, and oat groats (see Appendix A for substrate composition). Three hundred milligrams and one hundred and fifteen milligrams of the substrates were weighed and placed in the serum vials and Balch tubes, respectively. All weights were recorded. Aliquots (26ml) of fermentation medium were aseptically transferred under a CO_2 stream to the serum vials using 50 milliliter pipettes. Ten milliliters of medium were added to the Balch tubes in the same manner. The serum vials and Balch tubes were capped with butyl rubber stoppers and stored under refrigeration (4° C) for twelve hours to allow for hydration of the substrates. Tubes containing no substrates were also included in duplicate for each subject to allow for corrections of organic matter and short chain fatty acid content not arising from the substrates.

The procedure for the preparation of the fecal slurry is as follows. Each subject donated a fresh fecal sample from which the slurry was prepared within four hours. The

fecal samples were sealed in plastic bags and were held under refrigeration (4° C) until use. Twenty-eight grams of feces were blended using a Hamilton Beach blender under anaerobic conditions with two hundred and eighty milliliters of anaerobic dilution medium (1:10 dilution) for fifteen seconds. This mixture was filtered through four layers of cheesecloth into an Erlenmeyer flask and capped under carbon dioxide until use.

The sets of fermentations were staggered by two hours by subject to allow for completion of all tasks. The hydrated substrates in the serum vials and Balch tubes were anaerobically inoculated using ten milliliter pipettes with four milliliters and one and one half milliliters of the fecal slurry, respectively. The tubes were capped with butyl rubber stoppers and crimped closed using aluminum caps. Tubes were allowed to incubate at 37° C for twenty-four hours with periodic mixing.

After twenty-four hours, a one-milliliter aliquot was removed from the serum vials using 3 milliliter syringes for short chain fatty acid analysis. One hundred and twelve milliliters of 95% ethanol were added to the remaining twenty-nine milliliters in each serum vial and the solutions were allowed to precipitate for one hour. The contents of the serum vials were then vacuum filtered through tared Whatman 541 filter paper, and washed with 95% ethanol and acetone. The filter paper and residue were dried in a 105° C drying oven (Precision Scientific Model 124 drying oven) for twenty-four hours. After desiccation, the filter paper and residue was weighed and transferred to tared crucibles. The crucibles were placed in an ashing oven (500° C) for twelve hours. After desiccation, the crucibles and their contents were weighed. *In vitro* organic matter disappearance was calculated as the original organic matter weight minus (residue

organic matter weight minus the organic matter weight of the appropriate blank), divided by the original sample organic matter weight (Bourquin et al., 1996).

Fermentation in the Balch tubes was stopped after twenty-four hours by the addition of 0.4 milliliters of 20 g/L $CuSO_4$. Dialysis tubing (Spectra-Por 6, 45mm, 2000 dalton cut-off, Spectrum Medical Industries, Inc., Los Angeles, CA) was cut into 3" strips and pre-soaked for twelve hours in 0.5g/L NaN_3. The contents of the Balch tubes were transferred to dialysis tubing and clamped using dialysis clips. The clamped dialysis bags were placed in a solution containing 95g/L polyethylene glycol (MW=3500) and 0.5g/L NaN_3. The bags were dialyzed at room temperature with periodic mixing for forty-eight hours. The dialysis solution was changed at this time and the tubes were dialyzed for an additional twenty-four hours. The bags were then blotted dry with paper towel and cut open. The contents were transferred to tared crucibles and weighed. The crucibles were placed in a 105° C drying oven for 24 hours and reweighed. Water holding capacity was calculated as the grams of water lost during the drying process, divided by the dry residue weight. Potential water holding capacity was calculated as the water holding capacity multiplied by the percentage of dry matter remaining following the twenty-four hour fermentation period.

Fatty Acid Analysis

The one-milliliter samples extracted for SCFA analysis were treated with 0.25 mL 250 g/L metaphosphoric acid and centrifuged at 10,000 x g for 30 minutes (Beckman Allegra 6R Centrifuge). The supernatant was transferred to 2-ml glass GC vials and refrigerated (4° C) until analysis.

Analysis for acetate, propionate, and butyrate content of the samples was done using a Varian Model 3700 Gas Chromatograph (GC) equipped with a hydrogen flame-ionization detector. Branched chain amino acids were not quantified because several strains of anaerobic bacteria require these for growth and therefore they were added to the fermentation media (Yokoyama and Johnson, 1979). The two-meter glass column used for analysis was 10% SP1200/1% H_3PO_4 on 80-100 Chromosorb. The analysis was done using a temperature-timed program, in which the initial oven temperature setting was 120° C for one minute, with an increase of one degree per minute until the final temperature of 127° C was reached and held for one minute. Nitrogen was used as a carrier gas with a flow rate of 50 milliliters per minute. The injector temperature was 150° C. Two microliter quantities were used to inject samples in duplicate.

Statistical Analysis

Analysis for area under the curve data was done using Peak Simple II software, version 3.7. Areas under the curve for each injection were compared to a known standard and converted to millimoles per gram original organic matter. Molar proportions of each short chain fatty acid produced were calculated by dividing the respective amount by the total short chain fatty acid concentration.

The data analysis was done using a randomized complete block design using the General Linear Models procedure of SAS (SAS Institute, Inc., 1996). Inoculum donors were used as blocks. When significant substrate effects were detected ($p<0.05$), means were compared using the Least Significant Difference Method (Carmer and Swanson, 1974). Pearson's r correlations were computed using SPSS 7.5.

Chapter 3

RESULTS

The result for the fermentations with regard to short chain fatty acid production can be found in Table 3. Please see Appendix D for experimental raw data. The highest concentration of total short chain fatty acids was produced from the fermentation of high viscosity β-glucan. However, this yield was not statistically different from that of mixed fiber, oat flour, or inulin/FOS ($p<0.05$). The lowest short chain fatty concentration resulted from the fermentation of Solka-Floc® and oat groats, which were both significantly lower than the other fermented substrates ($p<0.05$). The rank order of substrates from highest SCFA production to lowest is as follows: high viscosity β-glucan> inulin/FOS > oat flour > mixed fiber > arabinogalactan > medium viscosity β-glucan > low viscosity β-glucan > oat groats > Solka-Floc®.

Table 3. Average short chain fatty acid production (mmol / gram original organic matter) of experimental substrates including all donors

	Acetate	Propionate	Butyrate	Total SCFA
Arabinogalactan	2.05	0.75	0.49	3.29
High Viscosity β-Glucan	3.22	0.75	1.32	5.30
Medium Viscosity β-Glucan	1.87	0.52	0.86	3.25
Low Viscosity β-Glucan	1.74	0.51	0.74	2.99
Solka-Floc	0.52	0.19	0.21	0.92
Inulin/FOS	2.27	0.44	2.26	4.97
Mixed Fiber	2.22	0.47	1.61	4.30
Oat Flour	2.56	0.55	1.55	4.67
Oat Groats	0.68	0.19	0.39	1.25
SEM	0.33	0.07	0.21	0.40
LSD*	1.00	0.23	0.65	1.31

* Least significant difference between any two mean values in the same column ($p<0.05$)

Fermentation of the high-viscosity β-glucan resulted in higher acetate production with respect to all substrates except inulin/FOS and oat flour ($p<0.05$). Fermentation of the oat groats and Solka-Floc® resulted in the least production of acetate. The yield of acetate from highest to lowest from the fermentation of the experimental substrates is as follows: high viscosity β-glucan > oat flour > inulin/FOS > mixed fiber > arabinogalactan > medium viscosity β-glucan > low viscosity β-glucan > oat groats > Solka-Floc®. The molar proportion (Table 4) of acetate produced was approximately the same for all substrates (~50-60%).

Table 4. Average percentage of short chain fatty acids produced post-fermentation of experimental substrates including all donors

	Molar Proportion Acetate [1]	Molar Proportion Propionate [1]	Molar Proporation Butyrate [1]
Arabinogalactan	62.4%	22.7%	14.9%
High Viscosity β-Glucan	60.5%	14.1%	25.4%
Medium Viscosity β-Glucan	57.4%	17.3%	25.3%
Low Viscosity β-Glucan	58.6%	18.1%	23.3%
Solka-Floc	56.4%	20.9%	22.8%
Inulin/FOS	46.7%	8.7%	44.6%
Mixed Fiber	51.9%	10.9%	37.2%
Oat Flour	52.0%	13.2%	34.8%
Oat Groats	52.3%	15.0%	32.7%
Standard Error of the Mean	4.10	1.90	3.78
LSD*	13.74	5.85	11.45

*Least significant difference between any two mean values in the same column ($p<0.05$)

Propionate was produced in the highest quantities during the fermentation of the high viscosity β-glucan and arabinogalactan, though these were not significantly different in comparison to oat flour ($p<0.05$). Again, oat groats and Solka-Floc® resulted in the least amount of propionate production ($p<0.05$). The production of propionate from highest to lowest concentration is as follows: high viscosity β-glucan > arabinogalactan > oat flour > medium viscosity β-glucan > low viscosity β-glucan > mixed fiber > inulin/FOS > Solka-Floc® > oat groats. The molar proportions of propionate ranged from the lowest for inulin/FOS to the highest for arabinogalactan, at 9 and 23% respectively.

Butyrate was produced in significantly higher concentrations during the fermentation of inulin/FOS in comparison to all other experimental substrates ($p<0.05$). The ranking from highest to lowest butyrate production is as follows: inulin/FOS > mixed fiber > oat flour > high viscosity β-glucan > medium viscosity β-glucan > low viscosity β-glucan >

arabinogalactan > oat groats > Solka-Floc®. Molar proportions of butyrate produced differed quite significantly with respect to substrate. The fermentation of inulin/FOS resulted in the highest molar proportion of butyrate (45%), while arabinogalactan fermentation resulted in the lowest molar proportion of butyrate (15%).

Table 5. Average organic matter disappearance (OMD) and dry matter disappearance (DMD) post-fermentation of experimental substrates including all donors

	OMD [1]	DMD [1]
Arabinogalactan	24.16	24.91
High Viscosity β-Glucan	36.49	38.08
Medium Viscosity β-Glucan	17.81	19.02
Low Viscosity β-Glucan	15.81	17.27
Solka-Floc	-1.79	-0.75
Inulin/FOS	76.93	78.49
Mixed Fiber	83.84	84.98
Oat Flour	45.31	46.45
Oat Groats	7.86	8.08
Standard Error of the Mean	4.00	4.13
LSD*	12.32	13.17

* Least significant difference between any two mean values in the same column ($p<0.05$)

[1] OMD = organic matter disappearance (%), DMD = dry matter disappearance (%)

Organic matter disappearance was significantly higher for mixed fiber and inulin/FOS than all other experimental substrates ($p<0.0001$). Oat groats and Solka-Floc® were the most poorly fermented substrates as measured by OMD. OMD from highest to lowest is as follows: mixed fiber > inulin/FOS > oat flour > high viscosity β-glucan > arabinogalactan > medium viscosity β-glucan > low viscosity β-glucan > oat groats >

Solka-Floc®. Organic matter disappearance was positively correlated with total short chain fatty acid production (r= 0.77, p=0.015).

Table 6. Average water holding capacity (WHC) and potential water holding capacity (PWHC) post-fermentation of experimental substrates including all donors

	WHC[1]	PWHC[1]
Arabinogalactan	6.20	4.66
High Viscosity β-Glucan	5.70	3.51
Medium Viscosity β-Glucan	5.25	4.34
Low Viscosity β-Glucan	5.65	4.60
Solka-Floc	4.46	4.51
Inulin/FOS	6.76	1.45
Mixed Fiber	6.34	0.97
Oat Flour	4.13	2.10
Oat Groats	3.79	3.46
Standard Error of the Mean	0.33	0.34
LSD*	0.69	0.83

* Least significant difference between any two mean values in the same column ($p<0.05$)
[1] WHC = g H_2O/g fermented residue dry matter, PWHC = g H_2O/g original substrate dry matter

Water holding capacity of fermented residues was highest for inulin/FOS, mixed fiber, and arabinogalactan. WHC was the lowest for oat flour and oat groats, both around 4 grams water per gram of residue. WHC from highest to lowest is as follows: inulin/FOS > mixed fiber > arabinogalactan > high viscosity β-glucan > low viscosity β-glucan > medium viscosity β-glucan > Solka-Floc® > oat flour > oat groats.

Potential water holding capacity was highest for arabinogalactan, medium and low viscosity β-glucan and Solka-Floc®. PWHC was lowest for mixed fiber and inulin/FOS. PWHC from highest to lowest is as follows: arabinogalactan > low viscosity β-glucan > Solka-Floc® > medium viscosity β-glucan > high viscosity β-glucan > oat groats > oat

flour > inulin/FOS > mixed fiber. Potential water holding capacity was inversely correlated with organic matter disappearance (r= -0.91, p=0.001).

Table 7. All parameters of fermentation measured by donor

	Donor [1]				
Item	A	B	C	D	SE
Total 24-h SCFA production	3.56 [b]	3.84 [b]	2.71 [a]	3.64 [b]	0.27
Acetate	1.75 [ab]	2.36 [a]	1.53 [b]	1.98 [ab]	0.22
Propionate	0.41 [b]	0.46 [b]	0.45 [b]	0.62 [a]	0.05
Butyrate	1.39 [a]	1.03 [ab]	0.74 [b]	1.04 [ab]	0.14
Percent of total - Acetate [2]	51.2% [a]	60.4% [b]	55.7% [ab]	54.0% [ab]	2.73
Percent of total - Propionate [2]	12.8% [a]	13.1% [a]	18.2% [b]	18.5% [b]	1.26
Percent of total - Butyrate [2]	36.0% [a]	26.5% [b]	26.1% [b]	27.5% [b]	2.52
DMD [3]	36.99 [a]	44.98 [a]	25.97 [b]	32.74 [ab]	2.76
OMD [3]	36.14 [a]	41.28 [a]	26.66 [b]	32.10 [ab]	2.67
WHC [3]	6.03 [a]	5.37 [b]	5.20 [b]	4.87 [b]	0.22
PWHC [3]	3.64 [a]	2.77 [b]	3.65 [a]	3.09 [ab]	0.23

[1] Values are means and standard error. Means within a row followed by the same symbol are not significantly different ($p<0.05$).
[3] DMD = dry matter disappearance (%), OMD = organic matter disappearance (%), WHC = water holding capacity (g H_2O/g fermented residue dry matter), PWHC = potential water holding capacity (g H_2O/g original substrate dry matter)

Donor C produced significantly fewer total short chain fatty acids than all other donors ($p<0.05$). Donor D produced significantly more propionate than all other donors ($p<0.05$), however the molar proportion of propionate produced was not significantly higher than donor C. The molar proportion of butyrate produced by donor A was significantly higher than all other donors ($p<0.05$).

Organic matter disappearance and dry matter disappearance followed a similar trend, with donor B contributing to the highest of both indices, though not significantly different from donors A and D. Donor C had the lowest OMD and DMD estimates, however was not significantly different from donor D.

Finally, donor A had the highest WHC ($p<0.05$) than all other donors. Donor D had the lowest WHC, averaging around 5 grams water per gram of residue dry matter. Potential water holding capacity was highest for donor C, at an average of 3.65 grams water held per gram of original dry matter. PWHC for donor C was not significantly different from donors A and D.

Chapter 4

DISCUSSION

The overall results of this study are in agreement with previously published data with regards to trends of fermentability and in most cases, absolute measurements of several indices. The nature of this research, however, makes absolute quantification and comparison between laboratories and substrates difficult. A comparative analysis of OMD and SCFA production and molar ratios reported significant differences among five laboratories using identical substrates and assigned to replicate the same batch fermentation study using human subjects (Barry et al., 1995). However, despite interlaboratory differences in the magnitude of various fermentation parameters, there was agreement with respect to ranking of fermentability among substrates. Because differences exist in comparisons of the same substrates fermented at different laboratories, it becomes even more difficult to make accurate comparisons with those studies following different procedural methods. Differences in the amount and brand of substrate added to the fecal inoculum may result in differences in end product fermentation measurements. The preparation of the fecal inoculum, fermentation time or the degree to which the environment for fermentation was kept anaerobic would also affect the results. Resazurin, a redox indicator, was added to the media and allowed for assurance that the fermentations remained anaerobic in this study. In addition, there are many studies that report SCFA and OMD results in swine or rat models. While comparisons between humans and these models have been shown to be relatively consistent, there is still some question as to the legitimacy of this practice (Barry et al.,

1995). Lastly, one must acknowledge that experimental error may be a factor that could explain the lack of agreement of certain measurements with previous studies.

The fermentation of the nine fibers used in this study resulted in a mean yield of short chain fatty acids in range with other published data acquired using similar techniques. In this fecal inoculation system, acetate was the primary short chain fatty acid produced, which agrees with many published data (Bourquin et al., 1996; Flickinger et al., 2000; McNeil, 1982; Vince et al., 1990). Total short chain fatty acid production was positively correlated to organic matter disappearance ($r = 0.77$, $p = 0.015$). Organic matter disappearance is an estimate of the extent to which the substrate is utilized by colonic bacteria. Therefore, as expected, the higher the organic matter disappearance, the greater the short chain fatty acid production. This finding is in agreement a previous finding (Bourquin, 1996). Potential water holding capacity was found to be inversely correlated to organic matter disappearance ($r = -0.91$, $p = 0.001$). This result was expected given that the more fermented the substrate is by bacteria, the less there will be available to hold water post-fermentation. Given this general agreement, each substrate will be discussed with respect to the indices measured.

Arabinogalactan is a highly branched water-soluble hemicellulose consisting of a galactan backbone with side chains of galactose and arabinose sugars (Vince et al., 1990). In this study, arabinogalactan was found to be moderately fermentable (3.29 mmol/g original organic matter), resulting in a high molar proportion of propionate (23%). This is in agreement with findings by other research groups (Bradburn et al., 1993; Cummings and Macfarlane, 1997; Wang and Gibson, 1993). Vince et al. (1990) reported a higher molar ratio of butyrate over propionate resulting from the fermentation of

arabinogalactan. However, this study differed in methodology, as the fermentation duration was forty-eight hours, and thus may account for the differing findings. Water holding capacity was relatively high for arabinogalactan (6.20 grams water per gram fermented residue dry matter). This finding correlates to the relatively low organic matter disappearance.

Three barley β-glucan substrates of differing viscosities were used in this study. β-glucan is a linear, unbranched polysaccharide composed of about 70% 4-O-linked β-D-glucopyranosyl units and 30% 3-O-linked β-D-glucopyranosyl units (Wood, 1990). β-glucan exists in varying amounts in the endosperm region of several plant products. The largest amount exists in barley (3-11%), followed by oats (3-7%), rye (1-2%), and wheat (<1%). Oat and barley β-glucans appear to have the same structural identity, however there may be some slight differences in the oligosaccharide fragments present (Mazza, 1998). There is some evidence that viscosity may play a role in the availability of the substrate for fermentation, as well as the rate of starch digestion and glucose absorption (Knudsen et al., 1990; Sundberg et al, 1996). Varying levels of viscosity are achieved during the extraction process, which reduces the molecular size relative to the native cell wall polymer of β-glucan (Wood, 1990).

In this study, the fermentation of the high viscosity β-glucan product produced significantly more short chain fatty acids than the two lower viscosity β-glucan products. However, the results from this study do not indicate that high viscosity β-glucan exceeds other known substrates in its ability to produce short chain fatty acids. The organic matter disappearance of all three β-glucans products was lower than expected the substances' characteristically high solubility. The OMD of the high viscosity β-glucan,

36.49%, is within range of previous findings of 46.2% OMD (McBurney, 1991). The medium and low viscosity β-glucans, at 17.81% and 15.81% OMD respectively, are not within range of previously published data. This may be a result of the processing of the barley β-glucan, making it chemically less available for fermentation. Experimental error may also play a role in these consistent, yet seemingly disparate findings. It should be noted, however, that the small amount of β-glucan fermented resulted in a high production of short chain fatty acids, in comparison to other substrates with higher organic matter disappearances. The fermentation of the high viscosity β-glucan resulted in the highest production of short chain fatty acids, at 5.30 mmol per gram original organic matter. None of the β-glucan substrates, however, were a significant source of butyrate, which agrees with previous findings in swine (Knudsen, 1993), but disagrees with other published data (McBurney, 1991). Due to the low disappearance of the β-glucan substrates, the WHC of all three was moderately high. Potential water holding capacity of all three substrates was inversely related to OMD.

The fermentation of Solka-Floc®, a crystalline cellulose product, led to the expected lowest production of short chain fatty acids (0.92 mmol/g original organic matter). This finding corresponds to those of many other studies of the same nature (Barry et al., 1995; Ehle et al., 1982; Penner and Liaw, 1990; Sunvold et al., 1995). The highly crystalline structure of Solka-Floc® makes this substrate resistant to enzymatic hydrolysis within the colon. The mean OMD for Solka-Floc® was −1.79%, illustrating the inert nature of this compound in this system. The negative value for substrate disappearance has been noted by other laboratories, and because it is not significantly different than zero, can be explained by experimental error (Barry et al., 1995; Sunvold et al., 1995). There is some

evidence that some individuals are able to transiently degrade this cellulose product, however this was not confirmed from this study (Ehle et al., 1982).

Inulin and fructooligosaccharide (FOS) were used in combination as an experimental substrate in this study. Both substances are naturally occurring polyfructans found in several commonly consumed vegetables as storage carbohydrates and have been shown to be resistant to digestive processes (Brighenti et al., 1999). The chemical structure of FOS consists of a glucose unit joined by an (α1-β2) linkage to two or more fructose units. Fructose units are joined to one another by β(2-1) linkages (Tashiro et al, 1997). The degree of polymerization characterizes inulin and FOS, which is 2-8 and 10-12, respectively. The OMD for the inulin/FOS product was 76.93%, suggesting virtually complete fermentation by colonic bacteria as the majority of the remaining organic matter is probably of bacterial origin. The high fermentability of inulin/FOS is supported by past research (Wang and Gibson, 1993). Total short chain fatty acids produced were also high in comparison to other substrates, though not significantly higher than that produced by the fermentation of high viscosity β-glucan or oat flour ($p<0.05$). The molar ratio of butyrate produced from the fermentation of inulin/FOS was significantly higher than all other experimental substrates (44%). This finding is in agreement with the findings of Tashiro et al. (1997) who found a significantly higher proportion of cecal butyrate in rats fed FOS in comparison to guar gum, cellulose, inulin, nystose, and a fiber free diet. In contrast to this finding, Wang and Gibson (1993) found inulin to be a relatively poor source of butyrate, reporting a molar ratio of 8%. However, other publications report inulin and FOS fermentation to result in significant productions of butyrate and propionate in both rats and humans (Levrat et al., 1991; Luo et al., 1996; Campbell et al.,

1997; Roland et al., 1995; Flickinger et al., 2000). The water holding capacity after fermentation of inulin/FOS was high (6.76 grams water per gram fermented residue dry matter), however due to its high OMD, probably reflects the water holding capacity of the bacteria present. Again, the potential water holding capacity (1.45 grams water held per gram of original substrate dry matter) was inversely correlated with OMD.

The mixed fiber substrate, BeFlora, used in this study is a proprietary blend of fructooligosaccharides (FOS), soy protein extract and glycolate (potato starch). Because the major fermentative component of this substrate is FOS, the results are similar to the above inulin/FOS substrate. The fermentation of the mixed fiber substrate resulted in a high yield of total short chain fatty acids in comparison to other substrates measured (4.30 mmol / gram original organic matter). The average organic matter disappearance of mixed fiber (83.84%) suggests virtually complete utilization by the bacteria present in the fecal inoculum. Similar to the findings for the inulin/FOS substrate, butyrate was a substantial proportion of the total short chain fatty acids produced (37%). Water holding capacity was high (6.34 grams water per gram fermented dry matter) and again probably reflects the water holding capacity of the bacteria present.

Oat flour was included as an experimental substrate in this study. Oat flour contains a high amount of digestible starch (see Appendix A) and therefore may not be of interest for *in vivo* colonic fermentation, as it is likely that the substrate would be digested prior to reaching the colon. This substrate was included in this study at the request of the funding corporation.

The oat groats used in this study were whole oats with the outer hull layer mechanically removed. The remaining outer aleurone cell wall layer on the groat is thick

and makes this product resistant to human digestion without further processing (Wood, 1990). The hull is a highly lignified and has a fibrous covering and therefore is not suitable for human digestion without processing (Mazza, 1998). The disappearance for this substrate due to fermentation was 7.86% and except Solka-Floc®, was significantly lower than all other substrates used in this study ($p<0.05$). The resulting short chain fatty acid production, 1.25 mmol/gram original organic matter, was also the lowest of all substrates except Solka-Floc® ($p<0.05$). The water holding capacity of this substrate was significantly lower than all substrates except oat flour ($p<0.05$). This occurrence is probably due to the unavailability of the soluble portion of the oat due to the surrounding insoluble hull. Bourquin et al. (1993) found similar results with the fermentation of oat bran fiber, reporting OMD to be less than 10% and WHC and PWHC to both be around four grams water per gram residue.

Significant differences among fermentation indices existed between donors. Variances in colonic colonization due to diet or genetics may explain this occurrence. Donor C appeared to have the least ability to ferment the fibers used in this study, as measured by individual and total short chain fatty acid production as well as organic matter disappearance.

Chapter 5

CONCLUSION

Several of the substrates analyzed in this study demonstrated the ability to effectively stimulate the production of short chain fatty acids, specifically butyrate, acetate, and propionate, as measured by *in vitro* batch fermentation. The fermentation of the high viscosity β-glucan, mixed fiber, and inulin/FOS substrates produced the greatest yields of short chain fatty acids. The fermentation of oat flour also yielded a high concentration of short chain fatty acids, however the likelihood of this substrate being fermented *in vivo* is relatively low due to its high starch content.

The high viscosity β-glucan was fermented to a greater extent than the two lower viscosity β-glucan products. From this study, it does appear that increasing molecular weight does increase fermentability of β-containing substances. However, the extent to which the high viscosity β-glucan was fermented was not as great as other substrates used in this study. The yield of short chain fatty acids was high, however not statistically significant from other substrates included in this study. Therefore, it is difficult to assess whether viscosity plays a large role in the extent of fermentability or production of short chain fatty acids in a β-glucan product. Further research is required to determine whether viscosity is a predictor of the fermentability of β-glucan products.

In vitro batch fermentations continue to be an effective method to predict the fermentability of dietary fibers *in vivo*. Emerging proprietary substrates can continue to be analyzed in this fashion to assess their contribution to factors suspected to maintain

colonic health. An additional strength of this study is that the results found confirmed previous research findings.

There are several limitations that must be considered when reviewing results from this type of research. First, the results from this study may not be generalized easily, as each subject may vary in their ability to fermented carbohydrate. Another limitation to this study is the small sample size used, as well as the lack of control of the diet ingested by the donors prior to the donation of fecal samples.

Future directions for this type of research may include the use of probiotics to condition the subject before an *in vitro* batch fermentation is done. This would allow the researcher to assess whether pre-conditioning with probiotics would significantly change the donor's ability to ferment carbohydrate. To strengthen this existing project, the use of breath hydrogen testing could be use to measure methane production. This information would help to confirm fermentation findings in this type of study.

APPENDICES

APPENDIX A

SUBSTRATE COMPOSITION

All substrates provided by General Mills, Inc., James Ford Bell Technical Center

Information included is limited due to proprietary nature of substrates.

Oat Flour

Composition:

Carbohydrate:	69.0%
Insoluble Fiber:	1.6%
Soluble Fiber:	0.9%
Total fat:	1.64%

Oat Groats

Composition:

Carbohydrate:	69.2%
Insoluble Fiber:	5.9%
Soluble Fiber:	3.0%
Protein:	12.9%
Total fat:	6.94%

β -Glucan 1

Megazyme International
Ireland Limited
Bray Business Park
Bray, Co. Wicklow
Ireland

Barley β-Glucan (High Viscosity) (Lot 40301)

Properties:

Viscosity:	114 cSt
Starch Content:	<0.1%
Protein Content:	0.65%

Moisture:	5.0%
Molecular Weight:	327,000

β Glucan 2

Megazyme International
Ireland Limited
Bray Business Park
Bray, Co. Wicklow
Ireland

Barley β-Glucan (Low Viscosity) (Lot 41005)

Properties:

Viscosity:	5.9 cSt
Starch Content:	<0.0%
Protein Content:	0.85%
Moisture:	6.0%
Molecular Weight:	137,000

β -Glucan 3

Megazyme International
Ireland Limited
Bray Business Park
Bray, Co. Wicklow
Ireland

Barley β-Glucan (Medium Viscosity) (Lot 60501)
Properties:

Viscosity:	25.0 cSt
Starch Content:	<0.1%
Protein Content:	0.10%
Moisture:	2.2%
Molecular Weight:	250,000

Inulin

Imperial Suiker Unie
8016 Hwy. 90A
P.O. Box 9

Sugar Land, TX 77487-0009
USA

Frutafit® Inulin/FOS-IQ (NG)

Composition:

Soluble dietary fiber (g):	90.00 ± 2.2
Total Carbohydrate:	
Inulin/FOS (g)	90.00 ± 2.2
Disaccharides	4.50 ± 0.7
Glucose	0.75 ± 0.25
Fructose	3.10 ± 1.3
Moisture (g):	3.00 ± 1.5

Mixed Fiber

Triarco Industries, Inc.
400 Hamburg Turnpike
Wayne, New Jersey 07470
USA

BeFlora™

A proprietary blend of FOS, soy extract and glycolate (potato starch)

Composition and properties unavailable

Arabinogalactan

Larex, Inc.
2852 Patton Rd.
St. Paul, MN 55113
USA

FIBERAID™

Composition:

Arabinogalactan (anhydrous)	95+%
Water	≤8%

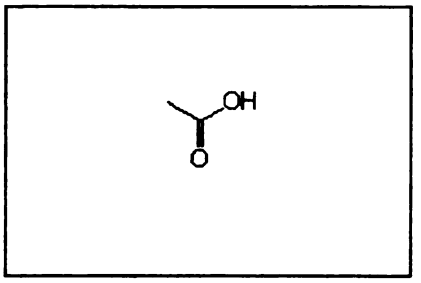
Cellulose

Fiber Sales and Development Corp.
PO Box 88940
St. Louis, MO 63166
USA

Solka-Floc® (crystalline cellulose)

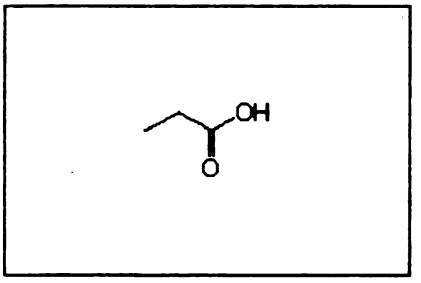
APPENDIX B

CHEMICAL STRUCTURES



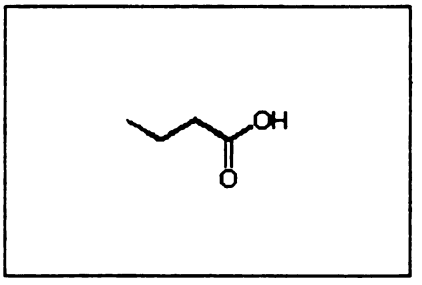
Molecular Weight = 60

Figure 1: Chemical Structure of Acetate



Molecular Weight = 74

Figure 2: Chemical Structure of Propionate



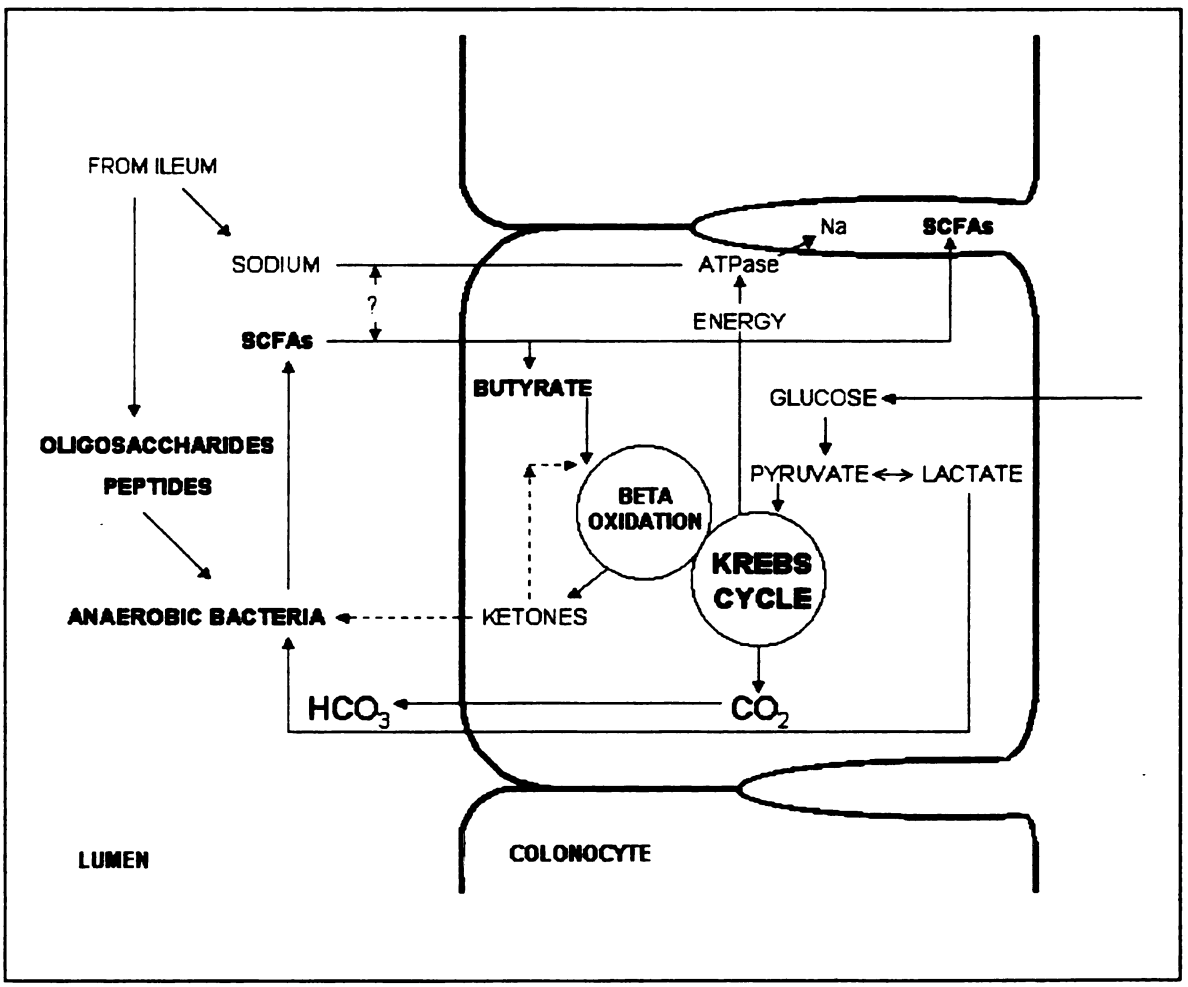
Molecular Weight = 88

Figure 3: Chemical Structure of Butyrate

APPENDIX C

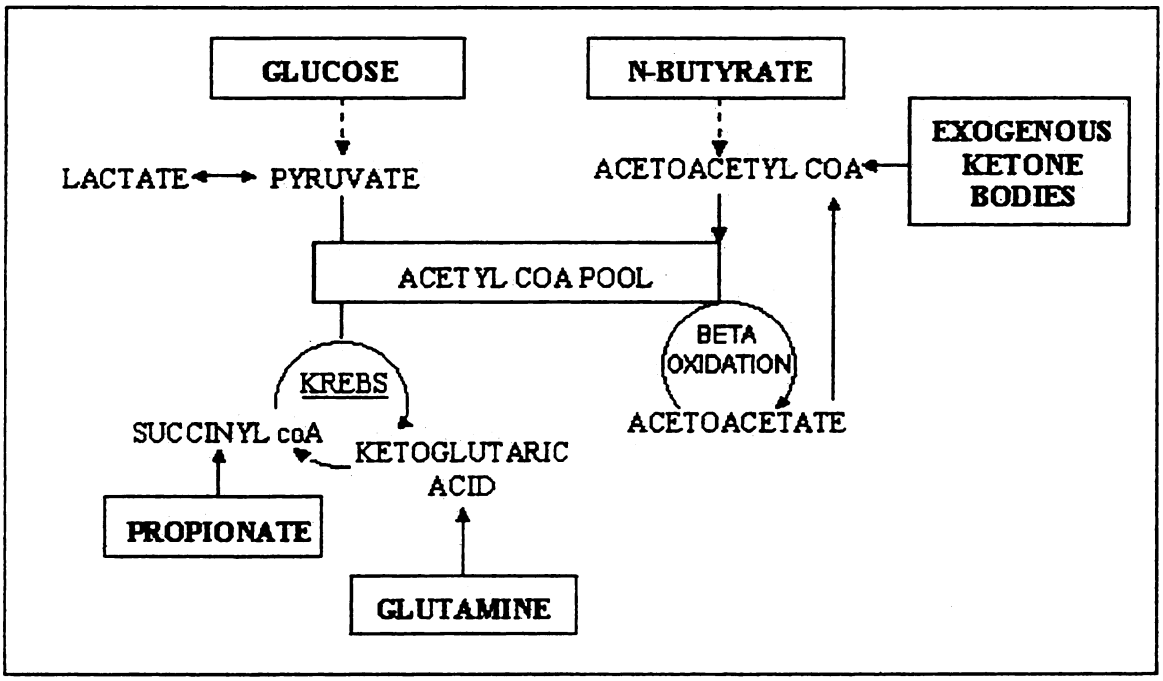
PROPOSED PRODUCTION, ABSORPTION AND UTILIZATION OF SCFA'S

Figure 1. SCFA production and metabolism



Adapted from Roediger, 1982.

Figure 2. Energy utilization of various respiratory fuels



Adapted from Roediger, 1982.

APPENDIX D

OMD, DMD, WHC, PHWC RAW DATA BY DONOR

Donor	Substrate	OMD	DMD	WHC	PWHC
A	Arabinogalactan	16.02	17.04	6.40	5.32
		17.35	16.62	6.66	5.54
	High Viscosity β-Glucan	44.27	49.15	7.42	3.92
		42.98	45.22	5.04	2.66
	Medium Viscosity β-Glucan	24.68	25.48	5.32	4.04
		21.80	22.65	5.01	3.81
	Low Viscosity β-Glucan	23.49	24.04	6.51	5.54
		5.97	5.73	6.91	5.88
	Solka-Floc	-1.46	1.03	4.54	4.63
		-3.92	-5.29	4.54	4.64
	Inulin/FOS	75.19	78.85	6.63	1.40
		77.18	78.86	7.27	1.54
	Mixed Fiber	82.98	83.14	8.00	1.43
		82.17	81.16	7.23	1.29
	Oat Flour	62.12	62.36	4.87	2.00
		55.15	55.46	4.97	2.04
	Oat Groats	14.64	14.28	5.77	5.07
		9.98	10.10	5.38	4.73
B	Arabinogalactan	30.48	33.31	6.16	4.21
		25.40	29.89	6.24	4.27
	High Viscosity β-Glucan	40.14	44.29	5.59	3.07
		42.13	45.84	5.78	3.17
	Medium Viscosity β-Glucan	39.98	45.74	5.81	3.45
		32.56	35.41	5.55	3.30
	Low Viscosity β-Glucan	35.68	39.93	4.60	2.71
		38.78	42.36	5.94	3.49
	Solka-Floc	1.41	4.46	4.50	4.36
		-0.99	1.42	4.77	4.63
	Inulin/FOS	72.15	79.50	8.21	1.52
		83.21	83.56	6.53	1.21
	Mixed Fiber	83.04	89.02	6.60	0.72
		84.88	89.18	4.22	0.46
	Oat Flour	61.63	61.97	4.07	1.51
		56.94	63.60	5.75	2.14
	Oat Groats	9.13	11.88	2.85	2.56
		6.50	8.20	3.45	3.11

OMD = organic matter disappearance (%), DMD = dry matter disappearance (%), WHC = g H_2O/g fermented residue dry matter, PWHC = g H_2O/g original substrate dry matter

Donor	Substrate	OMD	DMD	WHC	PWHC
C	Arabinogalactan	35.37	34.88	6.02	4.53
		16.13	14.64	5.64	4.24
	High Viscosity β-Glucan	25.70	24.22	6.27	4.83
		24.20	21.72	4.86	3.74
	Medium Viscosity β-Glucan	-1.66	-3.58	6.16	6.16
		2.62	3.47	5.85	5.85
	Low Viscosity β-Glucan	-3.37	-2.83	5.31	5.45
		1.10	-2.61	4.88	5.02
	Solka-Floc	-4.15	-3.79	4.95	5.29
		-7.83	-9.81	5.11	5.46
	Inulin/FOS	77.12	77.74	6.19	1.52
		73.03	73.28	7.26	1.78
	Mixed Fiber	84.08	83.89	5.96	0.91
		84.39	85.51	6.85	1.05
	Oat Flour	30.98	31.37	3.47	2.39
		32.40	31.10	3.18	2.19
	Oat Groats	2.18	3.13	2.57	2.47
		7.64	5.14	3.03	2.91
D	Arabinogalactan	26.89	28.47	6.53	4.81
		25.68	24.44	5.95	4.38
	High Viscosity β-Glucan	39.84	41.01	5.33	3.35
		32.62	33.22	5.31	3.34
	Medium Viscosity β-Glucan	11.76	11.71	5.92	5.24
		10.72	11.27	5.62	4.97
	Low Viscosity β-Glucan	13.69	17.36	3.06	2.58
		11.13	14.14	4.79	4.03
	Solka-Floc	-0.55	-0.35	3.24	3.14
		3.17	6.32	4.07	3.95
	Inulin/FOS	79.57	78.83	5.84	1.28
		78.02	77.27	6.17	1.35
	Mixed Fiber	85.30	84.90	6.14	0.99
		83.91	82.99	5.74	0.92
	Oat Flour	32.37	34.29	3.06	2.06
		30.90	31.43	3.71	2.49
	Oat Groats	2.29	1.39	5.00	4.70
		10.50	10.55	2.26	2.12

OMD = organic matter disappearance (%), DMD = dry matter disappearance (%), WHC = g H_2O/g fermented residue dry matter, PWHC = g H_2O/g original substrate dry matter

APPENDIX E

SCFA PRODUCTION RAW DATA BY DONOR

Donor	Substrate	SCFA Production (mmol/g original organic matter)			
		Acetate	Propionate	Butyrate	Total
A	Arabinogalactan	1.72	0.70	0.53	2.95
		1.83	0.74	0.47	3.04
		1.88	0.77	0.49	3.14
		1.74	0.72	0.46	2.93
	High Viscosity β-Glucan	2.96	0.35	1.27	4.59
		3.10	0.36	1.33	4.80
		4.46	0.61	1.97	7.05
		4.20	0.57	1.96	6.72
		3.17	0.35	1.36	4.89
		3.45	0.41	1.47	5.33
	Medium Viscosity β-Glucan	1.97	0.28	1.05	3.31
		1.82	0.26	0.96	3.05
		2.07	0.28	1.14	3.49
		2.35	0.31	1.28	3.94
	Low Viscosity β-Glucan	1.86	0.27	0.92	3.05
		1.55	0.24	0.77	2.56
		2.30	0.38	1.17	3.85
		1.90	0.32	0.95	3.16
		1.59	0.25	0.80	2.64
		1.59	0.25	0.81	2.65
	Solka-Floc	0.48	0.16	0.27	0.91
		0.59	0.19	0.24	1.02
		0.64	0.20	0.24	1.09
		0.62	0.20	0.23	1.04
	Inulin/FOS	2.21	0.56	3.37	6.14
		2.23	0.54	3.35	6.12
		1.85	0.53	3.15	5.53
		1.87	0.54	3.08	5.49
	Mixed Fiber	1.43	0.44	2.17	4.04
		1.69	0.52	2.50	4.71
		1.26	0.44	2.15	3.85
		1.35	0.49	2.36	4.20
	Oat Flour	1.94	0.67	2.30	4.91
		1.78	0.61	2.21	4.60
		2.28	0.73	2.70	5.71
		1.89	0.59	2.11	4.59
		2.32	0.72	2.59	5.63
		2.46	0.76	2.79	6.01
	Oat Groats	0.39	0.10	0.30	0.80
		0.38	0.09	0.28	0.75
		0.36	0.10	0.23	0.69
		0.33	0.09	0.22	0.64

Donor	Substrate	SCFA Production (mmol/g original organic matter)			
		Acetate	Propionate	Butyrate	Total
B	Arabinogalactan	2.24	0.87	0.56	3.67
		1.92	0.79	0.48	3.19
		2.45	1.11	0.64	4.20
		2.20	0.92	0.52	3.64
		2.19	0.93	0.53	3.65
	High Viscosity β-Glucan	2.03	0.47	1.41	3.91
		2.05	0.48	1.47	4.00
		2.69	0.67	1.94	5.30
		2.56	0.64	1.86	5.07
		2.26	0.55	1.61	4.42
		2.19	0.53	1.57	4.29
	Medium Viscosity β-Glucan	1.98	0.45	1.11	3.54
		2.17	0.49	1.19	3.84
		2.85	0.69	1.59	5.13
		2.81	0.69	1.57	5.07
		2.50	0.54	1.43	4.48
		2.27	0.48	1.29	4.05
	Low Viscosity β-Glucan	2.20	0.51	1.22	3.93
		2.69	0.69	1.58	4.97
		2.23	0.55	1.28	4.06
		2.24	0.46	1.29	3.99
		2.10	0.43	1.21	3.75
	Solka-Floc	0.58	0.16	0.21	0.96
		0.45	0.13	0.15	0.74
		0.54	0.19	0.20	0.92
		0.49	0.15	0.18	0.82
		0.53	0.18	0.18	0.89
	Inulin/FOS	2.09	0.31	1.46	3.85
		2.03	0.32	1.47	3.81
		2.50	0.36	1.70	4.56
		1.96	0.36	1.70	4.02
	Mixed Fiber	2.92	0.33	0.69	3.94
		3.28	0.37	0.80	4.45
		3.25	0.36	0.77	4.38
		2.90	0.31	0.70	3.91
	Oat Flour	5.02	0.41	1.29	6.72
		4.98	0.41	1.29	6.69
		4.96	0.42	1.34	6.72
		5.13	0.41	1.34	6.88
	Oat Groats	1.42	0.30	0.64	2.36
		1.41	0.30	0.62	2.34
		1.09	0.25	0.47	1.81
		1.11	0.25	0.47	1.82

Donor	Substrate	SCFA Production (mmol/g original organic matter)			
		Acetate	Propionate	Butyrate	Total
C	Arabinogalactan	1.90	0.57	0.40	2.86
		1.83	0.50	0.39	2.71
		1.79	0.53	0.35	2.67
		1.80	0.51	0.39	2.70
		1.86	0.58	0.38	2.82
	High Viscosity β-Glucan	3.12	0.77	0.85	4.75
		3.24	0.80	0.88	4.93
		3.07	0.95	0.89	4.91
		3.10	0.95	0.89	4.94
	Medium Viscosity β-Glucan	1.52	0.66	0.49	2.67
		1.21	0.52	0.40	2.13
		1.30	0.53	0.42	2.25
		1.36	0.56	0.44	2.36
	Low Viscosity β-Glucan	1.14	0.47	0.30	1.91
		1.07	0.44	0.28	1.79
		0.96	0.41	0.25	1.61
		0.78	0.33	0.20	1.31
	Solka-Floc	0.47	0.23	0.18	0.88
		0.45	0.22	0.18	0.85
		0.48	0.15	0.14	0.77
		0.32	0.14	0.12	0.57
		0.40	0.20	0.15	0.75
	Inulin/FOS	2.04	0.32	1.56	3.92
		2.13	0.32	1.69	4.14
		2.33	0.32	1.63	4.28
		2.22	0.31	1.64	4.17
	Mixed Fiber	1.98	0.48	1.44	3.91
		1.77	0.45	1.25	3.47
		1.69	0.43	1.28	3.40
		1.86	0.47	1.44	3.76
	Oat Flour	1.40	0.46	1.00	2.85
		1.51	0.51	1.13	3.15
		2.08	0.76	1.69	4.52
		1.97	0.73	1.59	4.29
	Oat Groats	0.25	0.09	0.17	0.51
		0.22	0.07	0.16	0.45
		0.31	0.10	0.22	0.63
		0.28	0.09	0.21	0.58

Donor	Substrate	SCFA Production (mmol/g original organic matter)			
		Acetate	Propionate	Butyrate	Total
D	Arabinogalactan	2.46	0.77	0.61	3.84
		2.40	0.82	0.60	3.82
		2.35	0.82	0.54	3.71
		2.52	0.90	0.56	3.98
		2.10	0.76	0.47	3.33
	High Viscosity β-Glucan	3.77	1.02	1.06	5.86
		3.64	1.08	1.11	5.83
		3.76	1.16	1.21	6.13
		4.12	1.22	1.27	6.61
		4.21	1.27	1.29	6.77
	Medium Viscosity β-Glucan	1.60	0.68	0.53	2.81
		1.53	0.64	0.50	2.67
		1.71	0.67	0.55	2.93
		1.69	0.73	0.61	3.04
	Low Viscosity β-Glucan	2.05	0.90	0.57	3.52
		2.03	0.87	0.55	3.46
		1.78	0.73	0.44	2.95
		1.70	0.69	0.42	2.81
	Solka-Floc	0.63	0.27	0.37	1.27
		0.51	0.19	0.28	0.98
		0.53	0.23	0.21	0.97
		0.49	0.19	0.17	0.85
	Inulin/FOS	2.80	0.60	2.66	6.06
		2.53	0.52	2.52	5.57
		2.81	0.54	2.64	5.99
	Mixed Fiber	3.07	0.71	2.25	6.04
		3.17	0.64	2.04	5.84
		2.01	0.45	1.82	4.27
		2.19	0.50	1.94	4.62
		2.30	0.66	2.15	5.10
	Oat Flour	1.35	0.47	0.99	2.81
		1.51	0.63	1.20	3.34
		1.36	0.55	1.06	2.98
		1.25	0.44	1.01	2.70
		1.44	0.47	1.13	3.04
	Oat Groats	0.70	0.24	0.47	1.41
		0.81	0.30	0.45	1.55
		0.86	0.30	0.63	1.79
		0.93	0.34	0.67	1.94

APPENDIX F

HUMAN SUBJECTS APPROVAL

MICHIGAN STATE
UNIVERSITY

June 1, 1999

TO: Dr.Norman HORD

RE: IRB# **99361** CATEGORY: 2-C

APPROVAL DATE:June 1, 1999

TITLE:WHOLE OAT UTILIZATION IN HUMANS

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the **UCRIHS approved this project.**

RENEWALS: UCRIHS approval is valid for one calendar year, beginning with the approval date shown above. Projects continuing beyond one year must be renewed with the green renewal form. A maximum of four such expedited renewals possible. Investigators wishing to continue a project beyond that time need to submit it again for a complete review.

REVISIONS: UCRIHS must review any changes in procedures involving human subjects, prior to initiation of the change. If this is done at the time of renewal, please use the green renewal form. To revise an approved protocol at any other time during the year, send your written request to the UCRIHS Chair, requesting revised approval and referencing the project's IRB# and title. Include in your request a description of the change and any revised instruments, consent forms or advertisements that are applicable.

PROBLEMS/CHANGES: Should either of the following arise during the course of the work, notify UCRIHS promptly: 1) problems (unexpected side effects, complaints, etc.) involving human subjects or 2) changes in the research environment or new information indicating greater risk to the human subjects than existed when the protocol was previously reviewed and approved.

If we can be of further assistance, please contact us at 517 355-2180 or via email: UCRIHS@pilot.msu.edu. Please note that all UCRIHS forms are located on the web: http://www.msu.edu/unit/vprgs/UCRIHS/

Sincerely,

David E. Wright, Ph. D.
UCRIHS Chair

DEW: db

cc: Julie Arnold
Kristen England

OFFICE OF
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