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BIOLOGICAL ANALYSIS OF PREBIOTICS IN VARIOUS PROCESSED FOOD MATRICES

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BIOLOGICAL ANALYSIS OF PREBIOTICS IN VARIOUS PROCESSED FOOD MATRICES

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The addition of prebiotic to a variety of food products has become a more common occurrence in recent years. Although research on the stability of prebiotics has been conducted, knowledge of the stability of prebiotics in processed foods is limited. The purpose of this research was to determine the biological stability of five prebiotics in a variety of food matrices when processed under various conditions. A biological test, the prebiotic activity assay, was used to test for stability of six food products (muffin, cookie, granola bar, breakfast cereal, sports drink, and bread) containing five different prebiotics (fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), resistant starch, and polydextrose). The prebiotic activity assay reflects the capability of a prebiotic to support the growth of a probiotic strain relative to an enteric strain and relative to growth of both on a non-prebiotic substrate. Due to the complex matrices of the food products as well as low concentrations of prebiotics, the prebiotic activity assay was not sufficiently sensitive to assess biological stability in these food products. Additional food products (cracker, granola, and sports drink) were produced without background sugars and included a higher concentration of prebiotic, 10%. The prebiotic activity assay was used to assess the biological stability of prebiotics within these food matrices. Overall, FOS

and inulin were stable when exposed to mild to moderate heating, but were biologically degraded when exposed to an acidic environment and moderate heat. GOS was stable when exposed to mild to moderate heat as well as when exposed to an acidic environment that was processed with moderate heat. Resistant starch posed problems with being accurately tested for prebiotic stability, and no strong conclusions were able to be made based on the results obtained through this method. The prebiotic activity assay was able to assess the biological stability of prebiotics in food matrices when exposed to several processing conditions, although the assay was better suited for certain prebiotics as well as certain food matrices.

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Chapter 1

Literature review

Introduction:

The gastrointestinal tract is inhabited by a vast array of microorganisms, the bulk of which are located in the colon (Tuohy et al., 2003). The colon is largely inhabited by anaerobic bacteria with cell numbers exceeding 10^{11} per gram (Flint et al., 2007). Colonization of the human gastrointestinal tract is initialized soon after birth, and after approximately two years, is relatively comparable to the adult microflora (Steer et al., 2000). Growth of this large bacterial population is supported, in part, by in-host secretions or from dietary carbohydrates that are not hydrolyzed and absorbed in the small intestine (Ito et al., 1990). The development and population of specific groups of gut bacteria may also be influenced by the diet consumed by the individual (Collins and Gibson, 1999). Importantly, the composition of the gut microbiota has been found to influence the health and nutrition of the host due to the supply of nutrients, conversion of metabolites, and interactions with host cells (Flint et al., 2007). The colonic microbiota has also been associated with certain diseases including inflammatory bowel disease, gastroenteritis, and colon cancer (Steer et al., 2000; Venter, 2007).

Therefore, there is now considerable interest in using the diet to manipulate the gut microflora (Gibson and Roberfroid, 1995; Flint et al., 2007). Beneficial changes within the gut microbiota have been attributed to increases in *Bifidobacterium* and *Lactobacillus*, which are generally thought to have health-promoting properties (Gibson and Roberfroid, 1995). The use of carbohydrates, known as prebiotics, that resist digestion and can be metabolized by certain gut bacteria has attracted most of this attention (Rastall, 2010). Prebiotics have been suggested to have several beneficial effects, including promotion of beneficial bacterial growth, stimulation of intestinal

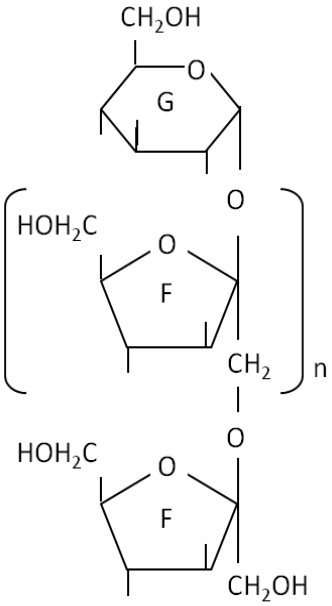
peristalsis, production of short chain fatty acids, and a shortened orofecal transit time (Cummings et al., 2001). Short chain fatty acids, along with carbon dioxide, hydrogen, and methane (CO_2, H_2 and CH_4, respectively) are products of fermentation by gut bacteria, and those may have beneficial effects both to the gut environment and the host. These effects include serving as sources of energy, regulation of gene expression and cell differentiation, and anti-inflammatory properties (Flint et al., 2007).

Prebiotics were originally defined as ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health’ (Gibson and Roberfroid, 1995). This definition has since been revised as ‘a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health’ (Gibson et al., 2004).

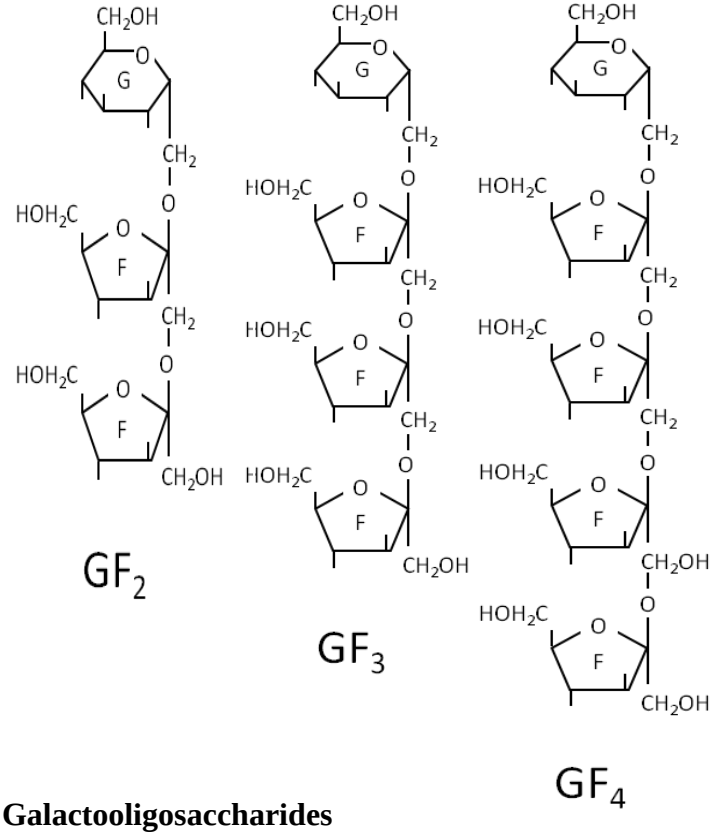
Dietary carbohydrates must also adhere to a list of criteria in order to be classified as prebiotics. These criteria are 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (Gibson et al., 2004). Dietary carbohydrates that show prebiotic ability include fructans - fructooligosaccharides (FOS) and inulin, galactooligosaccharides (GOS), polydextrose, resistant starch, soy-oligosaccharides, xylooligosaccharides, isomaltooligosaccharides, and lactulose (Gibson et al., 2004) (Fig. 1.1). The sources and properties of selected prebiotics will be described in the next section.

Figure 1.1 Prebiotic structures

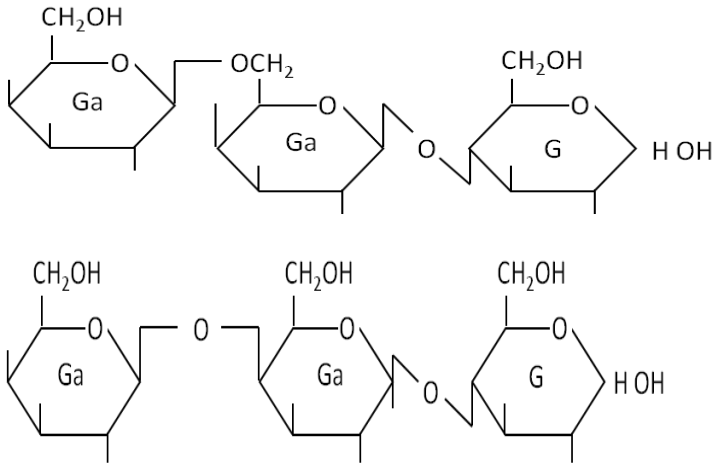
Inulin



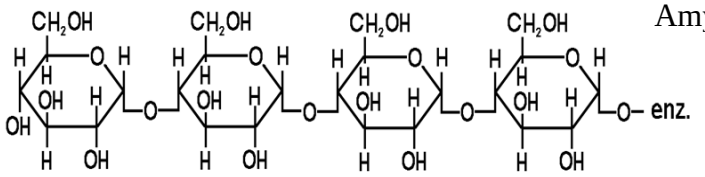
Fructooligosaccharides (GF_n)

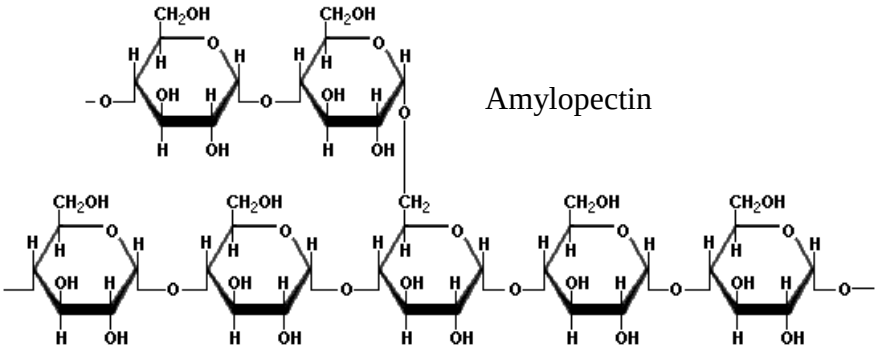


Galactooligosaccharides

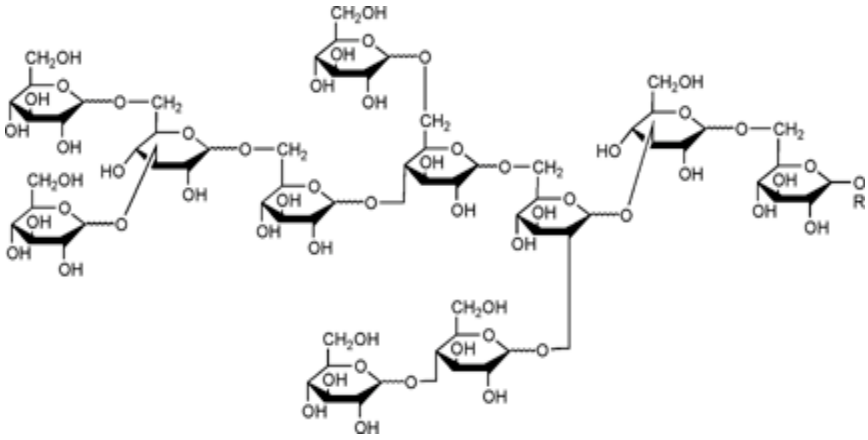


Resistant Starch





Polydextrose



Prebiotic oligosaccharides:

Fructooligosaccharides: Fructooligosaccharides (FOS) are linear fructose oligosaccharides consisting of a D-glucose monomer linked α-(1→2) to two or more β-(2→1)-linked D-fructosyl units (Yun, 1996). Fructooligosaccharides are composed of a mixture of 1-kestose (GF_2), nystose (GF_3), and 1^F-fructofuranosyl nystose (GF_4), also referred to as GF_n type FOS. Another form of FOS available is referred to as FF_n type FOS (Goh et al., 2006). This is produced from the partial hydrolysis of chicory inulin. The degree of polymerization can range from 2 to 10, but there is an average of 4 (Niness, 1999). The FF_n form is a linear chain of β-(2→1) glycosidic bonds of D-fructose. A terminal glucose may be linked by an α-(1→2) glycosidic bond (Makras et al., 2005).

FOS are approximately one-third as sweet as sucrose and calorie-free (Yun, 1996). Due to these characteristics, FOS can be used in foods where sucrose is too sweet as well as in foods for diabetics and lower calorie food products (Yun, 1996). Currently, FOS are added to several foods including dairy products, frozen desserts, baked goods, breakfast cereals, fillings, and fruit preparations (Franck, 2002).

Inulin: Inulin is a plant-derived polysaccharide consisting of fructose monomers. Commercially inulin is most commonly extracted from chicory roots using a hot water diffusion process (Niness, 1999). Inulin has an average degree of polymerization of 10 to12 and a distribution of molecules with chain length from 2 to 60 (Niness, 1999). Recently, a high performance (HP) inulin has become available. The shorter-chain molecules are removed, and the resulting product has an average degree of polymerization of 25 with molecular distributions from 11 to 60 (Niness, 1999).

Chemically, inulin can be a linear chain of either α-D-glucopyranosyl - [β-D-fructofuranosyl]$_{n-1}$ - β-D-fructofuranoside (GF_n) or β-D-fructofuranosyl - [β-D-fructofuranosyl]$_{n-1}$ - β-D-fructofuranoside (FF_n) (Roberfroid, 2007). The fructosyl-fructose linkages always occur as β-(2→1) in inulin while the fructosyl-glucose linkage is β-(2↔1) (Roberfriod, 2007). Inulin can escape digestion in the human gastrointestinal tract due to the β-(2→1) bonds, but once in the colon, β-fructosidase-producing bacteria can hydrolyze this bond (Makras et al., 2005).

In the food industry, inulin is used not only as a prebiotic, but also for its various functional properties. In particular, it is used as a fat-mimicker and a fiber enhancer (Niness, 1999). A wide variety of foods contain inulin including beverages, dairy products, baked goods, cereals, and frozen desserts (Franck, 2002; Hazen, 2011; Niness, 1999). Inulin is also found naturally in foods such as chicory, Jerusalem artichoke, onion, garlic, banana, asparagus, and leek (Venter, 2007).

Galactooligosaccharides: Galactooligosaccharides (GOS) are oligosaccharides that consist mainly of galactose monomers linked together through several different structural configurations (Playne and Crittenden, 2009). GOS mimic the oligosaccharides found naturally in human milk and selectively stimulate beneficial bacteria, primarily bifidobacteria, in infants (Akiyama et al., 2001; Ito et al., 1990). Typically, GOS consists of between 2 to 5 β-(1→6) galactopyranosyl monomers linked to a terminal glucopyranosyl residue by an α-(1→4) glycosidic bond (Playne and Crittenden, 2009).

GOS are often produced through transglycosylation during the enzymatic hydrolysis of lactose (Akiyama et al., 2001). The β-galactosidase enzymes are used to complete the transglycosylation reaction (Akiyama et al., 2001). β-galactosidase is

produced by certain yeasts and bacteria. The organism used for transglycosylation can have an effect on the linkages formed in the oligosaccharide (Playne and Crittenden, 2009).

GOS are typically added to infant formula to create a product with a greater similarity to human milk (Akiyama et al., 2001). Studies have shown that an intake of GOS leads to an increase of fecal bifidobacteria (Davis et al., 2010; Ito et al., 1990).

Resistant Starch: Resistant starch is a type of starch that is resistant to digestion. Like all starches, resistant starches are considered polysaccharides in which several monosaccharides, in this case glucose, are linked together by either α-D-(1→4) and/or α-D-(1→6) linkages (Sajilata et al., 2006). Two structural components form starch, amylose and amylopectin. Amylose consists of a mostly linear polymer with glucose monomers linked by α-D-(1→4) bonds, while amylopectin is a larger, highly branched molecule that consists of glucose monomers liked by both α-D-(1→4) and α-D-(1→6) (Sajilata et al., 2006). Resistant starches are classified as an incomplete digestion *in vitro* of starches in food products that have undergone cooking and cooling. Recently this definition has been expanded to include starch and starch degradation products that resist small intestinal digestion and enter the large bowel in healthy humans (Topping and Clifton, 2001). Resistant starch is classified into four fractions, RS_1 (type I), RS_2 (type II), RS_3 (type III), and RS_4 (type IV) (Sajilata et al., 2006). RS_1 is a physically inaccessible starch, typically found in partially milled grains and seeds. RS_2 is in a resistant granular form, therefore, is resistant to enzyme digestion. RS_3 is retrograded amylose formed during the cooling of gelatinized starch. RS_4 is chemically modified to contain bonds not typically found in starch (Sajilata et al., 2006).

Resistant starch is used in food products due to its physicochemical properties including swelling, increased viscosity, gel formation, and water-binding capacity (Sajilata et al., 2006). Due to the resistance to digestive enzymes, resistant starch can be used as a dietary fiber (Sajilata et al., 2006). The slow rate of digestion can be beneficial for controlled glucose release applications (Sajilata et al., 2006). There is also evidence that resistant starch may have prebiotic activity due to the resistance to digestion and subsequent fermentation, resulting in an increase in bowel health (Cummings and Englyst, 1987; Brown et al., 1995).

Polydextrose: Polydextrose is considered to be a resistant polysaccharide and, in certain countries, a soluble fiber (Craig et al., 1999). Polydextrose is produced through a vacuum thermal polymerization of glucose, using sorbitol as a plasticizer and citric acid as a catalyst. As a result of random polymerization and branching, a variety of glucosidic bonds are produced with α-(1→6) bond predominating (Craig et al., 2000). The R-groups (Fig 1.1) within the structure can be hydrogen, glucose, sorbitol, citric acid, or a continuation of the polydextrose polymer (Craig et al., 1999). The starting ratio of glucose:sorbitol:citric acid is 89:10:1 (Craig et al., 1994). Due to the complexity of the molecule, mammalian digestive enzymes are unable to readily hydrolyze the molecule (Craig et al., 2000). Polydextrose has an average degree of polymerization of 12 and an average molecular weight of ~2000 (Craig et al., 1999).

Polydextrose is added to foods for a variety of purposes. It increases the fiber content of food, and is thought to be fermentable in the large intestine increasing beneficial bacteria and their metabolic products (Craig et al., 2000). Polydextrose is not completely hydrolyzed, therefore, contains only 1 kcal per gram. It is often used in low

calorie food in place of sucrose and fat due to similarities in texture and body (Chinachoti, 1995).

Measurement of prebiotics in food:

Due to the increased use of prebiotics in foods, there is interest in assessing the level of prebiotic within a food product. Several methods exist to determine the amount and activity of a prebiotic including, chemical and biological methods. Biological methods include both *in vivo* and *in vitro* methods (Rastel, 2010). *In vivo* methods typically result in the most accurate results, but are time consuming and costly. An alternative to *in vivo* methods are *in vitro* methods, which provide a more rapid screening with an ease of testing and a lower cost (Hur et al., 2011). *In vitro* methods also have the advantage of not being hindered by biological variation that occurs with *in vivo* studies, there are no ethical constraints, and the food product being tested does not have to be GRAS (Minekus et al., 1995).

***In Vitro* methods:** *In vitro* methods are commonly used to determine if a specific food product will have prebiotic activity (Rastall, 2010). *In vitro* methods range from straightforward batch methods, to complex digestive and fermentation systems designed to mimic the human digestive system (Rastall, 2010) (Table 1.1).

Batch methods are the least complicated *in vitro* method. Batch methods consist of the test substrate, nutrient media, and either a fecal slurry or specific bacteria, incubated together in a suitable vessel (Rycroft et al., 2001). Typically, the only control applied to the fermentation system is temperature. pH and atmospheric control may be implemented depending on the experimental design (Rastall, 2010). Batch fermentation systems generate preliminary data that can provide insight to changes that occur to fecal

bacteria during the testing period (Rycroft et al., 2001). The disadvantage to batch methods is the lack of information on how the prebiotic survives the gut, as well as interactions with the host (Rastall, 2010).

Multi-stage fermentation systems are designed to mimic the human digestive system, including portraying a select few or all steps involved in the digestion and fermentation that would occur *in vivo*. Several multi-stage fermentation systems have been designed and validated to determine prebiotic activity (Rastall, 2010). The different systems vary in complexity; therefore, the results obtained from these systems also vary (Rastall, 2010).

Table 1.1: *In vitro* systems to test for prebiotics

In Vitro System	Properties	Benefits	Reference
Batch methods	Temperature control, possible pH and atmospheric control	Simple execution, low cost, preliminary data	Rycroft et al., 2001; Rastall, 2010
Three Stage Fermentation System	Temperature, pH and atmospheric control; Fermentation modeling	More extensive results than batch methods, but lacking digestion	Gibson et al., 1988
SHIME	Temperature, pH and atmospheric control; Digestion and small and large intestine modeling	Results mimicking *in vivo*, but lacking nutrient absorption	Molly et al., 1993
TIM I and TIM II	Temperature, pH, atmospheric, absorption, and waste removal control; complete digestive system modeling	Allows for nutrient absorption as well as waste removal to optimize fermentation growth	Minekus et al., 1995; Minekus et al., 1999; Meunier et al., 2008

A three stage continuous culture system to investigate changes in the microbial population using different substrates was developed (Gibson et al., 1988). This system had temperature control (37°C) and pH control, with vessels 1, 2, and 3 maintained at 5.5, 6.2, and 6.8, respectively. The three vessels were meant to represent the proximal and distal colon (Macfarlane et al., 1998). The system was not sparged with anaerobic gases, but each vessel was maintained with an oxygen free, nitrogen gas. The medium from vessel 1 was pumped at a controlled rate to vessel 2, medium from vessel 2 was pumped to vessel 3 in a continuous fashion, and the waste was then collected (Gibson et al., 1988). The medium was inoculated with fecal matter to start the fermentation. Samples are able to be extracted from the system when desired, including gases and bacteria (Gibson et al., 1988).

A more complex five-step multi-chamber simulated human intestinal microbial ecosystem (SHIME) was developed (Molly et al., 1993). This five step system represents both the small and large intestine. The first two steps represent the small intestine and were simulated by a two-step fill and draw system; the remaining three steps represent the large intestine and were a three-step reactor (Molly et al., 1993). The volume in the separate vessels was based on the *in vivo* residence time in the corresponding segments in the gastrointestinal tract. Pumps were used to move the contents of one vessel to the next vessel; the pumps for the two vessels representing the small intestine worked semi-continuously, while the pumps for the three vessels representing the large intestine worked continuously (Molly et al., 1993). This system had different ports in each vessel for input and output of medium, obtaining samples of the liquid phase and headspace

gases, pH, as well as ports to control the pH, and flushing the headspace gases (Molly et al., 1993).

Pancreas acetone powder dissolved in 150 mM sodium bicarbonate was added at a rate of 100 ml/h for one hour to vessel 1 to simulate the acidic effect of the stomach. The pH in this vessel started at 2.0 and increased to 7.5 due to the addition of the pancreas acetone powder solution. The pH of the remaining vessels was controlled between 6.5 and 7.0. The atmosphere of the vessels was controlled by sparging the headspace using nitrogen (Molly et al., 1993). Vessels 1 and 2 were inoculated with the food product undergoing testing and a fecal slurry was added to vessels 3, 4, and 5 (Molly et al., 1993). One limitation to this method is the lack of absorption of nutrients that would normally occur in a healthy human digestive tract (Molly et al., 1993).

Another system that has been developed is the *in vitro* model TIM. The major advantage to this model is a digestion process, with an absorption system using a dialysis membrane (Meunier et al., 2008). The TIM system consists of two separate systems TIM I and TIM II. TIM I represents the stomach and small intestine and TIM II represents the large intestine. Another advantage to this system is the removal of water and microbial metabolites, resulting in an increase in microbial cell densities (Minekus et al., 1999). This system was validated and found to have physiological levels of *Bifidobacterium*, *Lactobacillus*, Enterobacteriaceae and *Clostridium* (Minekus et al., 1999).

The stomach and small intestine system (TIM I) contains four compartments representing the stomach, duodenum, ileum, and jejunum (Minekus et al., 1995). The compartments consisted of glass tubes with flexible walls inside, enclosing water between the glass and flexible portion. The gastrointestinal transit time and the pH of the

different compartments were based on information gathered from the human digestion system. Bile salts were added at concentrations based on data from the literature, to the compartments of the small intestine (Minekus et al., 1995). One key feature of this system was a dialysis system used in the small intestine to absorb products of digestion (Minekus et al., 1995).

The large intestinal system (TIM II) contained four different glass units that were connected. Inside each of the glass units, there was a flexible wall (Minekus et al., 1999). The temperature of the system was regulated at 37°C using water pumped between the glass and the flexible wall (Minekus et al., 1999). The pressure of the water was computer controlled to mimic peristaltic movements. This was achieved by applying pressure onto the flexible wall, creating a peristaltic wave. As a result of this pressure, the chyme was able to move through the system (Minekus et al., 1999).

The pH was measured and controlled throughout the system to remain constant. The electrolyte and metabolite concentrations were controlled using a fresh dialysis liquid that was pumped into the lumen portion of the system; the used dialysis liquid was collected from the system (Minekus et al., 1999). The amount of chyme within the system remained at a set level using a pressure sensor (Minekus et al., 1999). The feeding medium was added to the system with a peristaltic valve system and the chyme was removed from the system using a peristaltic valve pump (Minekus et al., 1999). Anaerobic conditions were maintained throughout the system using nitrogen (Minekus et al., 1999).

Several characteristics of the TIM system including removal of metabolites, absorption of water separately from the microorganisms, and concentrated feeding,

allowed this model to maintain the number of microorganisms and metabolites at levels that likely occur within the human body (Minekus et al., 1999). Overall, the TIM *in vitro* system is a good alternative to *in vivo* studies due to the extensive control of the system, addition and removal of constituents, and use of a dialysis membrane.

Quantitative approaches

Quantitative approaches have been developed to compare prebiotics and their activity (Huebner et al., 2007; Palframan et al., 2003; Vulevic et al., 2004). All quantitative approaches are based on the selectivity of prebiotics. An increase in the populations of bifidobacteria and/or lactobacilli results in an increase in prebiotic activity, while an increase in enteric bacteria results in a decrease in prebiotic activity.

One of the first quantitative approaches as described in the literature is the prebiotic index (PI). This equation focuses on the changes of several microbial populations (Palframan et al., 2003). The PI equation takes into account bifidobacteria (Bif), bacteroides (Bac), lactobacilli (Lac), and clostridia (Clos) as follows:

Equation 1.2: Prebiotic index

$$PI = (Bif/Total) - (Bac/Total) + (Lac/Total) - (Clos/Total)$$

The numerator is obtained by the numbers at sample time / numbers at inoculation and the total bacteria is obtained by the total number of bacteria at sample time / total number of bacteria at inoculation (Palframan et al., 2003).

The second quantitative approach is an expansion of the prebiotic index referred to as the measure of the prebiotic effect (MPE) (Vulevic et al., 2004). This quantitative approach focuses on the growth of bacterial populations within the gut as well as the production of short chain fatty acids produced (Vulevic et al., 2004). This is achieved

through the use of three different equations. The first equation is focused on the concentration of substrate and how quickly the bacteria ferment the given substrate. The rate of assimilation is calculated by:

Equation 1.3: Rate of assimilation

$$S_t = S_0 - A_r t \quad (1)$$

where S_t is the substrate concentration after the time interval, t in hours, S_0 is the initial concentration and A_r is the rate of assimilation.

The second equation is based on the rate of growth for the bacterial populations using the following equation:

Equation 1.4: Rate of growth

$$\ln N_t = \ln N_0 + \mu t \quad (2)$$

where N is the total number of bacteria after the time interval, t in hours, N_0 is the initial number of bacteria and μ is the specific growth rate (Vulevic et al., 2004). This information is then used in an adjusted PI equation as follows:

Equation 1.5: Adjusted prebiotic index

$$PI_m = \mu_{max}Bif + \mu_{max}Lac + \mu_{max}Eub - \mu_{max}Bac - \mu_{max}Clos - \mu_{max}EC - \mu_{max}SRB \quad (3)$$

where Bif is bifidobacteria, Lac is lactobacilli, Eub is eubacteria, Bac is bacteroides, Clos is clostridia, EC is *Escherichia coli* and SRB is sulphate-reducing bacteria.

The third equation is based on short chain fatty acid (SCFA) production. The substrate used will promote the growth of certain bacteria and the growth of these bacteria will lead to a unique production of SCFA. The equation to determine total SCFA is as follows:

Equation 1.6: Total short chain fatty acids

$$T_{SCFA} = A + B + P + L \quad (4)$$

where A is acetate, B is butyrate, P is propionate and L is lactate (Vulevic et al., 2004). This equation could be modified to determine the ratio of lactate to total SCFA, since lactic acid is most often produced by lactobacilli and bifidobacteria. This modified equation is as follows:

Equation 1.7: Ratio of lactate to total short chain fatty acids

$$\text{Ratio} = \mathrm{d}L/\mathrm{d}T_{\text{SCFA}} \quad (5)$$

where d is the difference between the initial mass and the mass at the sampling time point (Vulevic et al., 2004).

The three equations (1, 3, and 5) can be combined to create the MRE equation.

Equation 1.8: Measure of the prebiotic effect

$$\text{MPE} = \frac{1}{2}\sqrt{x^2y^2 + x^2z^2 + y^2z^2}$$

where *x* is the rate of substrate assimilation (A_r) [Eq. (1)], *y* is the adjusted PI [Eq. (3)] and *z* is the ratio of lactate over the total SCFA [Eq. (5)] (Vulevic et al., 2004).

The third quantitative equation is the prebiotic activity score (Huebner et al., 2007). This equation is based on the ability of a specific substrate to support the growth of a specific organism relative to other organisms and relative to growth on a non-prebiotic substrate, such as glucose (Huebner et al., 2007). This equation results in a positive number for substrates that are both metabolized as well as glucose by probiotic bacteria, and that increase probiotic bacteria but not other intestinal bacteria (Huebner et al., 2007). The resulting score will be higher for substrates with more prebiotic activity. The prebiotic activity score is as follows:

Equation 1.9: Prebiotic activity score:

$$\left[\frac{(\text{probiotic}\log\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0 hr})}{(\text{probiotic}\log\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 0 hr})}\right] -$$

$$\left[\frac{(\text{enteric}\log\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0 hr})}{(\text{enteric}\log\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 0 hr})}\right]$$

(Huebner et al., 2007).

The use of an *in vitro* method along with a quantitative approach, allows for the prebiotic activity of a substrate to be determined. With the resulting score of activity, it is possible to compare prebiotics. It would also be possible to determine the level of prebiotic within a food matrix and, in turn, measure the biological stability of that prebiotic.

Stability of prebiotics:

When prebiotics are added to processed foods, stability during processing becomes an important factor. Processing conditions such as heat, acidity, and Maillard reactions may have an effect on the stability of prebiotics (Huebner et al., 2008). Several studies have been completed to determine the chemical stability of prebiotics when exposed to certain processing conditions. The stability of a prebiotic varies based on its properties. GOS appears to be relatively stable, although susceptible to Maillard browning; FOS and inulin are susceptible to acid hydrolysis; Polydextrose is stable, but can be degraded with extreme conditions (Beer et al., 1991; Blecker et al., 2002; Courtin et al., 2009; Klewicki, 2007; Playne and Crittenden, 1996). A handful of studies have

determined the effect of the food matrix on stability during processing (L'Homme et al., 2003; Keenen et al., 2011).

FOS and inulin were susceptible to acid hydrolysis when exposed to a low pH (3) and a moderate temperature (70°C) (Blecker et al., 2002). When released fructose molecules were monitored, there was an increase of fructose when chain lengths were shorter, thus FOS was more rapidly hydrolyzed than inulin. Inulin was hydrolyzed at a slower rate to begin and increased as the chain length was decreased (Blecker et al., 2002). Similar studies have concluded acid hydrolysis has occurred for FOS (Courtin et al., 2009; L'Homme et al., 2008)

When the stability of FOS was determined in processed fruit products, it was determined that the food matrix (products containing apple) had a greater effect on stability than exposure to normal processing conditions (L'Homme et al., 2003). The matrix of the food product may affect the prebiotic before any processing occurs on the product.

Hydrolysis of FOS and inulin during both thermal and high hydrostatic pressure processing was determined in apple purees (Keenen et al., 2011). There was hydrolysis of both FOS and inulin when processed using thermal and high hydrostatic pressure processing. However, a difference in stability was noticed between FOS and inulin for processing treatments. There was no difference in hydrolysis between thermal (~23%) and high hydrostatic pressure processing (~15%) for FOS, while thermal processing led to a greater hydrolysis (~30%) for inulin than high hydrostatic pressure processing (~10%) (Keenen et al., 2011).

When exposed to extreme dry heat (195°C), inulin was degraded substantially, to a degree of polymerization of less than 5, after 30 minutes (Bohm et al., 2005). Inulin may also encounter hydrolysis from inulinases. This hydrolysis was increased with an increase in temperature to a point, but once the temperature reaches a certain level, around 50°C, the inulinases started to denature (Cantana et al., 2007).

When exposed to high temperatures and low pH, GOS was relatively stable, especially when compared to FOS (Klewicki, 2007). When heated at temperatures as high as 95°C at pH levels as low as 2.7, GOS remained at levels near 100%. Whereas, FOS exposed to these same conditions was dramatically hydrolyzed, with remaining FOS varying between 13 and 80% depending on time exposed. Only when the temperature of exposure was increased to 120°C and the pH was lower than 3, was degradation observed for GOS (Playne and Crittenden, 1996). GOS was subject to browning when in the presence of amino acids, and occurred at levels greater than 80% at a pH of 7 (Playne and Crittenden, 1996).

Certain processing conditions can increase the level of resistant starches in foods due to retrogradation, although when high moisture and temperature are present in processing conditions the amount of resistant starch can be lowered (Sajilata et al., 2006). When exposed to a mild acidic environment, resistant starch can become hydrolyzed (Mun and Shin, 2006). Resistant starch was subjected to a mild acidic environment for an extended period of time (30 days) to observe hydrolysis. The extent of hydrolysis (5 to 44%) was dependent on type of resistant starch (RS_4 and RS_3, respectively) (Mun and Shin, 2006).

Polydextrose is stable under most processing conditions. However, extreme processing conditions, such as high temperature or low pH, may cause slight degradation. Polydextrose appeared to be stable until temperature of 85°C, then degradation occurred dependent on pH (Beer et al., 1991). This degradation was seen in a depolymerization of polydextrose producing glucose molecules (Craig et al., 1994). Free glucose molecules were present after high temperature combined with a low pH, as well as after storage of polydextrose in an acidic environment (Beer et al., 1991). Although the prebiotic is degraded chemically, biologically the prebiotic may still be available to colonic bacteria.

Biological studies have been conducted on stability of prebiotics, but are limited. When the prebiotic is chemically modified or degraded, there is a possibility that the degraded fractions of the prebiotic still provide biological activity in the large intestine (Huebner et al., 2008). The biological activity of FOS and inulin was determined using an *in vitro* batch method for samples that were exposed to heat, acidity, and Maillard reaction conditions (Huebner et al., 2008). The effect of low pH (3) alone did not change the prebiotic activity, but when combined with heat (85°C for 30 minutes), loss of activity was observed for FOS and inulin. The Maillard reaction conditions did not significantly affect the prebiotic activity for FOS and inulin (Huebner et al., 2008).

Another study examined the effect of heat treated inulin and how that degraded inulin affected intestinal bacteria (Bohm et al., 2006). Inulin was exposed to extreme dry heat (165° and 195° for 30 minutes) conditions. When used to stimulate intestinal bacteria, there was an increase in beneficial bacteria, suggesting that the degraded products still act as prebiotics (Bohm et al., 2006).

Although research has been completed on the stability of prebiotics when exposed to certain processing conditions, there is minimal research on the stability of prebiotics in processed foods. Therefore, this research will be focused on determination of stability of prebiotics in processed foods using a biological method. The processed foods used in this study will include common foods exposed to various processing conditions. Several prebiotics will be tested in the various food products.

To determine the biological stability of prebiotics in processed foods, a biological assay will be conducted. The biological assay will consist of a batch method due to the simplicity of the method and extensive amount of samples to be tested. The biological assay will follow the set up designed in the study by Huebner et al., (2007). This biological assay was chosen based on its ability to determine a prebiotic score that effectively portrayed functionality of several prebiotics and allowed comparison between samples in previous studies. To determine a quantitative score for the activity within the food sample, the prebiotic activity score (Huebner et al., 2007) equation will be used. This equation was chosen based on simplicity of the equation as well as standardizing the prebiotic activity against a non-prebiotic sugar, in this case glucose, and against a selected non-fermenting organism.

Chapter 2:

Materials and Methods

2.1. Prebiotics

The prebiotics used in this study included fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), resistant starch and polydextrose. The FOS used was Nutraflora P-95, obtained from GTC Nutrition (Golden, CO, USA). FOS is a white powder that contains 100% carbohydrate. The carbohydrates in the product are FOS (95.5%), sucrose (3.3%), and glucose and fructose (1.2%). The FOS within the product are all short chain oligosaccharides in the form of GF_2 (33.8 %), GF_3 (50.1%), and GF_4 (11.6%). FOS is a soluble product that is also dispersible.

The inulin used was Orafti GR, obtained from Orafti Group (Tienen, Belgium). This inulin originates from chicory and is in the form of white granulated powder. It is a mixture of oligo- and polysaccharides consisting of fructose molecules with a terminating glucose molecule. The degree of polymerization of chicory inulin is between 2 and 60, with an average greater than 10. The carbohydrate content of this inulin is greater than 99.5%. The purity of the inulin is greater than 92% with the remainder of the sugars consisting of less than 8% sucrose, fructose, and glucose. Inulin is soluble in water with good dispersability.

The GOS used was Purimune, obtained from GTC Nutrition (Golden, CO, USA). The appearance of GOS is a white powder. The purity of this GOS is 90%, with the remaining sugars consisting of lactose (7%), glucose (1%), and galactose (1%). GOS is a soluble product that is relatively stable in high temperatures and low pH.

The resistant starch used was Hi-Maize 260, obtained from National Starch (Bridgewater, NJ, USA). This resistant starch originated from corn and is a white

powder. It is classified as a type II resistant starch. It contains 98% carbohydrate, all of which are complex carbohydrates, and a minimum of 60% which is fiber.

The polydextrose that was used was Litesse Ultra, obtained from Danisco (Ardsley, NY, USA). The appearance of polydextrose is a white powder. Litesse Ultra contains 100% carbohydrate, of which less than 0.25% are others sugars. This polydextrose is a polymer of D-glucose with sorbitol at the terminal end. Polydextrose is soluble, up to 80% at room temperature.

2.2. Manufacture of initial food products

The products that were formulated with prebiotics included muffin, cookie, granola bar, breakfast cereal, sports drink, and bread. Frequently consumed products that were able to withstand the addition of prebiotic were criteria for food products used in this study. The products selected also portray a wide variety of food matrices that were exposed to several processing conditions, including baking, extrusion, low pH, and pasteurization. The food products were formulated to contain approximately 1% prebiotic. A 1% addition would allow the product to remain similar in appearance, texture and taste to a non-prebiotic product. The products were produced at the University of Nebraska - Food Science and Technology department by graduate students, Michelle Hoffman and Emily Ang. Three trials of each product were produced with the exception of one trial produced for bread.

2.2.1. The products and their formulations

1. The muffin was a chemically leavened baked product, with a matrix containing high fat and high moisture. The formulation for the muffins is described in Appendix A. The muffins were produced in batches of 24 muffins with each muffin weighing

approximately 59.5g. The muffins were stored in Ziploc bags (SC Johnson, Racine, WI, USA) at -17.8°C until sampled for the prebiotic activity assay. Two muffins from each trial were sampled along with two from the control trial.

2. The cookie was a sugar cookie, a chemically leavened product with a lower moisture content. The formulation of the sugar cookie is described in Appendix B. The cookies were placed in Ziploc bags and stored at room temperature until sampled for the prebiotic activity assay. Two cookies from each trial were sampled along with two from the control trial.

3. The granola bar was an unbaked product with a high sugar and high fat matrix. The granola bar was the only product where the prebiotics were subjected to heat. The formulation for the granola bar is described in Appendix C. The granola bars were divided, wrapped in plastic wrap, placed in Ziploc bags, and stored at room temperature until sampled for the prebiotic activity assay. Two granola bars from each trial were sampled along with two from the control trial.

4. The breakfast cereal was an extruded product with a low moisture matrix. The formulation of the cereal is described in Appendix D. The cereal was produced using five different variations including optimum screw speed and temperature, upper limit screw speed, lower limit screw speed, upper limit temperature, and lower limit temperature as shown in Appendix D. All other conditions remained unchanged. The various temperatures and screw speed may provide insight on how the prebiotic responds to extrusion and whether certain parameters retain different levels of prebiotic. The cereal was placed in Ziploc bags and stored at room temperature until sampled for

the prebiotic activity assay. Two samples were obtained from each trial along with two samples of a control trial.

5. The sports drink was a pasteurized product with a high sugar matrix and low pH. The resistant starch sports drink was the only sample tested. The formulation for the sports drink is located in Appendix E. The sports drink was stored in plastic bottles at room temperature until sampled for the prebiotic activity assay. Samples (6) were obtained from one bottle and two control samples were obtained from a control bottle.

6. The bread was a fermented product with a low fat, high moisture matrix. The formulation for bread is located in Appendix F. The bread was placed in Ziploc bags and stored at -17.8°C until sampled for the prebiotic activity assay. There was only one trial produced for bread; therefore, the sample size was lowered to two. Two samples of the control were also tested.

2.3. Manufacture of GOS chew

To determine if a high concentration of prebiotic would allow for a more accurate prebiotic activity score, GOS chews were tested. The GOS chews that were used for this experiment, are samples used in a previous study conducted at the University of Nebraska (Davis et al., 2011). The chews contained approximately 23% prebiotic in a high sugar, low moisture matrix. The formulation for the chews is located in Appendix G.

2.4. Manufacture of food products without sugar

To reduce the matrix effect on the prebiotic activity assay, products with limited background sugars were produced. These products included cracker, granola, and sports drink. These products were selected based on different matrices as well various processing conditions. The products were also able to withstand removal of sugars and

an addition of 10% prebiotic. The products were produced with four prebiotics, FOS, inulin, GOS, and resistant starch, which have been previously explained in section 2.1. Polydextrose was omitted in this part due to the lack of a probiotic strain of bacteria that is suitable for this bioassay. The products were made at the University of Nebraska – Food Science and Technology department.

2.4.1. Food products and their formulations

1. The cracker was a chemically leavened product with a low moisture matrix. The cracker was exposed to heat and there was a possibility of Maillard browning during processing. The formulation of the cracker is located in Appendix H. Two trials of each prebiotic were produced as well as two control trials. Two separate crackers were tested from each trial for both the prebiotic and control. The crackers were stored in Ziploc bags at room temperature.

2. The granola had a high fat and low moisture matrix. The granola was exposed to heat and there was a possibility of Maillard browning. The formulation of the granola is located in Appendix I. Two trials of each prebiotic were produced as well as two control trials. Two samples were tested from each trial for both the prebiotic and control. The granola was stored in Ziploc bags at room temperature.

3. The sports drink was an acidic product with a high protein matrix. The formulation for the sports drink is located in Appendix J. The sports drink was produced with two different levels of acidity, pH 6.00 and pH 3.00. The different levels of pH will help determine if an acidic matrix, with the addition of heat, leads to degradation of the prebiotic. The sports drink was stored in plastic bottles in a cooler at 1°C. Two samples were tested from each trial for both the prebiotic and control.

2.5. Bacteria Screening

To determine bacteria that were appropriate for the prebiotic activity assay (Huebner et al., 2007) growth curves were conducted. The growth of the bacteria on 1% glucose, 1% prebiotic, and media without carbohydrates was determined by growth curves using a spectrophotometer or by plating growth at 0 and 24 hours. The criteria for a probiotic bacterium to be selected was growth on the prebiotic that resembled the growth on glucose. The criteria for an enteric bacterium to be selected was minimal growth on the prebiotic when compared to growth on glucose. The bacteria that were selected for use in the prebiotic activity assay are shown in Table 2.1. Once the testing for the initial food products was completed, certain bacteria were deemed insufficient for the prebiotic activity assay for testing food products without sugar. This was due to either low growth on the prebiotic for the probiotic strain or extensive growth of the enteric strain, resulting in a low prebiotic activity. Additional screening was completed and new bacteria were chosen for GOS and resistant starch as shown in Table 2.2.

2.6. Procedure for prebiotic activity assay

2.6.1. Initial food products and GOS chews

The lactobacilli and bifidobacterium cultures were stored at -80°C in MRS Broth (Difco Laboratories, Detroit, MI, USA) containing 15% (wt/vol) glycerol. *Escherichia coli* and *Enterobacter aerogenes* were stored at -80°C in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) containing 15% (wt/vol) glycerol.

For the prebiotic activity assay, the lactobacilli cultures were streaked on MRS agar and incubated at 37°C for 48 hours, *Bifidobacterium animalis* subsp. *lactis* was streaked on MRS agar in anaerobic conditions using an anaerobic chamber (Bactron IV

Anaerobic Chamber, Shel Lab, Cornelius, OR) and incubated at 37°C for 72 hours anaerobically. *E. coli* and *E. aerogenes* were streaked on Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24 hours. One colony from the MRS plates was transferred into 10 ml MRS broth and incubated at 37°C for 16 hours. *Bifidobacterium animalis* subsp. *lactis* was transferred and incubated in the anaerobic chamber. One colony from the TSA plates was transferred into 10 ml TSB, then incubated at 37°C for 16 hours, and 100 µl of the TSB was transferred into 10 ml M9 minimal media then incubated at 37°C for 16 hours. The overnight cultures were diluted by 1/10 using either basal MRS or basal M9, with basal referring to preparation without carbohydrates.

The sample being tested was prepared by taking 5 g of the sample food and placing it in a stomacher bag (Nesco, Two Rivers, WI, USA). The samples were prepared in duplicate for each of the three trials of the food product produced, for both the probiotic and enteric samples. A control product was tested from one trial in duplicate, for both the probiotic and enteric samples. Liquid media was added to the samples, basal MRS for probiotic and basal M9 for enteric, in quantities of 10 ml for muffin, cookie, granola bar, and sports drink, 15 ml for bread, and 20 ml for cereal. The GOS chews were tested with the same procedure. 5 g of chew was sampled and 10 ml of either basal MRS or basal M9 was added. The amount of media added was determined on the least amount needed to saturate the sample and was still able to be transferred by pipette. Dilution of the prebiotic by adding media was a concern therefore media was added in various amounts based on the properties of the food product.

Approximately 100-200 µl of the diluted overnight culture was added to the sample mixture. This amount varied based on the amount of media added as well as the amount needed to start at 6.00 colony forming units per gram (cfu/g). The sample mixture was then homogenized using a stomacher. The samples were then diluted by taking 100 µl of the sample mixture and adding it to 900 µl 0.9% saline solution. Once the dilutions were made, 10 µl was placed on an agar plate, either MRS agar or TSA, and spread. The final dilutions plated for the 0 hour time point were 10^{-4} and 10^{-5}. The samples were then incubated at 37°C for 24 hours. The plates were incubated at 37°C for 24 - 48 hours. After 24 hours, the samples were diluted and plated for final dilutions of 10^{-7} and 10^{-8}. The plates were incubated at 37°C for 24 - 48 hours. The plates were counted in the dilution containing 25 - 250 colonies and recorded.

2.6.2. Calculations:

The colony forming units per gram (cfu/g) were calculated and then applied to the prebiotic activity equation (Huebner et al., 2007).

Equation 2.1: Prebiotic activity score - Initial food products and GOS chews

$$\left[\frac{(\text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 0 hr})}{(\text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on control at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on control at 0 hr})}\right] -$$

$$\left[\frac{(\text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 0 hr})}{(\text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on control at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on control at 0 hr})}\right]$$

The original equation used glucose as a control. Glucose was not a suitable standard due to the high starting weight, variation in sugar content and dilution factors. Due to this constraint, a control product without prebiotic was tested and used in place of glucose.

2.6.3. Food products without sugar

The cracker and granola were tested using the prebiotic activity assay as previously explained with certain alterations. The changes occurred during the sample preparation. A decrease in sample size will limit the amount of potential contaminating matrix, but will still allow a suitable amount of prebiotic to be available for the assay. The sample size was reduced to 0.5 g for cracker and granola and 1 ml for the sports drink. The cracker and granola were placed in a stomacher bag along with 9.5 ml of basal MRS or basal M9. 1 ml of the sports drink was added to sterile tubes along with 9 ml basal MRS or basal M9. The control products were also tested for prebiotic activity to determine if the assay was biased towards any food material besides the prebiotic.

Control samples were spiked with prebiotic to determine the activity of the prebiotic without processing. 0.45 g of the sample from either cracker or granola and 0.05 g of prebiotic were added to a stomacher bag along with 9.5 ml of basal MRS or M9. 10% prebiotic was added to the control sports drink and then 1 ml of the spiked sports drink was added to 9 ml basal MRS or basal M9.

For GOS, the frozen stock culture *Lactobacillus reuteri* DSM 20016 T was streaked on MRS agar and incubated at 37°C in an anaerobic chamber. After 48 hours, one colony was transferred to 10 ml MRS broth and incubated for 16 hours at 37°C

anaerobically. The culture was then removed from the anaerobic chamber and used in the assay remaining in aerobic conditions.

For resistant starch, the frozen stock culture *Bifidobacterium longum* ATCC 15708 was streaked on MRS agar with 0.05% cysteine (Sigma, St. Louis, MO, USA) added and incubated at 37°C for 48 hours in an anaerobic chamber. One colony was transferred to 10 ml MRS broth + 0.05% cysteine and incubated for 16 hours at 37°C anaerobically. The MRS portion of the resistant starch experiments was performed in the anaerobic chamber and the samples, as well as the plates, were incubated in the anaerobic chamber. The broth used in the assay was MRS + 0.05% cysteine and the plates were MRS agar + 0.05% cysteine. The remainder of the procedures were performed in the same manner.

The prebiotic activity assay was used to determine stability in this portion of the project. Glucose was used as the control due to similar starting amounts as well as a lack of background carbohydrates in the testing material. 1 ml of a 5% glucose solution was the standard for cracker and granola and 1 ml of a 10% glucose solution was the standard for the sports drink. The growth of the glucose solutions was determined at 0 and 24 hours for all bacterial strains used using the same dilutions and plating methods previously explained.

2.6.4. Calculation:

The colony forming units per gram were calculated and then applied to the prebiotic activity equation (Huebner et al., 2007).

Equation 2.2: Prebiotic activity score – Food products without sugar

$$\left[\frac{(\text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 0 hr})}{(\text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on glucose at 24 hr} - \text{probiotic}\log\frac{\text{cfu}}{\text{g}}\text{ on glucose at 0 hr})}\right] -$$

$$\left[\frac{(\text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on the prebiotic at 0 hr})}{(\text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on glucose at 24 hr} - \text{enteric}\log\frac{\text{cfu}}{\text{g}}\text{ on glucose at 0 hr})}\right]$$

2.7 Statistical Analysis

The first set of food products including muffin, cookie, granola, and sports drink were analyzed based on whether or not the prebiotic activity score was significantly different from a control activity score, 0. The control activity score is based on the principle that a non-prebiotic food will have equal growth for the probiotic and enteric sections of the calculation resulting in a score of 0. The procedure used was the univariate procedure. Bread was not included in the analysis due to the sample size being too low. P values of less than 0.05 were considered to be significant.

The cereal was analyzed based on differences within the parameters of processing on the cereal. The differences of least squares means, a factorial analysis of variance, was used to test significance. The mean of the three trials was used in the analysis. All five processing conditions, optimum, upper limit temperature, lower limit temperature, upper limit screw speed, and lower limit screw speed, were compared within the same prebiotic. There was no comparison between prebiotics. P values of less than 0.05 were considered to be significant.

The food products without sugars were analyzed using differences of least squares means as well. The mean of the two trials was used in the analysis. Three categories of products; (a) control, (b) processed prebiotic, and (c) prebiotic added after processing were compared against each other for all prebiotics and products. P values of less than 0.05 were considered to be significant.

Table 2.1: Bacterial strains used in the prebiotic activity assay for initial food products

Prebiotic	Probiotic	Enteric
FOS	*Lactobacillus paracasei* 1195[1]	*Escherichia coli* ECOR 22[5]
GOS	*Lactobacillus plantarum* 4008[2]	*Enterobacter aerogenes*[6]
Inulin	*Lactobacillus paracasei* 1195	*Escherichia coli* ECOR 22
Resistant Starch	*Lactobacillus plantarum* 299v[3]	*Escherichia coli* ECOR 22
Polydextrose	*Lactobacillus delbrueckii* subsp. *bulgaricus* 64, *Lactobacillus acidophilus* 14, *Lactobacillus rhamnosus* 32, and *Bifidobacterium animalis* subsp. *lactis* 04[4]	*Escherichia coli* ECOR 22

[1]University of Nebraska, Lincoln, NE, USA

[2]American type culture collection, Rockville, MD, USA

[3]ProViva, Skanemejerier, Sweden

[4]Danisco Global Culture Collection, Ardsley, NY, USA

[5] *Escherichia coli* Reference collection, University of Rochester, Rochester, NY, USA

[6]University of Nebraska, Lincoln, NE, USA

Table 2.2: Bacterial strains used in the prebiotic activity assay for products without sugar

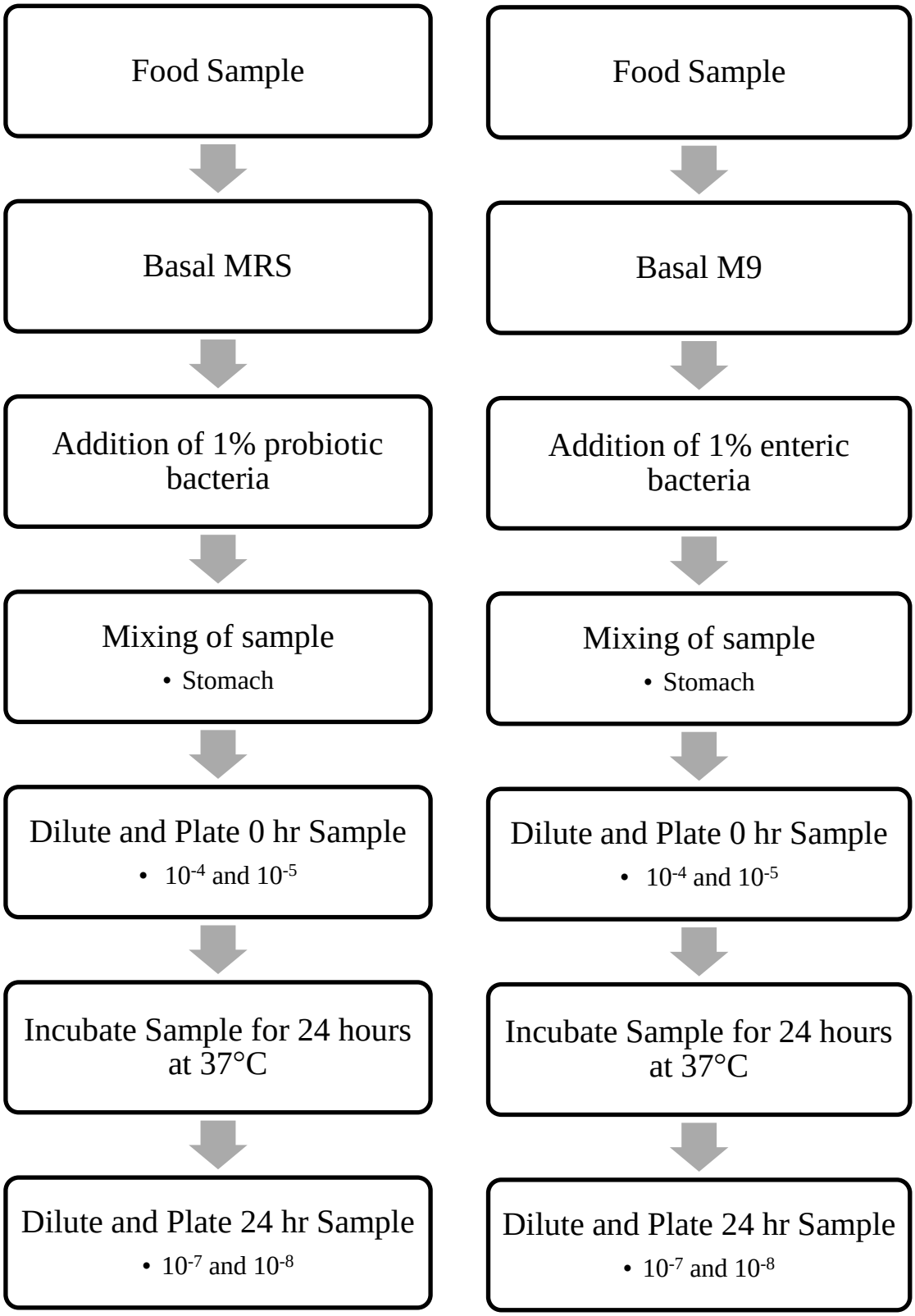
Prebiotic	Probiotic	Enteric
FOS	*Lactobacillus paracasei* 1195	*Escherichia coli* ECOR 22
GOS	*Lactobacillus reuteri* DSM 20016 T[1]	*Escherichia coli* JM 109[2]
Inulin	*Lactobacillus paracasei* 1195	*Escherichia coli* ECOR 22
Resistant Starch	*Bifidobacterium longum* ATCC 15708[3]	*Escherichia coli* ECOR 22

[1] German Collection of Microorganisms and Cell Cultures, Germany

[2] New England Biolabs, Ipswich, MA USA

[3] American type culture collection, Rockville, MD, USA

Figure 2.3: Flow chart of method



Chapter 3:

Results

3.1 Initial food products

The prebiotic activity assay was used to determine the stability of six prebiotics in a variety of food products. The food products portrayed a variety of processing variations including baking, extrusion, and pasteurization. The results from the analysis of these products provided insight on how the food matrices, as well as the processing conditions, affected the biological stability of the prebiotic.

In Table 3.1 the prebiotic activity score is listed for the following products, muffin, granola, cookie, bread, and sports drink. For the initial food products, the final prebiotic activity number was the average of six assays consisting of three trials performed in duplicate. The standard deviation was based on the average of the three trials. The prebiotic activity assay was performed in the same manner for each product, with the exception of the variation in the volume of media used. A control product was analyzed in the same manner as the food product and used in the control portion of the calculation.

In general, the scores were low. The highest score was 0.05 for the polydextrose cookie. The lowest score was -0.08 for the FOS cookie. The remainder of the scores fell in between these two scores and were relatively close to zero. The range of scores was low, indicating negligible prebiotic activity for most, although a few did show some significance statistically.

In Table 3.2 the prebiotic activity scores for all variations of cereal are listed. Three trials of the cereal were tested in duplicate for the prebiotic activity assay. The control product was also tested and used in the control portion of the assay. The scores for the cereal were also relatively low. The highest score was 0.07 for the FOS upper

limit screw speed and the lowest score was -0.07 for the resistant starch optimum cereal. Although there does not appear to be much variation between scores, statistically there is a difference between processing conditions for three of the prebiotics, FOS, resistant starch and polydextrose.

3.2. GOS chew

Due to the low scores, a chew with a high level of GOS was tested to determine a prebiotic activity score. The GOS chew was tested using the prebiotic activity assay. One trial was tested in duplicate for the chews with dilutions of 10 and 20 ml. The prebiotic activity scores for the GOS chews are shown in Table 3.3. The resulting activity for the chews were approximately 0. Increasing the concentration of prebiotic did not result in an increase in prebiotic activity, suggesting that the background sugars are contributing to the low scores.

3.3. Food products without sugar

To lower the matrix effect of the foods, new products without background sugars were produced. Two trials were produced for each product, which were tested in duplicate. The prebiotic activity of these products was tested using the same method as the initial food products with a few modifications. The sample size was lowered to either 0.5 g for the cracker and granola, or 1 ml for the sports drink. Glucose was used as the control at concentrations of 0.5% and 1% to correspond to the concentration of prebiotic in the sample tested. All the prebiotics used previously were tested with the exception of polydextrose.

The standard prebiotic activity of four prebiotics, FOS, inulin, GOS and resistant starch are listed in Table 3.4. The prebiotic activity of the pure prebiotic provides a

reference level of prebiotic activity that is expected without matrix effect or degradation. The standards were calculated at two concentrations of prebiotic, 1% and 0.5%. The assay was preformed as explained in chapter 2 with the pure prebiotic as the sample and glucose as the control. The test was conducted in quadruplicate. The activities of the prebiotics ranged from the activity of resistant starch at 0.12 at 0.5% to 0.49 at 1% for inulin. The activities of the prebiotics did not vary extensively between 0.5% and 1% concentrations.

All products were tested three ways, (a) with the prebiotic in the food matrix undergoing processing, (b) the control product with the prebiotic added post processing, and (c) a control product without any prebiotic. By testing each product three ways, the scores provide information on how the matrix affects the prebiotic activity score, if processing affects the prebiotic activity score, and what the score would be if the prebiotic was unprocessed. The control score also accounts for any bias the assay had for the food product. If, for example, activity was detected in the food product that was not due to the prebiotic, the control number would portray that activity.

The results for FOS are listed in Table 3.5. The scores reflect how processing affects the activity of FOS in the products. The scores ranged from -0.04 to 0.37 for the processed foods. FOS was stable throughout testing, but when the food matrix was exposed to heat and a low pH (sports drink, pH 3.00), the prebiotic activity score was -0.04 for the sports drink (pH 3.00). When the prebiotic was added after processing for the sports drink (pH 3.00) the score was 0.37

The results for inulin are listed in Table 3.6. The scores for inulin are similar to FOS. Like FOS, inulin was stable throughout processing, except when the food matrix

was exposed to a low pH and was processed with heat (sports drink, pH 3.00). The prebiotic activity score is 0.02 for the processed sports drink (pH 3.00), while the sports drink (pH 3.00) with prebiotic added after processing score is 0.33.

The results for GOS are listed in Table 3.7. The scores for GOS range from 0.02 for the processed cracker, to 0.41 for the processed sports drink (pH 3.00). Although the processed cracker score was low, the spiked product was also low suggesting a matrix effect rather than degradation. Two controls, granola and sports drink, resulted in negative activity scores. In the remainder of the products GOS appears to be stable.

The results for resistant starch are listed in Table 3.8. The scores for resistant starch were lower than expected. The scores were relatively constant between the processed product, control product and prebiotic added after processing with the exception of the sports drink (pH 6.00) processed with prebiotic, which is lower than both the control and the sports drink with unprocessed prebiotic. The sports drink (pH 3.00) processed with prebiotic was also lower than the control sports drink.

Although the activity of the initial food products were low, specific changes to the matrix of the food product along with an increase in concentration of the prebiotic allowed measurable activity scores. The resulting scores from the products produced without carbohydrate exhibited different levels of prebiotic activity. The matrix was a concern for cracker and granola, but less of a concern for the sports drink. The scores varied between prebiotics, suggesting different levels of selectivity and activity between prebiotics. The prebiotic activity scores provided information concerning processing. The scores observed for the sports drink showed degradation occurring for FOS and inulin within an acidic environment that was heated.

Table 3.1: Prebiotic activity scores for food products

Food Product	Prebiotic				
	FOS	GOS	Inulin	RS	PDX
Muffin	0.03 ± 0.03	0.00 ± 0.03	-0.04 ±0.03	-0.02 ±0.03	0.02 ±0.07
Granola Bar	0.00 ±0.01	0.00 ±0.03	0.00 ±0.04	0.02 ±0.02	0.03* ±0.01
Cookie	-0.08* ±0.02	0.00 ±0.01	0.03 ±0.03	0.03* ±0.01	0.05* ±0.01
Bread[1]	0.02 ±0.01	-0.05 ±0.04	0.03 ±0.01	-0.04 ±0.04	0.02 ±0.05
Sports Drink	x[2]	x[2]	x[2]	-0.04 ±0.04	x[2]

[1] Only one trial was produced; therefore the sample number was 2

[2] This product was not produced

* Significant difference from control (0) detected using the univariate procedure ($p < 0.05$)

Table 3.2: Prebiotic activity scores for cereal

Food Product	Prebiotics				
	FOS*	GOS	Inulin	RS*	PDX*
Extruded Cereal (Optimum)	0.01 ± 0.01	-0.05 ±0.11	0.03 ±0.03	-0.07[a] ±0.01	-0.01[a] ±0.06
Extruded Cereal (Upper Limit Screw Speed)	0.07[a] ±0.09	0.00 ±0.03	-0.01 ±0.04	0.02[b,d] ±0.04	0.01 ±0.02
Extruded Cereal (Lower Limit Screw Speed)	-0.04[b,d] ±0.04	0.03 ±0.06	0.06 ±0.06	-0.02[b,c] ±0.02	0.01 ±0.05
Extruded Cereal (Upper Limit Temperature)	0.03[c] ±0.03	-0.02 ±0.03	0.05 ±0.07	0.03[b,d] ±0.01	0.04[b] ±0.01
Extruded Cereal (Lower Limit Temperature)	-0.04[b,d] ±0.03	-0.02 ±0.01	-0.01 ±0.05	0.03[b] ±0.00	0.05[b] ±0.05

* Significant differences detected ($p < 0.05$) between extrusion parameters using least square means method

[a,b] Significant differences detected ($p < 0.05$) using least square means method

[c,d] Significant differences detected ($p < 0.05$) using least square means method

Table 3.3: GOS chew

Food Product	Dilutions	
	10 ml	20 ml
GOS chew	0.00 ±0.03	-0.06 ±0.03

Table 3.4: Standard prebiotic activity scores of prebiotics

Prebiotic	1% prebiotic	0.5% prebiotic
FOS	0.37 ±0.01	0.44 ±0.03
Inulin	0.49 ±0.05	0.45 ±0.02
GOS	0.21 ±0.06	0.25 ±0.10
Resistant Starch	0.14 ±0.02	0.12 ±0.00

Table 3.5: Prebiotic activity for FOS in food products

	Processed with prebiotic	Prebiotic added post-processing	Control
Cracker	0.14[2] ±0.03	0.21[2] ±0.02	-0.07 ±0.07
Granola	0.22[2] ±0.05	0.18 ±0.06	0.09 ±0.10
Sports Drink pH 6.00	0.37[2] ±0.05	0.43[2] ±0.07	-0.05 ±0.12
Sports Drink pH 3.00	-0.04[1,2] ±0.05	0.37[2] ±0.01	-0.14 ±0.14

[1] Significant differences ($p < 0.05$) detected using least square means methods between processed with prebiotic and prebiotic added post processing

[2] Significant differences ($p < 0.05$) detected using least square means methods from control

Table 3.6: Prebiotic activity for inulin in food products

	Processed with prebiotic	Prebiotic added post-processing	Control
Cracker	0.15[2] ±0.04	0.22[2] ±0.05	-0.07 ±0.07
Granola	0.23[2] ±0.06	0.22[2] ±0.08	0.09 ±0.10
Sports Drink pH 6.00	0.37[2] ±0.02	0.41[2] ±0.04	-0.05 ±0.12
Sports Drink pH 3.00	0.02[1,2] ±0.02	0.33[2] ±0.03	-0.14 ±0.14

[1] Significant differences ($p < 0.05$) detected using least square means methods between processed with prebiotic and prebiotic added post processing

[2] Significant differences ($p < 0.05$) detected using least square means methods from control

Table 3.7: Prebiotic activity for GOS in food products

	Processed with prebiotic	Prebiotic added post-processing	Control
Cracker	0.02 ±0.04	0.07 ±0.01	-0.01 ±0.07
Granola	0.15[2] ±0.03	0.08[2] ±0.04	-0.17 ±0.02
Sports Drink pH 6.00	0.39[2] ±0.04	0.35[2] ±0.12	-0.14 ±0.03
Sports Drink pH 3.00	0.48[2] ±0.04	0.41[2] ±0.03	0.00 ±0.03

[1] Significant differences ($p < 0.05$) detected using least square means methods between processed with prebiotic and prebiotic added post processing

[2] Significant differences ($p < 0.05$) detected using least square means methods from control

Table 3.8: Prebiotic activity for resistant starch in food products

	Processed with prebiotic	Prebiotic added post-processing	Control
Cracker	-0.14 ±0.03	-0.19 ±0.07	-0.10 ±0.02
Granola	-0.14 ±0.02	-0.13 ±0.14	-0.16 ±0.05
Sports Drink pH 6.00	0.03[1,2] ±0.01	0.18 ±0.03	0.26 ±0.04
Sports Drink pH 3.00	0.15 ±0.03	0.15 ±0.03	0.28 ±0.02

[1] Significant differences ($p < 0.05$) detected using least square means methods between processed with prebiotic and prebiotic added post processing

[2] Significant differences ($p < 0.05$) detected using least square means methods from control

Chapter 4:

Discussion

4.1. Initial food products

The initial food products were designed to contain approximately 1% prebiotic. This value was chosen based on the amount of prebiotic that could be added without drastically changing the integrity of the product. Although previous research using the prebiotic activity assay was based on a prebiotic concentration of 1%, the prebiotic was incorporated into microbial media and assayed directly. In contrast, the 1% prebiotic within these food products was further diluted and mixed with other food components, such as sucrose. The 5 g of sample was then diluted with media. The 5 g was considered an appropriate starting amount as it contained a testable amount of prebiotic once diluted, although background sugars in the products still remained a concern. For the products that were least diluted (muffin, cookie, granola bar, and sports drink), 10 ml of media was used creating a 1:3 dilution. At this point the prebiotic concentration was 0.33%. Bread required a dilution of 15 ml of media creating a 1:4 dilution and a prebiotic concentration of 0.25%. Cereal required a dilution of 20 ml of media creating a 1:5 dilution and a prebiotic concentration of 0.20%. The amount of sucrose and other sugars within these products far exceeded the amount of prebiotic within these products.

Due to the low starting concentration of prebiotic and high contaminating sugars, the prebiotic activity was very low (near 0.0). It is likely that the growth of the bacteria was based on the contaminating sugars within the food product since those sugars represented the majority of what was available. If there was growth on the prebiotic, it was likely very minimal and the prebiotic activity assay was not adequately sensitive to measure activity.

Breakfast cereal, however, presented differences between extrusion conditions for FOS, resistant starch, and polydextrose. Several of the activity scores included in the cereal results are below 0, which may influence the differences found in conditions. It is difficult to conclude whether or not these differences are due to the extrusion conditions or variation in the testing procedure.

To determine degradation of the prebiotic in these products, a more specific method of testing would be required, most likely a chemical method. The prebiotic activity assay could be a possible method to determine degradation within these products, but removal of background sugars before testing would need to occur. Even with the removal of background sugars, the low concentration of prebiotic might be problematic.

4.2. GOS Chew

In order to determine whether the low concentration of prebiotic or high contaminating sugars were the main problem, further testing was conducted. A GOS chew was tested for prebiotic activity to determine if a higher concentration of prebiotic would result in an increase in activity. However, as for the other food materials, the prebiotic activity of the chew was also near 0. Even with the high concentration of prebiotic within the chew, the enteric bacteria were still able to grow to levels comparable to the probiotic bacteria. From this experiment, it was concluded that the contaminating sugars were the main inhibitor to this method.

4.3. Food products without sugar

In order to determine if degradation was occurring in food matrices, three food products that could be made without sugar and included a high concentration of prebiotic were produced. These food products were: (a) cracker, (b) granola, and (c) sports drink

in both a neutral pH, 6.00 and a low pH, 3.00. These products were tested for prebiotic activity using the prebiotic activity assay.

Although the prebiotic activity assay was not sufficiently sensitive to detect appreciable prebiotic activity in the previous products, it was able to detect prebiotic activity in these reformulated products. With the removal of sugars the enteric bacteria should have minimal energy sources and growth should be limited. If prebiotic is degraded, the sugars will provide energy sources for the enteric bacteria and limit the activity of the prebiotic. With higher concentrations of prebiotic within the product, a smaller sample size could be used which would limit the amount of matrix included in the test. The dilution of the sample would not cause concern given that a tenfold dilution resulted in 1% prebiotic available for testing. The smaller sample size also allowed for glucose to be used as the control in the assay since the concentration of the prebiotic in the sample can be replicated for glucose.

The prebiotic activity scores for FOS depict that the assay was able to determine prebiotic within the sample. The levels of FOS detected varied within the products suggesting either degradation or a matrix effect had occurred. The FOS in the cracker was lower than the prebiotic activity determined for the FOS standard at a concentration of 0.5% as shown in Table 3.4. This difference in activity did not appear to be due to degradation. If degradation was occurring during processing, the prebiotic activity for FOS added to the cracker post-processing would be higher, resembling the score obtained for standard FOS. The low score obtained for the FOS cracker was most likely due to the matrix of the food product. The extraction of the FOS from the matrix of the cracker was likely inhibited. If the FOS was not available due to the matrix, complete fermentation of

the prebiotic by the bacteria in the assay would not occur and the prebiotic activity score would be uncharacteristically low. The matrix may have supported the growth of both bacteria equally causing a small decrease in activity from the standard FOS; where FOS was the only component added to the testing medium.

The matrix also seems to be inhibiting the prebiotic activity in the FOS granola sample as well. The control sample also portrayed a small activity, suggesting that there may have been some growth on the granola unrelated to the prebiotic. The matrix of both the cracker and granola contained a large amount of fat, which might have been able to bind the prebiotic and reduce the amount of prebiotic available for fermentation during the assay. From the activity scores obtained for FOS, it appears that FOS was stable when exposed to mild to moderate heating experienced in baking conditions.

The matrix of the sports drink was considerably less than both the cracker and granola. The prebiotic activity scores resemble those of the standard at 1% concentration. The fermentation of the prebiotic in this matrix seems to be complete. The activity of FOS in the sports drink (pH 3.00) is dramatically decreased when compared to the activity of the sports drink (pH 3.00) with prebiotic added after processing. This decrease in activity was likely due to the exposure of the prebiotic to a low pH while heating. When the prebiotic was exposed to only moderate heat, the prebiotic remained biologically active as seen in the sports drink (pH 6.00). FOS was biologically degraded when exposed to both a low pH and moderate heating. Similar results of acid hydrolysis in heat have been reported in previous studies (Blecker et al., 2002; L'Homme et al., 2003; Huebner et al., 2008; Keenen et al., 2011).

Inulin provided similar results to FOS. Like FOS, the activities for inulin in both the cracker and granola were low, around 0.2. The low activity seems to be a result of the matrix effect rather than degradation due to the similarity of activity between the processed prebiotic and prebiotic added after processing. Inulin appears to be biologically stable when exposed to baking conditions including mild to moderate heating. The degradation of inulin in an acidic environment while heated corresponds with results of similar studies (Blecker et al., 2002; Huebner et al., 2008; Keenen et al., 2011).

When inulin was added to a sports drink, moderate heat did not affect the activity, but when combined with a low pH the activity was decreased significantly. The activities of inulin within the sports drinks are similar to the activity of standard inulin. There does not appear to be a matrix effect in the sports drink. Inulin was also biologically degraded when exposed to a low pH and moderate heating.

The prebiotic, GOS, seems to be less selective when compared to FOS and inulin. When screening bacteria suitable for the enteric portion of the assay, several enteric bacteria were able to ferment GOS as well or better than glucose. The *E. coli* JM 109 strain found unable to ferment GOS and used in this portion of experiments, is a lactose negative strain which may be a reason that it is unable to ferment GOS.

Standard GOS had less prebiotic activity than the standards of FOS and inulin when tested at 1 and 0.5%. This decrease in activity could be related to the strains selected for the assay and/or the prebiotic nature of the carbohydrate. The activity of GOS in the cracker was low in all categories tested. There may have been more of a matrix effect for GOS within the cracker than FOS and inulin. Due to the low scores and no apparent correlation between the processed prebiotic and the prebiotic added after

processing, this assay may not be able to adequately determine if degradation was occurring during processing of the cracker. The scores for granola were slightly higher than the scores for the cracker and appear to be stable, but still suggest a matrix effect.

The sports drink was a more suitable matrix to determine prebiotic activity than either the cracker or granola for GOS. In the sports drink GOS appears to be relatively stable. There was no difference between the processed prebiotic sports drink and when the prebiotic was added after processing for both the neutral, pH 6.00, and acidic, pH 3.00, sports drinks. From this data, when GOS was exposed to heat as well as a low pH, the prebiotic remained biologically active.

Although GOS has been reported as stable during most processing conditions (Klewicki, 2007; Playne and Crittenden, 1996), as was seen from this data, Maillard browning was a concern for stability of GOS due to the reducing end of the molecule. When exposed to heat browning occurs at extremely high levels (Playne and Crittenden, 1996). Maillard reactions are a concern with the addition of GOS to food products. The product from this research that was subjected to browning reactions was the cracker. Although the cracker did show evidence of increased browning for the GOS sample, data on degradation was not as clear as desired. The prebiotic activity score was low for the GOS cracker, but no difference was observed between the processed GOS cracker and the cracker with GOS added after processing.

The prebiotic activity of resistant starch was the lowest of the prebiotics tested. This assay may not be suitable to determine the extent of the prebiotic activity for samples containing resistant starch. Fermentation of resistant starch may depend on a community of microbiota as seen in the large intestine. Since the molecule is larger than

FOS, inulin, and GOS, several bacteria could be required to break down individual glucose molecules used by probiotic bacteria. The use of only one organism would not ferment the entire molecule and portray the full prebiotic activity.

Another limitation of resistant starch was the insoluble nature of the prebiotic. When added to media for testing, resistant starch settled at the bottom of the vessel used. The bacteria may not be able to fully access the prebiotic. Solubility of resistant starch did not improve when heated, stirred or with adjusted pH.

The low activity of resistant starch may be attributed to the strain of bacteria used in this study. Originally, *L. plantarum* 299v was used for this assay due to a greater prebiotic activity compared to several strains tested. Although the activity of *L. plantarum* 299v was still low, the strain was utilized in the assay rather than screen more bacteria.

When *L. plantarum* 299v was used in the prebiotic activity assay with the newly developed products, the strain was not suitable for the assay due to low prebiotic activity of the products. In the newly developed products, the low growth could be contributed to the lack of activity of the prebiotic, whereas in the initial products the lack of fermentation of *L. plantarum* 299v was not as noticeable due to high growth on other constituents. New strains of bacteria were screened to determine if another strain of bacteria would provide a higher level of activity and the prebiotic activity assay could be used to assess degradation of resistant starch. *B. longum* ATCC 15708 was found to have the greatest activity on resistant starch, although this activity is still the lowest of the prebiotics used for this research.

Even with the increase in activity using *B. longum* ATCC 15708, the prebiotic activity assay does not seem to be a suitable method to test resistant starch. The low activity of the standard resistant starch also makes it hard to compare the various processing conditions. If degradation had occurred during processing, it would result in a small change in activity that may be overlooked and not detectable using statistical analysis.

The processed sport drink (pH 6.00) was found to be significantly different from both the sports drink (pH 6.00) with prebiotic added after processing and the control sports drink. Degradation could have occurred in that sample, however, the control product was unusually high. Due to the high control number, the assay seems to be biased towards the probiotic stain used. With the high control activity, it is difficult to determine if degradation of resistant starch is the cause of the low activity in the sports drink (pH 6.00). Based on the literature, resistant starch can be degraded in high moisture matrices with heat (Sajilata et al., 2006).

The prebiotic activity assay was able to detect the degradation of prebiotics within certain food matrices more so than others. The sports drink was the least complex matrix to determine prebiotic activity and determine if degradation was occurring. The only ingredient that may inhibit the assay in the sports drink was whey protein and the concentration used was far less than the amount of prebiotic.

The matrix of the cracker and granola were more complex. Both matrices were high in fat with other complex carbohydrates. These matrices were inhibiting the assay by either making the prebiotic inaccessible to the bacteria or there was some growth by both bacteria that was not due to the prebiotic thus reducing the score.

The matrix of the food product could potentially bind certain prebiotics to a greater extent, thus rendering them unavailable for use in the assay. This does not seem to be an issue within the matrix of the sports drink due to little variation between prebiotics within similar categories of food products. The matrix of the cracker and granola created approximately the same amount of matrix effect for both FOS and inulin. The activity of GOS, on the other hand, was significantly lower ($p < 0.05$) for the cracker compared to FOS and inulin. GOS could be more difficult to extract from the matrix of the cracker. It is difficult to determine if the low scores are due to an increased matrix effect, or from the lower prebiotic activity of the standard GOS.

Overall, the prebiotic activity assay was able to determine prebiotic activity in food products. This activity was able to determine if degradation had occurred during processing. The prebiotic being tested and the food matrix containing the prebiotic affected the level of activity that was able to be detected using this method. Since it was difficult to determine if low activity was due to degradation during processing or a matrix effect, testing of a sample that contains unprocessed prebiotic added to the control product was required. The prebiotic activity method could, however, be improved to better detect activity within food samples.

There was little variation between the two samples of food product through manufacture and testing, suggesting that this method of testing is able to test multiple samples with little variation. There is, however, variation in results due to the amount of bacteria used to inoculate the samples. The samples were inoculated with approximately 6.00 cfu/ml of bacteria but due to different concentrations of bacteria in overnight

cultures this number varied slightly. This could contribute to minor variations between products as well as prebiotics.

The sample preparation could be altered to better detect prebiotic activity. Better extraction of the prebiotic could result in higher activities for the food products. Certain techniques were attempted to decrease the matrix effect of the food product, in this case FOS cracker. The cracker was ground into a powder before sampling to increase surface area exposed during testing as well as to increase the likelihood of FOS removal from the matrix, but did not result in an increase of the prebiotic activity of the food product. Centrifugation of the sample after stomaching was conducted to remove any particles that may interfere with the assay. Since the FOS, inulin and GOS are soluble, they would remain in the liquid portion after centrifugation. Centrifugation did not increase the prebiotic activity of the cracker as expected. Lowering the inoculation of bacteria to see if an increase in activity due to a more complete fermentation of product was also conducted, but did not result in an increase of activity. For resistant starch, increasing the incubation of the samples from 24 to 48 hours to determine if a longer incubation period resulted in greater fermentation was tested for the standard resistant starch at both 0.5% and 1% concentrations. There was no difference seen in activity of the prebiotic with the additional time.

Screening additional bacteria to increase standard prebiotic scores of GOS or resistant starch may prove to be beneficial. Although a wide variety of both probiotic and enteric bacteria were screened, there remains the possibility of an organism that better ferments GOS or resistant starch. Also, a cocktail of bacteria may result in higher prebiotic activities, especially for resistant starch.

Overall, this research has been able to provide insight of the effects of food matrices as well as processing on stability of prebiotics. The data generated from this research is generally in agreement with previously published data using both chemical and biological methods to test for prebiotic.

Chapter 5:

Conclusions

Overall, the outcomes of this research are summarized as follows:

- The original prebiotic activity assay was not sufficiently sensitive to detect prebiotic activity within matrices containing sugars as well as matrices with a low concentration of prebiotic.
- The prebiotic activity assay was able to determine prebiotic activity within a food matrix that does not contain any background sugars.
- Certain food matrices allowed for greater detection of activity.
- The prebiotic activity assay was able to determine degradation of a prebiotic within a food matrix.
- Certain prebiotics had greater prebiotic activity and were able to be tested more accurately with the prebiotic activity assay.
- The food matrix was a concern when testing food products and was a factor when assessing the amount of prebiotic within the sample.
- Fructooligosaccharides and inulin were stable in food matrices exposed to mild to moderate heating (cracker, granola, and sports drink (pH 6.00)), but were degraded when incorporated into a sports drink (pH 3.00) with an acidic environment and processed with moderate heat.
- Galactooligosaccharides were not able to be clearly assessed for cracker due to a likely matrix effect, but appeared to be stable within granola and sports drink. GOS appears to be relatively stable when exposed to a variety of processing conditions.

- Resistant starch had a low standard prebiotic activity and low scores when tested in food products. Changes in the prebiotic activity assay would have to occur to assess stability in food products.

References

1. Akiyama, K., M. Takase, K. Horikoshi, S. Okonogi. 2001. Production of galactooligosaccharides from lactose using a β-Glucosidase from *Thermus* sp. Z-1. Biosci. Biotechnol. Biochem. 65:438-441.

2. Beer, M., E. Arrigoni, D. Uhlmann, D. Wechsler, R. Amado. 1991. Stability of polydextrose solutions to heat treatment and storage under acid conditions. Lebensm. -Wiss. Technol. 24:245-251.

3. Bohm, A., I. Kaiser, A. Trebstein, T. Henle. 2005. Heat-induced degradation of inulin. Eur. Food Res. Technol. 220:466-471.

4. Bohm, A., B. Kleessen, T. Henle. 2006. Effect of dry heated inulin on selected intestinal bacteria. Eur. Food Res. Technol. 222:737-740.

5. Blecker, C., C. Fougnies, J.C. Van Herck, J.P. Chevaleir, M. Paquot. 2002. Kinetic study of the acid hydrolysis of various oligofructose samples. J. Agric. Food Chem. 50:1602-1607.

6. Brown, I.L., K.J. McNaught, E. Moloney. 1995. Hi-maize: new directions in starch technology and nutrition. Food Aust. 47:272-275.

7. Cantana, R., M. Eloy, J.R. Rocha, B.S. Ferreira, J.M.S. Cabral, P. Fernandes. 2007. Stability evaluation of an immobilized enzyme system for inulin hydrolysis. Food Chem. 101:260-266.

8. Chinachoti, P. 1995. Carbohydrates: functionality in foods. Am. J. Clin. Nutr. 61:S922-S929.

9. Collins, M.D., and G.R. Gibson. 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nutr. 69:S1052-S1057.

10. Courtin, C.M., K. Swennen, P. Verjans, J.A. Delcour. 2009. Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides. Food Chem. 112:831-837.

11. Craig, S.A.S., J.M. Anderson, J.F. Holden, P.R. Murray. 1994. Bulking agents: polydextrose. In Carbohydrates as organic raw materials III. ed. van Bekkum H., H. Roper, F. Voragen. pp 217-230. New York:VCH.

12. Craig, S.A.S., J.F. Holden, J.P. Troup, M.H. Auerbach, H. Frier. 1999. Polydextrose as soluble fiber and complex carbohydrate. In Complex carbohydrates in foods. ed. Cho S.S., L. Prosky, M. Dreher. pp 229-248. New York: Marcel Dekker.

13. Craig, S.A.S., J.F. Holden, M.Y. Khaled. 2000. Determination of polydextrose as dietary fiber in foods. J. AOAC Int. 83:1006-1012.

14. Cummings, J.H., and H.N. Englyst. 1987. Fermentation in the human large intestine and the available substrates. Am. J. Clin. Nutr. 45:1234-1255.

15. Cummings, J.H., G.T. Macfarlane, H.N. Englyst. 2001. Prebiotic digestion and fermentation. Am. J. Clin. Nutr. 73:S415-S420.

16. Davis, L.M.G., I. Martinez, J. Walter, R. Hutkins. 2010. A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults. Int. J. Food Microbiol. 144:285-292.

17. Flint, H.J., S.H. Duncan, K.P. Scott, P. Louis. 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ. Microbiol. 9:1101-1111.

18. Franck, A. 2002. Technological functionality of inulin and oligofructose. Br. J. Nutr. 82:S287-S291.

19. Gibson, G.R., J.H. Cummings, G.T. Macfarlane. 1988. Use of a three stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenisis by mixed populations of the human gut bacteria. Appl. Environ. Microbiol. 54:2750-2755.

20. Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

21. Gibson, G.R., H.M. Probert, J.V. Loo, R.A. Rastall, M.B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17:259-275.

22. Goh, Y.J., C. Zhang, A.K. Benson, V. Schlegel, J.H. Lee, R.W. Hutkins. 2006. Identification of a putative operon involved in fructooligosaccharide utilization by *Lactobacillus paracasei*. Appl. Environ. Microbiol. 72:7518-7530.

23. Hazen, C. 2011. Better nutrient-fortified beverages. Food Prod. Design 21:48-52.

24. Huebner, J., R.L. Wehling, R.W. Hutkins. 2007. Functional activity of commercial prebiotics. Int. Dairy J. 17:770-775.

25. Huebner, J., R.L.Wehling, A. Parkhurst, R.W. Hutkins. 2008. Effect of processing conditions on the prebiotic activity of commercial prebiotics. Int. Dairy J. 18:287-293.

26. Hur, S.J., B.O. Lim, E.A. Decker, D.J. McClements. 2011. *In vitro* digestion models for food applicants. Food Chem. 125:1-12.

27. Ito, M., Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajima, T. Kan. 1990. Effects of administration of galactooligosaccharides on the human fecal microflora, stool weight and abdominal sensation. Microb. Ecol. Health D. 3:285-292.

28. Keenen, D.F., N. Brunton, F. Butler, R. Wouters, R. Gormley. 2011. Evaluation of thermal and high hydrostatic pressure processed apple purees enriched with prebiotic inclusions. Innovative. Food Sci. Emerg. Technol. 12:261-268.

29. Klewicki, R. 2007. The stability of gal-polyols and oligosaccharides during pasteurization at a low pH. Lebensm. -Wiss. Technol. 40:1259-1265.

30. L'Homme, C., M. Arbelot, A. Puigserver, A. Biagini. 2003. Kinetics of hydrolysis of fructooligosaccharides in mineral-buffered aqueous solutions: influence of pH and temperature. J. Agric. Food Chem. 51:224-228.

31. L'Homme, C., A. Puigserver, A. Biagini. 2003. Effect of food-processing on the degradation of fructooligosaccharides in fruit. Food Chem. 82:533-537.

32. Macfarlane, G.T., S. Macfarlane, G.R. Gibson. 1998. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. FEMS Microbiol. Ecol. 35:180-187.

33. Makras, L., G. Van Acker, L. De Vuyst. 2005. *Lactobacillus paracasei* subsp. p*aracasei* 8700:2 degrades inulin-types fructan exhibiting different degrees of polymerization. Appl. Environ. Microbiol. 71:6531-6537.

34. Meunier, J.P., E.G. Manzanilla, M. Anguita, S. Denis, J.F. Perez, J. Gasa, J.M. Cardot, F. Garcia, X. Moll, M. Alric. 2008. Evaluation of a dynamic *in vitro* model to stimulate the porcine ileal digestion of diets differing in carbohydrate composition. J. Anim. Sci. 86:1156-1163.

35. Minekus, M., P. Marteau, R. Havenaar, J.H.J. Huis in't Veld. 1995. A multicompartmental dynamic computer controlled model simulating the stomach and small intestine. Altern. Lab Anim. 23:197-209.

36. Minekus, M., M. Smeets-Peeters, A. Bernalier, S. Marol-Bonnin, R. Havenaar, P. Mateau, M. Alric, G. Fonty, J. Huis in't Veld. 1999. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl. Microbiol Biotechnol. 53:104-114.

37. Molly, K., M. Vande Woestyne, W. Verstraete. 1993. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl. Microbiol Biotechnol. 39:254-258.

38. Mun, S.H., and M. Shin. 2006. Mild hydrolysis of resistant starch from maize. Food Chem. 96:115-121.

39. Niness, K.R. 1999. Inulin and oligofructose: what are they? J. Nutr. 129:S1402-S1406.

40. Palframan, R., G.R. Gibson, R.A. Rastall. 2003. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. Lett. Appl. Microbiol. 37:281-284.

41. Playne, M.J., and R. Crittenden. 1996. Commercially available oligosaccharides. Bull. Int. Dairy Fed. 313:10-22.

42. Playne, M.J., and R.G. Crittenden. 2009. Galacto-oligosaccharides and other products derived from lactose. Adv. Dairy Chem. 3:122-150.

43. Rastall, R.A. 2010. Functional oligosaccharides: application and manufacture. Annu. Rev. Food Sci. Technol. 1:305-339.

44. Roberfroid, M. 2007. Prebiotics: the concept revisited. J. Nutr. 137:S830-S837.

45. Rycroft, C.E., M.R.M. Jones, G.R. Gibson, R.A. Rastall. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol. 91:878-887.

46. Sajilata, M.G., R.S. Singhal, P.R. Kulkarni. 2006. Resistant starch – a review. Compre. Rev. Food Sci. Food Saf. 5:1-17.

47. Steer, T., H. Carpenter, K. Tuohy, G.R. Gibson. 2000. Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. Nutr. Res. Rev. 13:229-254.

48. Topping, D.L., and P.M. Clifton. 2001. Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81:1031-1055.

49. Tuohy, K.M., H.M. Probert, C.W. Smejkal, G.R. Gibson. 2003. Using probiotics and prebiotics to improve gut health. Drug Discovery Today 8:692-700.

50. Venter, C.S. 2007. Prebiotics: an update. J. Fam. Ecol. Consum. Sci. 35:17-25.

51. Vulevic, J., R.A. Rastall, G.R. Gibson. 2004. Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. FEMS Microbiol. Lett. 236:153-159.

52. Yun, J.W. 1996. Fructooligosaccharides – occurrence, preparation, and application. Enzyme Microb. Technol. 19:107-117.

Appendix A

Muffin

Formula

Ingredient	Weight (g)
All-purpose flour	250
Sucrose	75
Baking powder	15
Salt	3.1
Eggs	50
Butter	75
Milk	200
Water	50
Prebiotic	7.5

Appendix B

Cookie[1]:

Formula

Ingredients (at 75°F)	Weight (g)
Shortening	64.0
Sugar	130.0
Salt	2.1
Bicarbonate of soda	2.5
Dextrose solution (8.1 g dextrose hydrous, USP in 150 ml water)	33.0
Distilled water	16
Flour 14% mb	225

[1] AACC Method 10-50D

Method

1. Cream shortening, sugar, salt, and soda on low speed 3 min. Scrape down after each min.
2. Add dextrose soln and distd water. Mix 1 min at low speed. Scrape. Mix 1 min at medium speed. Add all the flour and mix 2 min at low speed, scraping down after each ½ min.
3. Place six portions of dough at well-spaced points on cookie sheet. Lay gauge strips along each side on top of sheet. Flatten dough mounds lightly with palm of hand and roll doughs to proper thickness with rolling pin on gauge strips. Cut cookies on sheet, lifting scrap dough up from around cutter and discarding.

4. Get dough wt and bake immediately.
5. Bake cookies 10 min at 400°F on improvised hearth in oven chamber.
6. On removal from oven, lift cookies from baking sheet with wide spatula and place them on absorbent paper. Wipe cookie sheet with dry paper towel to remove grease and crumbs.

Appendix C

Granola Bar

Formula

Ingredient	Weight (g)
Rolled Oats	420
Granola cereal	420
Margarine	50
Honey	350
Peanut butter	50
Sucrose	100
Salt	5
Peanuts (dry roasted)	75
Prebiotic	15

Appendix D

Cereal

Formula

Ingredient	Weight (g)
Degerminated Corn flour	1010
Oat flour	800
Granulated Sucrose	160
Salt	20
Calcium Bicarbonate	10
Prebiotic	20

Method

1. Mix all ingredients together in mixer for 2 minutes
2. Water was added until the moisture content was 17%
3. The cereal was extruded using various parameters

Conditions	Temperature (°C)	Screw Speed (RPM)
Optimum	140	170
Upper Limit Temperature	170	170
Lower Limit Temperature	110	170
Upper Limit Screw Speed	140	220
Lower Limit Screw Speed	140	120

Appendix E

Sports Drink

Formula

Ingredient	g/10 L
Granulated Sucrose	250
High fructose corn syrup	250
Citric acid	Added until desired pH is achieved
Sodium chloride	10
Sodium citrate	1
Prebiotic	100
Red food Color	---

Method

1. All dry ingredients were mixed in a 20 liter tank.
2. Sufficient amounts of distilled water were measured into the tank to reach a final liquid volume of 10 liters
3. Optimum pH is 3.5 and was adjusted accordingly for each batch using additional citric acid or 1N sodium hydroxide.
4. The batches were heated to a minimum temperature of 175°F using a Groen steam-jacketed kettle (model No. TDB/7-40) and a stainless steel coil.
5. The drink product was hot-filled into PET bottles and allowed to cool.
6. The product was stored at ambient temperature.

Appendix F

Bread[1]:

Formula

Formula	Flour Basis (%)
Flour 14% mb	100.0
Salt	1.5
Yeast	5.0
Water	Variable
Sugar	6.0
Shortening	3.0
NFDM solids	4.0
Malt, dry powder	0.3
Ascorbic acid	40.0 ppm

[1] AACC Method 10-10A

Method

1. Place dry ingredients (flour, NFDM, shortening) into mixing bowl. Make small pocket in center of mix for addn of liquids.
2. Add liquid simultaneously or in order: yeast, sugar/salt, malt, bromated/ascorbic acid, remainder of water.
3. Place bowl on mixer, set estimated mixing time on automatic timer, and start mixer, recording clock time for fermentation schedule.
4. After few sec, brush any flour from sloping edge of bowl back into mixing dough.

5. Watch dough closely during development to judge optimum mixing time. Optimum development occurs when dough strands no longer break off short but flow out in strings tending to sheet and a sheen appears.
6. If necessary, stop mixer before optimum development to scrape down sides of bowl and to judge addnl mixing needed.
7. Mix to optimum. Record total mix time and remove bowl from mixer.
8. Insert dough thermometer and record temp after 30-60 sec.
9. Round dough by hand, keeping smooth skin on top side. Place seam side down in lightly greased fermentation bowl and place in fermentation cabinet (30°C and 85% RH).

Fermentation and Punching Schedule

First punch (55 min after the start of mixing)

1. Remove dough from cabinet and carefully invert onto lightly floured surface. Pull exposed surface together to form smooth skin, slightly elongating dough piece.
2. Pass through sheeter lengthwise.
3. Fold sheeted dough in thirds or folded in half and in half again. Place folded dough, crease down, in bowl and return to fermentation cabinet.

Second punch (25 min later)

1. Repeat all steps of first punch, continuing to observe and record dough characteristics.

Molding and panning (10 min later)

1. Repeat step 1 of first punch.
2. Pass thru sheeter lengthwise twice.

3. Starting from first end out of sheeter, curl dough by hand with slight pressure to eliminate air bubble, place in wooden rollers of moulder, applying gently pressure to elongate to size of pan or roll under palms of hands for 10 back and forward movements.
4. Place seam side down in lightly greased baking pan. Put paper label on side, end, or bottom of dough to identify sample. Return to fermentation cabinet.

Proofing

Proof 30-38 min, or to desired height, usually 2.0-2.5 cm above top rim of pan.

Baking

Oven temp at 218°C. Bake 24 min.

Appendix G

GOS Chew

Formula

Ingredient	Composition (%) of chew
Water	11.54
Sugar	19.42
GOS (Purimune)	23.40
Corn syrup	31.83
Palm kernel oil	5.41
Chocolate liquor	7.44
Lecithin	0.53
Vanilla	0.43

Appendix H

Cracker

Formula

Ingredients	Weight (g)
All purpose flour	135
Salt	0.7
Baking powder	0.4
Butter	26
Water	89
Prebiotic[1]	27.5

[1] Prebiotic was not added to control cracker

Method

1. Combine flour, salt, baking powder and prebiotic
2. Cut in butter until crumbly
3. Add water, stir until just mixed
4. Knead dough on lightly floured surface
5. Roll out dough to a thickness of 1/8 inch
6. Cut into 2” squares
7. Prick with fork 2 or 3 times
8. Bake in preheated toaster oven at 350°F for 18 minutes

Appendix I

Granola

Formula

Ingredients	Weight (g)
Rolled oats	100
Butter	14.5
Peanut butter	90
Wheat germ	7.5
Prebiotic[1]	23.5

[1] Prebiotic was not added to control

Method

1. Mix oats, wheat germ, and prebiotic
2. Mix butter and peanut butter
3. Heat the peanut butter and butter mixture for 45 seconds in the microwave
4. Combine peanut butter mixture with oat mixture mixing well
5. Press mixture into shallow pan
6. Bake in toaster oven at 350°F for 8 minutes

Appendix J

Sports Drink

Formula

Ingredients	Weight (g)
Whey Protein Isolate	5
Citric acid	20% solution was added until desired pH was reached
Sodium chloride	0.25
Sodium citrate	0.025
Prebiotic[1]	28.3
Water	250

[1] Prebiotic was not added to control

Method

1. Mix dry ingredients together
2. Add water
3. Adjust pH to 6.00 or 3.00 depending on version desired with citric acid
4. Heat to a temperature of 175°F
5. Pour sports drink into sterile container to store