

SURVIVAL OF PROBIOTICS IN PEANUT BUTTER, AND THEIR INFLUENCES  
ON SELECTED FOODBORNE BACTERIAL PATHOGENS IN SIMULATED  
GASTROINTESTINAL FLUIDS

by

YAA ASANTEWAA KAFUI KLU

(Under the Direction of JINRU CHEN)

ABSTRACT

Most children in developing countries die before their 5th birthday from numerous causes including infectious diarrhea which presents the second highest number of deaths. In recent years, the administration of probiotics is being used as an adjuvant therapy with rehydration and nutritional intervention for the management of diarrhea. Probiotic bacteria need a suitable food matrix as carrier to exert the proposed health benefits when ingested. This study was undertaken to determine the survivability of four selected commercial probiotic products in full-fat peanut butter and reduced-fat peanut butter during a year-long storage study at 4, 25 or 37 °C. Additionally, the ability of the probiotics in peanut butter to survive simulated gastrointestinal conditions and eventually inhibit the growth of *Salmonella enterica* and *Listeria monocytogenes* was studied. It was observed that a higher temperature of 37 °C was more detrimental to probiotic viability and a single probiotic strain had a significantly lower survival rate compared to multiple probiotic strain mixture. It was also observed that within a multi-strain probiotic

product, probiotic survival during storage was strain specific. In general, *Bifidobacterium* species used in the study had a better survival rate than *Lactobacillus* and *Streptococcus/Lactococcus*. In a 6 h assay, peanut butter had a significant protective effect on the viability of probiotic bacteria when they were exposed to simulated gastrointestinal conditions. Additionally, probiotics in the peanut butter survived simulated gastrointestinal study and they were able to inhibit the growth of *S. enterica* and *L. monocytogenes* in a 24 h study under simulated gastrointestinal conditions. Furthermore, the fat content of full-fat peanut butter did not exhibit a significant protective effect for probiotics during storage or simulated gastrointestinal passage. Results of the study suggest that peanut butter, either full-fat or reduced-fat is an appropriate vehicle to carry probiotics to children prone to diarrhea.

INDEX WORDS: Peanut butter, Probiotics, Diarrhea, Malnutrition, RUTF, Pre-school children, Gastrointestinal passage, *L. rhamnosus* GG, *Salmonella enterica*, *Listeria monocytogenes*

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## DEDICATION

Forever, I honor my work to Jesus Christ for favoring me to pursue graduate studies and for bringing it to a divine fulfillment. This is dedicated to my mother, Patricia Akosua Klu, an educationist who taught me the importance of education at a tender age, instilled essential godly principles into my life and constantly prays for me. This is dedicated to my unborn children as well; my prime energy and drive for this journey, was the incessant beautiful dream of having you invaluable gifts in the future. I did not give up because I want you kids to be proud to have me as your mother just as I am proud of my mother, a rare combination of brains, beauty and virtues.

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## CHAPTER 1

### INTRODUCTION

The issue of childhood mortality is a global public health concern, especially in the developing world (WHO 2011, 2013) and mortality due to infectious diarrhea is about 760,000 each year, resulting in the second highest number of deaths in pre-school children (WHO, 2013). A major risk for diarrheal diseases is malnutrition, which unfortunately is a consequence of diarrhea, and creates a vicious cycle between these two conditions in children under 5 years of age (Caufield et al., 2004; Gorospe and Oxentenko, 2012; Manary et al., 2012; WHO, 2013).

For many years, rehydration has been the major therapy for the management of diarrhea as dehydration is highly linked to the deaths related to diarrhea (WHO, 2005). In addition to rehydration, other therapies have been used including pharmacological, micronutrient supplement, dietary, and probiotics (Podewils et al., 2004; WHO, 2005; UNICEF/WHO, 2009). Improvement of the nutritional status of children affected with diarrhea is one of the main objectives of the treatment package for diarrhea (WHO, 2005) and thus, dietary therapy is essential for the appropriate recovery of children. In recent years, the use of probiotics as an adjuvant therapy in diarrhea management is becoming popular although there are no standards regarding its administration (Kelly, 2011, Whyte and Jenkins, 2012). Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). There are numerous proposed health benefits of probiotics but of high interest to the

public health sector with regards to the health of preschool children is their role in diarrheal management (McFarland, 2009; Butel, 2013). Even though there are no standards regarding the use of probiotics as a therapy for diarrheal diseases, there is documentation on their effectiveness in shortening the duration of diarrhea, reducing the severity of the symptoms and even reducing the incidence of diarrhea in clinical observations (Isolauri, 2004; Nomoto, 2005; Binns and Lee, 2010).

For probiotics to survive harsh conditions in the gastrointestinal tract, attach to intestinal walls and colonize them, and exert the proposed health benefits, food matrices is reported to be one of the most important factors that regulate these functions (Mattila-Sandholm et al., 2002; Ranadheera et al, 2010). For many years, cultured milk products have been widely used as carriers for probiotics (Champagne et al., 2011) but in recent years, other dairy food products and non-dairy products including cheese, chocolates, vegetable products, soy products and ice-cream are being recommended as probiotic carriers (Nebesny et al., 2005; Prado et al., 2008; Granato et al., 2010; Rivera-Espinoza and Gallardo-Navarro, 2010; Karimi et al., 2011). Research has shown that food matrices with high protein and fat contents, high buffering capacity and high pH are good carriers of probiotic bacteria; they protect bacteria during storage as well as during transit in the gut (Boylston et al., 2004; Karimi et al., 2011). Peanut butter is a dense food matrix with high protein and fat content as well as close to neutral pH (Burnett et al., 2000) which makes it a suitable vehicle for probiotic delivery. Peanut butter is also a major ingredient in Ready to Use Therapeutic Foods which are used in the treatment of severe cases of childhood malnutrition (Manary, 2006). The nutritional and physical properties of peanut butter presents a likely suitable vehicle for probiotic bacteria and

thus could be used to address malnutrition and diarrhea concurrently since both conditions are usually presented together in pre-school children.

The objectives of this study were:

1. To observe the survival of selected single strain or multiple strains of probiotic bacteria in full fat and reduced fat peanut butter during a 1 year storage study
2. To observe the fate of selected strains of probiotic bacteria in peanut butter during a simulated gastrointestinal passage
3. To observe the survivability of selected foodborne pathogens in the presence of probiotics in a simulated gastrointestinal study

## CHAPTER 2

### LITERATURE REVIEW

#### **1. Diarrheal diseases in pre-school children**

##### **1.1. Their causes and public health burden**

Diarrhea is from the Greek word “flow through” and is defined from that word as the rapid transit of gastric contents through the bowel (Whyte and Jenkins, 2012). The World Health Organization (WHO), describes diarrhea as the passage of 3 or more loose or watery stools, or passage more frequently than normal for a given individual within a period of 24 hours (UNICEF/WHO, 2009). The issue of diarrhea is a worldwide problem and even in industrialized nations, diarrhea is a considerable cause of morbidity in the first year of life (Whyte and Jenkins, 2012). In most developing countries, children under 3 years of age experience an average of 3 episodes of diarrhea yearly with high mortality rates (UNICEF/WHO, 2009). Diarrhea is prevalent in the developing world, largely due to the lack of safe drinking water, poor hygiene and sanitation, poor health and poor nutrition status (UNICEF/WHO, 2009). A UNICEF/WHO (2009) report stated that, Africa and South Asia account for more than 50% of childhood diarrhea cases and more than 80% of deaths that occur in children in these continents is due to diarrhea. Statistics show that globally, approximately 20% of all mortality occur in children under 5 years of age (WHO, 2011) and an estimated 2.5 billion cases of diarrhea occur yearly among pre-school children (UNICEF/WHO, 2009). In 2012, 6.6 million pre-school children died from many causes with diarrhea related causes being the second highest (WHO, 2013).



Furthermore, according to the 2013 WHO facts, an estimated 760,000 deaths occurring yearly amongst children less than 5 years old is attributed to diarrhea. Diarrhea causes loss of lives in pre-school children more than malaria, AIDS and measles combined. The burden of diarrhea on the persons affected, the public health sector and the total economic development of a nation is so large that it is estimated that 13% of all Disability Adjusted Life-Year (DALY) are caused by diarrhea (WHO, 2011). DALY is defined by the WHO as the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability. Simply, one DALY is defined as one lost year of healthy life. According to the United Nations (UN) Millennium Development Goals, child mortality is to be reduced by two thirds between 1990 and 2015. Progress has been made by reducing childhood mortality to 47%, from 12.4 million in 1990 to 6.6 million in 2012 (UN, 2013). The average rate of death from diarrhea has also decreased in recent years from about 1.5 million in 2009 (UNICEF/WHO, 2009) to about 760,000 in 2012 (WHO, 2013). However, 6.6 million total deaths and 760, 000 deaths due to diarrhea is still an alarming number of young human resources being lost each year; especially in sub-Saharan Africa, where 1 in 10 children die before their 5<sup>th</sup> birthday (UN, 2013).

## **1.2. Causes and mechanisms**

The major causes of diarrhea are gastrointestinal infections and malnutrition (UNICEF/WHO, 2009). In cases of diarrhea caused by gastrointestinal infections, the major pathogen involved is rotavirus which is responsible for 40% of diarrheal cases (UNICEF/WHO, 2009). The other most common causative agents which are of bacterial origin include the species *Shigella*, *Campylobacter*, *Salmonella* and *E. coli*. Diarrheal

infections from protozoan sources are rare but in some cases, *Cryptosporidium* species have been isolated from patients (Manary et al., 2012). It is a common assumption that cholera causes numerous childhood mortalities due to diarrhea however, such deaths occur more in adults and older children than in pre-school children (UNICEF/WHO, 2009).

All populations are susceptible to diarrhea but pre-school children are at a greater risk of life-threatening dehydration caused by diarrhea because water constitutes a bigger part of a child's body. Additionally, children have a higher metabolic rate and their kidneys cannot conserve much water which leads to a more harmful effect when they are dehydrated (UNICEF/WHO, 2009). The secretion and absorption of water and electrolytes in the gastrointestinal system is a highly dynamic and balanced process and the intestines handle large volumes of electrolytes, water and nutrients daily with the secretion and absorption of fluids occurring simultaneously (Whyte and Jenkins, 2012). If the amount of fluid secreted from the small intestines exceeds the absorptive capacity of the large intestines and the efficiency of reabsorption of fluids in the intestines reduces from 99% to even about 98%, diarrhea could occur (Schiller, 2012; Whyte and Jenkins, 2012). Excess water in feces occurs through one of the following mechanisms: poorly absorbed ingested materials with high osmotic activity that hold excess fluid, reduction in the rate of absorption of fluid from the intestines, and increase in the secretion rate of fluids (Schiller, 2012). In general, two forms of diarrhea are usually seen in patients; osmotic and secretory diarrhea (Schiller, 2012; Whyte and Jenkins, 2012; Pezzella et al., 2013).

Osmotic diarrhea is caused by excess osmotically active particles in the gut lumen which causes inflammation within the gut mucosa and also drives water into the lumen *via* osmotic force (Whyte and Jenkins, 2012). The overload of malabsorbed osmotically active substances is what causes the water to be retained in the intestinal lumen, causing a watery diarrhea (Dinesen and Harbord, 2013). In the event of osmotic diarrhea, the osmotically active substances retain water within the intestines because water is freely permeable in the intestines (Schiller, 2012). Excessive intake of laxatives and magnesium containing drugs can result in osmotic diarrhea (Dinesen and Harbord, 2013). Additionally, malabsorption of solutes like lactose, motility disorders and damage to absorptive area of the mucosa can result in osmotic diarrhea (Whyte and Jenkins, 2012).

Secretory diarrhea on the other hand is characterized by the secretion of excess amount of fluids by the bowel mucosa into the lumen; causes include toxins, pathogenic infections, and also abnormalities in absorptive mechanisms (Dinesen and Harbord, 2013). Toxins and peptides cause secretory diarrhea by abnormal ion transport across intestinal epithelial cells (Dinesen and Harbord, 2013). In the intestines, the cells in the Lieberkuhn's crypts function as net secretors of electrolytes and water and also contain a bidirectional sodium/chloride channel which opens up when there are higher levels of cyclic AMP (cAMP) and calcium ions (Whyte and Jenkins, 2012). When these channels open, there is a net movement of chloride, sodium and water into the lumen and a slight change in the flow across the channel causes an enormous increase in secretion. Toxins including cholera toxin and pathogenic bacteria like *E. coli* causes increase in cAMP levels, which drive chloride across the brush border membrane into the lumen, causing a net movement of excess water, resulting in watery secretory diarrhea (Whyte and Jenkins,

2012). Furthermore, abnormal motility, which causes intestinal contents to rush past absorptive sites before absorption is completed, can result in either osmotic and secretory diarrhea; it causes secretory diarrhea when water and electrolytes are incompletely absorbed and it cause osmotic diarrhea when the digestive process for osmotically active substances is partial (Schiller, 2012). Osmotic diarrhea can be controlled when a child is fasted; however secretory diarrhea is aggravated when food is withdrawn from the child. Additionally, there are situations where both osmotic and secretory diarrhea can occur concurrently (Whyte and Jenkins, 2012, Pezzella et al., 2013).

### **1.3. Clinical presentation**

Diarrhea in children is mostly caused by infectious agents of viral, bacterial and parasitic origins (WHO, 2013) and is transmitted through the fecal-oral route (UNICEF/WHO, 2009). The condition exhibits in three main clinical forms which include acute watery diarrhea, bloody diarrhea and persistent diarrhea (WHO, 2005, UNICEF/WHO, 2009). Other clinical presentations will also be discussed in addition to the three mentioned.

#### **1.3.1. Acute diarrhea**

Acute watery diarrhea is usually caused by *Vibrio cholerae*, rotavirus and *E. coli* and is characterized by large amounts of fluid losses and usually causes rapid dehydration in affected children (UNICEF/WHO, 2009; Kelly, 2011). This type of diarrhea can last for several hours up to about 7 days (UNICEF/WHO, 2009; Kelly, 2011). The key risk for this type of diarrhea is dehydration and weight loss in children who are not well fed (WHO, 2005). In the case of severe dehydration, if prompt rehydration therapy is not

undertaken, metabolic acidosis occurs, consciousness may be impaired and can eventually lead to coma (Kelly, 2011).

### **1.3.2. Bloody diarrhea (dysentery)**

Bloody diarrhea also known as dysentery is characterized by blood in the watery stools. There is intestinal damage and rapid nutrient losses in affected individuals; and bacterial pathogens involved include *Shigella spp*, *Salmonella spp*, *Campylobacter jejuni* and enterohemorrhagic *E. coli* (Kelly, 2011). Dehydration may also occur with this type of diarrhea (WHO, 2005).

### **1.3.3. Persistent diarrhea**

Individuals suffer from episodes of diarrhea which lasts for at least 14 days and feces may or may not have blood. Persistent diarrhea is very common in malnourished children or children with AIDS, and enteropathogenic *E. coli* and enteroaggregative *E. coli* are mostly associated with it. This type of diarrhea aggravates malnourishment and disease conditions in these children (WHO, 2005; UNICEF/WHO, 2009).

### **1.3.4. Chronic diarrhea**

Chronic diarrhea refers to diarrhea that persists for more than 3 (Whyte and Jenkins, 2012) to 4 (Gorospe and Oxentenko, 2012) weeks.

### **1.3.5. Antibiotic Associated Diarrhea (AAD)**

In certain situations, antibiotic therapy is used in the treatment of infectious diarrhea (WHO, 2005; Kelly, 2011; Guarino et al., 2012; Dinesen and Harbord, 2013). Another form of diarrhea that is present in children is known as Antibiotic-Associated Diarrhea (AAD). AAD is defined as otherwise unexplained diarrhea that occurs with the administration of antibiotics, and its occurrence in the pediatric population is 11 – 40%

between the initiation of therapy and up to 2 months after cessation (Szajewska and others 2006). Beaugerie and Petit (2004) also defined AAD as diarrhea that develops from few hours after the onset of antibiotic therapy to 6-8 weeks after therapy has ended. It is documented that antibiotic usage disrupts the integrity of the gut barrier which results in an individual's susceptibility to pathogen colonization and growth until the normal microflora is re-established (McFarland, 2009). AAD may occur due to the overgrowth of pathogenic microorganisms, direct toxic effect of antibiotics on the intestines or the alteration of digestive functions which results from low populations of normal gut flora (Beaugerie and Petit, 2004).

#### **1.3.6. Diarrhea with severe malnutrition (kwashiorkor or marasmus)**

This form of diarrhea causes high rates of mortality with symptoms of heart failure, protein, vitamin and mineral deficiency, severe dehydration and systemic infections (WHO, 2005). Furthermore, persistent diarrhea and bloody diarrhea also accounts for increasing numbers of deaths with these conditions (WHO, 2005; de Mattos et al., 2009).

## **2. The relationship between diarrhea, malnutrition and gastrointestinal health in pre-school children**

Diarrhea is in actual fact a combination of a nutritional disease with fluid and electrolyte loss. Severe acute malnutrition creates a vulnerable fluid and nutrition homeostasis and also, diarrhea reduces fluid and nutrient absorption (Manary et al., 2012). For several years in times past, malnutrition has been known to exacerbate the burden of life-threatening diseases in developing countries (Pelletier et al., 1995). Even

though risk of dying is high amongst severely malnourished children, because the prevalence of moderate malnourishment is high, many of the deaths that occur as a result of malnutrition are attributable to moderate under-nutrition (Pelletier et al., 1995). In recent years, malnutrition in young children is still a major contributing factor to disease prevalence in children with diarrhea being no exception (Caufield et al., 2004). Diarrhea is both a cause and a consequence of malnutrition in pre-school children; a vicious cycle exists between malnutrition and diarrhea (UNICEF/WHO, 2009; Manary et al., 2012; WHO, 2013). When children are undernourished, they are at a higher risk of frequent episodes of diarrhea which are very severe and go on for several days. Unfortunately, when these children go through repeated bouts of diarrhea, their nutritional and health statuses worsen because they are not able to feed well and nutrient absorption is decreased which exacerbates malnourishment. For this reason, diarrhea has been documented to be one of the causes of stunted growth (UNICEF/WHO, 2009). A high risk for death and disability occurs when children with severe acute malnourishment have numerous incidence of diarrhea because high loss of water from diarrhea can lead to dehydration, shock, electrolyte imbalance and poor mental health (Suh et al., 2010). Most malnourished children who do not die from diarrhea face chronic problems in their physiological and intellectual development (Whyte and Jenkins, 2012). Furthermore, when children suffer from severe and numerous bouts of diarrhea, they become susceptible to morbidity and mortality from other infections and diseases (Moore et al., 2010).

There is a bidirectional causal relationship between chronic diarrhea and malnutrition, and the nature and severity of malnourishment depends on cause of the

chronic diarrhea coupled with the location and extent of gastrointestinal involvement (Gorospe and Oxentenko, 2012). Chronic diarrhea predisposes a child to a poor nutritional status, and malnutrition increases the risk of having prolonged diarrhea due to poor immune response and other adverse mucosal conditions (Gorospe and Oxentenko, 2012). Malnutrition broadly refers to an imbalance of nutritional intake and utilization, and the type of malnutrition presented in developing and under developed countries is under-nutrition and/or protein-energy malnutrition. Malnutrition in pre-school children is mostly due to inadequate intake of appropriate nutrients and/or the inability to maintain adequate stores of calories and essential nutrients due to underlying causes such as chronic diarrhea (Gorospe and Oxentenko, 2012). Malabsorption refers to defective mucosal absorption of nutrients mostly in the small intestines and is an underlying process for both malnutrition and chronic diarrhea. Chronic diarrhea may also result in lethargy and increased nutrient losses which eventually leads to malnutrition; this vicious cycle continues with impairment to intestinal recovery, and consequently, increasing rates of malabsorption and, aggravating malnutrition and diarrhea. Fig. 2.1 (adapted from Gorospe and Oxentenko, 2012) depicts the simple vicious cycle of malnutrition and chronic diarrhea. There also exists a relationship between gastrointestinal infections, chronic diarrhea and malnutrition (Gorospe and Oxentenko, 2012). Infections directly cause malnutrition by increasing catabolism and caloric loss and indirectly cause malnutrition by inducing mucosal injury and inflammation, subsequently resulting in chronic diarrhea. Malnutrition can also prolong the period an infection persists as it impairs the host's immune response and gut mucosal barrier functions (Gorospe and Oxentenko, 2012). Fig. 2.2 shows the relationship between gastrointestinal infections



and malnutrition as adapted from (Gorospe and Oxentenko, 2012). It is impossible to discuss diarrhea without considering intestinal health. To combat malnutrition and reduce the mortality of young children, the importance of intestinal health cannot be overlooked (Sekirov et al., 2010). In 400 B.C., Hippocrates, made a phenomenal statement: “death sits in the bowels” and “bad digestion is the root of all evil” (Sekirov et al., 2010). Colonization of the gastrointestinal tract starts during the birthing process and an individual’s microbiota is influenced by genetic factors, antibiotic usage, neonatal nutrition, adult nutrition, mode of infant delivery and hygienic factors (Brown et al., 2012). Research suggests that, the numbers and composition of microbes in the gut is related to disease; it has an influence on the susceptibility of an individual to chronic diseases like irritable bowel syndrome, obesity, diabetes, celiac disease and Crohn’s disease and has been implicated in several cases of these diseases (Sekirov et al., 2010; Brown et al., 2012). A number of studies has shown that there are differences in the microbiota of individuals based on their diet which directly influences their susceptibility to both infections and chronic ailments; and the microbiota of the gastrointestinal tract is one of the most critical factors in determining the susceptibility of an individual to gastrointestinal infections (Sekirov et al., 2010; Brown et al., 2012). Diet and nutrition, especially relating to neonates and in children less than 5 years of age play one of the most important roles in the diversity and function of the gut microflora (Brown et al., 2012). de Lange et al. (2010) reported that, feeding young pigs with dietary fiber, crude protein, plasma proteins, feed enzymes, probiotics, prebiotics and essential oils stimulated the establishment of a healthy gut microbiota, improved growth and reduced the incidence of infections especially diarrhea. A study conducted in Bangladesh

revealed that the gut microbiota of healthy and malnourished children differed significantly especially with the malnourished children having higher numbers of pathogenic bacteria from the genera *Escherichia* and *Klebsiella* (Monira et al., 2011). Results from that study confirm that diet plays a very unique and important role in the balance and function of the gut microbiota and the overall health of children less than 5 years of age. The relationship between diet and gastrointestinal health suggests why severely and/or moderately malnourished children are more prone to diarrheal diseases.

### **3. Current intervention methods and the role of probiotics**

Regardless of whichever type of clinical diarrhea may persist, one or more of the following interventions may be involved; they can be broadly categorized into rehydration therapy, pharmacologic therapy, micronutrient supplementation and dietary therapy (Podewils et al., 2004; WHO, 2009).

#### **3.1. Rehydration therapy**

Loss of water and electrolytes occur during diarrhea, is a situation that is highly linked to death. The WHO has recommended oral rehydration therapy since 1970 to prevent severe dehydration associated with diarrhea; rehydration is the first aid in the treatment plan for all forms of diarrhea (WHO, 2005). In the event of severe dehydration intravenous rehydration is required (Kelly, 2011); normal saline or lactated ringer solution should be administered intravenously immediately at a rate of 30 mL/kg of body weight for 30 min to 1 h and then 70 mL/kg body weight for 2.5 to 5 h until the perfusion, pulse and mental status normalizes, thereafter oral therapy is administered (Podewils et al., 2004; WHO, 2005). In the event of mild to moderate dehydration, Oral

Rehydration Therapy (ORT) should be initiated (Podewils et al., 2004) and the most common rehydration therapy used is the Oral Rehydration Salt (ORS) administered at a rate of 75 mL/kg of body weight per h for 4 h (Podewils et al., 2004; WHO, 2005). The WHO (2005) reported that an improved ORS has been developed after 20 years of research. The new formula is called low or reduced osmolarity ORS. Its use reduces the need for supplemental intravenous fluid therapy by 33% after initial rehydration. The WHO findings recorded that, upon administration of the low osmolarity ORS, the incidence of stool volume is reduced by 20% and the incidence of vomiting by 30%; this new formula is currently recommended by UNICEF/WHO. There are other available commercial rehydration fluids and homemade fluids which include gruels of very thin consistency made from maize, rice, potato, millet or sorghum with salt and some sugar added; generally foods or fluids with very thin consistency which contains salt are recommended (WHO, 2005). Furthermore, breastfeeding is a good rehydration therapy for young children (WHO, 2005; Kelly, 2011). The administration of ORS does not directly stop diarrhea but rather controls dehydration. In 1966, scientists discovered that the sodium glucose transporter is not affected by the pathogens responsible for diarrhea and thus in the presence of sodium and glucose in the lumen, the transport mechanism continues to work even as the chloride channel continues to cause secretion of fluids (Whyte and Jenkins, 2012). Thus, because ORS contains both sodium and glucose in the correct proportions, when administered, the absorption of sodium is increased, thereby promoting passive absorption of water. Therefore by administering ORS to affected children, they are hydrated until the causative pathogen is eliminated from the body (Whyte and Jenkins, 2012).

### **3.2. Pharmacologic therapy**

Antimicrobials are not part of the normal routine for diarrheal treatment and its use is controversial as diarrheal cases is self-limiting (Podewils, 2004). The WHO recommends that antimicrobials should not be used routinely because it is a hurdle to clinically distinguish between cases caused by enterotoxigenic *E. coli* from those caused by rotavirus and *Cryptosporidium*, which cannot be eliminated by antimicrobials (WHO, 2005). According to the WHO (2005), the sensitivity of the causative agent is needed for proper antimicrobial administration and such information is usually unavailable. Additionally, antimicrobial usage increases the risk of adverse reaction and promotes the development of resistant bacteria (Servin, 2004). However, antimicrobial treatment decreases the duration of the ailment and also reduces the fluid requirements (Podewils, 2004). Antimicrobials are used in some cases including *Campylobacter* infections, dysenteric shigellosis, cholera, and some protozoal infection in severe immunocompromised patients and patients with bacterial overgrowth in the intestines (Podewils et al., 2004; WHO, 2005; Kelly, 2011; Guarino et al, 2012; Whyte and Jenkins, 2012).

### **3.3. Micronutrient supplementation**

Zinc and vitamin A are the most common micronutrients given during episodes of diarrhea as an adjunct therapy (Manary et al., 2012). Treatment package recommended by UNICEF/WHO for diarrhea control includes two main components which are rehydration and zinc supplementation (UNICEF/WHO, 2009), thus making zinc supplementation a very important factor in diarrhea management. The WHO recommends the administration of zinc (10-20 mg/day administration for 10-14 days) to

children suffering from acute or persistent diarrhea (Podewils et al., 2004; WHO, 2005; UNICEF/WHO, 2009) and it has been documented that zinc shortens the duration of diarrhea and lessens the severity. The administration of zinc has also proven to reduce mortality rates from persistent diarrhea by 75% (Podewils et al., 2004). Zinc is both preventive and therapeutic in diarrheal management and stimulates immune response, ion absorption, and promotes the repair and proliferation of epithelial cells (Guarino et al., 2012). Podewils et al. (2004) reported poor consistent research results on the effectiveness of Vitamin A for reducing the severity of diarrhea. However, WHO reports that vitamin A supplementation has been shown to decrease the duration, severity and complications associated with diarrhea (UNICEF/WHO, 2009).

### **3.4. Dietary therapy**

The main objective of any treatment package in the event of diarrhea is to prevent dehydration and also to improve the nutritional status of the affected child (WHO, 2005) and thus the importance of food in diarrheal management cannot be underestimated. Food consumption in the form of a soft bland diet during the period of diarrhea is recommended as it helps in fluid absorption and consequently rehydration, speeds up recovery of intestinal function, and aid in the repair of enterocytes (Whyte and Jenkins, 2012; Dinesen and Harbord, 2013). As a rule, the WHO recommends that the usual diet of a child should not be withheld or diluted but continued during diarrhea and increased after the diarrhea clears and children being breastfed should continue breastfeeding (WHO, 2005). When a child suffering from diarrhea does not feed well, the duration of illness increases and restoration of normal intestinal functions prolongs. In addition, because most malnourished children suffer from diarrhea, withholding food from them

worsens their nutritional status and increases mortality rate (UNICEF/WHO, 2009). The importance of food in diarrhea management was reported in a study by de Mattos et al. (2009). They observed the duration of persistent diarrhea in children when fed with four different diets; children fed with a yoghurt-based or amino acid-based diet, experienced a significant reduction in the duration of diarrhea and in the stool output. However, soy-based and casein-based diets did not have any benefit in the management of persistent diarrhea in the children. Their findings suggest that indeed diet plays a role in the occurrence and management of diarrhea.

### **3.5. Probiotics**

Some authors have reported that the role of probiotics in the management of diarrhea is unclear and thus administration is not routinely recommended (Podewils et al, 2004; Kelly, 2011; Whyte and Jenkins, 2012). A report by Manary et al. (2012) indicates that specific probiotics (*L. rhamnosus* GG, *L. bulgaricus* and *S. thermophilus*) decreased diarrhea frequency and duration in well-nourished children but data from malnourished children was mixed. Regardless, numerous preliminary experiments and clinical studies show probiotics as an important new therapy for the prevention and treatment of infectious diarrhea (Saavedra, 2000). There are numerous documentations from clinical studies that suggest the effectiveness of probiotics in reducing the incidence of diarrhea, shortening the duration and/or reducing the severity of diarrhea (Marteau, 2001; Cremonini et al., 2002; Tuohy et al., 2003; Isolauri, 2004; Nomoto, 2005; Binns and Lee, 2010).

#### **4. What probiotics are**

In recent years, there is more awareness on the relationship between diet and health and this has birthed functional foods. The term functional food is not defined by law or in the dictionary and is an emerging term for foods that have health benefits beyond basic nutrition. Different organizations have different definitions for the term but they all have a common root. IFT's functional food experts define functional foods as foods and food components that provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects (IFT, 2014). Functional foods are placed between foods which supply basic physiological needs and drugs that treat diseases and are used to sustain good health and counterbalance small physiological changes that may occur in a healthy individual (Jankovic et al., 2010). In recent times, most functional food products target gastrointestinal health because the gut acts as an interface between diet and metabolic pathways in the human body (Zubillaga et al., 2001; Reid, 2008) and hence, probiotic organisms are of much interest in the functional food industry. As early as 1992, Roy Fuller defined probiotic foods as foods containing live microorganisms believed to actively enhance health by promoting the balance of microflora in the gut (Shah, 2007). Probiotics are classified as functional food components and the current definition is live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). It must also be noted that probiotics are not commensal microorganisms found in the gut (Reid, 2008). Prior to this definition, a very popular one existed which was live microbial supplements that beneficially affect the host by improving its intestinal

microbial balance (Fuller, 1989). Both the past and recent definitions propose probiotics as microorganisms which improve the health status of the consumer. The word 'probiotic' is originally from the Greek word 'pros bios' which means 'for life' (Gismondo et al., 1999). Members of the genera *Bifidobacterium* and *Lactobacillus* are the mostly used probiotics and with many documentation on their effectiveness (Gomes and Malcata 1999; Shah, 2007; Alegre et al., 2011; Butel, 2013). Furthermore, some species from the genera *Leuconostoc*, *Enterococcus* and *Pediococcus* also exhibit probiotic properties (Shah, 2007; Butel, 2013). Many probiotic microorganisms are lactic acid producing bacteria which are gram positive, nonmotile, non-sporeforming rod and coccus-shaped organisms that ferment carbohydrates and alcohol to predominantly produce lactic acid (Stiles and Holzapfel, 1997). To be considered as a probiotic organism, a bacterium must be resistant to acids and bile, attach to human epithelial cells, colonize in the human intestines, produce antimicrobial substances, have good growth characteristics and also have some proposed health benefits (Prado et al., 2008; Ranadheera et al., 2010; Sarkar, 2010). The intestinal microbiota plays a role in homeostasis, as well as metabolic, nutritional, physiological and immunological processes in the human body (Sekirov et al., 2010; Gerritsen et al., 2011). The gut microbiota is rich with a diversity of microorganisms (about 100 trillion of over 1000 species) with differences in numbers and composition at different sections of the gastrointestinal tract (Sekirov et al., 2010; Gerritsen et al., 2011; Jost et al., 2012). The gastrointestinal tract is a large organ and harbors a large percentage of the human microflora with the colon alone containing over 70% of the microbes in the body (Sekirov et al., 2010). As already stated, there is an important relationship between the microbiota of an individual and the function of the



intestines so it is vital that the microbiota functions normally to maintain a balance immunity and homeostasis (Brown et al., 2012) which thus makes probiotics indispensable in maintaining good gastrointestinal health. Recently, there have been much research on probiotics and numerous health benefits have been proposed.

Although the mechanisms of action are not very understood there are few proposed one and are listed below.

#### **4.1. Proposed mechanisms of action**

##### **4.1.1. Modulation of host's microbiota**

Originally, the purpose for probiotics was to change the composition of the normal intestinal microflora from a potentially harmful composition into a microflora that would be beneficial towards an individual (Ouwehand et al., 2002). This statement suggests that the modulation of host's microbiota is one of the important and/or initial mechanisms by which probiotics exert their health benefits. The microbiota is an important constituent of the intestine's defense barrier because it induces and maintains specific immune responses and hypo-responsiveness to antigens (Tuohy et al., 2003). Modulation of the host's microbiota produces what is called the "barrier effect" which is the resistance to colonization by pathogenic microorganisms (Butel, 2013). Inhibition of pathogens may be due to the production of metabolites such as short chain fatty acids that reduces lumen pH, production of bio-surfactants and other substances with antimicrobial activity, competition for binding and adhesion sites, production of broad spectrum bacteriocin, and inhibition of adhesion (Ouwehand et al., 2002; Nomoto, 2005; Oelschlaeger, 2010; Butel, 2014).

#### **4.1.2. Enhanced gut barrier functions**

Intestinal mucosa provides protection to the host against antigens in the gut lumen that originate from ingested food as well as the normal microflora (Ouwehand et al., 2002). The permeability of the intestines is a reflection of the gut-barrier function and an immature gut barrier could lead to severe intestinal permeability, immune responses and abnormal antigen transfer which increase the host's vulnerability to inflammation and infections (Isolauri, 2001). Some of gut barrier functions include peristalsis, production of gastric acid and mucus, intestinal proteolysis and intracellular junction complexes (Ouwehand et al., 2002). Probiotics are known to improve gut barrier functions and one mechanism is by increasing the expression of mucins that leads to improved barrier effects by preventing direct contact of pathogens with the intestinal lumen (Isolauri, 2001; Butel, 2014). Additionally, probiotics are suggested to improve the junctions between intestinal epithelial cells as a physiological barrier function and improve the production of antimicrobial peptides (Butel, 2014).

#### **4.1.3. Immune modulation**

More than 70% of immune cells are present in the gut, making the intestines one of the largest organs that controls immune functions in the body (Butel, 2013). The gastrointestinal barrier controls antigen transport and the generation of immunologic phenomena in the gut and an immature or poor gut barrier may cause an increase in intestinal permeability and irregular antigen transfer and immune responses which makes a person susceptible to infections, inflammation and hypersensitivity (Isolauri, 2001). Probiotics may indirectly influence the body's immune functions by changing the activity and of composition of the intestinal flora (Ouwehand et al., 2002; Shah, 2007). Isolauri

(2001) elaborated that consumption of probiotics reinforces immune regulation, immune elimination and immune exclusion. Interaction of probiotics with host epithelial cells via adhesion can activate a signaling cascade that could lead to immune modulation (Oelschlaeger, 2010). In addition, stimulation of secretory IgA is increased (Butel, 2014) and a proposed mechanism of stimulation is: some probiotics can liberate low molecular weight peptides that trigger an immune response that stimulates the production of IgA in response to antigenic stimulus (Tuohy et al., 2003). *L. casei shirota* has been reported to promote the proliferation of macrophages and neutrophils in the bone marrow and spleen (Nomoto, 2005). Data also suggest that in neonates when the gut microbiota delays in development, there is delayed maturation of circulating IgA and IgM secreting cells (Isolauri, 2001), hence confirming the importance of a gastrointestinal health in immune function.

## **4.2. Proposed health benefits**

### **4.2.1. Improvement in lactose metabolism**

One of the most widely accepted health benefits of probiotic microorganisms is the relief of the symptoms of lactose malabsorption (Shah, 2007). Lactose intolerance is a digestive problem, which is very common amongst many populations worldwide and is caused by the deficiency of the enzyme  $\beta$ -D-galactosidase. This enzyme is responsible for the cleaving of lactose into glucose and galactose. Probiotic cultures produce  $\beta$ -D-galactosidase which is released into the intestines when the bacteria are lysed by bile salts (Tuohy et al., 2003). Thus, probiotics can help in alleviating lactose malabsorption (Gismondo et al., 1999; Kailasapathy and Chin, 2000; Zubillaga et al., 2001; Shah, 2007).

#### **4.2.2. As an adjunct therapy in inflammatory bowel disease (IBD) and irritable bowel syndrome**

One of the well documented clinical applications of probiotics is its use as adjunct therapy in gut-inflammatory conditions (Isolauri, 2001). Some authors have proposed that although the mechanism of IBD is not clear, it is assumed that the disease is a result of dysfunction of the host immune response towards normal gastrointestinal microbiota or from a defective mucosal barrier (Anukam, 2007). Because research has shown that the numbers of *Lactobacillus* and *Bifidobacterium* in patients with Crohn's disease and ulcerative colitis is very low (Shah, 2007), a possible relationship between microbiota and mucosal inflammation is suggested (Butel, 2014). The role of probiotics in inflammatory bowel disease is not curative rather, the use of probiotics prolongs periods of remission after treatment with corticosteroids, hence preventing frequent relapse (Ouwehand et al., 2002; Sullivan, 2002; Tuohy et al., 2003; Shah, 2007; Butel, 2014). The prevention of relapse is believed to be either through the regulation of inflammatory response or modulation of the gut microbiota composition or its activities (Tuohy et al., 2003). Butel (2014) believed after conducting some clinical control trials that, probiotics are a potential therapy for the management of irritable bowel syndrome.

#### **4.2.3. Control of *Helicobacter pylori* infections**

*H. pylori* is associated with the development of peptic ulcer, gastric cancer and chronic gastritis and it produces urease which hydrolysis urea to ammonium thus increasing the pH of the stomach, consequently promoting colonization of the pathogen (Sullivan and Nord, 2002; Shah, 2007). This infection is typically treated with antibiotics which usually have side effects (Shah, 2007). Several *in vitro* studies have shown that

some strains of *Lactobacillus* and *Bifidobacterium* inhibit the growth and attachment of *H. pylori* and reduce the load of the bacteria (Zubillaga et al., 2001; Sullivan and Nord, 2002; Shah, 2007). Nomoto (2005) reported that in a clinical study that involved 53 individuals infected with *H. pylori*, the patients who were administered with fermented milk containing *L. johnsonii* La1 had lower populations of *H. pylori* in the stomach at the end of the study. *S. boulardii* has also been shown to be a promising adjuvant for antibiotic therapy in treating *H. pylori* infections (Butel, 2014).

#### **4.2.4. Reduction in serum cholesterol**

Some studies have shown that probiotic bacteria are able to de-conjugate bile salts and de-conjugated bile salts does not absorb lipids as readily as conjugated bile salts and consequently reduces cholesterol levels (Gomes and Malcata, 1999; Kailasapathy and Chin, 2000; Shah, 2007; Oelschlaeger, 2010).

#### **4.2.5. Management of diarrhea**

One of the well proven and best documented benefits of the administration of probiotics is the prevention and treatment of infectious diarrhea and antibiotic associated diarrhea (Gorbach, 2002) especially as occurs in children. Probiotics have been proven to be effective against diarrheal causing pathogens including rotavirus, *Salmonella*, *Clostridium difficile*, and *E. coli* (Shah, 2007). Probiotics reduce the incidence, duration and severity of diarrhea, and prevents the occurrence of diarrhea in children (Marteau, 2001; Cremonini et al., 2002; Tuohy et al., 2003, Isolauri, 2004; Sazawal et al., 2006; Szajewska et al., 2006; McFarland, 2009; Binns and Lee, 2010; Butel, 2013). *L. rhamnosus* GG, *L. reuteri*, *L. casei* and *B. lactis* Bb12 are some of the strains which have been adequately studied as effective in managing diarrhea though well controlled clinical

trials (Sullivan and Nord, 2002; Tuohy et al., 2003; Isolauri, 2004; Shah, 2007). In a review of 63 clinical studies of about 10,000 participants, of which 56 of the studies involved infants and young children it was recorded that, notwithstanding the study endpoints and the nutritional status of the patients, the frequency and duration of acute infectious diarrhea was significantly reduced in patients administered with probiotics (Hom, 2011).

Of all the probiotic strains, *L. rhamnosus* GG is the most widely used and also the one with lots of positive clinical reports on their effectiveness in the control, treatment and prevention of diarrhea in children (Gismondo et al., 1999, Gorbach, 2002; Tuohy et al., 2003; Nomoto, 2005). *L. rhamnosus* GG has been reported to be more effective in the treatment of rotaviral diarrhea than *L. bulgaricus* and *S. thermophilus* (Shah, 2007). As early as in the late 90s, Guarino et al. (1997) reported that administration of *L. rhamnosus* GG reduced the duration of rotaviral diarrhea and decreased the shedding of the virus in infected children. In another study, *L. rhamnosus* GG was able to significantly reduce rotavirus diarrhea in more than 100 children in 3 randomized controlled trials (Butel, 2014). *L. rhamnosus* GG was proven to be effective when its administration significantly shortened the duration of rotaviral diarrhea and reduced the risk of nosocomial diarrhea in neonates (Nimoto, 2005). Apart from rotaviral infection, *L. rhamnosus* GG is shown to be useful in the management of AAD. The simultaneous administration of *L. rhamnosus* GG with erythromycin decreased the risk of AAD with the occurrence of diarrhea being 17% in treatment group and 48% in the control group (Cremonini et al., 2002). Researchers of another study observed the effects of probiotic consumption on AAD and they observed that the frequency of AAD in 269

children was 3.4% in treatment group and 17.3% in placebo group (McFarland, 2009). The reduction in the duration of AAD with the administration of *L. rhamnosus* GG was also reported by Gismondo et al., (1999) and Tuohy et al., (2003). Several studies have also shown that *L. rhamnosus* GG was effective in reducing the duration of infantile and acute diarrhea (Sullivan and Nord, 2002; Tuohy et al., 2003; Shah, 2007).

#### **4.2.6. Other proposed functions**

Probiotics are also proposed to have some other functions including: alleviating of allergic reactions, anti-inflammatory response, anti-mutagenic and anti-cancerous effects (Zubillaga et al., 2001; Gorbach, 2002; Ouwehand et al., 2002; Sullivan and Nord, 2002; Touhy et al., 2003; Isolauri, 2004; Shah, 2007; Butel, 2014).

### **5. Role of food matrix in probiotic survival and actions**

#### **5.1. What is a probiotic food?**

Probiotic food product is defined as a food product that contains viable microorganisms in sufficient populations incorporated in a suitable matrix (Cruz et al., 2009). To provide health benefits related to probiotics, recommendations for minimum viable cells in a food product vary and there are no official or specific set standards (Reid 2008; Karimi et al., 2011). Some authors suggest a minimum level of  $10^6$  CFU/g or CFU/mL (Thamaraj and Shah 2004; Helland et al., 2004; Possemiers et al., 2010). However, others recommend a daily consumption of at least  $10^8 - 10^{10}$  cells in about 100 g/mL of products; equivalent to  $10^6 - 10^7$  viable cells in a g or mL of food (Angelov et al., 2005; Cruz et al., 2009; Champagne et al., 2011; Karimi et al., 2011). Furthermore,

the Canadian Food Inspection Agency recommends an arbitrary number of  $10^9$  CFU per serving (Champagne et al. (2011)).

The type of food matrix used as a carrier for probiotic delivery has a significant influence on their survival during the storage of the food product and on their efficacy when consumed (Ranadheera et al., 2010). Food substrate is one of the major factors regulating the colonization of microorganisms in the gastrointestinal tract and protecting probiotic bacteria during transit in the stomach; food may contain ingredients that could interact with probiotics to improve their functionality (Ranadheera et al., 2010). The nature of a food matrix or its formulation is a major technological factor that influences the functionality of probiotics (Mattila-Sandholm et al., 2002). Probiotics are not new products and for centuries, mainly have been carried in cultured/fermented milk (Boylston et al., 2004; Rivera-Espinoza and Gallardo-Navarro, 2010; Champagne et al., 2011; Nousia et al., 2011) which appear to be very good vehicles (Champagne and Gardner, 2005; Ranadheera et al., 2010).

## **5.2. Current trends in probiotic products**

Recently, a lot of research is being done on the possibility of using other dairy products and non-dairy products as potential carriers of probiotic cultures. Consumers are having increasing demand for non-dairy probiotic products because of taste for variety, increase in numbers of vegetarians, cholesterol content of dairy foods, and lactose intolerance issues (Heenan et al., 2004; Prado et al., 2008; Granato et al., 2010; Rivera-Espinoza and Gallardo-Navarro, 2010). It is essential that probiotic foods and beverages are part of regular and daily diet of a population so that therapeutic levels are easily achieved (Rodgers, 2008; Ranadheera et al., 2010) supporting the need for more



variety of both dairy and non-dairy probiotic foods and beverages. Probiotic bacterial cells are required to survive processing steps used to incorporate them into food products and should be stable during storage by maintaining viable numbers and functionality (Knorr, 1998; Ubbink and Krüger, 2006; Cruz et al., 2009; Jankovic et al., 2010; Champagne et al., 2011; Karimi et al., 2011). It is important to examine factors that affect the viability of probiotics during storage separately from those that affect survival of probiotics during processing because the ability to survive during processing is not linked to the ability to survive during storage (Champagne et al., 2005). A key consideration when selecting foods as vehicles for probiotics is the types and concentration of proteins, fat and sugars, as well as the pH of the food product (Ranadheera et al., 2010). It is essential because these contribute to the buffering capacity of foods, an important factor which affect probiotic survival and growth in the gut and stability during storage (Ranadheera et al., 2010; Rivera-Espinoza and Gallardo-Navarro, 2010). There are several studies on successful development different probiotic products, of which some are dairy-based and some are not.

### **5.2.1. Cheese products**

Cheese is one of the most promising carriers for probiotic delivery as it has a dense solid matrix, a relatively high fat content, a higher pH, lower titratable acidity, and a good buffering capacity which protects probiotic cells against harsh gastric and intestinal conditions (Karimi et al., 2011). Several studies have thus been conducted on cheese as a plausible carrier for probiotics. Liong et al. (2009) incorporated *L. acidophilus* FTCC 0291 into a soy-based cream cheese and reported that the probiotic organism maintained the proposed therapeutic level of  $10^6$  CFU/g during a 20 d storage

period at both 4 °C and 25 °C. Argentinian Fresco cheese was used to carry different strains of *Bifidobacterium* and *Lactobacillus* and in 60 d, the probiotic microorganisms survived satisfactorily (Vinderola et al., 2000). Cheese-based dips were found to be an ideal carrier for three strains of *Lactobacillus*, one strain of *Bifidobacterium* and *Propionibacterium* (Tharmaraj and Shah, 2004). Cheddar cheese proved to be a great vehicle for probiotic delivery as therapeutic levels of cells were maintained for 32 wk (Phillips et al., 2006). Fortin et al. (2011) also reported that cheddar cheese as a suitable matrix for *B. longum* and white cheese was successfully made with *L. acidophilus* by Kasimoğlu et al. (2004).

### **5.2.2. Chocolate**

Dark and milk chocolate matrices served successfully as vehicles for the delivery of *L. helveticus* and *B. longum*; and after a simulated stomach and intestinal passage, the chocolate matrix had 5 fold more viable probiotic cells compared to milk matrix. (Possemiers et al., 2010). Sucrose-free milk and dark chocolates were processed with yoghurt bacteria which were shown to survive during the production of the chocolate and remain viable during storage (Nebesny et al., 2005).

### **5.2.3. Vegetable, cereal and soy based products**

Rathore et al. (2012) reported that a strain of *L. plantarum* and *L. acidophilus* was able to ferment cereal substrate to produce a potential probiotic beverage, and Coda et al. (2011) were also able to produce a fermented probiotic beverage from cereals. Soy bar was found to be a suitable matrix for the delivery of some probiotic microorganisms (Chen and Mustapha, 2012). A series of successful probiotic incorporation has been

done with other soy products including: soy yoghurt (Bedani et al, 2014), soy beverage (Champagne et al., 2010), and fermented soy product (Bedani et al., 2013).

#### **5.2.4. Ice cream products**

Some authors have also effectively incorporated different probiotic bacteria into ice-cream which is one of matrices considered as suitable for probiotic delivery. Good survival data have been documented for probiotics during processing and storage of probiotic ice-cream (Hekmat and McMahon, 1992; Salem et al., 2005; Cruz et al., 2009; Mohammadi et al., 2011; Nousia et al., 2011).

#### **5.2.5. Characteristics of peanut butter that makes it a possible vehicle for probiotic delivery as a target towards diarrhea management in children**

Peanut butter is a colloidal suspension of lipid and water in a peanut meal phase (Burnett et al., 2000). It is shelf stable, energy dense, low moisture and nutrient dense product and is one of the major ingredients in Ready to Use Therapeutic Foods (RUTFs) which are used to treat acute and chronic malnutrition (Manary, 2006; Diop et al., 2003; Ndekha et al., 2005). Peanut butter is also one of the natural rich sources of arginine (*ca.* 2.7g/100g peanut butter). Arginine is a conditionally essential amino acid but in neonates and young children, it is essential (Tapiero et al. 2002). Arginine plays a key role in intestinal health. It modulates a lot of metabolic activities including growth and immune function (Nieves and Langkamp-Henken, 2002). Arginine supplementation is effective in improving intestinal barrier function and integrity (Viana et al. 2010; Wang et al. 2009). Several studies with animal models have revealed the function of arginine in gut integrity. When the diets of weaned piglets were supplemented with arginine, their intestinal morphology was improved and the incidence of diarrhea was reduced compared

to control groups (Shan et al. 2012). The physical and nutritional properties of peanut butter make it an ideal vehicle to deliver probiotics and maintain probiotic viability. Therefore probiotic peanut butter can be used to deliver probiotic cultures to manage diarrhea and malnutrition concurrently.

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**Figure Legends**

Fig. 2.1. Vicious cycle of malnutrition and chronic diarrhea (Adapted from Gorospe and Oxentenko, 2012)

Fig. 2.2. Relationship between gastrointestinal infections and malnutrition (Adapted from Gorospe and Oxentenko, 2012)



Fig. 2.1

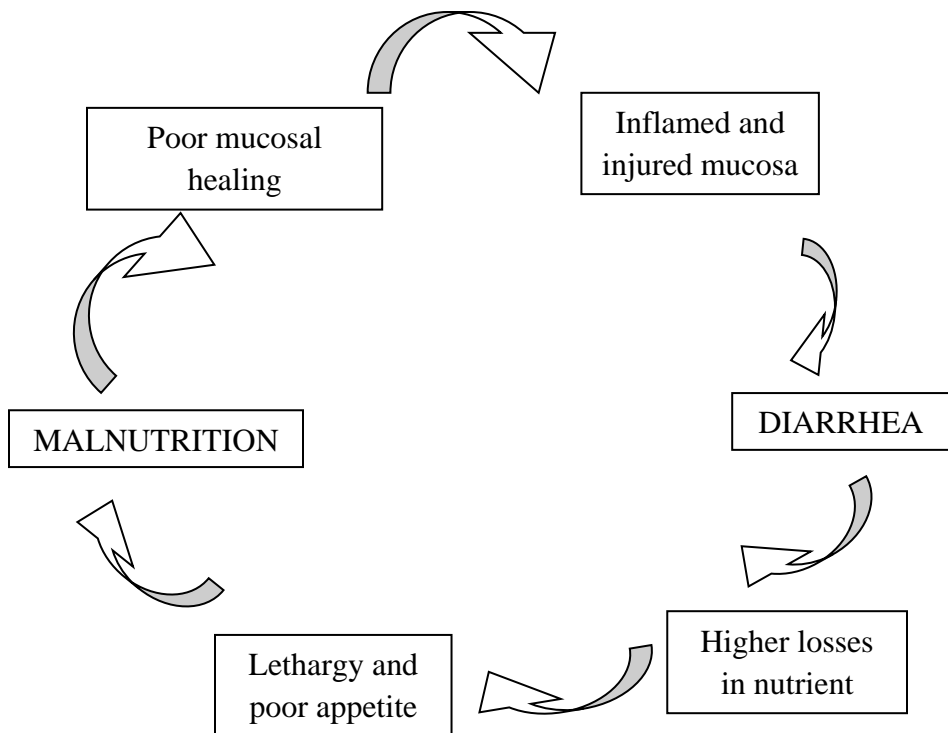
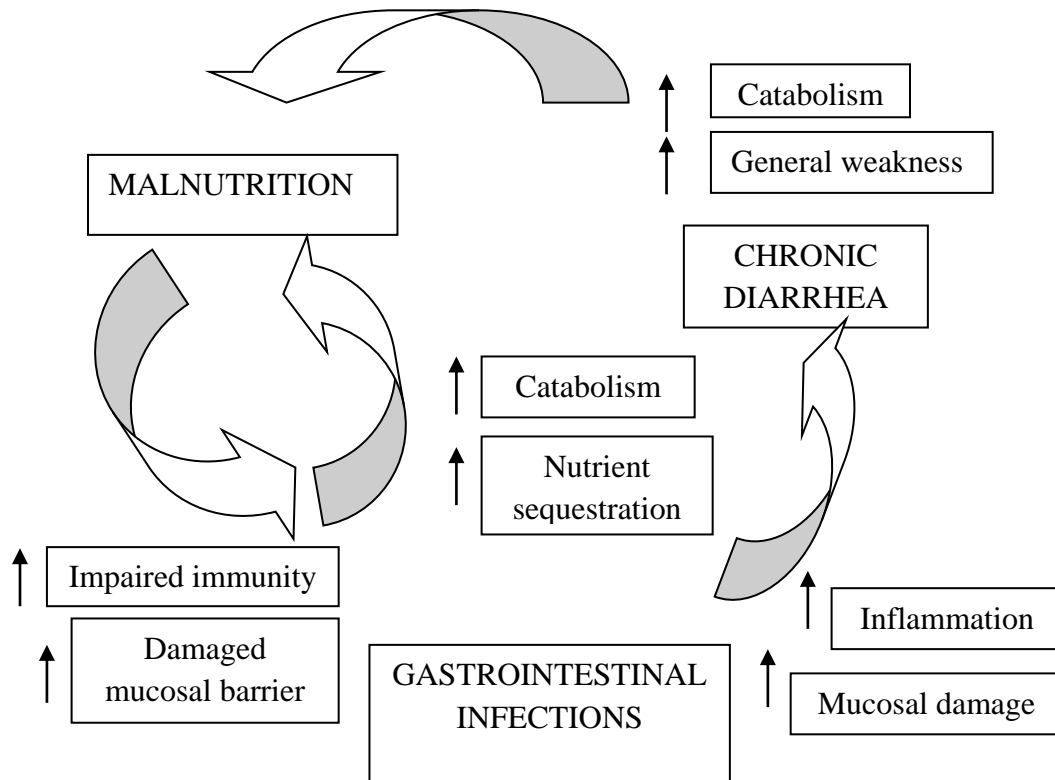


Fig. 2.2



CHAPTER 3

SURVIVAL OF *LACTOBACILLUS RHAMNOSUS* GG AS INFLUENCED BY  
STORAGE CONDITIONS AND PRODUCT MATRIXES<sup>1</sup>

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<sup>1</sup> Klu, Y.A.K., J.H. Williams, R.D. Phillips and J. Chen. 2012. *Journal of Food Science*.  
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**Abstract:** Mortality resulting from diarrhea especially that occurs in children younger than 5 years of age ranks 3rd among all deaths caused by infectious diseases worldwide. Probiotics such as *Lactobacillus rhamnosus* GG are clinically shown to effectively reduce the incidence of diarrhea in children. A food substrate is one of the major factors regulating the colonization of microorganisms in human gastrointestinal tracts. Peanut butter is a nutritious, low-moisture food that could be a carrier for probiotics. In this study, we observed the influence of storage conditions and product matrixes on the survival of *L. rhamnosus* GG. Cells of *L. rhamnosus* GG were inoculated into full fat or reduced fat peanut butter at  $10^7$  CFU/g. Inoculated peanut butter was stored at 4, 25 or 37 °C for 48 wk. Samples were drawn periodically to determine the populations of *L. rhamnosus* GG. Results showed that there was no significant decrease in the viable counts of *L. rhamnosus* GG in products stored 4 °C. The survivability of *L. rhamnosus* GG decreased with increasing storage temperature and time. Product matrixes did not significantly affect the survival of *L. rhamnosus* GG except at 37 °C. Populations of *L. rhamnosus* GG were preserved at > 6 logs in products stored at 4 °C for 48 wk and at 25 °C for 23-27 wk. At 37 °C, the 6 log level could not be maintained for even 6 wk. The results suggest that peanut butter stored at 4 and 25 °C could serve as vehicles to deliver probiotics.

**Keywords:** Survival, probiotics, *Lactobacillus rhamnosus* GG, peanut butter

## Introduction

The World Health Organization (WHO) statistics shows that globally approximately 20% of all deaths are related to children younger than 5 years of age (WHO 2011). Diarrheal diseases are a common contributing factor of childhood mortality. The morbidity rate of diarrheal diseases in the developing world is 3.2 cases per year per child. Approximately 1.7 – 2.5 million annual deaths occur due to diarrhea, and most of these deaths occur in children under the age of 5.

Podewils and others (2004) stated that the causative agents of diarrhea in young children include bacteria, viruses and parasites with bacteria and viruses being responsible for majority of the cases. Therapeutic interventions for diarrheal diseases include rehydration, dietary [including micro-nutrient supplementation (zinc)] and pharmacological (antibiotic usage) remedies (Podewils and others 2004). Administration of sub-therapeutic levels of antibiotics is currently one of the major preventive interventions in developing countries, which unfortunately promotes the development of antibiotic resistance among pathogens and induces antibiotic-associated diarrhea (AAD). AAD is defined as otherwise unexplained diarrhea that occurs with the administration of antibiotics, and its occurrence in the pediatric population is 11 – 40% between the initiation of therapy and up to 2 months after cessation (Szajewska and others 2006). Several clinical studies have shown that diarrheal incidence particularly in children younger than 5 years of age can be controlled or reduced by the administration of probiotics (McNaught and Macfie 2001; Sullivan and Nord 2002; Nomoto 2005; Szajewska and others 2006; Sazawal and others 2006). Probiotics are live

microorganisms which when administered in adequate amounts confer a health benefit on the host (Karimi and others 2011).

Many health benefits have been proposed including immune modulation and maintenance of gut integrity especially through the prevention and control of gastrointestinal malfunctions and infections (Oelschlaeger 2010; Jankovic and others 2010). *Lactobacillus rhamnosus* GG can readily colonize the gastrointestinal tracts of children (Galpin and others 2005), aid in the treatment of *Clostridium difficile* infections, and prevents AAD (Gismondo and others 1999; MacFarland 2009). The bacterium has been shown to reduce the duration of diarrhea by about 50% (Tuohy and others 2003). McNaught and MacFie (2001) reported that in a study of 100 children admitted to a hospital with acute diarrhea, the duration of the illness was 3 days in children receiving *L. rhamnosus* GG compared with 6 days in the control group. A meta-analysis of masked randomized, placebo-controlled trials conducted by Sazawal and others (2006) concluded that consumption of *L. rhamnosus* GG as well as other probiotic strains reduced the incidence of AAD by 52% and the risk of acute diarrhea among children by 57%. In another meta-analysis of randomized controlled trials, Szajewska and others (2006) confirmed that some probiotic strains including *L. rhamnosus* GG could reduce the risk of AAD among children.

To provide health benefits related to probiotics, recommended minimum number of cells in a food product varies with no set standards (Reid 2008; Karimi and others 2011). Some suggest a minimum level of  $10^6$  CFU per g/mL (Thamaraj and Shah 2004; Helland and others 2004; Possemiers and others 2010), and others recommend a daily consumption level of at least  $10^8 - 10^9$  CFU per 100 g/mL of products, equivalent to  $10^6 -$

$10^7$  CFU per g/ml (Angelov and others 2005; Cruz and others 2009; Karimi and others 2011). Mostly recently, higher daily intake levels such as  $10^8$  –  $10^{10}$  CFU have also been recommended (Champagne and others 2011). Generally, the efficacy of probiotics is enhanced when the cultures are ingested with a food product (Gardiner and others 1999; Alegre and others 2011). The fat content, concentration and type of proteins, sugars and product pH are some of the factors that could affect probiotic growth and survival in food (Ranadheera and others 2009). It was stated that a food substrate is one of the major factors in regulating colonization of microorganisms in human gastrointestinal tracts.

Poor nutrition is a predisposing factor to diarrhea morbidity and mortality in children in the developing world; recovery from all kinds of ailments is promoted under non-immune compromised, healthy body systems, and diarrhea in children is no exception. Peanut butter is a relatively inexpensive, shelf stable, energy dense and highly nutritious product, and a handful of peanut contains at least 17% of the daily requirement of each indispensable amino acid. This study was undertaken to observe the survival trends of *L. rhamnosus* GG in full fat and reduced fat peanut butter under refrigeration (4 °C), ambient (25 °C), and abusive (37 °C) storage conditions and to determine the influence of food matrixes on the survival of *L. rhamnosus* GG.

## **Materials and Methods**

### **Materials**

A full fat peanut butter product and a reduced fat peanut butter product were obtained from the American Blanching Company (Fitzgerald, Ga., U.S.A.). The full fat peanut butter contains peanuts, sugar, hydrogenated vegetable oil (rapeseed, cottonseed

and soybean), salt and molasses. The ingredients for the reduced fat peanut butter include peanuts, partially defatted peanut flour, sugar, hydrogenated vegetable oil (rapeseed and cottonseed), salt, molasses, monoglycerides, tocopherol, acetate and pyridoxine HCl. Nutrition labels revealed that each 100 g of full fat product contains 21.31 g of protein, 26.31 g of carbohydrate, 10.57 g of sugar, 7.20 g of fiber, 431.61 mg of sodium and 595.00 mg of potassium. Each 100 g of reduced fat product has 28.12 g of protein, 27.51 g of carbohydrate, 11.67 g of sugar, 8.57 g of fiber, 479.04 mg of sodium and 707.69 mg of potassium. Both products have a total solid content of about 99.00%. *L. rhamnosus* GG [Culturelle® (Natural Health and Wellness; 30 capsules per package)] was purchased from Amerifit, Inc. (Cromwell, Conn., U.S.A.). Each capsule is claimed by the manufacturer to have 10 billion live cells and contains microcrystalline cellulose and milk proteins as carriers. Polyethylene terephthalate (PET) jars (4 oz) and smooth polypropylene (PP) lids with pressure sensitive liner were purchased from Container and Packaging Supply (Eagle, Idaho, U.S.A.).

### **The fat content of peanut butter products**

The fat contents of the two peanut butter products were analyzed using the Goldfish extraction method 948.22a (AOAC, 2000) and a Goldfish Fat and Oil Extractor (Labconco Co., Kansas City, Mo., U.S.A.). Briefly, 4 g samples were placed into Oil Extraction Cellulose Thimbles (Fisher Scientific, Pittsburg, Pa., U.S.A.). The samples in the thimbles were fixed on the Goldfish Fat and Oil Extractor. A 50 mL volume of petroleum ether (J. T. Baker, Phillipsburg, N.J., U.S.A.) was used to extract the fat for about 24 h. Fat content of the samples was derived based on the following calculation: % total fat = (weight of extracted fat × 100) / weight of dry sample.



### **The water activity and pH of peanut butter products**

The pH of peanut butter homogenate (25 g of peanut butter in 50 mL of water) was estimated using a pH meter (model 8000; VWR International, Pa., U.S.A.). The water activity of the peanut products was measured using the Pawkit Water Activity Meter according to the manufacturer's instructions (Decagon Devices, Wash., U.S.A.).

### **The inoculation level of *Lactobacillus***

The actual counts of *L. rhamnosus* GG in each capsule was determined by averaging the cell populations in 5 individual capsules. A 0.1 g of the culture in each capsule was mixed in 9.9 mL of 0.1% sterile peptone water which was pre-heated to 37 °C. After mixing, 1 mL aliquots of the sample was serially diluted in 9 mL of 0.1% sterile peptone water, and 0.1 mL of last three dilutions were spread plated in duplicate onto de Man, Rogosa and Sharpe (MRS) agar (Becton, Dickinson and Co., Sparks, Md., U.S.A. ) and *Lactobacillus* Selection (LBS) agar (Becton, Dickinson and Co.). Inoculated plates were incubated under anaerobic condition for 72 h at 37 °C using the BD GasPak™ EZ in a BBL GasPak® System (Becton, Dickinson and Co.). Cell colonies were enumerated using the Leica Quebec Darkfield Colony Counter (Leica, Buffalo, N.Y., U.S.A.). Average colony counts from LBS and MRS were reported since the two media gave comparable results. From the enumeration results, the actual amount of *L. rhamnosus* GG cells per capsule was calculated by multiplying the cell counts in 0.1 g of capsule content with the average weight of the contents in 20 different capsules. This information is important for verifying the manufacturer's claims and determining the amount of capsule content needed for peanut butter inoculation.

### **Inoculation of peanut butter with *L. rhamnosus* GG**

The PET jars and PP lids, commonly used packaging materials for commercial peanut butter were sterilized under UV light for 15 min in a Level II Biosafety Cabinet (NuAire Laboratory Equipment Supply, Plymouth, Minn., U.S.A.). The full fat and reduced fat peanut butter described above were pre-heated in a Stabil-Therm Electric Oven (Blue M Electric Co., Blue Island, Ill., U.S.A.) at 37 °C for 6 h to reduce product viscosity and aid in uniform mixing. Precisely 2.5 kg of peanut butter was placed into a KitchenAid® mixer, and a pre-determined amount of *L. rhamnosus* GG culture in the dry form was inoculated into the product to achieve an inoculation level of 10<sup>7</sup> CFU/g. The peanut butter and probiotic culture was mixed at room temperature for 15 min at 66 and 148 rpm for orbital and beater speeds, respectively. Nitrogen gas was incorporated into the product during the mixing to remove oxygen that might cause excessive rancidification of fats in the products during storage. Peanut butter exhibits thixotropic behavior and thus after mixing, samples of the inoculated peanut butter (10 mL) were easily dispensed into the PET jars using a sterile syringe (Becton, Dickinson and Co.) which had its end altered to aid product flow. The headspace of the jars was flushed with nitrogen for 30 s to remove oxygen, and the jars were then tightly closed with the PP lids. The weights of peanut butter in each container (22.14 ± 1.75 g) were determined by subtracting the average weight of 20 individual empty containers (23.90 g) from the combined weights of the product and the container. Inoculated peanut butter was stored at 4, 25 or 37 °C for 48 wk to mimic refrigeration, ambient and abusive storage condition, respectively. Un-inoculated peanut butter was used as negative controls.

### **Enumeration of *Lactobacillus***

The initial population of the *Lactobacillus* was determined immediately after the inoculation. Subsequently, samples were drawn monthly from each storage condition for enumeration during the 48 wk storage period. Previously unopened containers were used at each sampling interval. Sterile 0.1% peptone water pre-warmed to 37 °C was added to a sample in the container to achieve a 2-fold dilution based upon the weight of peanut butter in each container. The samples were mixed by vigorous manual shaking for 1 min. After mixing, 1 mL aliquot of the sample was serially diluted in 9 mL 0.1% sterile peptone water. A 0.1 mL of appropriate dilutions was plated using the method described above.

### **Statistical analysis**

Two replicate experiments were conducted. Version 9.1 of SAS (SAS Inst. Inc., Cary, N.C., U.S.A.) was used for data analysis. A 3-way Analysis of Variance (ANOVA) F-test was conducted, and the General Linear Model (GLM) procedure was used to analyze *L. rhamnosus* GG counts as influenced by storage temperature, storage time, food matrix and the interactions between them. The Fisher's Least Significant Difference (LSD) was employed to compare the significant differences between the counts of *L. rhamnosus* GG in the two peanut products stored at three different temperatures for an extended period of time. All analyses were conducted at a 95% confidence level.

## Results and Discussion

### Fat content, water activity and pH of full fat and reduced fat peanut butter

The average fat content of full fat peanut butter was  $50.10 \pm 1.16\%$  and that of reduced fat peanut butter was  $39.90 \pm 0.62\%$ . The average water activity of both products was 0.44 while the pH of the products ranged from 6.10 to 6.35. Product pH is an important parameter that affects the survivability of probiotics (Nebesny and others 2007; Abe and others 2009; Nualkaekul and Charalampopoulos 2011). Champagne and Gardner (2008) found that probiotics lose their viability during storage at pH 4.0-5.0. Water activity is another important factor influencing the survival of probiotic cultures; Abe and others (2009) observed that at four different storage temperatures, 25, 37, 45 and 60°C, the inactivation rate of *Bifidobacterium* increased proportionally in powdered food products with increasing water activity. Low water activity reduced the extent of viability loss of freeze dried *Lactobacillus* (Champagne and others 1996; Weinbreck and others 2010). The near neutral pH and low water activity of peanut butter makes it a promising vehicle for probiotic delivery.

### Survival of *L. rhamnosus* GG

At all sampling points, the control samples had no background flora on MRS and LBS agar (data not shown). The initial counts of *L. rhamnosus* GG in full fat and reduced fat peanut butter ranged between 7.07 and 7.15 log CFU/g (data not shown). Figure 3.1 shows the survival trends of *L. rhamnosus* GG in full fat and reduced fat peanut butter during the 48 wk storage period under the three different storage conditions used in the study. It was observed that by the end of the experimental period *L.*

*rhamnosus* GG decreased less than 1 log CFU/g at 4°C in both full fat and reduced fat peanut butter. At 25°C, the counts of *L. rhamnosus* GG decreased 3 to 4 log CFU/g during the same time period. At 37 °C, the counts of *L. rhamnosus* GG in full fat peanut butter reached to an undetectable level (< 8 CFU/g) by wk 19. A different trend was observed with the bacterium inoculated in reduced fat peanut butter, and the counts of *L. rhamnosus* GG decreased to 3.35 log CFU/g at the same sampling point.

Results of statistical analysis showed that the type of peanut butter, storage temperature, storage time (Table 3.1 and Table 3.2) and the interactions between them (data not shown) significantly ( $P < 0.0001$ ) affected the survival of *L. rhamnosus* GG. Greater survival rates were observed at 4°C, followed by 25 °C and then 37 °C (Tables 3.2 and 3.3). Although the results of overall statistical analysis suggest that *L. rhamnosus* GG had a better survivability in reduced fat peanut butter under all three storage conditions (Table 3.3), significant population differences at individual sampling points were only observed at 37 °C from wk 10 till the end of the storage period (Table 3.1). The counts of *L. rhamnosus* GG in full fat and reduced fat products were not significantly different when stored at 4 °C (Table 3.1) and at 25 °C except at the 27 and 40 wk sampling points (Table 3.1).

Bruno and Shah (2003) stated that appropriate storage temperature was essential to maintain viable populations of probiotic bacteria. Nebesny and others (2007) observed that approximately 89 – 94% of *Lactobacillus* cells inoculated into dark chocolates survived a 12 mo storage period at 4 °C. Saarela and others (2006) detected a better survival rate of *Bifidobacterium* in milk and fruit juices stored at 4 °C compared to products stored at 20 °C. Similar phenomenon was also observed by Bruno and Shah

(2003). After 5 mo, *Bifidobacterium* stored at 20 °C could not be detected while the counts of *Bifidobacterium* stored at 4 °C had declined from 10.61 to 6.02 log CFU/mL over a 20 mo storage period. Results of this and some previous studies have shown that storage of food at low temperature helps preserve the viability of probiotic cultures. Relatively lower rate of probiotic survival at 25 °C is contributed to the increased metabolic activity of the organisms compared to the activity at refrigeration temperature (Bruno and Shah 2003). At 37 °C, loss of viability was highest, and this was consistent with the findings of Ananta and others (2005) who observed more pronounced loss in viability of sprayed-dried *L. rhamnosus* GG stored for 5 wk at 37 °C compared to cultures stored at 25 °C.

The relatively lower survivability of *L. rhamnosus* GG in full fat peanut butter at 37 °C could also be attributed to the reactive oxygen species formed during lipid oxidation. Although lipid oxidation could take place in both full fat and reduced products, it is expected to occur at elevated rates in the full fat product due to its higher fat content. During extended storage of peanut products, oxidation of peanut fat could lead to the formation of primary and secondary oxidative compounds such as peroxy, alkoxy radicals, aliphatic aldehydes, ketones, and alcohols (Wambura and Yang 2010; Nepote and others 2006). These compounds could damage cell membranes, proteins and nucleic acids, leading to the death of probiotic bacterial cells (Storz and Imlay 1999; Dowds 1994; Storz and others 1990). Nepote and others (2006) studied lipid oxidation that occurred in roasted peanuts at 15, 23 or 40 °C and found that greater lipid oxidation occurred as storage temperature and storage time increased. Additionally, the protein, carbohydrate, sugar, fiber and salt contents of reduced fat peanut butter were all slightly

higher than those in full fat peanut butter. It is not clear whether these ingredients impacted the survival of probiotic bacteria at 37 °C.

As stated previously, there is no recognized number of probiotic bacteria for efficacy (Reid, 2008). There are instances where, to reach expected health benefits, probiotics only need to be above 6 log CFU/g of a food product at the time of consumption (Nebesny and others 2007; Liong and others 2009). In the present study, this level was preserved in products stored at 4 °C for 48 wk, at 25 °C for 23 wk in full fat peanut butter and for 27 wk in reduced fat peanut butter. At 37 °C, the 10<sup>6</sup> CFU/g level could not be maintained in both products for even 6 wk.

## Conclusions

The present study shows that the survival of *L. rhamnosus* GG was influenced by storage temperature and storage time. Generally, the viability of *L. rhamnosus* GG decreased in both product types with increasing storage temperature and storage time. Higher survivability of *L. rhamnosus* GG was observed in reduced fat peanut butter as compared to full fat peanut butter only at 37°C. Products stored at 4 °C for 48 wk and at 25 °C for 23 or 27 wk could maintain probiotic counts of at least 10<sup>6</sup> CFU/g. These results suggest that peanut butters could be used to deliver probiotic organisms. However, if one considers the recommendation of 10<sup>9</sup> CFU as the minimum daily intake, a higher level of probiotic bacteria will have to be inoculated into peanut butter at the initial stage of the project. This can be easily accomplished without significant challenges. Results of the study also suggest that probiotic peanut butter has the potential to be used as one of the strategies to control diarrhea and malnutrition in developing

countries. In developed countries where malnutrition is not such a problem, peanut butter, which is rich in arterial dilating substances, can be used to offer health benefits to aged populations and individual with cardiovascular diseases.

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**Table 3.1. Average *Lactobacillus rhamnosus* GG populations in full fat and reduced fat peanut butter at each storage temperature**

Temperature (°C)	Cell population (log CFU/g)	
	Full Fat peanut butter	Reduced fat peanut butter
4	6.87aB	6.95aA
25	5.71bB	6.00bA
37	1.51cB	3.81cA
Average	4.70B	5.59A

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of storage temperature

Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of peanut butter type)

**Table 3.2. Average *Lactobacillus rhamnosus* GG populations as affected by storage temperature, storage time and product type (full fat or reduced fat peanut butter)**

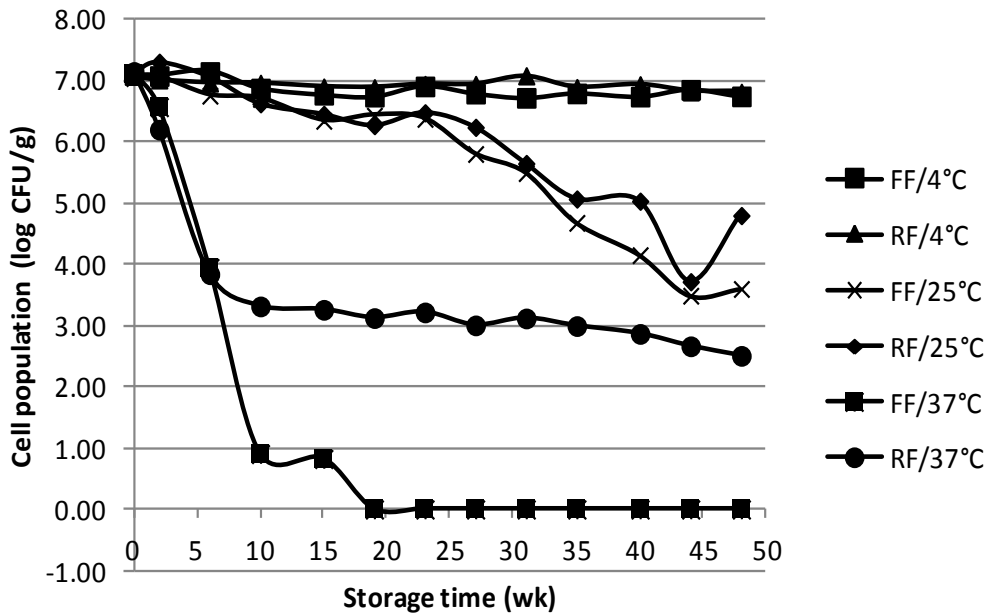
Variable	Cell population (log CFU/g)
<b>Temperature</b>	
4 °C	6.91a
25 °C	5.85b
37 °C	2.66c
<b>Peanut butter type</b>	
Full fat	4.70b
Reduced fat	5.59a
<b>Storage time (wk)</b>	
0	7.11a
2	6.88a
6	5.97b
10	5.28c
15	5.15cd
19	4.95def
23	5.01cde
27	4.84ef
31	4.72fg
35	4.45gh
40	4.36hi
44	3.97j
48	4.13ij

Means in the same column within the same category (storage temperature, storage time or product type) not followed by the same letters are significantly different ( $P < 0.05$ )

**Figure Legend**

Figure 3.1. Survival of *Lactobacillus rhamnosus* GG populations in full fat peanut butter (FF) and reduced fat peanut butter (RF) stored at 4, 25 and 37 °C for 48 wk.

Figure 3.1. Klu et al.





CHAPTER 4  
SURVIVAL OF FOUR COMMERCIAL PROBIOTIC MIXTURES IN FULL FAT  
AND REDUCED FAT PEANUT BUTTER

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<sup>1</sup> Klu, Y.A.K., R.D. Phillips and J. Chen. 2014. Accepted by *Food Microbiology*.  
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**HIGHLIGHTS**

Two evaluated probiotic mixtures survived better ( $P<0.05$ ) than two other mixtures.

Increase in storage temperature and time adversely affected probiotic viability.

Fat content of peanut butter had no significant impacts on probiotic viability.

*Bifidobacterium* survived better than *Lactobacillus* and *Streptococcus/Lactococcus*.

**Abstract**

A well-documented health benefit of probiotics is their ability to reduce the incidence of diarrhea in young, malnourished children in the developing countries. This study was undertaken to determine whether peanut butter, a nutritious, low-moisture food could be a carrier for probiotics by observing the survivability of selected probiotic mixtures in peanut butter under different storage conditions. Commercial probiotic mixtures (B, U, N and S) comprising of multiple strains of *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus* were inoculated into full fat or reduced fat peanut butter at  $10^7$  CFU/g. Resulting products were stored at 4, 25 or 37 °C for 12 months. Populations of *Lactobacillus*, *Bifidobacterium* and *Streptococcus/Lactococcus* were determined periodically. The average viable cell counts of N and S were significantly lower than those of B and U ( $p < 0.05$ ). In all probiotic products stored at different temperatures, *Bifidobacterium* had the greatest survivability, followed by *Lactobacillus* and *Streptococcus/Lactococcus*. The probiotics used in the study had different surviving patterns, and their survival was influenced by storage conditions. Fat content of peanut butter had no significant impacts on probiotic viability. Results suggest that peanut butter can be a vehicle to deliver probiotics for preventing diarrhea among malnourished children.

## 1. Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). A well substantiated health benefit of probiotics is the management of diarrhea (Boylston et al., 2004) which is second to pneumonia as the highest cause of mortality in children under 5 years of age in the developing countries (WHO, 2011). Numerous reports of clinical studies have documented the effectiveness of probiotic consumption in the prevention, control and treatment of diarrhea amongst children in this age group (Binns and Lee, 2010; Isolauri, 2004; Sazawal et al., 2006).

To achieve expected health benefits, the survivability of probiotic organisms is essential since only viable cells at the time of consumption have therapeutic values. Currently, the precise therapeutic level or dosage of probiotics for expected health benefits has not been established (Reid, 2008). However, it has been proposed that the viable numbers of probiotic cells at the time of consumption should be at least  $10^6$  -  $10^7$  CFU/g or mL of food product in order to reach the  $10^8$  –  $10^9$  CFU minimum daily intake level through the consumption of 100 g or mL of a food product (Cruz et al., 2009; Karimi et al., 2011; Rathore et al., 2012).

The survival and colonization of probiotics are regulated by food substrates with which probiotics are consumed. Probiotic cultures have been recently incorporated into cheese, ice-cream and butter as well as meat, cereal, fruit and vegetable based products (Cruz et al., 2009; Rivera-Espinoza and Gallardo-Navarro, 2010; Ranadheera et al., 2013). Compared to yoghurt which is the widely used vehicle for probiotic delivery, cheese was found to be a more suitable carrier for probiotics due to its denser matrix,

higher fat content and pH, and lower titratable acidity (Boylston et al., 2004; Karimi et al., 2011).

Peanut butter is a shelf stable, low moisture, energy and nutrient dense product and has been used as the major ingredient of Ready to Use Therapeutic Foods (RUTFs; Diop et al., 2003; Manary, 2006; Ndekha et al., 2005) for treatment of severe childhood malnutrition which could be the consequence of chronic diarrhea (Caulfield et. al, 2004). In a previous study, we observed that at an inoculation level of  $10^7$  CFU/g of peanut butter, a single probiotic strain, *L. rhamnosus* GG maintained a viability of  $10^6$  CFU/g for 48 wk at 4 °C and for 27 wk at 25 °C (Klu et al., 2012). The aims of this study were to observe the survivability of four commercial probiotic mixtures, each containing 4 to 16 different probiotic strains, and to examine the interaction of different probiotic strains in full fat and reduced fat peanut butter at 4, 25 or 37 °C during a 12 month storage period. The ultimate goal of the research is to use probiotic peanut butter or peanut based probiotic RUTFs to control malnutrition and diarrhea concurrently.

## **2. Materials and methods**

### **2.1. Materials**

A full fat peanut butter product and a reduced fat peanut butter product were graciously provided by the American Blanching Company (Fitzgerald, GA, USA). Products were stored during the experiment in clear polyethylene terephthalate (PET) jars (4 oz.) tightly covered with pressure-sensitive lined polypropylene (PP) lids (Container and Packaging Supply, Eagle, ID, USA). The full fat peanut butter contains peanuts, sugar, hydrogenated vegetable oil (rapeseed, cottonseed and soybean), salt and molasses.

The ingredients for the reduced fat peanut butter include peanuts, partially defatted peanut flour, sugar, hydrogenated vegetable oil (rapeseed and cottonseed), salt, molasses, monoglycerides, tocopherol, acetate and pyridoxine HCl. Information from the manufacturer indicates that the full fat peanut butter had a protein content of 21.31% and reduced peanut butter 28.12%. The total carbohydrate content is 26.31% for full fat peanut butter and 27.51% for reduced fat peanut butter. The sugar contents for full fat and reduced fat peanut butter are 10.57% and 11.67%, respectively, and the amounts of fiber are 7.20% and 8.57% for full fat peanut butter and reduced fat peanut butter, respectively.

Four commercial probiotic mixtures, designated as B, U, N and S were used in the study. Mixtures B and U had the same probiotic strains including *Lactobacillus acidophilus* (CUL 60), *L. acidophilus* (CUL 21), *Bifidobacterium bifidum* (CUL 20) and *Bifidobacterium lactis* (CUL 34). The only known difference between the two products is that mixture B had a manufacturer's claim of 25 billion CFU of viable cells per capsule while U contained 50 billion CFU of viable cells per capsule. Mixture N had a manufacturer's claim of 16 billion live cells per g of powder and contained 16 different bacterial strains including *B. bifidum*, *Bifidobacterium breve*, *B. lactis*, *B. lactis* Bif Relief 24-7™, *Bifidobacterium longum*, *L. acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactococcus lactis* and *Streptococcus thermophilus*. Mixture S had 15 billion viable cells per capsule. According to the manufacturer, its constituents included *L. acidophilus* (45%), *L. rhamnosus* (25%), *S. thermophilus* (10%), *L. plantarum* (7%), *B. bifidum* (6%),

*L. bulgaricus* (3%), *B. longum* (3%) and *L. salivarius* (1%). The exact strain ratios of product B, U and N are not available since they are considered proprietary information by the manufacturers.

## 2.2. The fat content of peanut butter products

The fat contents of the two peanut butter products were analyzed using the AOAC Goldfish extraction method with a Goldfish Fat and Oil Extractor (Labconco Co., Kansas City, MO, USA); triplicate assays were performed. Briefly, 4 g samples were placed into Oil Extraction Cellulose Thimbles (Fisher Scientific, Pittsburg, PA, USA) and were fixed on the instrument. A 50 mL of petroleum ether (J. T. Baker, Phillipsburg, NJ, USA) was used to extract the fat at the boiling temperature of petroleum ether (35 - 60 °C) for about 18 h. Fat content of the samples was derived based on the following calculation: % total fat = (weight of extracted fat/weight of dry sample × 100).

## 2.3. The water activity and pH of peanut butter products

The pH of peanut butter homogenate (25 g of peanut butter in 50 mL of water) was determined using a pH meter (model 8000; VWR International, PA, USA). The water activity of the peanut products was measured using the Pawkit Water Activity Meter according to the manufacturer's instructions (Decagon Devices, WA, USA). Triplicate measurements were performed.

#### 2.4. Hexanal analysis

Hexanal contents of peanut butter products were analyzed at the beginning as well as the end of the 12 month storage period and at each sampling point, triplicate analysis was conducted. A 3 g sample was placed into a 10 mL sample vial (Sigma-Aldrich, St. Louis, MO, USA). Exactly 50  $\mu$ L of a 30 ppm 4-Heptanone standard solution (Sigma-Aldrich) was added to the sample, and the vial was closed with a PTFE/Silicone septum (Sigma-Aldrich) sealed screw cap. Vials were heated on a heating block (Barnstead Thermolyne, Dubuque, IA, USA) at 35 °C for 15 min. A 100  $\mu$ m polydimethylsiloxane stable-flex solid phase micro-extraction fiber assembly was exposed to the headspace of the vial for 30 min, after which the needle in the fiber assembly was injected into a Varian 3400 Gas Chromatograph with a flame ionization detector (Varian Analytical Instruments, Walnut Creek, CA, USA). Desorption of hexanal occurred at 200 °C for 5 min, and Helium (20 cm/sec at 125 °C) was used as a carrier. The oven temperature was initially maintained at 50 °C for 5 min and then increased to 200 °C at 10 °C increment per min. The temperatures of the injector and detector were 200 °C and 300°C, respectively.

#### 2.5. Inoculation of peanut butter with probiotic mixtures

The jars and lids described previously were sterilized under UV light for 15 min in a Level II Biosafety Cabinet (NuAire Laboratory Equipment Supply, Plymouth, MN, USA). The peanut butter products were pre-heated in a Stabil-Therm Electric Oven (Blue M Electric Co., Blue Island, IL, USA) at 37 °C for 6 h to reduce product viscosity and aid in uniform mixing. Precisely 2.5 kg peanut butter was placed into a sterile



KitchenAid® mixer, and a pre-determined amount of each probiotic mixture was inoculated into the product to achieve an inoculation level of *ca.*  $10^7$  CFU/g. The peanut butter and probiotic culture were mixed at room temperature for 15 min at 66 and 148 rpm for orbital and beater speeds, respectively. Nitrogen gas was incorporated into the product during the mixing to remove oxygen that might cause excessive rancidification of fats in the products during storage. Peanut butter exhibits thixotropic behavior and thus after mixing, samples of the inoculated peanut butter (10 mL) were easily dispensed into the jars using a sterile syringe (Becton, Dickinson and Co., Sparks, MA, USA) which had its end altered to aid product flow. The headspace of the jars was flushed with nitrogen for 30 s to remove oxygen, and the jars were then tightly closed with lids. Inoculated peanut butter was stored at 4, 25 or 37 °C for 12 months to mimic refrigeration, ambient and abusive condition, respectively. Un-inoculated peanut butter was used as negative controls.

## 2.6. Enumeration of probiotic bacteria

The initial population levels of *Lactobacillus*, *Bifidobacterium* and *Streptococcus/Lactococcus* species were confirmed immediately after the inoculation. To ensure that the inoculated probiotic cells were evenly distributed, multiple samples of peanut butter were taken from various locations of the mixer. Samples stored at each temperature were drawn monthly during the 12 month storage period. Previously unopened containers that had been brought to room temperature were used at each sampling interval. Sterile 0.1% peptone water (Becton, Dickinson and Co.) warmed to 37 °C was added to samples in the containers to achieve a 2-fold dilution based upon the

weight of peanut butter in each container. The samples were mixed by vigorous manual to-and-fro shaking at an arm angle of about 45° for 1 min. After mixing, 1 mL aliquot of the sample was serially diluted in 9 mL of 0.1% sterile peptone water. A 0.1 mL of appropriate dilutions was plated, and inoculated plates were incubated and colonies enumerated. All media used in the study were purchased from Becton, Dickson and Co. *Lactobacillus* Selection (LBS) agar and de Man, Rogosa and Sharpe (MRS) agar were used for the enumeration of *Lactobacillus* species. LBS agar supplemented with tomato juice (Kroger, Cincinnati, OH, USA; 200 mL/L; LBST) which had been filtered with a cheese cloth was used to enhance the growth of *L. acidophilus*. Both LBS and LBST were prepared with the addition of 1.3 mL of glacial acetic acid (Fisher Scientific, Fair Lawn, NJ, USA) per L of media. *L. lactis* and *S. thermophilus* were enumerated on M17 agar supplemented with 0.5% (final concentration) of lactose. *Bifidobacterium* species were enumerated on modified *Bifidobacterium* agar which include 42.5 g Columbia agar base, 2.5 g glucose, 0.01 riboflavin (Fisher Scientific), 2.5 g lactulose (EMD Chemicals Inc. San Diego, CA, USA) and 0.5 g L-Cysteine HCl (Sigma-Aldrich) per liter of media. The pH of the media was adjusted to  $5.5 \pm 0.2$  by the addition of propionic acid (J.T. Baker). *Lactobacillus* and *Bifidobacterium* species were incubated under anaerobic condition for 72 h at 37 °C using the BD GasPak™ EZ in a BBL GasPak® System (Becton, Dickinson and Co.). *L. lactis* and *S. thermophilus* were incubated at 30 °C and 37 °C, respectively under anaerobic condition for 48 h. Since LBS, MRS and LBST gave comparable results, the average colony counts from the three media were used as the final *Lactobacillus* counts. Enumeration results for *Lactococcus lactis* and *Streptococcus thermophilus* was averaged. Counts of *Lactobacillus*, *Bifidobacterium* or

*Lactococcus/Streptococcus* were either used separately for analysis on individual genera in probiotic mixtures or added up for analysis on total populations within each probiotic mixture.

## 2.7. Statistical analysis

Two replicate experiments were conducted for bacterial enumeration. Data were analyzed using a 3-way Analysis of Variance F-test and the General Linear Model of Statistical Analysis Software (SAS Inst. Inc., Cary, NC, USA). At a confidence level of 95%, Fisher's Least Significant Difference Design was used to compare the significance of differences among populations of probiotic mixtures (B, U, N and S) and individual groups of probiotic bacteria (lactobacilli, bifidobacteria and streptococci/lactococci) in different type of peanut butter products (full fat and reduced fat) and under various storage conditions (time and temperature). The same statistical protocol was used to determine the differences in the hexanal content of peanut butter products with respect to probiotic mixtures, type of peanut butter and storage conditions.

## 3. Results and discussion

### 3.1. Physical and chemical properties of full fat and reduced fat peanut butter products

The water activity and pH of the two peanut butter products used in the present study were comparable. The average water activity was  $0.31 \pm 0.03$  and pH was  $6.23 \pm 0.12$ . According to the FDA, the water activity of a typical peanut butter or peanut spread should be 0.35 or less (USFDA, 2009). The average fat content of full fat peanut butter used in the present study was  $50.10 \pm 1.16\%$  and that of reduced fat peanut butter

was  $39.90 \pm 0.62\%$ , and these values were consistent with FDA standards (USFDA, 2012).

Results of hexanal analysis showed that there was no statistically significance difference between the hexanal contents in samples stored at 4 °C and 25 °C ( $p > 0.05$ ). However, a significantly higher hexanal content was detected in samples stored at 37 °C (Table 4.1). Although the hexanal content in full fat vs. reduced fat peanut butter stored at this temperature varied 11.12 ppm, the difference was statistically insignificant ( $p > 0.05$ ; Table 4.1). Total hexanal content was significantly higher in samples inoculated with probiotic mixtures N and S than those inoculated with mixture B ( $p < 0.05$ ; Table 4.1). The mean hexanal content in samples inoculated with mixture U was not significantly different from those inoculated with the other three probiotic mixtures (Table 4.1). The mean hexanal content of all samples tested in the present study increased from 2.15 ppm at the beginning of the experiment to 71.27 ppm after the 12 month storage period (Table 4.1).

Hexanal is a sensitive and reliable indicator of fatty acid oxidation that occurs during product storage, and the amount of hexanal formed in a product has a direct correlation with its storage temperature (Holse et al., 2012; Panseri et al., 2011). In peanuts, hexanal is formed mostly from the oxidation of linoleic acid (Wambura and Weihua, 2010). The present study detected more hexanal in peanut butter products stored at 37 °C than at 25 and 4 °C (Table 4.1). Similar to what was observed in the present study, Nepote et al. (2006) reported that during storage, higher levels of lipid oxidation products were formed in dry roasted and honey roasted peanuts at 40 °C compared to -15 and 23 °C. Hexanal and other products of lipid oxidation could damage cellular protein

and nucleic acid as well as cell membrane, thereby impacting the viability of probiotic cells (Dowds 1994; Storz and Imlay 1999). In the present study, however, a higher hexanal content of peanut butter product did not always co-relate to lower probiotic cell viability (Table 4.1).

### 3.2. Influence of storage conditions on the populations of different probiotic mixtures

Statistical analysis revealed that storage temperature and storage time had a significant influence on the populations of the four probiotic mixtures during storage ( $p < 0.05$ ; Table 4.1). The average cell counts of the four probiotic mixtures decreased with increasing storage temperature (Table 4.1). By the end of the 12 month storage period, the average counts of the four probiotic mixtures in the two peanut butter products stored at the three temperatures had a 2.65 log CFU/g decrease (Table 4.1). Although the mean probiotic bacterial count of the four probiotic mixtures in reduced fat peanut butter was significantly higher than the count in full fat peanut butter ( $p < 0.05$ ; Table 4.1), the difference between the two counts was only 0.14 log CFU/g. No background lactic acid bacteria were found in the negative controls at all storage conditions.

The graphs in Fig. 4.1 show the survival trends of the probiotic mixtures in peanut butter products stored at different temperatures. At 4 °C, all four probiotic mixtures maintained their viabilities with the exception of mixture S whose counts decreased approximately 1 log CFU/g in both full fat and reduced fat peanut butter by the end of the 12 month storage period. Probiotic bacterial populations in samples stored at 25 °C decreased approximately 1 - 3 log CFU/g at the end of 12 month storage period. The counts of the probiotic mixtures were relatively lower in samples stored at 37 °C at the

same sampling point; largely between 2.50 – 3.75 log CFU/g except for mixtures N and S. Cell counts of mixture N in full fat peanut butter fell below the detectable limit (< 8 CFU/g) at the end of 11 months, and those of mixture S dropped from the initial 7 log CFU/g to approximately 1 log CFU/g in full fat peanut butter at the end of the 12 month storage period.

Previous literatures have documented a reverse relationship between storage temperature and probiotic viability. Wang et al. (2004) observed that the viabilities of *S. thermophilus* and *B. longum* decreased with increase in storage temperature in dried fermented soymilk stored at 4 °C and 25 °C. Higher populations of viable probiotic cells were observed in products held at 4°C (68.8%) compared to 25 °C (60.8%) after a 4 month storage period. Furthermore, Champagne et al. (1996) observed significant differences in probiotic survival rate at -20, 4 and 20 °C; and as storage temperature increased, mortality of probiotic cultures also increased during storage especially at 20 °C. Abe et al. (2009) documented a decrease in the survivability of *Bifidobacterium* with increasing storage temperature from 5, 25, 37, 45 to 60 °C. In a previous study in our laboratory, viability of *L. rhamnosus* GG in full fat and reduced fat peanut butter decreased as temperature increased from 4 °C to 25°C and 37 °C (Klu et al., 2012). It is believed that a high storage temperature results in an increases in metabolic and cellular activities which leads to the exhaustion of nutrients stored within probiotic cells and eventually cell death (Bruno and Shah, 2003).

The average counts of mixture U were higher than the counts of the other three probiotic mixtures in both peanut butter products ( $p < 0.05$ ; Table 4.2). The average count of mixture B was significantly higher than the counts of mixtures N and S in full

fat peanut butter. However, the average counts of mixtures N and S in full fat peanut butter were not significantly different as were the counts of mixtures B, N and S in reduced fat peanut butter ( $p > 0.05$ ; Table 4.2). Food component such as fat is expected to protect probiotic cells during storage (Karimi et al., 2011; Possemiers et al., 2010). However, this phenomenon was not clearly observed in the present study (Table 4.2) which is consistent with the findings of Tharmaraj and Shah (2004) who reported that the addition of oil to cheese-based dips did not offer any additional protective effect for probiotics.

Previous studies have shown that *L. acidophilus* and *Bifidobacterium* species are the normal inhabitants of human gastrointestinal tracts (Shah, 2007; Champagne et al., 2005). They are widely used as probiotics in food because of their health benefits (Shah, 2007; Gueimonde et al., 2004) and tolerance to acid and bile (Gomes and Malcata, 1999). However, since all four probiotic products used in this study contained *Bifidobacterium* and *L. acidophilus*, the greater survival rates of U and B must have been attributed by other factors. It is suggested that the survival rate of a particular probiotic depends on the co-existence of other probiotic bacteria in a same mixture. Tharmaraj and Shah (2004) reported that *L. acidophilus* survived better, in cheese based dips, in combination with *Bifidobacterium animalis* and *L. paracasei* than with *B. animalis*, *P. shermanii* and *L. paracasei*. *B. animalis* had a better survivability when in combination with *L. acidophilus*, *L. paracasei* and *P. shermanii* than when combined with a *L. acidophilus* and *L. paracasei*. Additionally, the authors observed a mutual antagonistic effect of *B. animalis* on *L. rhamnosus* and *L. paracasei*. In the present study, products N and S had 16 and 8 different probiotic bacteria, respectively. It is not clear whether the co-existence

of different bacterial strains in the probiotic mixture had an effect on their viabilities during storage.

### 3.3. Survival of lactobacilli, bifidobacteria and streptococci/lactococci

As shown in Table 4.3, the average counts of bifidobacteria were significantly higher than the counts of lactobacilli in mixtures U, B and N ( $p < 0.05$ ). In mixture S however, the two populations were similar ( $p > 0.05$ ). The average populations of streptococci/lactococci were significantly lower than those of bifidobacteria and lactobacilli in mixtures N and S ( $p < 0.05$ ). It should be kept in mind that although the total probiotic inoculation level was kept at  $10^7$  CFU/g, the initial counts of *Bifidobacterium*, *Lactobacillus* and *Streptococcus/Lactococcus* were not exactly the same.

Fig. 4.2 shows the ratios of bifidobacteria or lactobacilli to total probiotic population (sum of the counts of all present probiotic strains) in probiotic mixtures B and U in full fat and reduced fat peanut butter under different storage conditions. After 1 month storage at different conditions, *Bifidobacterium* species accounted for 25-32% of the total probiotic population in mixture B in full fat peanut butter (Fig. 4.2A) and 22-29% of the total probiotic population in reduced fat peanut butter (Fig. 4.2B). At the same time interval, the species accounted for 10% of the total probiotic population in mixture U in full fat peanut butter (Fig. 4.2C) and 45% in reduced fat peanut butter (Fig. 4.2D). At the 6 month sampling point the proportion of *Bifidobacterium* species in mixture B ranged from 50-74% in full fat peanut butter (Fig. 4.2A) and 48-75% in reduced peanut butter (Fig. 4.2B), while in mixture U, *Bifidobacterium* species comprised



47-74% of total probiotic bacterial populations in full fat peanut butter (Fig. 4.2C) and 48-78% of the probiotic population in reduced fat peanut butter (Fig. 4.2D). At the 12 month sampling point, *Bifidobacterium* proportions increased in mixture B stored at 4 and 25 °C compared with the results collected at the end of 6 month of the storage (Figs. 4.2A and 4.2B). In samples stored at 37 °C, however the proportions of *Bifidobacterium* either remained steady or slightly decreased. *Bifidobacterium* proportions in mixture U remained mostly steady at 25 °C between the 6 and 12 month sampling points (Figs. 4.2C and 4.2D). However, the proportions in reduced fat peanut butter slightly increased at 4 °C and decreased at 37 °C (Fig. 4.2D) while in full fat peanut butter the proportions increased slightly at both storage temperatures (Fig. 4.2C). After the 12 month storage period, the overall proportions of *Bifidobacterium* from all storage temperatures had increased from the initial 25-32% to 57 –76% in mixture B. In mixture U, the increment was from the initial 10-45% to the final 51-80%.

These results suggest that *Bifidobacterium* strains in mixtures U and B were more persistent than *Lactobacillus* in peanut butter products. Similar observations have been made in earlier studies. Heenan et al. (2004) studied the survival of probiotics in a frozen vegetarian dessert (pH 7). At the end of a 25 wk storage at -20 °C, the two *B. lactis* strains had a higher percentage of survival (88.0 and 84.7% respectively) than did the *L. acidophilus* and *L. paracasei* strains (59.4 and 44.6% respectively) used in the study. In another study, *L. acidophilus* La-5 and *B. lactis* Bb-12 were incorporated into ice cream (pH 6.51) either separately or as a mixture and the resulting products were stored for 60 d at -25 °C (Magariños et al., 2007). When the probiotic bacteria were incorporated separately, *L. acidophilus* had a survival rate of 87% and *B. lactis*, 90%. When the two

bacteria were added to the ice cream as a mixture, *L. acidophilus* and *B. lactis* had 82 and 92% survivability, respectively after the 60 d of storage. Hekmat and McMahon (1992) inoculated *B. bifidum* and *L. acidophilus* into a fermented ice cream mix (pH of 4.7). It was observed that, after a 17 wk storage period at -29 °C, *L. acidophilus* had reduced to  $3 \times 10^6$  CFU/mL while *B. bifidum* to  $1 \times 10^7$  CFU/mL from the initial number of  $5 \times 10^8$  CFU/mL for both probiotic bacteria.

As described previously, probiotic mixture N and S contained not only *Lactobacillus* and *Bifidobacterium* but also *Streptococcus/Lactococcus*. Interactions among the members of each mixture in peanut butter products were complicated and no clear and consistent trend was observed.

#### **4. Conclusions**

Storage conditions played a key role in maintaining the viability of probiotic cultures, and cell survival rate decreased with increasing storage temperature and time. Overall, probiotic mixture U has the greatest survival rate followed by B, N and S. *Bifidobacterium* species had the highest survivability followed by *Lactobacillus* species and then *Streptococcus/Lactococcus*. Peanut butter is a suitable food matrix to deliver probiotics, and the fat content of peanut butter did not significantly influence the survivability of the probiotic mixtures included in the present study.

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**Table 4.1.**

Results of statistical analysis - Average probiotic bacteria populations and hexanal contents in samples inoculated with B, U, N or S as affected by storage temperature, peanut butter type, and storage time during a 12 month storage period at 4, 25 and 37 °C.

<b>Variable</b>	<b>Cell population (log CFU/g)</b>	<b>Hexanal (ppm)</b>
<b>Temperature (°C)</b>		
4	7.05a	4.71b
25	6.24b	6.43b
37	4.54c	189.83a
<b>Peanut butter type</b>		
Full fat peanut butter	5.87b	68.88a
Reduced fat peanut butter	6.01a	65.10a
<b>Probiotic mixtures</b>		
B	5.99b	23.55b
U	6.75a	78.90ab
N	5.55c	97.63a
S	5.48c	85.00a
Control	- *	49.87ab
<b>Storage time (mo)</b>		
0	7.33a	2.15b
1	6.94b	ND
2	6.58c	ND
3	6.34d	ND
4	6.16e	ND
5	6.10ef	ND
6	5.97fg	ND
7	5.82g	ND
8	5.59h	ND
9	5.50h	ND
10	5.22i	ND
11	5.01j	ND
12	4.68k	66.99a

Means in the same column not followed by the same letter are significantly different ( $P < 0.05$ ).

ND: Not determined

-\*: Below detectable level ( $< 8$  CFU/g)

**Table 4.2.**

Results of statistical analysis - Average probiotic populations of B, U, N and S as affected by peanut butter type during a 12 month storage period at all three storage temperatures (4, 25 and 37 °C).

Probiotic Mixture	Cell population (log CFU/g)	
	Full fat peanut butter	Reduced fat peanut butter
B	5.99bA	5.98bA
U	6.71aA	6.79aA
N	5.47cA	5.62bA
S	5.30cA	5.66bA

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of probiotic mixture.

Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of type of peanut butter.

**Table 4.3.**

Results of statistical analysis - Average cell populations of different probiotic species in each probiotic mixture during a 12 month storage period at all three storage temperatures (4, 25 and 37°C).

	Cell population (log CFU/g)			
	B	U	N	S
<b>Bacteria type</b>				
<i>Lactobacillus</i>	5.82bB	6.62bA	5.40bC	5.53aC
<i>Bifidobacterium</i>	6.10aB	6.81aA	5.61aC	5.66aC
<i>Streptococcus + Lactococcus</i>			4.86cA	4.83bA

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of probiotic mixture.

Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of type of bacteria.

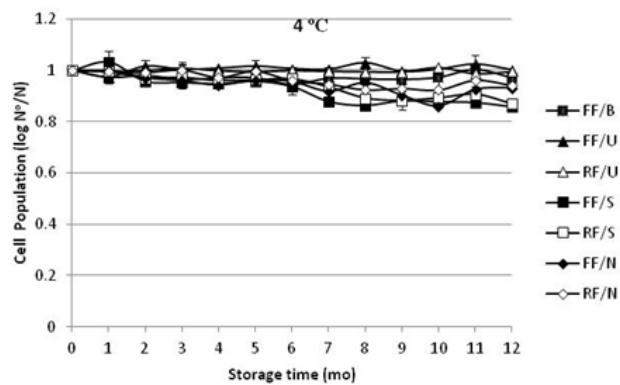
**Fig. legends**

Fig. 4.1.- Survival of probiotic bacteria in mixtures B, U, N and S in full fat peanut butter (FF) or reduced fat peanut butter (RF) at 4 °C (A), 25 °C (B), and 37 °C (C)

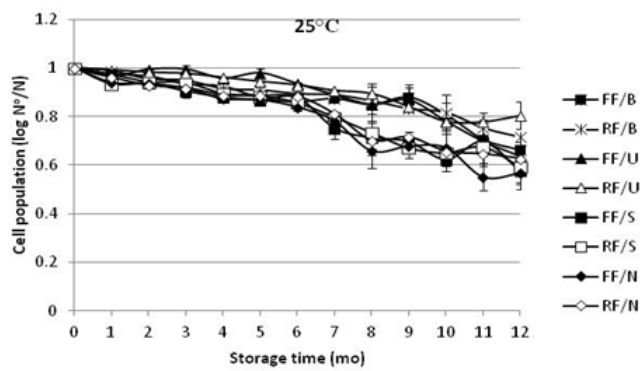
Fig. 4.2.- Ratios of probiotic species, *Lactobacillus* (Lacto) or *Bifidobacterium* (Bifi) to total probiotic population in mixture B in in full fat peanut butter (A) and reduced fat peanut butter (B) and in mixture U in full fat peanut butter (C) and reduced fat peanut butter (D) during a 12 month storage period at 4, 25 or 37 °C

Figure 4.1 Klu et al.

A



B



C

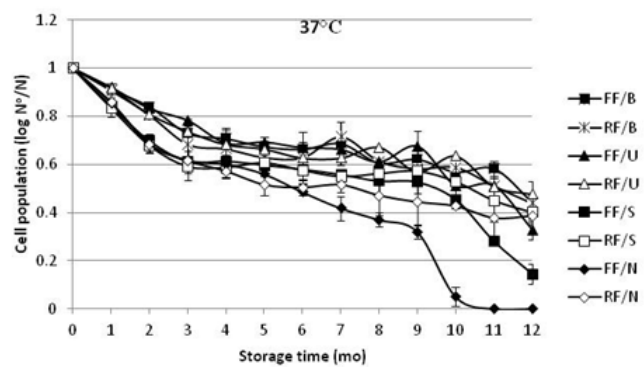
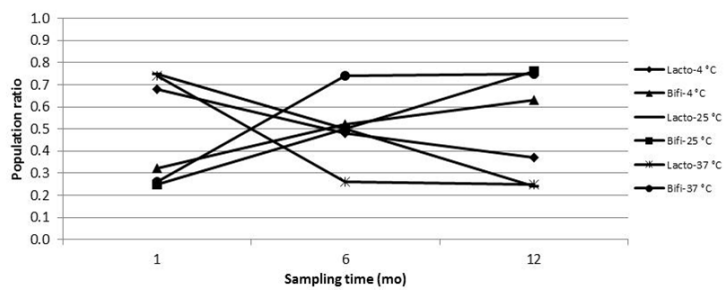
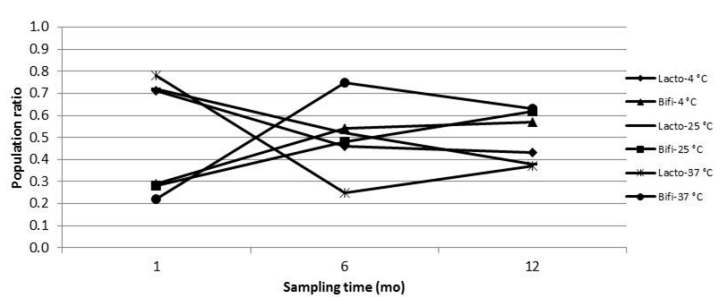


Figure 4.2 Klu et al.

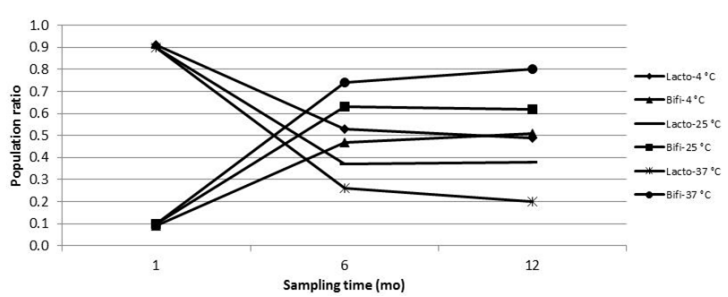
A



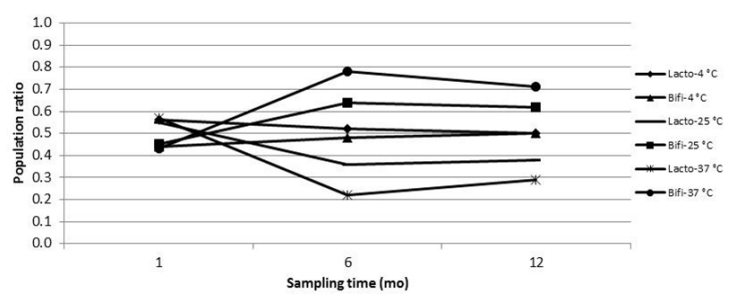
B



C



D



CHAPTER 5  
EFFECT OF PEANUT BUTTER MATRICES ON THE FATE OF PROBIOTICS  
DURING SIMULATED GASTROINTESTINAL PASSAGE

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<sup>1</sup> Klu, Y.A.K., R.D. Phillips and J. Chen. 2014. To be submitted to *International Journal of Food Microbiology*

## Abstract

Diarrheal diseases are very common in pre-school children especially malnourished ones in developing countries. Studies have found that probiotics are effective in controlling diarrhea in these children and peanut butter is a suitable matrix for preserving the viability of some probiotics under different storage conditions. This study was undertaken to observe the ability of peanut butter in enhancing the viability of selected probiotic bacteria during a simulated gastrointestinal passage. Full-fat and reduced-fat peanut butter were inoculated with lyophilized commercial probiotic product U (4-strain mixture), N (16-strain mixture) or C (single-strain culture) at *ca.*  $10^7$  CFU/g. Inoculated peanut butter products were homogenized in 0.5% NaCl solution, and a same population of lyophilized probiotics suspended in 0.5% NaCl solution served as a control. To mimic digestion in the stomach, the pH (*ca.* 6) of the samples was reduced to 1.6 and pepsin and lipase were added. Samples were incubated for 2 h with agitation at 37 °C. Bile and pancreatin were subsequently added to simulate digestion in the upper small intestine. Samples were incubated at 37 °C with agitation for 2 h after sample pH was increased to 4.7. Finally, the pH of the samples was adjusted to 7.1 to simulate conditions in the lower small intestines. Samples were incubated under the condition described above. Aliquots were collected after incubation for 30 min and 2 h (gastric phase) as well as 4 h (enteric phase 1) and 6 h (enteric phase 2). Probiotic bacteria in collected samples were enumerated. A lower cell population was observed in samples inoculated with probiotic culture C, followed by probiotic mixture U and mixture N ( $p < 0.05$ ). At the end of the 6 h incubation period, in peanut butter homogenates, probiotic bacteria population from product N (5.67 log CFU) was higher than that in product U



(3.48 log CFU) and product C (3.00 log CFU). On average, *Streptococcus/Lactococcus* species had the highest survival rate followed by *Bifidobacterium* and *Lactobacillus* species during the simulated passage. Overall, the fat content of peanut butter did not have a significant impact on probiotic survivability. The study suggests that the peanut butter matrices are able to protect probiotic bacteria during simulated gastrointestinal passage, making them possible vehicles for probiotic delivery to children prone to diarrhea.

**Keywords:** Gastric, enteric, *L. rhamnosus* GG, peanut butter, reduction

## 1. Introduction

In most developing countries, a major public health concern is the alarming rate of diarrhea and related mortality in pre-school children (WHO, 2013). Malnutrition is a major cause of diarrhea which subsequently worsens malnourishment in these children (WHO, 2013). In addition to several intervention strategies including good nutrition, zinc supplementation, safe drinking water, rehydration and improved environmental sanitation (WHO, 2013), many clinical studies have found that probiotics, live microorganisms which confer a health benefit to the host when administered in adequate amounts (FAO/WHO, 2001), are effective in preventing, controlling and treating diarrhea in pre-school children (Binns and Lee, 2010; Isolauri, 2004; Nomoto, 2005; Sazawal et al., 2006; Sullivan and Nord, 2002).

To exert proposed health benefits, probiotic bacteria must be available in high numbers at the time of consumption and be viable after passing through gastrointestinal tract (Jensen et al., 2012; Ranadheera et al., 2010; Valerio, et al, 2006). Several authors have addressed the importance of food in maintaining probiotic viability during storage and in the digestive tract (Alegre et al., 2011; Karimi et al., 2011; Ranadheera et al., 2010). Chocolate (Nebesny et al., 2005; Possemiers et al., 2010), ice-cream (Hekmat and McMahan, 2002; Magariños et al., 2007; Ranadheera et al., 2012; Salem et al., 2005), fermented soy product (Bedani et al., 2013) and cheese (Boylston et al., 2004; Karimi et al., 2011; Madureira et al., 2005) have been documented as suitable vehicles for probiotic delivery. Ranadheera et al. (2012) stated that foods with high pH and a high buffering capacity could reduce the acidity of the human stomach and enhance the viability of probiotic cells. In a laboratory study, Ross et al. (2005) observed that the addition of

milk proteins to gastric juice or microbiological media significantly increased their pH and subsequently enhanced the survival of some *Bifidobacterium* and *Lactobacillus* species. In addition to directly impacting cell viability, the physical property of food was reportedly affect the transit time of probiotics through human stomach; with liquid foods moving faster than solid foods (Huang and Adams, 2004). Furthermore, the functional properties of probiotics are influenced by the food ingredients used for their delivery, and these include prebiotic substances such as inulin, fructooligosaccharides, galactooligosaccharides and lactulose (Ranadheera et al., 2010). Thus, it is the tolerance of probiotic bacteria, in their delivering food matrix, towards gastrointestinal conditions that is important in maintaining the health benefits of probiotics. Most recently, Klu et al. (2012) reported that probiotic bacterium *Lactobacillus rhamnosus* GG could maintain its viability in peanut butter for as long as 48 wk at refrigeration temperature and up to 27 wk under ambient storage condition. The purpose of this study was to observe whether peanut butter could protect probiotic bacteria during a simulated gastrointestinal passage. Since peanut butter based Ready-to-Use Therapeutic Foods have been widely used to treat malnutrition amongst children in developing countries (Manary et al., 2006; Ndekha et al., 2005), ultimately, the goal is to use probiotic peanut butter to address the problem of malnutrition and diarrhea concurrently.

## **2. Materials and methods**

### **2.1. Materials**

Full-fat and reduced-fat peanut butter products, both having a solid content of 99.00% and a fat content of  $50.10 \pm 1.16\%$  and  $39.90 \pm 0.62\%$ , respectively were

provided by American Blanching (Fitzgerald, GA, USA). Both products contained peanuts, hydrogenated vegetable oils (rapeseed, cottonseed and soybean), sugar, molasses and salt. In addition, the reduced-fat peanut butter also contained partially defatted peanut flour, monoglycerides, tocopherol, acetate and pyridoxine HCl. Information from the manufacturer reveals comparable total carbohydrate, sugars, fiber, sodium and potassium for both products. However, the reduced-fat peanut butter had a protein content of 28.12% comparing to a protein content of 21.31% for the full-fat peanut butter.

Three commercial probiotic products, designated as C, N and U were used in the study. Product C contained *L. rhamnosus* GG, and each capsule had a manufacturer's claim of 10 billion viable cells. Product N had a manufacturer's claim of 16 billion live cells per g of powder and was made up of 16 different probiotic bacteria which included *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *B. lactis* Bif Relief 24-7™, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactococcus lactis* and *Streptococcus thermophilus*. Product U contained a mixture of strains including *L. acidophilus* (CUL 60), *L. acidophilus* (CUL 21), *B. bifidum* (CUL 20) and *B. lactis* (CUL 34). Each capsule was claimed by the manufacturer to contain 50 billion live cells.

## 2.2. Inoculation of peanut butter with probiotics

The probiotic bacteria were inoculated into the peanut butter products as previously described by Klu et al. (2012). Specifically, the full-fat and reduced-fat

peanut butter were pre-heated in a Stabil-Therm Electric Oven (Blue M Electric Co., Blue Island, IL, USA) at 37 °C for 6 h to reduce product viscosity and aid in uniform mixing. Precisely 1.5 kg peanut butter was placed into a sterile KitchenAid® mixer, and a pre-determined amount of each probiotic product was inoculated into the peanut butter to achieve an inoculation level of *ca.* 10<sup>7</sup> CFU/g. The peanut butter and probiotics were mixed at room temperature for 15 min at 66 and 148 rpm for orbital and beater speeds, respectively. Samples of the inoculated peanut butter were dispensed into pre-sterilized (15 min under UV light in a Level II Biosafety Cabinet- NuAire Laboratory Equipment Supply, Plymouth, MN, USA) clear polyethylene terephthalate jars (4 oz.) and were covered with pressure-sensitive lined polypropylene lids (Container and Packaging Supply, Eagle, ID, USA).

### 2.3. Survival of probiotic bacteria under simulated gastrointestinal conditions

Gastrointestinal conditions were simulated by adopting a method described by Buriti et al. (2010). Based on the weight of product, full-fat or reduced-fat probiotic peanut butter products were suspended in 0.5% NaCl solution (Fisher Scientific, Fair Lawn, NJ, USA). The samples were mixed by vigorous manual to-and-fro shaking at an arm angle of about 45° for 1 min. Ten mL of each homogenate was transferred into a sterile 50 mL polypropylene conical tube (Becton, Dickinson and Co., Sparks, MA, USA). To mimic digestion in the stomach (gastric phase), the pH (*ca.* 6) of the samples was adjusted to 1.6 with 1 M HCl solution (Fisher Scientific). Pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO, USA) and lipase from *Rhizopus arrhizus* (Sigma-Aldrich) were added to reach a concentration of 0.03 g/10 mL and 0.009 mg/10

mL, respectively. Samples were incubated for 2 h at 37 °C with an agitation of 150 rpm in a G24 Environment Incubator Shaker (New Brunswick Scientific Co., Edison, NJ, USA) to mimic gastric phase. After the incubation, the pH of the samples was adjusted to *ca.* 4.7 using a sterile alkaline solution (1 liter contains 150 mL of 1M NaOH and 14 g of NaH<sub>2</sub>PO<sub>4</sub>). Bile of bovine source (Sigma-Aldrich) and pancreatin from porcine pancreas (Sigma-Aldrich) were subsequently added to reach a concentration of 0.1 g/10 mL and 0.01 g/10 mL, respectively. Samples were incubated for 2 h to simulate digestion in the upper small intestines (enteric phase 1) under the conditions specified above. After the 2 h incubation, the alkaline solution was used to further increase the pH of the samples to 7.1. In addition, the concentrations of bile and pancreatin were adjusted to 0.1 g/10 mL and 0.01 g/10 mL, respectively. The samples were incubated for another 2 h to simulate digestion in the lower small intestines (enteric phase 2) at aforementioned conditions, to complete a total of 6 h assay. At 30 min, 2 h, 4 h and 6 h intervals, aliquots were collected and probiotic bacteria were enumerated as described below. Probiotic culture/mixtures suspended in 10 ml of 0.5% NaCl solution that did not have the protection of peanut butter matrices were used as controls (10<sup>7</sup> CFU/mL). The pH of peanut butter homogenate and “no peanut butter control” was determined using a pH meter (model 8000; VWR International, PA, USA). All enzymatic solutions used in the experiments were sterilized using sterile syringes (Becton, Dickinson and Co.) and 0.45 µm sterile nylon filter units (Fisher Scientific).

#### 2.4. Enumeration of probiotic bacteria

The 1 mL aliquots taken at each sampling interval were serially diluted, when necessary and 0.1 mL of appropriately diluted samples was plated on microbiological

media purchased from Becton, Dickson and Co. (Sparks, MA, USA). Rogosa (LBS) agar and de Man, Rogosa and Sharpe (MRS) agar were used for the enumeration of *Lactobacillus* species. LBS agar modified with filtered tomato juice (200 mL/L; LBST) was used to enhance the growth of *L. acidophilus*. Both LBS and LBST were prepared with the addition of 1.32 mL of glacial acetic acid (Fisher Scientific) per L of media. *L. lactis* and *S. thermophilus* were enumerated on M17 agar supplemented with 0.5% (final concentration) of lactose. *Bifidobacterium* species were enumerated on modified *Bifidobacterium* agar, each liter of which includes 42.5 g Columbia agar base, 2.5 g glucose, and 0.01 g riboflavin (Fisher Scientific), 2.5 g lactulose (EMD Chemicals Inc. San Diego, CA, USA) and 0.5 g/L cysteine HCl (Sigma-Aldrich). The pH of the media was adjusted to  $5.5 \pm 0.2$  by the addition of propionic acid (J. T. Baker, Phillipsburg, NJ, USA). All probiotic bacteria species were incubated under anaerobic conditions using the BD GasPak™ EZ in a BBL GasPak® System (Becton, Dickinson and Co.).

*Lactobacillus* and *Bifidobacterium* species were incubated at 37 °C for 72 h while *L. lactis* and *S. thermophilus* were incubated at 30 °C and 37 °C, respectively for 48 h. All cell colonies were counted using the Leica Quebec Darkfield Colony Counter (Leica, Buffalo, N.Y., U.S.A.). Log reductions of probiotic cell populations were computed by subtracting the cell populations enumerated at each sampling point from the initial bacterial counts. To avoid confusions that might be caused by sample volume increase due to the adjustment of pH and enzyme concentrations during the course of the experiment, results were expressed as logarithm values of colony forming units (log CFU) in total volume of peanut butter homogenate at each sampling point.

## 2.5. Statistical analysis

Experiments were conducted in three trials with replicate samples at each trial. Data was analyzed using a 3-way Analysis of Variance F-test and the General Linear Model of Statistical Analysis Software (SAS Inst. Inc., Cary, NC, USA). At a confidence level of 95%, Fisher's Least Significant Difference Design was used to compare the significance of differences among bacterial populations in different probiotic products (C, N and U) and individual groups of probiotic bacteria (lactobacilli, bifidobacteria streptococci/lactococci) in different type of peanut butter products (full fat and reduced fat) as influenced by length of simulated gastrointestinal passage.

## 3.0 Results

### 3.1. Survival of different probiotic products during gastrointestinal passage

Table 5.1 shows the overall survival of bacteria in all three probiotic products during the simulated gastrointestinal passage. The average bacterial populations from the three probiotic products decreased significantly ( $p < 0.05$ ) after 30 min in simulated gastric phase and remained at similar levels subsequently ( $p > 0.05$ ; Table 5.1). More probiotic bacteria survived in the two peanut butter homogenates than in the "no peanut butter control", and additionally, the fat content of peanut butter products had no significant influence on probiotic survival during simulated gastrointestinal passage ( $p < 0.05$ ; Table 5.1). Higher cell population was observed in samples inoculated with probiotic product N, followed by product U and product C ( $p < 0.05$ ; Table 5.1).

Fig 5.1 shows cell population reductions in samples inoculated with probiotic products C, N and U at different phases of simulated gastrointestinal passage. The



number of *L. rhamnosus* GG cells in samples inoculated with product C demonstrated a steady decline in both peanut butter homogenates and in the “no peanut butter control” within 30 min into the gastric phase (Fig 5.1A). However, a higher reduction of *ca.* 6 log CFU was observed with the “no peanut butter control” sample (Fig 5.1A). Between the 30 min and 2 h sampling points a slight decline of *ca.* 1 log CFU in *L. rhamnosus* population was observed in the three samples (Fig 5.1A). From the 2 h to 4 h sampling points, *L. rhamnosus* populations increased from 0.29 to 1.47 log CFU in the three samples (Fig 5.1A). At the end of the 6 h incubation period, the average cell populations of *L. rhamnosus* GG in the “no peanut butter control” had decreased 6.97 log CFU. In full-fat and reduced-fat peanut butter homogenates however, the populations decreased by 3.90 and 4.09 log CFU, respectively from the initial counts (Fig 5.1A).

Fig 5.1B shows the survival trend of probiotic bacteria in samples inoculated with probiotic mixture N during simulated gastrointestinal passage. A population reduction of 0.78 log CFU was observed at the 30 min sampling interval in both peanut butter homogenates; in “no peanut butter control” however, *ca.* a 3 time higher decline of 2.56 log CFU was observed. Cell populations in peanut butter homogenates and “no peanut butter control” sample were stable thereafter throughout the experiment. At the end of the 6 h experiment, the average log reduction of probiotic bacteria from the initial counts was 1.23 and 1.43 log CFU in full-fat and reduced-fat peanut butter homogenate, respectively while in the “no peanut butter control” the probiotic population had decreased 3.41 log CFU (Fig. 5.1B).

Fig. 5.1C shows how probiotic bacteria survived in samples inoculated with product U during the experimental period. As the experiment progressed, there was a

steady reduction in cell populations of *ca.* 1 log CFU in both peanut butter homogenates at most sampling points. As observed with product C, comparatively a greater population reduction of U was observed in “no peanut butter control” sample. At the end of the study, probiotic cell populations in full-fat and reduced-fat peanut butter decreased by 3.93 and 3.11 log CFU, respectively from the initial counts. In “no peanut butter control” however, a reduction of 4.57 log CFU was observed at the end of 2 h incubation period, and by the end of 4 h incubation period, the probiotic counts had reduced 7.05 logs (Fig. 1C Fig 5.1C).

Many authors have reported the protective effect of different food matrices on probiotic bacterial survival during simulated gastrointestinal passage (Bedani et al., 2013; Blaiotta et al., 2013; Buriti et al., 2010; Huang and Adams, 2004; Pitino et al., 2012; Ranadheera et al., 2012). It is evident that peanut butter homogenates offered protection to the bacteria from each probiotic product used in the present study (Fig. 1). The two peanut butter products in this work had similar ingredients however; the full-fat peanut butter had more fat and reduced-fat peanut butter had more protein. Although fat has been reported to protect probiotic cells in gastrointestinal tracts (Karimi et al., 2011; Possemiers et al., 2010; Ranadheera et al., 2012), a similar phenomenon was not observed in the present study. The low fat content in the reduced-fat peanut butter was compensated by the high protein content in the present study. Proteins have been proposed to improve the tolerance of probiotic bacteria to gastrointestinal conditions (Ranadheera et al., 2010, 2012). Buriti et al. (2010) reported that substitution of milk fat by inulin in guava mousses increased the survival of *L. acidophilus* in simulated gastric and enteric conditions, suggesting that fat is not the only food ingredient that is capable

of providing protection to probiotic bacteria in simulated gastrointestinal environment. The fat and protein constituents of the peanut butter products in combination may have offered equal protection to probiotic bacteria as they went through the simulated gastrointestinal passage in the present study.

It was observed in the present study that on the average, exposure of probiotic cells to acidic conditions and gastric enzymes had an adverse effect on the viability of probiotic bacteria (Fig. 5.1). Other researchers have also observed high rates of probiotic inactivation at gastric pH levels (Bove et al., 2013; Mainville et al., 2005; Marteau et al., 1997; Pitino et al., 2012). Ranadheera et al. (2012) reported that during a simulated gastrointestinal study, a greater probiotic bacterial inactivation occurred when the pH was 2 compared to pH at 3 or 4. Bove et al. (2013) studied the effect of a food matrix on the survival of *L. plantarum* through an oro-gastro-intestinal tract simulator. The lower probiotic survival occurred when the food matrix was exposed to pH 1.5 as compared to higher pH used in the study.

In the present study, it is evident that *L. rhamnosus* GG from probiotic product C was relatively intolerant of gastric conditions compared to the bacteria from the other two probiotic products and an average of 5.40 log reduction in peanut butter homogenates and 6.92 log reduction in "no peanut butter control" were observed during the gastric phase of the experiment (Fig. 5.1). However, the number of *L. rhamnosus* GG in peanut butter homogenates increased by 1.40 log CFU by the end of enteric passage (Fig. 5.1). Buriti et al. (2010) noticed that *L. acidophilus* cells in guava mousse reached an undetectable level in the gastric phase but viable cells were recovered in the enteric phase when pH

(1.6) of the sample was increased to 7.1. It is possible that some sub-lethally injured probiotic bacterial cells had recovered during the enteric phase of the study.

Comparing to the bacterial cells from probiotic product C, those from product N exhibited higher tolerance to both simulated gastric conditions. At the end of the 6 h experiment, the populations of probiotic bacteria from product N were *ca.* 3 logs higher than the population of *L. rhamnosus* GG in the peanut butter homogenates (Figs. 5.1A and 5.1B). Product N had 16 probiotic strains, and the presence of multiple bacterial strains could have contributed to the observed phenomenon as different researchers have reported that the ability of probiotics to tolerate gastric pH and bile salts is strain specific (Madureira et al., 2005; Mainville et al., 2005; Papamanoli et al., 2003; Pitino et al., 2010). Product U, nevertheless, had fewer bacterial strains compared to product N, and at the end of the 6 h simulated study peanut butter homogenate inoculated with the bacteria from product U only had *ca.* 0.5 log more viable cells compared to those in samples inoculated with *L. rhamnosus* GG (Figs. 5.1A and 5.1C). In the control samples probiotic cells from product U were below the detectable limit by the 4<sup>th</sup> h of the experiment. These results suggest that both the number and the type of probiotic species and strains are important for their overall survival under simulated gastrointestinal conditions.

It is evident that peanut butter homogenates protected the bacteria from all three probiotic products used in this study. However, this protective effect was more evident for probiotic strains from product U judging by the size of population difference between peanut butter homogenates and the control samples (Fig. 5.1C). This result suggests that

ingestion with food might be necessary in order to receive expected health benefits from the probiotic bacteria from product U.

### 3.2. Survival pattern of lactobacilli, bifidobacteria and streptococci/lactococci during simulated gastrointestinal passage

Table 5.1 shows that *Lactobacillus* had the highest cell population loss during the 6 h simulated gastrointestinal passage, followed by *Bifidobacterium*. In comparison, *Streptococcus/Lactococcus* had the lowest cell population loss among the three different probiotic bacterial species used in the study.

Table 5.2 shows the survival trends of the three bacteria species from products N and U. It was observed that at the end of the gastric phase (2 h), *Lactobacillus* cells from product N had a population reduction ( $p < 0.05$ ) of 1.61 and 1.89 log CFU in full-fat and reduced-fat peanut butter homogenates, respectively, but in the “no peanut butter control” an approximate 2 times higher reduction of 3.80 log CFU was observed. A similar phenomenon was observed at the end of the enteric phase (6 h), the reductions in *Lactobacillus* populations were 2.86 and 2.43 log CFU, respectively in the two peanut butter homogenates while the reduction in the control was 3.40 log CFU ( $p < 0.05$ ).

At the 30 min sampling point, *Bifidobacterium* cells from product N in both peanut butter homogenates had a 0.72 log CFU reduction while the reduction in the “no peanut butter control” was approximately 3 times higher (2.38 log CFU). From this sampling point forward to the 2 h sampling interval, *Bifidobacterium* cell counts in the two peanut butter homogenates remained fairly stable ( $p > 0.05$ ; Table 5.2). The same counts in the control sample however, had a 3.79 log CFU reduction ( $p < 0.05$ ) from the

initial count. At the end of 6 h incubation period, *Bifidobacterium* populations in peanut butter homogenates were 2.44 to 2.73 logs higher ( $p < 0.05$ ) compared to those in the “no peanut butter control”.

*Streptococcus/Lactococcus* species did not exhibit any clear survival trend in the present study. The reductions of *Streptococcus/Lactococcus* population were 0.42 or 0.34 log CFU in full fat or reduced fat peanut butter homogenate, respectively at the 30 min sampling interval while a reduction of 1.80 log CFU was observed in the control sample. At the end of the enteric phase (6 h), similar log reductions of 0.84 and 0.80 CFU ( $p > 0.05$ ) were seen in samples with peanut butter products but in the control sample, the reduction was 2.84 logs higher ( $p < 0.05$ ) (Table 5.2). Comparatively, *Streptococcus* and *Bifidobacterium* species from product N had a greater survival ( $p < 0.05$ ) than *Lactobacillus* species in the peanut butter homogenates at most sampling points (Table 5.2).

After 30 min into gastric phase, *Lactobacillus* populations in product U in the full-fat and reduced-fat peanut butter homogenates declined 0.83 and 0.65 log CFU, respectively. But in the control sample, the reduction of the same population was as high as 3.59 log CFU. Furthermore, cell population reductions were similar ( $p > 0.05$ ) in both peanut butter homogenates at this sampling point but they were significantly different ( $p < 0.05$ ) from the same population in the “no peanut butter control” sample. A steady decline in *Lactobacillus* populations was observed in all three types of samples at all phases of the experiment; At 4 h, *Lactobacillus* cells in “no peanut butter control” declined to the undetectable level ( $< 0.6$  log CFU/mL), giving an average log reduction of

7.05 log CFU. At the end of 6 h experiment, cell populations in the two peanut butter products were 4.16 to 4.88 log CFU higher than those in the control samples ( $p < 0.05$ ).

Similar to the trend observed with *Lactobacillus*, *Bifidobacterium* in product U declined steadily from the initial count through all phases of the experiment ( $p < 0.05$ ); yet after 4 h into the experiment, *Bifidobacterium* cells were undetectable in the control samples. At 6 h, *ca.* 5 to 6 log CFU more reductions were observed in “no peanut butter controls” when compared to cell populations in full fat and reduced peanut butter homogenates (Table 5.2). Similar to what was observed in peanut butter homogenates containing product N, *Lactobacillus* had relatively greater cell population loss than *Bifidobacterium* ( $p < 0.05$ ; Table 5.2).

With the exclusion of the samples collected at 30 min and 2 h, *Bifidobacterium* cells from product N survived better than the cells of *Lactobacillus* in the two peanut butter homogenates ( $p < 0.05$ ; Table 5.2). However, the populations of *Lactobacillus* and *Bifidobacterium* species from product U were significantly higher in reduced-fat peanut butter homogenate compared to full fat peanut butter homogenate with the exception of samples collected at 30 min ( $p < 0.05$ ; Table 5.2)

*S. thermophilus* is reported to have poor survival rate in gastrointestinal environments when consumed without food matrices (Conway et al., 1987), but Lick et al. (2001) reported that it had improved survival in the intestines of Gottingen minipigs when fed with yoghurt. Similar protective effect of peanut butter homogenates towards the cells of *S. thermophilus* and *L. lactis* was observed in the present study (Table 5.2). The two bacteria survived better than *Bifidobacterium* and *Lactobacillus* (Table 1), which is in contradiction to what was reported by Marteau et al. (1997) who observed that *B.*

*bifidum* and *L. acidophilus* survived better at all stages of a simulated gastrointestinal passage than *S. thermophilus* and *L. bulgaricus* when administered with fermented milk products. Unlike the study of Marteau et al., the present work sampled a combination of *L. lactis* and *S. thermophilus*. *S. thermophilus* was accompanied by other probiotic strains in our study, and it is possible that some strains have a higher tolerance to low pH and bile salts as what was observed by Blaiotta et al. (2013). Furthermore, the present study and the study of Marteau et al. used different type of foods as matrices, and Ranadheera et al. (2012) suggested that the tolerance of probiotics to gastric pH and bile salts can be influenced by carrier foods. Possemiers et al. (2010) observed a 5-fold higher viability of *L. helveticus* and *B. longum* when the probiotic bacteria were delivered by chocolate compared to milk.

The different survival trend of *Lactobacillus* and *Bifidobacterium* from probiotic products N and U (Table 5.2) is a clear indication of the importance of probiotic species/strain variability in the survival of probiotic cells under simulated gastric and enteric conditions. Using 7 strains of *L. rhamnosus* in cheese during a simulated human digestion, Pitino et al. (2012) found that the survival of *L. rhamnosus* was strain specific at each stage of the digestion. Strain specific tolerance to gastric acidity and bile salts was also observed in two other studies by same authors (Pitino et al. 2010; Lo Curto et al, 2011).

Our results showed that on the average, *Bifidobacterium* strains had higher survival rates than *Lactobacillus* strains and this observation was supported by several previous studies. Bedani et al. (2013) reported that *Bifidobacterium animalis* Bb-12 in a fermented soy product was more robust and tolerant during *in vitro* simulated



gastrointestinal passage, maintaining a mean population of *ca.* 8 log CFU/g while *L. acidophilus* La-5 could only maintain a mean population below 5 log CFU/g. Similar observation was made by Madureira et al. (2011). *B. animalis* was found to have better resistance than *L. acidophilus* and *L. casei* to simulated stomach and intestinal conditions.

Salaün et al., (2005) reported that a major factor affecting changes in pH in any given system is the buffering capacity of the food product. Foods like cheese, ice-cream and chocolate have good buffering capacities, thereby enhancing the survival of bacterial cells. In addition to the buffering capacity of food, physical encapsulation of probiotic cells by food components is another possible protective mechanism for probiotic cells (Työppönen et al., 2003). Future research is needed to elucidate the molecular basis of the protective mechanisms observed in the present study.

## **Conclusion**

Full-fat and reduced-fat peanut butter homogenates offered equal protection to probiotic bacteria from products C, N and U during simulated gastrointestinal passage. Overall, streptococci and lactococci had the highest survival, followed by bifidobacteria and lactobacilli. At the end of 6 h gastrointestinal passage, in both peanut butter homogenates, probiotic bacteria from product N had the highest survival, followed by probiotic bacteria from products U and C. Results from this study suggests that peanut butter matrices are capable of protecting probiotic bacteria under simulated gastrointestinal conditions and are possible vehicles to deliver probiotic to children prone to diarrhea.

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**Table 5.1**

Average reduction (log CFU) of total probiotic bacteria populations and probiotic species (*Lactobacillus*, *Bifidobacterium* and *Streptococcus/Lactococcus*) in probiotic products C, N and U as observed in full fat-peanut butter, reduced-fat peanut butter and “no peanut butter control” during a 6 h (360 min) simulated gastrointestinal study

	<b>Reduction in cell population (logCFU)</b>
<b>Probiotic culture/mixture</b>	
<b>C</b>	4.10c
<b>N</b>	1.50a
<b>U</b>	2.48b
<b>Probiotic bacteria</b>	
<i>Lactobacillus</i>	2.38c
<i>Bifidobacterium</i>	1.93b
<i>Streptococcus/Lactococcus</i>	0.98a
<b>Food matrix</b>	
<b>Full fat peanut butter</b>	2.14a
<b>Reduced fat peanut butter</b>	2.13a
<b>No peanut butter control</b>	3.99b
<b>Time in simulated gastrointestinal fluid (min)</b>	
<b>0</b>	0.00a
<b>30</b>	2.55b
<b>120</b>	3.59c
<b>240</b>	3.60c
<b>360</b>	3.78c

Means in the same column not followed by the same letters are significantly different ( $P < 0.05$ )



**Table 5.2**

Average reduction (log CFU) of *Lactobacillus* (Lacto), *Bifidobacterium* (Bifi) and *Streptococcus/Lactococcus* (Strep) in probiotic mixtures N and U in full-fat (FF) and reduced-fat (RF) peanut butter and “no peanut butter control” (C) during a 6 h (360 min) simulated gastrointestinal study

Reduction in cell population (log CFU)															
	Mixture N									Mixture U					
	FF			RF			C			FF		RF		C	
	Lacto	Bifi	Strep	Lacto	Bifi	Strep	Lacto	Bifi	Strep	Lacto	Bifi	Lacto	Bifi	Lacto	Bifi
<b>0 min</b>	0.00aA	0.00aA	0.00abA	0.00aA	0.00aA	0.00abA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA
<b>30 min</b>	0.91abB	0.72bB	*0.42aA	0.92abB	0.72bB	*0.34aA	2.90bEF	2.38bDE	1.80bCD	0.83aB	1.12bBC	0.65abB	1.01bB	3.59bF	3.11bF
<b>120 min</b>	1.61bcBCD	0.96bABC	0.22abA	1.89bcDEF	0.85bAB	0.26abA	3.80cHI	3.79cHI	3.06cGH	2.53bFG	2.43cEFG	1.74bDE	1.68bcCDE	4.50cIJ	4.87cJ
<b>240 min</b>	2.38bcCDE	0.95bAB	1.05bABC	2.67cDEF	0.84bAB	0.46bcA	3.59cEF	3.62cEF	3.32cDEF	3.91cF	3.00cdDEF	3.56cDEF	2.20cdBCD	7.03dG	7.10dG
<b>360 min</b>	2.86cBC	0.74bA	0.84abA	2.43cB	1.03bA	0.80cA	3.40bcBCD	3.47cCD	3.64cCD	4.38cD	3.58cCD	3.66cCD	2.75dBC	7.03dE	7.10dE

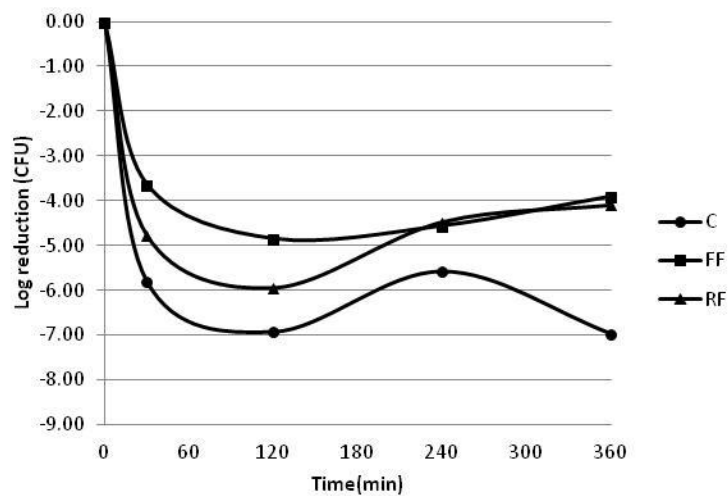
Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of treatment time  
 Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of probiotic mixture, probiotic species and food matrix  
 Numbers with an asterisk (\*) indicates a log CFU increment, not a log CFU reduction

**Fig. legends**

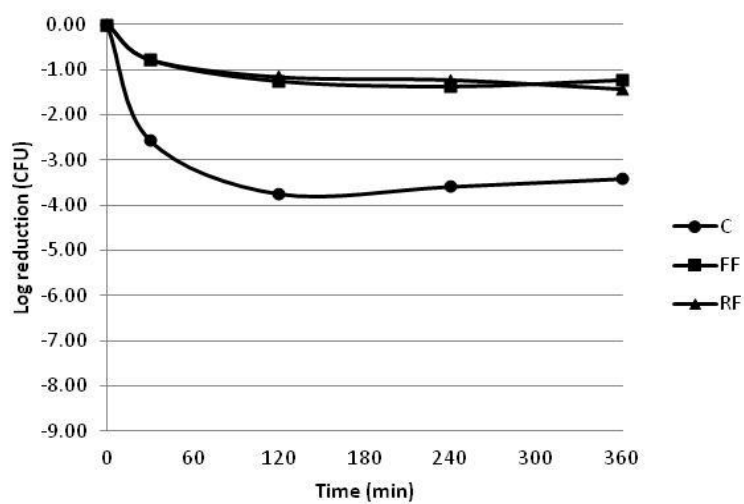
Fig. 5.1.- Reduction of mixed cell populations (log CFU) of probiotic products C (A), N (B) and U (C) in full-fat peanut butter (FF), reduced-fat peanut butter (RF) and “no peanut butter control” (C) during a 6 h (360 min) simulated gastrointestinal study

Fig. 5.1. Klu et al.

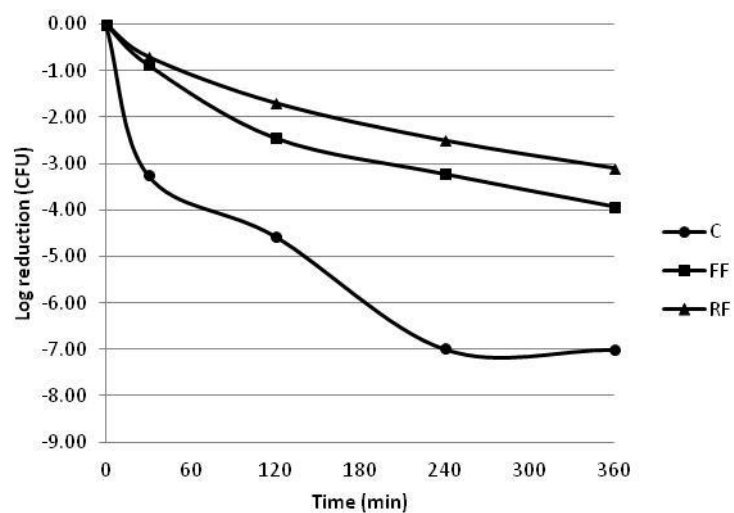
A



B



C



CHAPTER 6

INFLUENCE OF PROBIOTICS IN PEANUT BUTTER ON THE FATE OF  
SELECTED *SALMONELLA* AND *LISTERIA* STRAINS UNDER SIMULATED  
GASTROINTESTINAL CONDITIONS

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<sup>1</sup> Klu, Y.A.K., R.D. Phillips and J. Chen. 2014. To be submitted to *Applied and Environmental Microbiology*.

**ABSTRACT**

This study sought to observe the behavior of probiotics and *Salmonella enterica* or *Listeria monocytogenes* co-inoculated into peanut butter during a simulated gastrointestinal study. Homogenates of peanut butter co-inoculated with *Salmonella* or *Listeria* (5 log CFU/mL) and lyophilized or cultured probiotics (9 log CFU/mL) was exposed to simulated gastrointestinal conditions, and samples inoculated with only pathogens served as controls. Sample pH, titratable acidity and pathogen populations were determined during a 24 h period at 37°C. An agar diffusion assay was performed to determine the inhibitory effect of probiotic culture supernatants with either natural (3.80, 3.78 or 5.17) or neutralized (6.0) pH and fresh broths with their pH acidified to levels of the probiotic culture supernatants. Antibacterial activity of crude bacteriocin extracts was evaluated against *Salmonella* and *Listeria*. After the 24 h assay period, samples with probiotics had lower pH and higher titratable acidity than those without probiotics. Overall, the presence of probiotics caused a significant reduction ( $p < 0.05$ ) in pathogen populations in simulated gastrointestinal fluid. Filter-sterilized supernatants of *Bifidobacterium* and *Lactobacillus* cultures inhibited pathogen growth. However, the elevation of pH diminished their antibacterial activities. When acidified fresh broths were used, the effect of low pH on pathogen inhibition was observed. Crude bacteriocin extracts had a stain-specific inhibitory effect only towards *L. monocytogenes*. Probiotic bacteria in the "probiotic peanut butter" survived simulated gastrointestinal conditions and were able to inhibit the growth of *Salmonella* and *Listeria*. Results suggest peanut butter is a plausible carrier to deliver probiotics to improve gastrointestinal health.

Approximately 760,000 pre-school children in the developing countries die from diarrhea related causes each year (1). The burden of diarrhea on the persons affected, the public health sector and the total economic development of a nation is so large that it is estimated that 13% of all Disability Adjusted Life-Year, defined as the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability, are caused by diarrhea (2). Another devastating medical condition, severe acute malnutrition affects almost 20 million preschool children worldwide and is a causative factor of a third of the deaths that occur in these children (1). A vicious cycle exists between malnutrition and diarrhea; and diarrhea is both a cause and a consequence of malnutrition in pre-school children (1, 3, 4).

Reports of numerous clinical studies suggest that probiotics are effective in controlling, managing and preventing diarrhea in children. The proposed mechanisms of actions of probiotics include immunomodulation, barrier function enhancement, cytoprotective effects and antimicrobial functions (5). Specific benefits of probiotics in terms of promoting gastrointestinal health include inhibition of pathogen growth, prevention of attachment and colonization *via* antagonism (5-9). There is much research interest on the effectiveness of specific genus and strains of probiotics, and how their usage as single strains or mixtures exhibit proposed health benefits (10-13). For these mechanisms of actions to occur, it is important that the probiotic bacteria survive in the gastrointestinal tract and colonize the intestinal walls. It has been documented that food matrixes help in probiotic survival and colonization (14, 15) thus the type of food matrix may influence probiotic actions. Peanut butter is being used as major ingredient of Ready to Use Therapeutic Foods (RUTFs) for the treatment of severe malnutrition.

Studies conducted in our laboratory have showed that peanut butter matrix is able to protect different probiotic bacteria during storage (16, 17) and under simulated gastrointestinal conditions (18). Thus, "probiotic peanut butter" has been identified as a possible intervention to address malnutrition and diarrhea concurrently in the developing countries. The purpose of this study was to observe the behavior of probiotics and *Salmonella enterica* or *Listeria monocytogenes* co-inoculated in peanut butter during a simulated gastrointestinal study and the ability of probiotics to inhibit the growth of the two pathogens.

## **MATERIALS AND METHODS**

**Peanut butter, probiotics and pathogens.** Peanut butter was kindly provided by the American Blanching Company (Fitzgerald, GA, USA). A commercial probiotic product designated as N was used in the project. The product had a manufacturer's claim of 16 billion live cells per g of powder and was made up of 16 different probiotic bacteria which included *Streptococcus thermophiles*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *B. lactis* Bif Relief 24-7™, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius* and *Lactococcus lactis*. The pathogens used in this study included *Salmonella* Typhimurium 4-1, *Salmonella* Heidelberg 4-2, *Salmonella* Enteritidis 4-56, *Listeria monocytogenes* 7764, *L. monocytogenes* 7969 and *L. monocytogenes* 8733. All the pathogen strains are from our laboratory culture collections.

**Preparation of bacterial inocula.** *Bifidobacterium* species in probiotic mixture N were grown on modified *Bifidobacterium* agar which include 42.5 g Columbia agar base, 2.5 g glucose, 0.01 riboflavin (Fisher Scientific, Fair Lawn, NJ, USA), 2.5 g lactulose (EMD Chemicals Inc. San Diego, CA, USA) and 0.5 g L-Cysteine HCl (Sigma-Aldrich, St. Louis, MO, USA) per liter of media. The pH of the media was adjusted to  $5.5 \pm 0.2$  by the addition of propionic acid (J.T. Baker, Phillipsburg, NJ, USA). The de Man, Rogosa and Sharpe (MRS) agar was used to grow *Lactobacillus* species. *L. lactis* and *S. thermophilus* were grown on M17 agar supplemented with 0.5% (final concentration) of lactose. The media were purchased from Becton, Dickinson and Co. (Sparks, MA, USA). All probiotic bacteria were incubated under anaerobic conditions for 48-72 h using the BD GasPak™ EZ in a BBL GasPak® System (Becton, Dickinson and Co.). After the incubation, each probiotic culture was harvested with sterile distilled water. Equal numbers of each culture were mixed using a vortex mixer (Fisher Scientific) to form a single, uniform probiotic inoculum (*ca.*  $10^{10}$  CFU/mL). Inoculum of lyophilized probiotics was prepared by mixing 1 g of lyophilized probiotic powder in 9 mL of sterile water, and the bacterial cell population in the inoculum was *ca.*  $10^{10}$  CFU/mL.

Each strain of *Salmonella* or *Listeria* was grown for 16-18 h in tryptic soy broth (TSB; Becton, Dickinson and Co.) under aerobic condition. To form a 3-strain mixture, equal number of cells of each strain was mixed. The two pathogen mixtures were serially diluted in sterile water to a level of *ca.* 6 log CFU/mL.

**Simulated gastrointestinal studies.** Gastrointestinal conditions were simulated by adopting a method described by (19). The peanut butter was suspended in 0.5% NaCl



solution, and the samples were mixed by vigorous manual to-and-fro shaking at an arm angle of about 45° for 1 min. Resulting peanut butter homogenate was inoculated with the three-strain mixture of *S. enterica* or *L. monocytogenes* to a level of *ca.* 5 log CFU/mL. Ten mL of each inoculated peanut butter homogenate was transferred into a sterile 50 mL polypropylene conical tube (Becton, Dickinson and Co.) and subsequently inoculated with either lyophilized probiotic powder or fresh grown probiotic cultures of product N at 9 log CFU/mL. The control sample was only inoculated with *Salmonella* or *Listeria* at *ca.* 5 log CFU/mL.

To mimic digestion in the stomach, the pH (*ca.* 6) of the samples was adjusted to 1.6 with 1 M HCl solution (Fisher Scientific). Pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO, USA) and lipase from *Rhizopus arrhizus* (Sigma-Aldrich) were added to reach a concentration of 0.03 g/10 mL and 0.009 g/10 mL, respectively. Samples were incubated for 2 h at 37 °C with an agitation of 150 rpm in a G24 Environment Incubator Shaker (New Brunswick Scientific Co., Edison, NJ, USA) to mimic the gastric phase. After the incubation, the pH of the samples was adjusted to *ca.* 4.7 using a sterile alkaline solution (1 liter contains 150 mL of 1M NaOH and 14 g of NaH<sub>2</sub>PO<sub>4</sub>). Bile of bovine source (Sigma-Aldrich) and pancreatin from porcine pancreas (Sigma-Aldrich) were subsequently added to reach a concentration of 0.1 g/10 mL and 0.01 g/10 mL, respectively. Samples were incubated for 2 h (enteric phase 1) under the conditions specified above. After the 2 h incubation, the alkaline solution was used to further increase the pH of the samples to 7.1. In addition, the concentrations of bile and pancreatin were adjusted to 0.1 g/10 mL and 0.01 g/10 mL respectively. The samples were incubated for 20 h at the abovementioned conditions, to achieve a total of 24 h

assay. At 2 h, 4 h, 6 h, 9 h, 12 h and 24 h intervals, aliquots were collected and pathogen and probiotic populations were enumerated as described below. All enzymatic solutions used in the experiments were suspended in water and sterilized using sterile syringes (Becton, Dickinson and Co.) and 0.45 µm sterile nylon filter units (Fisher Scientific).

**Bacterial enumeration.** Collected samples were serially diluted and when necessary and 0.1 mL of appropriately diluted samples were plated on the following microbiological media. Bismuth sulfite agar was used for the enumeration of *Salmonella* whiles Modified Oxford Agar (MOX) supplemented with moxalactam sodium (20 mg/L) and colistin sulphate (10 mg/L) was used to enumerate *Listeria*. *Lactobacillus* selection (LBS) agar and MRS agar were used for the enumeration of *Lactobacillus* species. LBS agar modified with filtered tomato juice (200 mL/L; LBST) was used to enhance the growth of *L. acidophilus*. Both LBS and LBST were prepared with the addition of 1.32 mL of glacial acetic acid (Fisher Scientific) per L of media. *L. lactis* and *S. thermophilus* were enumerated on M17 agar supplemented with 0.5% lactose solution and *Bifidobacterium* species were enumerated on modified *Bifidobacterium* agar as described above. The media were purchased from Becton, Dickinson and Co. *Salmonella* and *Listeria* were incubated at 37 °C for 24–48 h. Probiotic bacteria species were incubated under anaerobic conditions as described above. All cell colonies were counted using the Leica Quebec Darkfield Colony Counter (Leica, Buffalo, N.Y., U.S.A.).

**Changes in sample pH and acidity during simulated gastrointestinal study.**

At each sampling points, aliquots were collected and the pH and titratable acidity was measured. Sample pH was determined using a pH meter (model 8000; VWR

International, PA, USA). Titratable acidity was performed according to the method described by (20).

**Organic acid profiles in probiotic culture supernatants.** To determine the major type and amount of organic acids produced by the probiotic bacteria, supernatants of 72 h probiotic cell cultures in M17, MRS, and MRS supplemented with 5% (v/v) cysteine HCl were analyzed (Covance, Madison, WI, USA) using a reversed phase HPLC with UV detection specified in the AOAC Official Method 986.13.

**Inhibition of pathogens by probiotic culture supernatants.** MRS broth supplemented with 5% (v/v) cysteine HCl (Sigma-Aldrich) was used to grow bifidobacteria in probiotic mixture N while MRS and M17 broths (Becton, Dickinson and Co.) were used for cultivating lactobacilli and streptococci/lactococci, respectively. The lyophilized probiotic mixture was inoculated into each broth and incubated at 37 °C for 48-72 h under anaerobic conditions as described above. After incubation, the broths were centrifuged at 10,000 g for 15 min at 4 °C using an Eppendorf 5810 R Centrifuge (Eppendorf, Hamburg, Germany). Thereafter, the cell-free supernatants obtained were filter sterilized as described above. The pH of a portion of the supernatant was adjusted to 6.0 using sterile NaOH (Fisher Scientific). In addition, fresh, sterile MRS and M17 broths with their pH acidified, with acetic acid and lactic acid, to levels of probiotic culture supernatants (3.80 from *Lactobacillus* culture, 3.78 from *Bifidobacterium* culture and 5.17 from *Streptococcus/Lactococcus* cultures) were included in the agar diffusion assay. Inorganic acid HCl was used as a comparison.

Each strain of *S. enteric* and *L. monocytogenes* were grown overnight in tryptic soy broth (Becton, Dickinson and Co.). Confluent bacterial lawns were made by

inoculating the overnight cultures (diluted to 5 log CFU/mL) on tryptic soy agar (Fisher Scientific) using a sterile cotton swab. Holes of 4 mm in diameter were punched and added into the holes were a 100  $\mu$ L aliquot of probiotic culture supernatants with natural or neutralized pH as well as acidified MRS and M17 broths. The resulting plates were incubated upright at 37 °C for 24 h. The diameter of inhibition zones formed on the bacterial lawn was measured after the incubation.

**Inhibition of pathogens by crude bacteriocin extracts.** Lactobacilli, bifidobacteria and streptococci/lactococci from probiotic mixture N were incubated in their respective broths as described above. Cell-free supernatants prepared according to procedures described above were precipitated at 25 °C with 80% ammonium sulfate (Fisher Scientific). Precipitated materials were collected by centrifugation at 10,000 g for 20 min at 4 °C and the precipitate was re-suspended in dipotassium phosphate buffer (pH 6.0; Fisher Scientific) and dialyzed against sterile water using a 1000,  $M_r$  Spectra/Por® 7 Membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) at 4 °C for 20 h with a change of sterile water within 2 h and 4 h of the dialysis. Crude bacteriocin extract of each probiotic species was subsequently used in an agar diffusion test as described above.

**Statistical analysis.** Experiments were conducted in four trials with replicate samples at each trial. The data obtained was analyzed using a 3-way Analysis of Variance F-test and the General Liner Model of Statistical Analysis Software (SAS Inst. Inc., Cary, NC, USA). At a confidence level of 95%, Fisher's Least Significant Difference Design was used to compare the significance of differences among pathogen populations in different treatment groups (pathogen alone, pathogen with cultured

probiotics, pathogen with lyophilized probiotics) during simulated gastrointestinal passage. Additionally, the differences among the zones of inhibition formed by individual strains of bacterial pathogens or crude bacteriocin extracts and among various treatment groups were compared using the same statistical protocol.

## RESULTS

**Changes in pathogen populations during simulated gastrointestinal passage.** Table 6.1 depicts cell populations of *L. monocytogenes* and *S. enterica* in peanut butter homogenates with or without probiotics during the simulated gastrointestinal passage. On the average there were no significant differences in the average populations of the two pathogens. Furthermore, by average, the populations of the pathogens in peanut butter homogenates without probiotic mixture N were significantly higher ( $p < 0.05$ ) than the pathogen populations in peanut butter homogenates with inoculated probiotics. There was no significant difference ( $p > 0.05$ ) between the populations of pathogens co-inoculated with cultured probiotics or lyophilized probiotics.

Changes in the cell populations of *Salmonella* strains during the 24 h simulated gastrointestinal study are presented in Table 6.2. *S. enterica* had relatively lower populations in samples without probiotics at the first 4 h of the simulated study ( $p < 0.05$ ; Table 6.2). Thereafter, a significant increase in *Salmonella* counts was observed at each sampling point till 24 h sampling interval ( $p < 0.05$ ). When *S. enterica* was co-inoculated with cultured probiotics, no specific trend was observed during the first 6 h of the experiment. At the 9 and 12 h sampling points, however, the populations of *Salmonella* increased and were not significantly different from the initial counts ( $p > 0.05$ ). At the

end of the experiment, *ca.* a 3 log cell count decrease was observed comparing to the results obtained at 12 h sampling point ( $p < 0.05$ ).

*S. enterica* population in samples co-inoculated with lyophilized probiotics had a similar trend of change in population that occurred in samples co-inoculated with cultured probiotic ( $p > 0.05$ ; Table 6.2). Although at each sampling point, there were differences in *S. enterica* counts among the 3 treatment groups, statistically, there were no significant differences in the counts apart from populations observed at the end of the experiment. The counts of *S. enterica* in samples co-inoculated with the two types of probiotics had similar ( $p > 0.05$ ) counts which was significantly higher than *Salmonella* populations in samples without the supplementation of probiotics.

In Table 6.3, *L. monocytogenes* populations in samples without the probiotics decreased throughout all sampling points but increased rapidly at the 24 h sampling point. There were no significant differences in the population of *L. monocytogenes* co-inoculated with lyophilized probiotic bacteria throughout the entire study period. Similar to what was observed with samples inoculated with *S. enterica* in Table 6.2, throughout the study period, *L. monocytogenes* populations across the three treatment groups at all sampling points were statistically similar except at the 4 h and 24 h sampling points. At 4 h sampling point, the counts of *L. monocytogenes* co-inoculated with cultured probiotic bacteria was lower ( $p < 0.05$ ) than those in the other two samples. At the end of the study, *L. monocytogenes* populations in samples without the probiotics had significantly higher cell counts ( $p < 0.05$ ) than those in samples co-inoculated with probiotics.

**Organic acid profile in probiotic culture supernatants.** The organic acid profile in the supernatants of probiotic bacterial cultures is shown in Table 6.4. All three

probiotic species produced significantly higher amount of lactic acid than acetic acid ( $p < 0.05$ ). *Lactobacillus* species produced the highest amount of organic acids, followed by *Bifidobacterium* and then *Streptococcus* ( $p < 0.05$ ).

**Changes in sample pH and titratable acidity.** Fig. 6.1 gives a graphical representation of the changes in sample pH and titratable acidity as simulated gastrointestinal study proceeded. The Figures show that samples inoculated with *S. enterica* and *L. monocytogenes* followed similar trends in changes in pH and titratable acidity during the simulated gastrointestinal study. The pH of the samples were the lowest (*ca.* 2.5) at the beginning of the study and a gradual increase in pH was observed till the 6 h sampling point (*ca.* 6.80). From the sampling point at 9<sup>th</sup> h to the 24<sup>th</sup> h, there was a gradual decrease in sample pH (Figs. 6.1A and 6.1B). From the onset of the experiment through to the 6 h sampling points, samples with and without probiotics had similar pH values. However, from 12 h onwards, a smaller decrease in pH was observed in the control samples (Figs. 6.1A and 6.1B). Samples inoculated with *S. enterica* and cultured or lyophilized probiotics had similar pH values from 12 to 24 h (Fig. 6.1A), however samples inoculated with *L. monocytogenes* and cultured probiotics had lower pH values than those with *L. monocytogenes* and lyophilized probiotics (Fig. 6.1B).

Fig. 6.1 also shows the trend in titratable acidity as the simulated gastrointestinal study proceeded. At 2 h sampling point, the titratable acidity observed from samples inoculated with both *S. enterica* and *L. monocytogenes* increased from the initial values (Figs. 6.1A and 6.1B) except for the samples inoculated with *Listeria* and lyophilized probiotics (Fig. 6.1A). At the 4 h sampling point, titratable acidity decreased in all samples (Fig. 6.1). Thereafter, gradual increases were observed in titratable acidity for

all samples. At the end of 24 h assay, the titratable acidity of samples inoculated with *S. enterica* or *L. monocytogenes* alone were lower than that of samples co-inoculated with probiotics (Fig. 6.1). Additionally, the titratable acidity value in samples with lyophilized probiotics was lower than samples with cultured probiotics.

**Inhibition of pathogens by probiotic culture supernatants.** The supernatants of *Bifidobacterium* and *Lactobacillus* cultures with natural pH values were effective against the 3 *Salmonella* (Table 6.5) and *Listeria* (Table 6.6) strains used in the study. When the pH of the supernatant was adjusted to 6.0, the antibacterial activity of probiotic culture supernatants diminished. When the pH of fresh broths was lowered to the level of probiotic cell culture supernatant, zones of inhibition of various sizes were observed. Broths with their pH adjusted by HCL exhibited a greater inhibition towards *S. Heidelberg* and *S. Enteritidis* than to *S. Typhimurium*, and acetic acid had a greater inhibition against *S. Typhimurium* than to the other two *Salmonella* strains (Table 6.5). Lactic acid was equally inhibitory to all 3 *Salmonella* strains.

The supernatants of *Bifidobacterium* and *Lactobacillus* cultures with natural pH had a greater inhibition was observed with *L. monocytogenes* strain 7764 compared with the other two strains used in the study (Table 6.6). As observed with *Salmonella*, supernatants with neutralized pH lost the inhibitory activity against *Listeria*. The pH of fresh broths lowered by HCL only inhibited the growth of strain 8733 while the pH of the broths lowered by the other two acids were equally inhibitory to all 3 *Listeria* strains. The supernatants of *Streptococcus/Lactococcus* cell culture had no inhibitory effects on *S. enterica* and *L. monocytogenes* (Table 6.5 and Table 6.6). However, those of *Lactobacillus* and *Bifidobacterium* cell culture had similar inhibitory effects ( $p > 0.05$ ).



**Inhibition of pathogens by crude bacteriocin extracts.** Crude bacteriocin extracts from the cell cultures of *Lactobacillus* and *Bifidobacterium* exhibited a similar ( $p > 0.05$ ) inhibition against *L. monocytogenes* 8733 and 7969 which were unsusceptible to the extract from *Streptococcus/Lactococcus* (Table 6.7). The growth of *L. monocytogenes* strain 7764 was inhibited by all three crude bacteriocin extracts. However, the level of inhibition from the extract from *Streptococcus/Lactococcus* cell culture supernatant was significantly lower than the supernatants of *Lactobacillus* and *Bifidobacterium* cultures. None of the extracts had any activity against the 3 *Salmonella* strains used in the study (data not shown). It must also be noted that the zones of inhibition formed by bacteriocin were fainter and less distinct than zones formed by cell free supernatants.

## DISCUSSION

Results in Table 6.1 show that probiotic bacteria in peanut butter were able to inhibit pathogen growth under simulated gastrointestinal conditions after a 24 h incubation period. These results were consistent with those of an earlier study by (20) who incubated *S. Enteritidis* ( $10^5$  CFU/mL) with two strains of *L. paracasei* and a strain of *L. acidophilus* ( $10^9$  CFU/mL) in a laboratory system without food matrix for a period of 24 h. In comparison to control, the presence of probiotics resulted in a 7 log reduction in the *Salmonella* counts. It was observed by (21) that the growth inhibition of *Salmonella*, *E. coli* and *Campylobacter* strains by co-inoculated probiotics only occurred after a 9-24 h incubation period. A similar observation was made in this study as inhibition of *Salmonella* and *Listeria* was only observed at the 24 h sampling point (Tables 6.2 and 6.3). The observations made in this and the previous studies suggest that

probiotic bacteria need to have an active metabolic activity within a certain time period in order to be able to inhibit the growth of pathogens. It was stated by (23) that the resident time for intestinal contents in the colon is 20-32 h whereas it is only 2-4 h from the stomach up to the ileum. Since probiotics are proposed to exert their beneficial effects in the large intestines, the results of the present study suggest that probiotic bacteria in peanut butter should have sufficient time to interact with co-ingested foodborne pathogens in human gastrointestinal tracts.

In the present study lactic acid and acetic acid were the major contributors to the growth inhibition of *S. enterica* and *L. monocytogenes*. Similar results have been reported by (24). The samples included in the simulated gastrointestinal study all had a gradual increase in titratable acidity and decrease in pH (Fig. 6.1). However, samples with probiotics had a greater pH reduction because the production of organic acids is a major metabolic activity of lactic acid bacteria (25). A greater inhibition in pathogen growth occurred in samples with lower pH and high titratable acidity (Tables 6.2 and 6.3; Fig. 6.1). When sample pH was neutralized the inhibitory effects of probiotic culture supernatants against *Salmonella* and *Listeria* diminished (Tables 6.2 and 6.3; Fig. 6.1). In a previous study, cell free supernatants of *Lactobacillus* or *Bifidobacterium* with pH 4.0-4.9 inhibited the growth of enteroaggregative *E. coli*; however no inhibitory effect was observed when the pH of the supernatant was adjusted to 7.0 (26). In another study, cell free supernatants of four strains of *Lactobacillus* and one strain of *Bifidobacterium* conferred a significantly greater inhibition on *S. Enteritidis* and other enteropathogens than did neutralized cell free supernatants (22). A growth inhibition of *S. Typhimurium* by cell free culture supernatants of *Bifidobacterium* was reported but when the pH of the

control medium and cell free culture supernatants was raised to 6.5, weaker growth inhibition was observed (24). Similarly, it was reported by (21) that when cell free supernatants were neutralized, no inhibitory effect against *Salmonella* was observed. The ability of spent culture supernatants of *Lactobacillus* strains to inhibit the growth of some pathogenic bacteria including *S. Typhimurium* was observed by (27). Furthermore, (12) reported a reverse relationship between the pH of cell free supernatants and growth inhibition of *S. Typhimurium* by some strains of *Bifidobacterium*, *Lactobacillus*, *Lactococcus* and *Streptococcus*.

According to results shown in Tables 6.5 and 6.6, HCl had a poorer inhibition to the growth of *Salmonella* and *Listeria* comparing to the two organic acids. Similar phenomenon was observed by (24). The antibacterial activity of organic acids is mainly from the undissociated form of the acids (12, 28, 29). The non-ionized form of the acids passes through bacterial cell membranes and then becomes ionized in the cytoplasm to release protons that decrease intracellular pH, inhibit the metabolic activity of bacteria and eventually cause cell death (24, 29). Strong inorganic acids like HCl are completely dissociated into anions and protons when in solution. Cell membranes have poor permeability to protons. However, some protons are capable of entering the cytoplasm by interacting with the proton transport system of the cells and thus HCl has a weaker inhibition towards pathogens (30).

Presence of bacteriocins is another likely reason for the observed growth inhibition of *Listeria* when it co-existed with probiotics under simulated gastrointestinal conditions. Bacteriocins are small peptides or proteins, produced by lactic acid bacteria that possess activity against other gram positive bacteria such as *L. monocytogenes* and

*Staphylococcus aureus* (31, 32). Although the mechanism of bacteriocin against bacterial pathogens is not fully understood, it was proposed that bacteriocins are able to recognize and interact with receptor molecules on bacterial surfaces. This action destabilizes bacterial cell membrane by a detergent-like or pore making action (32, 33). The changes that occur in the membrane induces the depletion of proton motive force which is an electrochemical gradient serving as driving force for bacterial cellular and metabolic activities, thus a depletion of it induces bacterial death. The disruption of cell membrane by bacteriocin also causes a depletion in intracellular ATP levels and release of intracellular ions and other cytoplasmic contents which eventually causes the death of bacterial cells (34). In a well diffusion assay with neutralized cell free supernatants of several lactic acid bacterial strains, (35) it was also observed that inhibition zones were formed on lawns of *L. monocytogenes*. In a further test the inhibition zones were reduced in the presence of proteinase K, suggesting that inhibition of *Listeria* was due to a proteinaceous molecule, probably a bacteriocin. Some authors have however reported results contradictory to that of this study. In a study by (36) their finding was that, the antibacterial activity of 17 strains of lactobacilli against some pathogens including *L. monocytogenes* was largely due to the action of organic acids and bacteriocin activity contributed little to the antibacterial activity. Interestingly, in the present study we found that cell free supernatants of *Streptococcus/Lactococcus* did not exhibit any inhibitory effects on *L. monocytogenes* 7764 (Table 6.3) whereas crude bacteriocin extract from the same species inhibited its growth (Table 6.7). Further studies are needed to confirm the identity of functional molecules in the crude bacteriocin extract and how they inhibited the growth of *Listeria*.

In the present study, the growth of *Listeria* was inhibited by crude bacteriocin extracts prepared from cell free supernatants of *Bifidobacterium* and *Lactobacillus* (Table 6.7), but the growth of *Salmonella* was not affected (Data not shown). Previous studies have shown that bacteriocins produced by probiotics are usually not effective towards gram negative bacteria such as *Salmonella* and *E. coli* but organic acids produced by the same probiotics have been shown to have inhibitory effects towards these pathogens (31, 37). The outer membrane of Gram-negative bacteria comprises a complex lipopolysaccharide which provides a rigid barrier. This outer membrane is hydrophilic in nature and it acts as a protective outer barrier to the influx of foreign substances including macromolecules and hydrophobic solutes. Thus, bacteriocins do not generally inhibit the growth of Gram-negative bacteria unless their cell membrane is compromised (38-40).

The growth inhibition of *Clostridium difficile*, *E. coli* and *S. Typhimurium* by the overnight cultures of different mixtures of probiotic bacteria was reported by (41). A *Lactobacillus* mixture comprising *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantatum*, *L. rhamnosus*, *L. salivarius*, *L. fermentum* and *L. helveticus* produced the biggest zone of inhibition followed by a *Bifidobacterium* mixture (*B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*) and then *L. lactis* and *S. thermophilus* mixture. Similar results were observed in the present study with the exception of what was observed with *L. lactis* and *S. thermophilus* mixture. Even though the cell free supernatants of streptococci and lactococci cultures contained some amounts of lactic and acetic acids, results from Tables 6.5 and 6.6 show that the supernatants did not inhibit either *Salmonella* or *Listeria*. It is possible the concentration of organic acids in the streptococci/lactococci supernatants was not high enough to inhibit the growth of the pathogens. A study by (12) has shown

that concentrated cell free supernatants of selected probiotics provided greater inhibitions towards pathogenic microorganisms than non-concentrated supernatants.

In conclusion, results from the present study suggests that, selected probiotics in peanut butter is able to inhibit the growth of *S. enterica* and *L. monocytogenes* under simulated gastrointestinal conditions. The production of organic acids by the probiotics inhibited the growth of both *Salmonella* and *Listeria*, however, bacteriocin production is likely to have further contributed to growth inhibition of *Listeria*. Results also suggest that peanut butter is a plausible carrier for probiotics and could be used in the management of ailments caused by foodborne pathogens.

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**Table 6.1.**

Average cell populations of *Salmonella enterica* and *Listeria monocytogenes* during simulated gastrointestinal passage

	Pathogen population (log CFU)	
	<i>Listeria monocytogenes</i>	<i>Salmonella enterica</i>
<b>Without probiotics</b>	4.95aA	5.05aA
<b>With cultured probiotics</b>	3.33bA	3.33bA
<b>With lyophilized probiotics</b>	4.09abA	3.42bA

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of treatment group

Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of pathogen type

**Table 6.2.**  
Changes in populations of *Salmonella* during a 24 h simulated gastrointestinal passage with probiotics

Time (h)	<i>Salmonella</i> population (log CFU)		
	Without probiotics	With cultured probiotics	With lyophilized probiotics
0	5.67cA	5.67abA	5.67aA
2	0.89eA	1.24cdA	1.24bcA
4	0.56eA	0.67dA	0.44cA
6	3.07dA	1.64cdA	1.40bcA
9	5.53cA	4.12abA	3.84abA
12	8.87bA	6.51aA	6.16aA
24	10.79aA	3.46bcB	5.19aB

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of study time  
 Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of treatment group

**Table 6.3.**  
Changes in populations of *Listeria monocytogenes* during a 24 h simulated gastrointestinal passage with probiotics

Time (h)	<i>Listeria monocytogenes</i> population (log CFU)		
	Without probiotics	With cultured probiotics	With lyophilized probiotics
0	5.57bA	5.57aA	5.57aA
2	4.41bcA	4.28abA	4.45aA
4	3.62cAB	2.97abB	3.78aA
6	3.38cA	3.56bA	3.20aA
9	3.60cA	2.48bA	3.61aA
12	3.39cA	3.02abA	3.92aA
24	10.67aA	2.44bB	4.10aB

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of study time  
Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of treatment group

**Table 6.4.**  
Organic acid constituent of broths after incubation

	Cell culture supernatant		
	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Streptococcus</i>
<b>Organic Acid (ppm)</b>			
Lactic Acid	19200aA	17900aB	5010aC
Acetic Acid	3520bA	2640bB	2190bC

Means in the same column not followed by the same lowercase letters are significantly different ( $P < 0.05$ ) in terms of type of organic acid

Means in the same row not followed by the same uppercase letters are significantly different ( $P < 0.05$ ) in terms of species of probiotic bacteria

**Table 6.5.**

Inhibition of *Salmonella* by the supernatants of probiotic cell cultures with natural or neutralized pH and fresh broths with their pH lowered by different acids

Treatment	Zone of inhibition (mm)					
	<i>Salmonella</i>			Probiotics		
	4-1	4-2	4-56	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Streptococcus/ Lactococcus</i>
Probiotic culture supernatant with natural pH	9.17a	8.83a	8.50a	13.17a	13.33a	0.00b
Probiotic culture supernatant with pH neutralized to 6.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Fresh broth with pH lowered by HCl to 3.80, 3.78 or 5.17	3.17b	6.33a	6.50a	8.17a	7.83a	0.00b
Fresh broth with pH lowered by acetic acid to 3.80, 3.78 or 5.17	9.33a	7.67b	7.67b	12.50a	12.17a	0.00b
Fresh broth with pH lowered by lactic acid to 3.80, 3.78 or 5.17	6.17a	6.50a	5.83a	9.33a	9.17a	0.00b

Means in the same row under *Salmonella* (left column) or Probiotics (right column) not followed by the same letters ( $P < 0.05$ ) are significantly different in terms of treatment

The left column (*Salmonella*) are zones of inhibition formed by different treatments on different strains of *Salmonella*

The right column (probiotics) are zones of inhibition formed by different treatments on *Salmonella* by different species of probiotics



**Table 6.6.**

Inhibition of *Listeria monocytogenes* by the supernatants of probiotic cell cultures with natural or neutralized pH and fresh broths with their pH lowered by different acids

	Zone of inhibition (mm)					
	<i>Listeria</i>			Probiotics		
	8733	7764	7969	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Streptococcus/ Lactococcus</i>
Probiotic supernatant with natural pH	14.50b	15.67a	14.50b	22.00a	22.67a	0.00b
Probiotic supernatant with pH neutralized to 6.0	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Fresh broth with pH lowered by HCl to 3.80, 3.78 or 5.17	9.00a	0.00a	0.00a	4.33a	4.67a	0.00b
Fresh broth with pH lowered by acetic acid to 3.80, 3.78 or 5.17	19.33a	19.33a	19.33a	28.00a	30.00a	0.00b
Fresh broth with pH lowered by lactic acid to 3.80, 3.78 or 5.17	10.33a	9.67a	9.00a	13.67a	15.33a	0.00b

Means in the same row under *Listeria* (left column) or Probiotics (right column) not followed by the same letters ( $P < 0.05$ ) are significantly different in terms of treatment

The left column (*Listeria*) are zones of inhibition formed by different treatments on different strains of *Listeria*

The right column (probiotics) are zones of inhibition formed by different treatments on *Listeria* by different species of probiotics

**Table 6.7.**  
Inhibition of *Listeria monocytogenes* by crude bacteriocin extracts

Strain	Zone of inhibition (mm)		
	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Streptococcus/ Lactococcus</i>
8733	10.50a	10.00a	0.00b
7764	11.00a	11.00a	5.50b
7969	6.50a	2.50a	0.00a

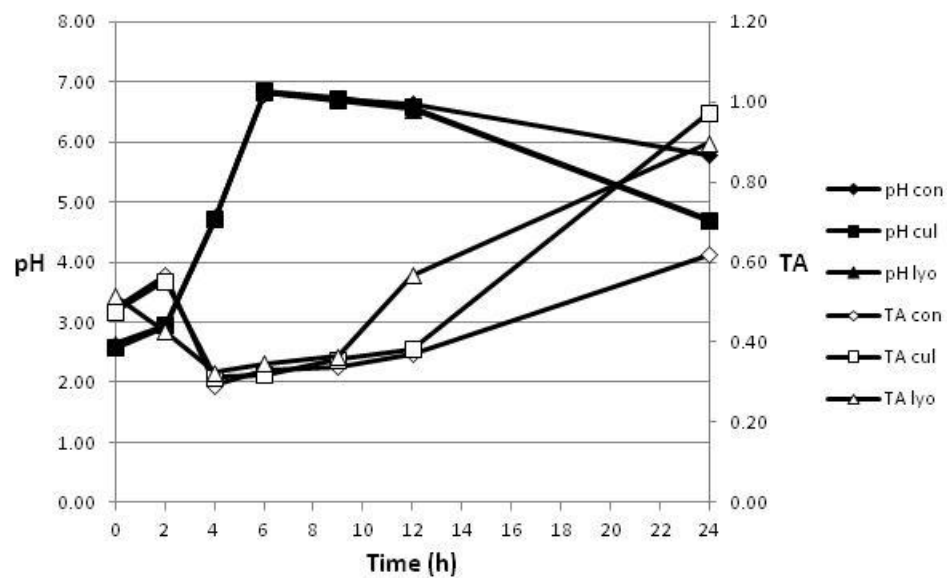
Means in the same row not followed by the same letters ( $P < 0.05$ ) are significantly different in terms of strain

**Fig. legends**

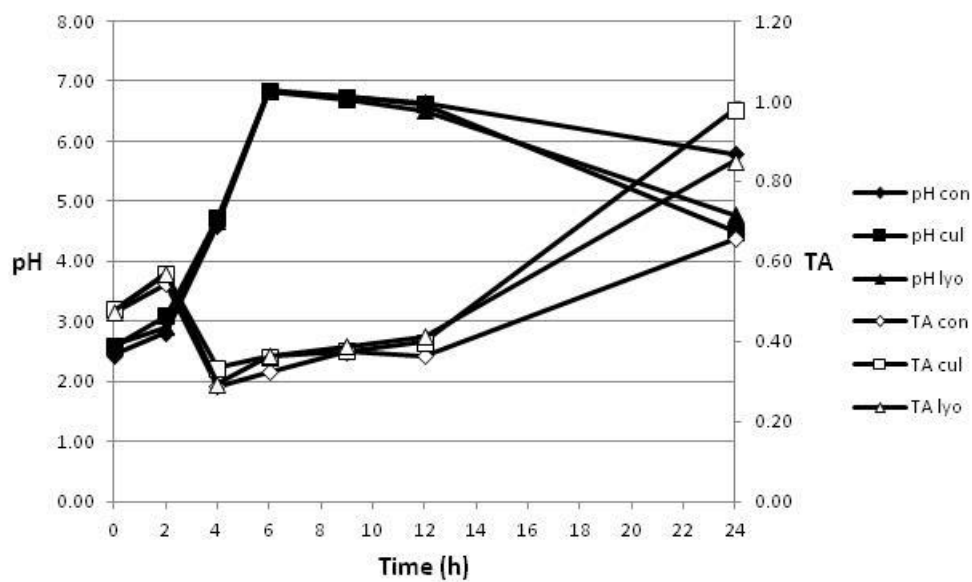
Fig. 6.1.- Changes in pH and titratable acidity (TA; expressed as % acidity) of samples inoculated with *Salmonella enterica* (A) or *Listeria monocytogenes* (B) during a 24 h simulated gastrointestinal passage. Designation “pH con” and “TA con” refer to samples inoculated with *Salmonella* or *Listeria* only. Designation “pH cul” and “TA cul” are samples co-inoculated with *Salmonella* or *Listeria* and cultured probiotics; and “pH lyo” and “TA lyo” are samples co-inoculated with *Salmonella* or *Listeria* and lyophilized probiotics.

Fig. 6.1. Klu et al.

A



B



## CHAPTER 7

### CONCLUSIONS

1. The survival of *L. rhamnosus* GG was influenced by storage temperature and storage time. Generally, the viability of *L. rhamnosus* GG decreased in both product types with increasing storage temperature and storage time. Higher survivability of *L. rhamnosus* GG was observed in reduced fat-peanut butter as compared to full-fat peanut butter only at 37°C. Products stored at 4 °C for 48 wk and at 25 °C for 23 or 27 wk could maintain probiotic counts of at least 10<sup>6</sup> CFU/g. These results suggest that peanut butters could be used to deliver probiotic organisms. However, if one considers the recommendation of 10<sup>9</sup> CFU as the minimum daily intake, a higher level of probiotic bacteria will have to be inoculated into peanut butter at the initial stage of the project. This can be easily accomplished without significant challenges. Results suggest that probiotic peanut butter has the potential to be used as one of the strategies to control diarrhea and malnutrition in developing countries.

2. Storage conditions played a key role in maintaining the viability of probiotic cultures, and cell survival rate decreased with increasing storage temperature and time. Overall, probiotic mixture U has the greatest survival rate followed by B, N and S.

*Bifidobacterium* species had the highest survivability followed by *Lactobacillus* species and then *Streptococcus/Lactococcus*. Peanut butter is a suitable food matrix to deliver probiotics, and the fat content of peanut butter did not significantly influence the survivability of the probiotic mixtures included in the study.

3. Full-fat and reduced-fat peanut butter homogenates offered equal protection to probiotic bacteria from products C, N and U during simulated gastrointestinal passage. Overall, streptococci and lactococci had the highest survival, followed by bifidobacteria and lactobacilli. At the end of 6 h gastrointestinal passage, in both peanut butter homogenates, probiotic bacteria from product N had the highest survival, followed by probiotic bacteria from products U and C. The study suggests that peanut butter matrices are capable of protecting probiotic bacteria under simulated gastrointestinal conditions and are likely vehicles to deliver probiotic to children prone to diarrhea.

4. Selected probiotics in peanut butter are able to inhibit the growth of *S. enterica* and *L. monocytogenes* under simulated gastrointestinal conditions. The production of organic acids by the probiotics inhibited the growth of both *Salmonella* and *Listeria*. However, bacteriocin production is likely to have further contributed to growth inhibition of *Listeria*. Results suggest that peanut butter is a plausible carrier for probiotics and could be used in the management of ailments caused by foodborne pathogens.