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Impact Of Medical Drugs, Commonly Administered To The Elderly, On Viability And Functionality Of Commercially Available Probiotic Supplements

Desarae Nicole Johnson
North Carolina Agricultural and Technical State University

Impact of Medical Drugs, Commonly Administered to the Elderly, on Viability and Functionality of Commercially Available Probiotic Supplements

Desarae Nicole Johnson

North Carolina A&T State University

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Desarae Nicole Johnson

has met the thesis requirements of
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Approved by:

Dr. Salam A. Ibrahim Major Professor	Dr. Mulumebet Worku Committee Member
Dr. Meeshay Williams-Wheeler Committee Member	Dr. Valerie Giddings Department Chair

Dr. Sanjiv Sarin
Dean, The Graduate School

Biographical Sketch

Desarae Nicole Johnson is a Food Microbiology and Biotechnology graduate research assistant. She is currently studying Food and Nutritional Sciences under the advisement of Dr. Salam A. Ibrahim. Desarae is the oldest of three daughters born to Alan and Deborah Johnson. She received a Bachelor of Arts and Science (BAS) degree in Biology and Humanities from Jacksonville University; where she was granted a full Division 1 athletic scholarship to play basketball. In the spring of 2010, Desarae was initiated into the Rho Omega Chapter of Delta Sigma Theta Sorority Incorporated on the campus of Jacksonville University. This community service driven organization is charged with an uncompromising commitment to communities through service, leadership, and empowerment. Following graduation Desarae played basketball professionally overseas in the European country of Malta. She was honored with the March 2012 Player of the Month award.

Desarae returned to academia in the spring of 2013, entering North Carolina A&T State University‘s Masters of Science, Food and Nutritional Sciences program. After maintaining a 4.0 GPA for over 3 semesters, she was initiated into Gamma Sigma Delta (Honorary Society of Agriculture) and received membership into Golden key international honors society. During the 2013- 2014 academic year Desarae joined the Food and Nutritional Sciences Club and held an officer position (Food Science liaison); as a member of IFT. In 2014-2015 she served as the president of Food and Nutritional Sciences Club. Recently, Miss Johnson was initiated into the Kappa Omicron Mu Chapter of Kappa Omicron Nu, the leading honor society for students in the human sciences.

While completing her studies Desarae also published a review article in an industry journal, and is currently working on two additional manuscripts to submit for publication. She

has also presented posters for the American Association of Family & Consumer Sciences (NCAAFCS) and NC A & T State University Board of Visitors.

During the summer of 2014 Desarae excelled in her position as an Abbott Quality and Regulatory intern in Chicago, Illinois with Abbott. This internship led to an opportunity to continue working with Abbott as an Abbott Campus Ambassador representing NCAT. Upon graduation Desarae will begin the Abbott Professional Development Program. This two year leadership development program will allow her the opportunity to experience several divisions and positions within the company including managerial positions.

Desarae enjoys extracurricular activities which include active participation in her sorority Delta Sigma Theta Sorority Inc. and club sports at A&T. During the 2014-2015 academic year she became a mentor and player for the club flag football team (Elite) and basketball team (Legacy).

Dedication

I dedicate this thesis to my mother Deborah M. Johnson. Your unparalleled strength and unwavering faith in God during trials and tribulations has been the example that empowered me to become the woman I am today. I am forever thankful.

To my sisters, Chelsey and Baylor, and father, Alan Johnson, whose words of encouragement and comedic relief were invaluable I have a special feeling of gratitude.

I also dedicate this work and give special thanks to DeUnna R. Hendrix whose constant support and motivation continuously pushes me to reach new heights.

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My sincere appreciation goes to my committee members Dr. Mulumebet Worku and Dr. Meeshay Williams-Wheeler for their support and contributions to my thesis work. I would also like to thank our Department Chair, Dr. Valerie Giddings, who played an integral role in my decision to continue pursuing the thesis track and a Ph.D. in the future.

I would like to thank all of the faculty members in the Food and Nutritional Sciences program who have all had a major impact on me and have provided exceptional guidance during my degree. Finally, I would like to thank my colleagues Saeed, Rabin, Temitayo, Nadia, Samorya, and Tarik, Alani, and Ugo for their help and support.

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Abstract

The elderly population is inherently more susceptible to gastrointestinal problems and diseases due to significant age-related gastrointestinal changes experienced in gut physiology, reactivity of the immune system, and/or diet. These factors, coupled with increased occurrence of disease and corresponding medication use, could also modify the composition of gut microbiota. The impact of non-steroidal anti-inflammatory drugs (NSAIDs) on the physical gastrointestinal tract has already been shown. However, the impact of these factors on the microbiota inhabiting the GI tract has not been well established. A better understanding of the relationship between medication use and gut microbiota composition may have beneficial implications for general and elderly health. Increasing knowledge on the health benefits of probiotics among consumers has dramatically surged the use of probiotics in the recent decades. The objective of this study was to determine the impact of commonly administered medical drugs and commonly consumed caffeine on the viability and functionality of commercially available probiotic supplements. Ten probiotic supplements containing various strains of bifidobacteria (*B. longum*, *B. bifidum*, *B. lactis*, *B. breve, B. infantis*) and lactobacilli (*L. acidophilus*, *L. rhamnosus*, *L. reuteri, L. gasseri, L. plantarum, L. casei, L. brevis, L. salivarius, L. paracasei*) were individually grown in laboratory medium de Man, Rogosa and Sharpe (MRS) broth at 37°C for 24 h. Batches of 10 mL MRS broth were mixed with 100 µL of aspirin stock, inoculated with 1 mL overnight grown probiotic culture, and then incubated at 37ºC for 2h. Bacterial populations were determined at 0 and 2h of incubation. In addition, bile resistance, β-galactosidase activity, reducing power, and protein expression were examined. Our results showed that six out of the ten commercial probiotic supplements contained bacterial populations as claimed on their respective labels. The β- galactosidase activity of each supplement was determined. The enzyme activity ranged from 1

to 1,120 Miller units. A single supplement was selected for continued testing in objective 2. Exposure to one tablet of aspirin was found to decrease bacterial population approximately 6.75 log CFU/ml, and exposure to caffeine (0.5% w/v) decreased population approximately 0.23 log CFU/ml. Determination of β- galactosidase activity resulted in reductions in enzyme activity post aspirin exposure and caffeine enhanced enzyme activity.

CHAPTER 1

Introduction

As the aging process proceeds, the elderly naturally encounter a decrease in the number of beneficial bacterial species present in the microbiota. The stability of gut microbiota in the elderly is affected by physiological changes in the gastrointestinal tract stimulated by increasing age. Combined with factors such as changes in lifestyle and diet, diminished performance of the immune system could negatively impact the presence of microbes (Biagi et al., 2010). Because natural declines in physiological function commonly occur with age, this decline may indirectly alter the composition of gut microflora due to the influence of absorption and/or metabolism of nutrients. Decreased muscle bulk, coupled with tooth loss, causes mastication, and, in some cases, swallowing difficulties (Karlsson, Persson, & Carlsson, 1991; Newton, Yemm, Abel, & Menhinick, 1993). Diminished sensory sharpness leads to increased blandness in food flavor (Weiffenbach, Baum, & Burghauser, 1982). The combination of these factors can result in nutritionally imbalanced diets in the elderly population.

The reduction of beneficial bacteria in the gut microbiota of the elderly is often exacerbated by an increase in the consumption of medications consumed as a result of an increase in the occurrence of illnesses commonly associated with aging. Common ailments suffered by the elderly are often treated with a range of medications, from non-steroidal anti-inflammatory drugs (NSAID), to acetaminophen (Tylenol). Individuals consuming probiotic products sparingly and inconsistently are not equip to withstand the potential decline in bacterial population. Therefore, medication use could alter their microbiota composition, and cause potential gastrointestinal problems and diseases.

In recent years, considerable effort has been made to investigate the positive impact of probiotics on health. Probiotics are live microorganisms administered in adequate amounts that confer a beneficial health effect on the host. Traditionally probiotics have been consumed via dairy products such as milk, cheese, and yogurt. The most commonly used probiotics in foods are *Lactobacillus* and *Bifidobacterium*. For centuries, lactic acid bacteria (LAB) have been utilized in food fermentation, and the functions of these food fermenting agents have expanded to also include potential as conferrers of health benefits (Song, Ibrahim, & Hayek, 2012). Lactic acid bacteria are nonpathogenic, and are generally recognized as safe (GRAS). Recent studies have demonstrated that the effects of probiotics can potentially be further improved (Gyawali & Ibrahim, 2012). Plants rich in micronutrients such as manganese and zinc have been shown to enhance the ability of probiotics to produce organic acids. These organic acids offer potential as a natural antimicrobial, thus improving the safety of foods and supporting human health (S. Ibrahim, Dharmavaram, Seo, & Shahbazi, 2003; Nakashima, 1997). Enzymes also enhance the production of organic acids such as lactic and acetic acids, which act as antimicrobial agents and suppressors of pathogenic bacteria growth (S. Ibrahim et al., 2003; S. A. Ibrahim & Salameh, 2001; Nakashima, 1997).

The promotion of health via probiotic supplements, however, has not been well established. A broader selection of probiotic supplements to choose from benefits consumers and has the potential to increase the likelihood of purchase. Currently probiotics are available as dietary supplements in the form of capsules, tablets, powders, liquids, and chews. The elderly population benefit from the convenience of multiple probiotic supplements as well because the assortment of forms allows them to select a product that caters to their mastication or swallowing

abilities. Elderly with weak swallowing abilities are now able to choose a liquid or powder form of a probiotic supplement for increased ease of administration.

Despite the negative impact medical drugs have on the microbiota, these medications are necessary in order to mitigate pain and other health problems experienced by the elderly. This has prompted scientists to look for alternative means to combat these negative effects and enhance the beneficial bacteria of the microbiota. In recent years, considerable effort has been made to identify dietary therapeutics for this purpose. The most practical application for elderly individuals to combat the negative impact of medications is to maintain a well-balanced daily diet that also includes probiotics each day. The first objective of this study was to examine the viability of probiotics in commercial dietary supplements. The second objective was to determine the impact of aspirin and caffeine on the functionality of a probiotic supplement. In this project, functionality was determined by performing β- galactosidase activity, bile resistance, reducing power, and protein expression.

CHAPTER 2

Literature Review

2.1 Aging

2.1.1 The Current Situation

In recent years there has been a dramatic rise in the elderly population as a result of an increased life expectancy that can be contributed to innovations in science and medicine (E. Woodmansey, 2007). However, most elderly individuals are not necessarily experiencing longevity, which is the potential to live a long life in a healthy state (Saunier & Doré, 2002). The elderly population is inherently more susceptible to gastrointestinal problems and diseases due to significant age-related gastrointestinal changes experienced in gut physiology, reactivity of the immune system, and/or diet. Studies have demonstrated the need for an emphasis on gut microbiota homeostasis as a means of experiencing longevity, given the importance of the microbiota in elderly health. Factors commonly associated with aging often lead to compromised microbial balance of the elderly microbiota. When dietary habits cannot support this balance, alternative methods, such as the consumption of probiotic products is an option for promoting gut microflora homeostasis.

Aging is a significant topic of concern throughout the world. By definition, aging is the _regression of physiological function accompanied by advancement of age‘ (Imahori, 1992). By 2040, the US Census Bureau estimates that the elderly population will double from 7 percent to 14 percent of the total world population; for the first time in history, within the next 10 years, there will be more people in the world aged 65 and over than children under 5 (Kinsella, 2009). In the state of North Carolina alone, the number of people age 65 and up has increased 25.7 percent between 2000 and 2010; 12.9 percent of the state‘s total population. According to the

UNC Institute on Aging, between 2010 and 2030 North Carolina‘s elderly population will increase by 400,000 people per decade, reaching 2.14 million or about 18 percent of the state‘s total population by 2030 (Aging, 2011). In 2010the U.S. Bureau of the Census calculated individuals‘ 65 years and up as a percentage of total population (Fig. 2.1) in the United States. Increases in the elderly population correlates with increases in financial burdens caused by healthcare needs, treatment costs, and end of life expenses.

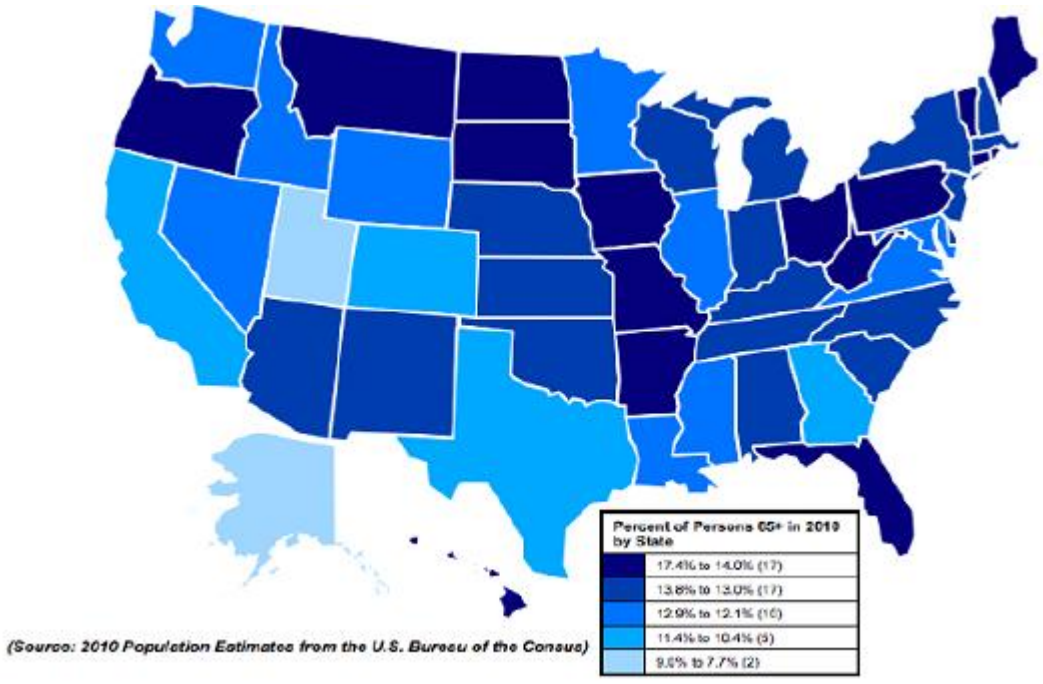

Figure 1 The percentage of elderly individuals in 2010 by state.

Bacteria colonize all of the physically available space along the gastrointestinal tract, with varying distribution. These bacteria have invaluable functions in the human body. ―Human beings have been recently reviewed as _metaorganisms‘ as a result of a close symbiotic relationship with the intestinal microbiota. This assumption imposes a more holistic view of the aging process where dynamics of the interaction between environment, intestinal microbiota and host must be taken into consideration.” (Biagi et al., 2010) There are several genetic and metabolic attributes provided by gut bacteria that humans remain unable to evolve independently (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The relationship between human host and the composition of its gut microflora is primarily mutually beneficial. The metabolic activity of the gut microbiota provides the human host metabolic energy and absorbable substrates and nutrients, while the human host provides the microbiota a source of energy and nutritious products for growth and development.

An essential feature of a healthy gut microbiota is the production of short chain fatty acids (SCFA) such as acetate, butyrate, and propionate(Barcenilla et al., 2000; Louis & Flint, 2009). SCFA have a nutritious effect on the intestinal epithelium. Each of the three major short-chain fatty acids stimulate epithelial cell proliferation and differentiation in the large and small intestine in vivo (Butler, Sun, Weber, Navarro, & Francis, 2000). The microbiota also ferments carbohydrates, which provides energy to the colon; serving 50% of the daily energy requirements, for colonic epithelial cells (Tuohy, Probert, Smejkal, & Gibson, 2003).This energy production is accomplished by fermenting butyrate, a carbohydrate, into organic acids (Tuohy et al., 2003). Butyrate is active in the physical separation of the microbiota and enterocytes; as a result of this separation the release of mucins, the protein component of the mucus layer that forms gel, is stimulated (Barcelo et al., 2000; Petersson et al., 2011).

Additional general health benefits conferred to the host by the gut microflora include immunostimulation, synthesis of vitamins, improved digestion and absorption, cholesterol reduction, lowered gas distension, and inhibition of potentially pathogenic bacterial growth (Wallace et al., 2011). Health benefits conferred to the host are contingent upon maintaining a homeostatic state amongst the network of microflora.

Common declines in health, specifically gut microbiota health, occur with aging. "Age-related physiological changes in the gastrointestinal tract, as well as modification in lifestyle, nutritional behavior, and functionality of the host immune system, inevitably affect the gut microbial ecosystem." (Biagi et al., 2010) Increased occurrence of disease and corresponding medication use in the elderly also modify the composition of the gut microbiota (Tiihonen, Ouwehand, & Rautonen, 2010). Members of the elderly community encounter unfavorable changes in microbiota, which can lead to health problems.

The microorganisms of the gastrointestinal tract are not indestructible, and the positive attributes provided by the bacteria can be overcome by pathogens. Some pathogens have evolved specifically for gastrointestinal infection, examples include *Salmonella* spp., *Escherichia coli* strains and *Campylobacter jejuni* (Tuohy et al., 2003). The balance can be delicate, and many age related physiological issues potentially lead to harmful effects caused by the gut microflora; examples include intestinal putrefaction, carcinogen production, liver damage, production of toxins, diarrhea and/or constipation, and intestinal infections (Wallace et al., 2011).

2.1.2 Development of gut microflora

The human intestinal tract is home to over 14 log microorganisms; 10 times greater than the total number of somatic and germ cells found in the human body (Bäckhed et al., 2005). "Our gut microbiota can be pictured as a microbial organ placed within a host organ: It is

composed of different cell lineages with a capacity to communicate with one another as well as the host; it consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations; and it can maintain and repair itself through self-replication." (Bäckhed et al., 2005)

At birth the gastrointestinal tract is a sterile environment (Wallace et al., 2011). This assemblage of bacteria begins colonizing immediately after birth. Factors such as vaginal versus caesarean birth, as well as breast versus formula feeding are among the first to impact gut flora(Guarner & Malagelada, 2003). The only significant changes in the gut microbiota occur during infancy, after which a relatively stable environment is maintained throughout childhood and adult life (Favier, Vaughan, De Vos, & Akkermans, 2002; Zoetendal, Akkermans, & De Vos, 1998). The initial colonization heavily influences the final composition of the permanent flora in adults (Guarner & Malagelada, 2003).

Healthy individuals typically have an intestinal habitat of 300 to 500 different species of bacteria (Guarner & Malagelada, 2003). Bacteria colonize all of the physically available space along the gastrointestinal tract, with varying distribution. Each species occupies a niche in a particular habitat. The gastrointestinal tract is deceptively long. It has been calculated to be 150-200 m^2 (Waldeck, 1990). Surface area of the gastrointestinal tract, specifically the small intestine is increased via folding, e.g.: three-fold by forming circular folds, 7-10 fold by folding of the epithelium (intestinal villi) and 15-40 fold by the formation of microvilli in the enterocyte resorptive luminal membrane.' (Holzapfel, Haberer, Snel, & Schillinger, 1998). The gut microflora increases in quantity and diversity as one moves along the gastrointestinal tract. In the stomach, the bacterial count is about 10^1cells/gram, and includes *Lactobacillus*, *Veillonella*, and Helicobacter. The duodenum, jejunum and ileum of the small intestine possess bacterial counts

of 10^3, 10^4, 10^7cells/gram respectively which include Bacilli, Streptococcaceae, Actinobacteria, Actinomycinaeae, and Corynebacteriaceae. Lastly, the colon houses a bacterial count of 10^{12}cells/gram consisting of Lachnospiraceae and Bacteroidetes.

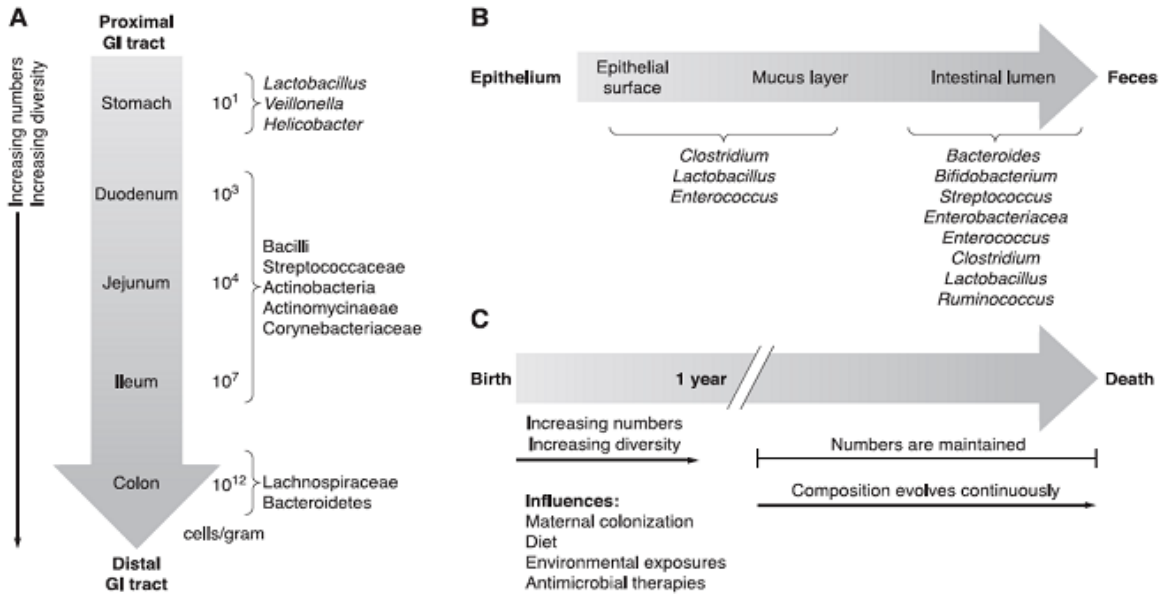

Figure 2 Gut microbiota. a) The numerical breakdown of gut microorganisms along the GI tract. b) The bacterial composition of the intestinal wall. c) The evolution of bacteria during aging.

Current sample collection methods used to analyze diversity of the microbiota are limited by the difficulty of access. Fecal samples are commonly used due to the convenience of collection. However, bacteria from these samples do not fully represent the total population of the gut microbiota. Investigators have begun utilizing methods such as molecular finger printing and 16S ribosomal DNA (rDNA) to explore this ecosystem. Various studies have shed light upon the composition as it has been accessed. The gut microbiota is predominately strict anaerobes,

but facultative anaerobes and aerobes are also present. To date, over 50 bacterial phyla have been identified in the human gut, but two remain in the forefront of importance Bacteroides and Firmicutes; occurring in lower magnitude are Proteobacteria, Verrucomicobia, Actinobacteria, Fusobacteria, and Cyanobacteria (Eckburg et al., 2005; Schloss & Handelsman, 2004).

Each phyla, species, and strain of bacteria has an individual role that often time only they specifically are able to perform. Certain bacteria are able to metabolize a wide variety of substrates, while others have more specialized abilities.

About 30% of the gut microflora is composed of *Bacteroides* of the phylum Bacteroidetes (Salyers, 1984). Notable species to mention include *B. thetaiotaomicron*, *B.vulgatus*, *B. distasonis* and *B. fragilis* (Salyers, 1984). The Bacteroides species are recognized as starch degraders. Additional strains have been found to degrade certain types of structural polysaccharides as well. The high population of *Bacteroides* in the gut is believed to be caused by the import of oligosaccharides for continued hydrolysis.

2.1.3 Changes in the composition of gut microflora

The adult gut microbiota is primarily composed of non-sporing anaerobes, the most prominent being *Bacteroides* spp., *Eubacterium* spp., *Clostridium* spp., *Lactobacillus* spp., *Fusobacterium* spp., as well as a variety of other gram-positive cocci (Wallace et al., 2011). Present in fewer numbers are Enterobacteriaceae, methanogens and dissimilatorysulphate-reducing bacteria (Wallace et al., 2011). The anaerobic environment, characteristic of the gastrointestinal tract, is produced by the metabolism of oxygen by the microbiota (Wallace et al., 2011). The composition of the gut is individualized, and varies based on one‘s history and encounters with conditions such as acute diarrheal illness, diet, and antibiotic treatment (Guarner & Malagelada, 2003).

The organization of the symbionts remain relatively stable during adult life, however age-related changes, such as changes in diet, environment and host immune system reactivity, influence the microbiota population composition (Hooper & Gordon, 2001; E. Woodmansey, 2007). The changes that occur in microfloral bacterial populations can be seen in Figure 3. ~~–~~Recent studies indicate shifts in the composition of the intestinal microbiota, potentially leading to detrimental effects for the elderly host. Increased numbers of facultative anaerobes, in conjunction with a decrease in beneficial organisms such as the anaerobic lactobacilli and Bifidobacteria, amongst other anaerobes, have been reported." (E. Woodmansey, 2007). Additional studies have also concluded that with increasing age the number of viable *Bacteroides* decreases (Hopkins & Macfarlane, 2002; E. J. Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004). This is notable due to the nutritional versatility of the *Bacteroides* species. *Bacteroides* are capable of using a broad assortment of carbon sources and are believed to conduct the majority of the polysaccharide digestion in the colon (Salyers, 1984).

The normal microbial balance of the elderly gut is very delicate therefore malfunctions in gastric function, or intestinal motility have a major impact on health. ~~–~~Changes at species level of such a nutritionally important sub-population could have considerable consequences for the elderly host, because of alteration in metabolic activities, and for other bacteria in the ecosystem that rely on a complex cross-feeding network within the gut." (Gibson, Cummings, & Macfarlane, 1988) Reports of the changing microbiota of the elderly include increased numbers of lactobacilli, clostridia, and facultative anaerobes (T Mitsuoka, Hayakawa, & Kimura, 1974; Tomotari Mitsuoka, 1990). Decreases in bacteria such as *F. prausnitzii*, *E. allii* and other bacteria belonging to the *E. rectal*/ *Roseburia* group have been correlated with functional declines in the elderly microbiota production of butyrate (Biagi et al., 2010). These butyrate

producing bacteria also have anti-inflammatory properties, and population declines of these bacteria might support the inflamm-aging process in the gastrointestinal tract of elderly people. Loss in butyrate production may also play a role in the development of degenerative diseases and anorexia (Donini, Savina, & Cannella, 2010; Guigoz, Doré, & Schiffrin, 2008).

Essentially, the stability of the gut microbiota of the elderly is affected by the physiological changes of the gastrointestinal tract stimulated by increasing age; coupled with factors such as changes of lifestyle and diet, and diminished performance of the immune system (Biagi et al., 2010).

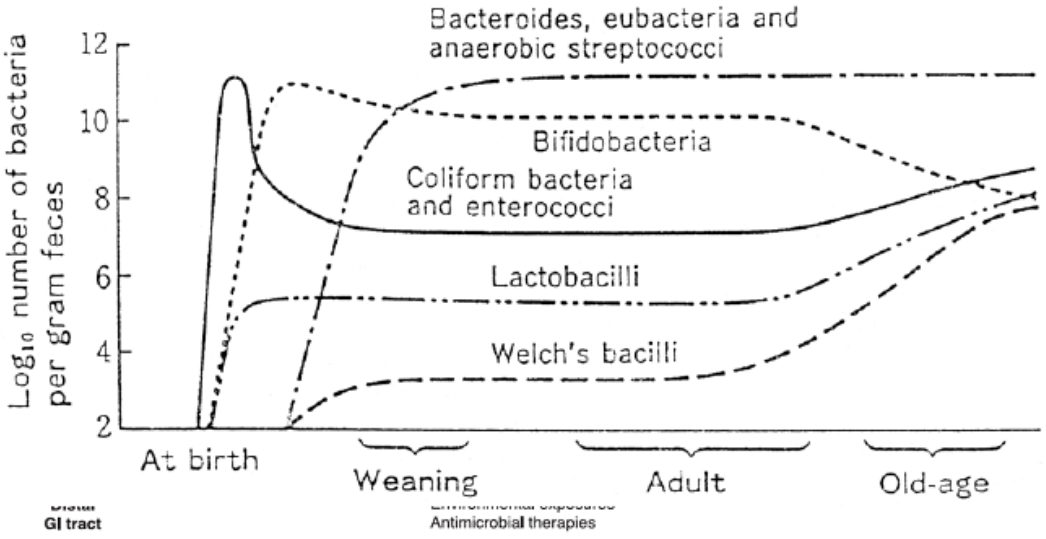

Figure 3 Changes of intestinal microflora with age. *(Tomotari Mitsuoka, 1978)*

2.1.4 Physiological changes of the gut with age

Natural declines in physiological function commonly occur with age. This decline may indirectly alter the composition of the gut microflora due to the influence of absorption and/or metabolism of nutrients. Decreased muscle bulk coupled with tooth loss causes mastication, and in some cases, swallowing difficulties (Karlsson et al., 1991; Newton et al., 1993). Diminished

sensory sharpness leads to foods tasting bland (Weiffenbach et al., 1982). The combination of which can bring about the nutritionally imbalanced diet of the elderly population.

A major physiological change, reported by the elderly, in the gastrointestinal tract is the reduction in fecal weight (E. J. Woodmansey et al., 2004). "Low fecal weights have been correlated to slow intestinal transit times and reduced excretion of bacterial matter ." (Stephen, Wiggins, & Cummings, 1987) Furthermore, increased retention time is associated with an increase in bacterial protein fermentation and consequently, the levels of ammonia and phenols generated in putrefactive processes in the gut." (Macfarlane, Cummings, Macfarlane, & Gibson, 1989)As a consequence of the decreased intestinal motility constipation occurs (Kleessen, Sykura, Zunft, & Blaut, 1997). The inability to effectively excrete proper levels of harmful bacteria unfavorably alters the fermentative processes of the gut (Brocklehurst, 1972; Macfarlane et al., 1989). Thus affecting the intestinal ecosystem homeostasis (Biagi et al., 2010).

Barrier function is another component of the gut potentially compromised with increasing age. Evidence of barrier importance has been demonstrated via germ-free animals, whose susceptibility to infections was lessened by the protection of the intestinal barrier (Wallace et al., 2011). The function of the barrier is carried out by a network of the physical components of the intestine; villi height, crypt depth, and thickness of mucus. The size and surface area of villi determine the amount of nutrients able to be absorbed (Yang et al., 2009). Therefore, larger surface areas, allow larger amounts of nutrients to be absorbed. Similarly, increases in crypt depth are consistent with digestive efficacy (Guan, 2000). The role of crypts in the intestines is believed to be associated with facilitating digestion and absorption. Increased crypt depth means increased crypt cell number, thus increasing digestive efficacy (Guan, 2000).

2.1.5 Effect of gut microflora on the immune function

The three components of the GI ecosystem, the microbiota, the intestinal epithelium, and the mucosal immune system, are all vital to the functional and developmental maturity of the system (Tiwari, Tiwari, Pandey, & Pandey, 2012). The convergence of the microbiota and immune system begins at infancy, and aids in the development of the immune system. This partnership lasts throughout an individual‘s lifetime. This network protects against potentially harmful antigens and microorganisms the human body may encounter from external exposure. The gut microbiota and gut mucosal surface coexist in close proximity to one another. The single cell epithelial layer must differentiate this high microbial presence from a persistent threat of microbial invasion. Humans have developed a specific gastrointestinal immune system in order to process this risk; to limit tissue invasion by intestinal microorganisms and to preserve the symbiotic nature of this interaction (Hooper & Macpherson, 2010). The intestinal microbiota is monitored by the lymphoid tissue, which maintains control by a _constitutive low-grade physiological inflammation‘. This low grade inflammation is based on a network of positive and negative biological feedback processes. The organization of the gastrointestinal mucosal immune system facilitates the distinction between harmful pathogens and symbiotic microorganisms. Thus, generating a strong effector response towards the former and remaining tolerant to the latter. (Biagi, Candela, Fairweather-Tait, Franceschi, & Brigidi, 2012).

Live bacteria manage the function of the immune system at systemic and mucosal levels. The management role of the microbiota entails guiding intestinal immune cells into their proper arrangement. Intestinal immune cells mediate tolerance-inducing responses and are active in host defense. The cells congregate at inductive sites such as Peyer‘s patches found in the small intestine, and lymphoid follicles, and colonic patches found in the large intestine; as well as

effector sites which include the epithelium of the intestine (Garrett, Gordon, & Glimcher, 2010). The gut epithelial is constantly communicating with the immune cells via intracellular signaling pathways (Collier-Hyams & Neish, 2005; Rumbo et al., 2004). Innate immune responses are activated by intestinal epithelial cells, which release chemokines and cytokines. These signaling proteins control dendritic cell and macrophage responses (Collier-Hyams & Neish, 2005). The coordination communication and placement of all the components within the network of the immune system contribute to the proper development of immune cell responses. These responses will be able to appropriately remove infectious agents and damaged tissues from the body, and maintain the state of homeostasis within the gastrointestinal tract.

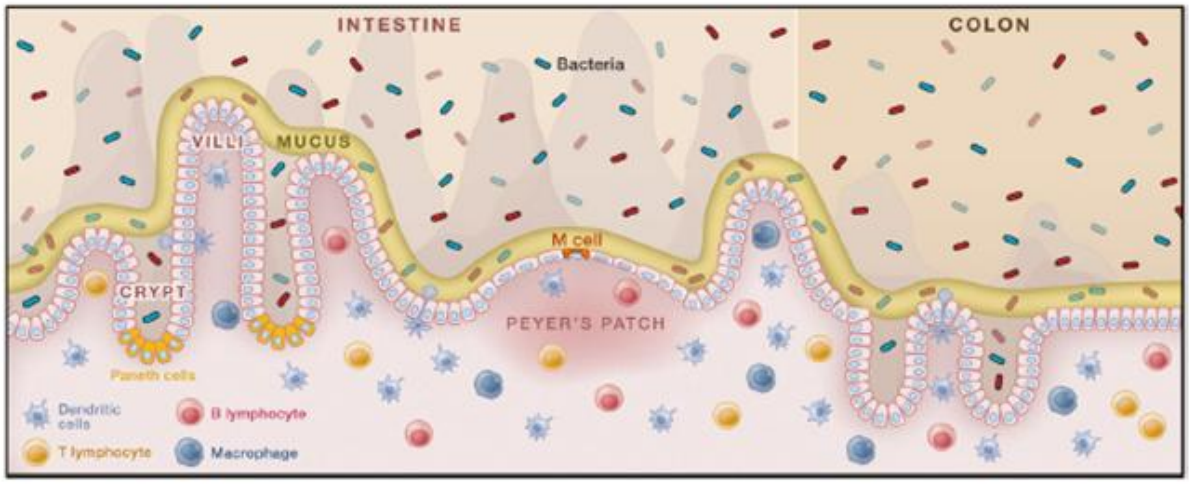

Figure 4 A detailed view of the intestinal and colon walls.

The barrier formed by the intestinal epithelium and the gut microbiota is designed to prevent the invasion or uptake of potentially pathogenic microorganisms, antigens, or other harmful compounds that enter the gut lumen (Holzapfel et al., 1998). Not only are the bacteria of the microflora efficient as a physical barrier, but are also efficient at digesting antigens. Specialized transport mechanisms in the villus epithelium and Peyer‘s patches are used to trigger specific immune responses to target antigens. This barrier remains stable in healthy individuals, and the human host is provided with protection as well as normal intestinal and immune function.

Attachment of *Lactobacillus* and *Bifidobacterium*, specifically, onto the natural mucosal barrier prevents the attachment of potentially pathogenic microorganisms (Garcia-Lafuente, Antolin, Guarner, Crespo, & Malagelada, 2001). The gut microflora not only provides protection physically, using competitive exclusion by occupying attachment sites, but also by consuming the nutrients that pathogens would potentially use. This assemblage of microorganisms also produces antimicrobial compounds (Sekirov et al., 2010).

2.1.5.1 Functional reduction of the immune system with advancing age

Due to the term *elderly* being used in reference to a population with varying health status‘ it becomes difficult to accurately define an age at which the gastrointestinal environment begins to experience age-related decline (Biagi et al., 2012).

Immunosenscence is the process in which the functionality of the gastrointestinal immune system declines (Ostan et al., 2008; Shanley, Aw, Manley, & Palmer, 2009). Inflammageing is another health decline experienced by elderly individuals characterized by a chronic low-grad inflammatory status (Franceschi, 2007; Franceschi et al., 2007; Larbi et al., 2008). The inflammageing process occurs at a localized area of the intestinal mucosa.

Continuous inflammation is believed to contribute to the systemic inflammation, which may affect or be affected by the gut microbiota. It is believed that the inflammatory process could be caused and/or cultivated by an immune response activated unusually by the gut microbiota; this could be due to decreased mucosal tolerance or age related microbiota composition changes, or possibly both (Guigoz et al., 2008). Moreover, deficiencies in nutrition, age-related tissue weakness, and injuries may also contribute to the onset of a pathogenic inflammatory response to the presence of normally harmless symbiotic bacteria (Schiffrin, Morley, Donnet-Hughes, & Guigoz, 2010).

2.1.6 Common factors of aging that can possibly affect gut microflora

A variety of factors, or in some cases a combination of factors, onset by old age are believed to contribute to alterations in gut microflora composition as well as increased incident of illness/disease experienced by the elderly. Deterioration in dentition, salivary function, and digestion; changes in living arrangement, and/or socioeconomic status; decreased mobility and prevalence of disease may all affect the intestinal microbiota upon aging.

Deterioration in dentition is often caused by the lost or reduction in the strength of the muscles necessary for mastication as well as tooth lose (Karlsson et al., 1991; Newton et al., 1993). When older people experience weakness in chewing and swallowing their food it leads to a high likelihood of diet modification to conform to their mastication abilities. However, for muscle function the diet must include adequate amounts of protein, vitamins and minerals.

Studies observing frailty in the elderly found a relationship between diet and physical strength, and correlated dietary magnesium with muscle capacity (Fiatarone et al., 1990). Food sources highest in dietary magnesium include dark leafy greens, nuts and seeds, fish (mackerel), soy beans, whole grains, bananas and dried fruits. It was also found that subjects with the

weakest muscle strength had the lowest levels of circulating vitamin D. Food sources highest in dietary vitamin D include fatty fish such as salmon, tuna, and mackerel; beef liver, eggs, and fortified fruit juices. For elderly persons with preexisting mastication issues many of these food products become difficult to break down. Therefore some of the very food products that would provide the key nutrients needed for maintaining muscle strength are not consumable. As a result of this problem occurring with a variety of hard to eat foods, the diet becomes nutritionally imbalanced. This is important because the microorganisms of the gut use the nutrients consumed for fuel for the host as well as themselves. A nutritionally imbalanced diet leads to both host and microflora lacking vital nutrients. Ervin, 2008 found elderly individuals who had suffered tooth lose and rated their health as fair or poor generally consumed fewer servings of fruits and vegetables, ate a less varied diet and had a poorer quality diet than people with teeth(Ervin, 2008).

Changes in living arrangements and/or socioeconomic status after which elderly persons move to assisted living facilities are another major contributing factor. In 2009 Dean et al. reported that factors including appetite, knowledge of food, perceived distance to grocery stores, access to kitchen amenities or quality food items, and support from family and friends all contribute to the variety in the diet and nutritional balance of many elderly persons. Depending on the family circumstances some older individuals live alone, in day hospitals, or in long term assisted living facilities. Claesson et al. completed a study investigating the microbiota of elderly individuals of diverse living environments. Individuals residing in long term assisted- living facilities were found to have less diverse microbiota than those living independently. Results from this study also indicated that healthy diverse diets promote a more diverse gut microbiota.

Lastly and most commonly, elderly persons typically experience amplified frequency of disease. The difficulty of disease in this population is exacerbated by co-morbidity.

2.1.6.1 Diet of the elderly

The influence of diet on the gut microflora is seen as early as birth. As previously mentioned, the microflora of newborns differs depending on the consumption of breast milk or formula. Breast fed babies develop a simple microflora composed primarily of bifidobacteria. Formula fed babies have large numbers of bifidobacteria in addition to species such as bacteroides and clostridia (Hopkins, Sharp, & Macfarlane, 2002). The impact of one‘s diet continues into adulthood. The proportion of the macronutrients carbohydrates, protein and fat, in the diet directly influences the composition of the microbiota.

Diet is the most controllable of all factors encountered by the elderly. Animal and small scale human studies have shown diet to influence microbiota composition over long periods of time (Hildebrandt et al., 2009; Mai, McCrary, Sinha, & Glei, 2009; Muegge et al., 2011). Incorporating a healthy diet is often the first strategy to preserve health among the elderly (Biagi et al., 2012). The diet provides nutrition and alters the environment for microbes in the gastrointestinal tract (Lee, 2013). A healthy diet can also impact gut transit time and pH. ~~–~~Scientific and clinical evidence available to date indicates that diet is a major driving factor for the establishment of the gut microbiome. Slow digestible carbohydrates (human milk glycan, inulin and fructooligosaccharide), insoluble complex carbohydrates and protein diets favor the growth of *Bacteroides*, *Clostridium*, and *Bifidobacterium.* Fat on the other hand suppresses the number of *Bacteroides*, *Clostridium*, and *Bifidobacterium*; whereas polyphenols in general suppress *Bacteroides*, *Clostridium*, but enhance *Bifidobacterium*.” (Lee, 2013)

Living arrangements must be taken into account when discussing the diet of the elderly. For those living in nursing homes or other assisted living facilities the composition of diet vary from elderly persons living independently at home or in the home of a relative. The healthiest of the elderly population have been found to be those living in a community setting, eating a balanced and variety diet leads to a distinct microbiota from those in long-term residential care (Claesson et al., 2012).

2.1.6.2 Medical Drug Use of the elderly

In 2004 Gurwitz completed a comprehensive national survey of the non-institutionalized adults in the United States and found that more than 90 percent of individuals 65 or older were using at least one medication per week. More than 40 percent use 5 or more different medications per week, and 12 percent use 10 or more different medications per week (NHTSA), 2008). Of all the administered prescription drugs, the elderly receive 30 percent of these medications (Genser, 2008).

2.1.6.2.1 Health benefits of medical drugs

The positive attributes of medical drugs are evident by the extended lives elderly persons with diseases are able to live. These compounds successfully treat or alleviate ailments that at one time were considered fatal. Medication therapies are often used as primary and/or secondary defenses in disease prevention. Primary prevention occurs before the individual acquires a disease. The objective of primary prevention is to keep individuals from contracting the disease. This is accomplished via lifestyle modification and/or reducing exposure to disease causing agents. These primary prevention procedures typically reduce incidence and prevalence of disease. Secondary prevention occurs after disease has arisen, and serves to control disease progression. In these instances the disease is found early and is often treatable if not curable.

Aspirin, or acetylsalicylic acid, has a proven benefit for the secondary prevention of cardiovascular disease. An estimated 50 million people take aspirin daily as a means of treating or preventing cardiovascular disease (Chan & Graham, 2004).

2.2 Medical drugs commonly taken by the elderly

Common ailments suffered by the elderly are often treated with a range of medications, from non-steroidal anti-inflammatory drugs (NSAID), to acetaminophen (Tylenol). Medications are easily accessed over the counter, increasing the likelihood of purchase and self medicating.

Non-steroidal anti-inflammatory drugs or NSAIDs are one of the most widely prescribed medications in the world, and provide analgesic, antipyretic and anti-inflammatory effects. This group of medications can be acquired by prescription or over the counter. The types of NSAIDs available over the counter (OTC) include aspirin such as Bayer ©, Bufferin ©, or Excedrin©; ibuprofen such as Advil© or Motrin IB©; or naproxen such as Aleve. These are also the prominent members NSAIDs.

Many are commonly used to treat musculoskeletal and arthritic diseases, at higher prescription doses, by reducing joint inflammation. At a lower dose NSAIDs also treat a wide array of pain related issues such as headaches, muscle aches, trauma, dental extraction, and surgery. Of the three types of non-steroidal anti-inflammatory drugs, all block prostaglandins. Prostagladins are hormone like substances that triggers pain, inflammation, muscle cramps and fever.

The active ingredient, acetylsalicylic acid, used in the first non-steroidal anti-inflammatory drug, was synthesized by Felix Hoffman in 1897. Aspirin is not only commonly prescribed to elderly patients in its pure form, but it is also found as an ingredient in many other drugs.

2.2.1 Adverse effects of medical drugs on the gut microflora of the elderly

The homeostasis of the intestinal microflora must be maintained in order for good health to prevail. Advancing age is commonly associated with rising prevalence of disease, the corresponding medication use for treatment can alter the composition of the gut microflora (Tiihonen et al., 2010). "Elderly patients are the recipients of more than 30% of all prescription drugs, often given as multiple treatments, so are at higher risk of compromised nutritional status because of drug-nutrient interactions, for example, loss of body electrolytes." (Genser, 2008) The elderly are twice as likely to experience an adverse reaction to medical drugs as their younger counter parts.

There are many reported adverse effects associated with the drugs commonly prescribed to elderly patients. NSAID drugs are linked to gastric and duodenal damage (Bjarnason & Takeuchi, 2009; Laine, 1996; Soll, Weinstein, Kurata, & McCarthy, 1991) and opioids to constipation (Pappagallo, 2001). The more common locations effected by adverse reaction of drugs are the small and large intestine, which represent 20 to 40 percent of drug side effects (Zeino, Sisson, & Bjarnason, 2010). "The high incidence of small and large intestinal side effects of drugs is due to the fact that both organs are controlled by a highly delicate interaction between the autonomic sympathetic and parasympathetic nervous system, the high metabolic activity of cells involved in absorption and secretion and the intestine is exposed to the greatest concentration of microbes and is exposed to high drug concentration. The potential for a drug to adverse affect these pathways is therefore substantial." (Zeino et al., 2010)

Extensive use of NSAID medications is known to affect intestinal health. Damage to the mucosa of the gastrointestinal tract is the most common adverse effect of non-steroidal anti-inflammatory drugs (Bjarnason, Hayllar, MacPherson, & Russell, 1993). Other side effects

include gastric pain, heartburn, nauseas, vomiting, bleeding, perforation, ulceration, dyspepsia, and in severe instances hemorrhage and death (Rampton, 1987). It has been shown that 60% of patients regularly taking NSAIDs have developed intestinal inflammation associated with protein and blood loss; even upon discontinuing use of the medication patients continued to experience intestinal inflammation for up to 16 months (Bjarnason et al., 1987).

One study found that NSAID users have a three time greater risk to develop serious adverse gastrointestinal effects than those that do not use NSAID medication. Researchers also found that additional risk factors for these potential hazards include being age 60 years or above, having a previous history of gastrointestinal problems, or are simultaneously using corticosteroids (Gabriel, Jaakkimainen, & Bombardier, 1991). More than 7000 hospitalizations and 7000 deaths annually in the United States can be attributed to NSAID induced gastrointestinal pathology (Davies & Wallace, 1997).

A very common NSAID drug is aspirin. Endoscopic evidence for the damage aspirin can cause the gastrointestinal tract was found as early as the 1930s by Douthwaite and Lintott. Later in the 1970s investigators proved that aspirin inhibited prostaglandin production, which is involved in inflammation. The standard dosage of aspirin is estimated at 325 mg/day. This dosage has been linked to higher risks of gastrointestinal bleeding, including fatal bleeding, in comparison to the lower dose of 75 mg (Campbell, Smyth, Montalescot, & Steinhubl, 2007). Even when administered at low doses aspirin has been found to cause peptic ulceration, ulcer bleeding, anemia, and the need for transfusion(Roderick, Wilkes, & Meade, 1993; Slattery, Warlow, Shorrock, & Langman, 1995). Individuals are continually directed to take high doses of aspirin, despite studies which have shown that higher doses confer no greater benefit in thrombotic event prevention than the lower doses (Reilly & FitzGerald, 1987).

2.3 Probiotics

In the early 20th century Eli Metchnikoff made the first observation of the positive role microorganisms play in human health. Upon studying rural populations in Europe, such as Bulgaria and the Russian Stepps, Metchnikoff discovered these populations living long lives by means of diets consisting largely of milk fermented by lactic acid bacteria (Ogueke, 2010). Officially named "probiotics" in 1953, these microorganisms were a group of bacterial strains found to positively enhance the function of the digestive tract (Tiwari, 2012). Later in 2002 the Food and Agriculture Organization (FAO) of the United Nations defined probiotics as "Live microorganisms administered in adequate amounts that confer a beneficial health effect on the host."

The term probiotic is not synonymous with the naturally occurring beneficial bacteria of the gastrointestinal tract, although species may be isolated from this source (Douglas & Sanders, 2008). There are guidelines published by the FAO defining probiotics at the strain level (Nations, 2006). Identification of the probiotic strain must properly include the genus and species level in accordance with current scientific practice, and be named in accordance with current nomenclature. With the guidelines in place the term *probiotic* cannot be used as a "catch all" to describe undefined combinations of microorganisms (Surawicz, 2004). Once defined, probiotic strains should be submitted into an international culture collection (Euzéby, 2008). This allows members of the scientific community the opportunity to replicate published studies.

It is important to take note of the difference between a live, active culture and a probiotic. Live, active cultures are commonly found in fermented foods, especially fermented dairy products, but also vegetable products such as sauerkraut. Products containing live active cultures are not tested for their ability to confer health benefits, as is required of probiotics. Live active

culture containing products are only tested for their fermentation properties. Therefore, unless live cultures contribute documented health benefits, they should not be called *probiotic* (Sanders, 2009).

Probiotic bacteria have an array of biological effects, which include, enhanced: antibody production, natural killer cell activity, epithelial barrier function, tight junction protein phosphorylation, and epithelial cell glycosylation; modulation of: host immune response, dendritic cell phenotype and function, and apoptosis; altered cytokine release; induction of: regulatory T cells and PPAR-g; inhibition of: proteasome activity and pathogenic bacterial invasion; and blockade of bacterial adhesion to epithelial cells (Tiwari, 2012). The previously mentioned barrier created by the gastrointestinal tract and the gut-associated immune system are vital components of human health. The gut microbiota is an active element in this mucosal barrier (Kalliomäki, Salminen, & Isolauri, 2008).

2.3.1 Functionality (Viability) of Probiotics

In order for bacteria to be considered for use as a probiotic there are specific functional characteristics it must possess. Probiotic bacteria should be first and foremost nonpathogenic and nontoxic. There must be a beneficial effect exerted on the host as a result of the bacteria. It is essential the bacteria be able to survive the harsh environment of the gut and metabolize there. By remaining alive in large numbers when reaching the intestines, bacteria should be able to maintain the balance of the microflora; promoting the growth of friendly bacteria and inhibiting harmful ones, which is ideal (Tiwari et al., 2012). A good sensory quality is another sought out virtue if the bacteria it is to be incorporated into a food.

In order for the claimed health effects of the probiotic bacteria to be sustained, the microorganisms must remain viable during the processing and storage stages (Song et al., 2012).

This refers to an effective number of viable cells available once the product reaches consumers. Labeling is also important; probiotic labeling must provide truthful and adequate information to consumers (Sanders, Gibson, Gill, & Guarner, 2007).

In preparing microorganisms for probiotic use, manufacturers must also take precautions. When infusing foods with probiotic bacteria, specifically lactic acid bacteria (LAB), the organisms are present as active microorganisms. This limits the shelf life of the food product to about a month. ―Strains should be adapted to a suitable carrier or fermentable substrate (e.g. milk), and the final product should have an acceptable shelf-life and sensory attributes such as color, taste, aroma, and texture." (Holzapfel et al., 1998). The bacterial strains present in the product should remain viable in large numbers and maintain their metabolic activity even after the expiration date. Dietary supplements, contain the microorganisms in a dormant desiccated state, which increases shelf life to up to 24 months given that the water activity is kept low (<0.20) (Ouwehand, Salminen, & Isolauri, 2002). Water activity, in addition to temperature and pH are major factors in producing probiotic food products and other supplements.

Identifying bacteria worthy of probiotic use is a rigorous process, scientists must test all bacteria that are to be considered, because probiotic effects are often strain specific, the benefits associated with one species or strain is not necessarily true for others (Williams, 2010). This applies even within the same species. It is also important to note that any bacterial strain to be used for human consumption be tested and approved especially if the strain comes from a non human source.

2.3.1.1 Safety of Probiotics

2.3.1.1.1 Consumers

Consumers should not take probiotic consumption lightly. It is important to recognize the dynamics of bacterial strains present in the product as well as the specific health promotion the product confers. Variances in genera, species, and strains of bacteria from product to product, lead to differences in the intent of the product. Claims of efficacy labeled on probiotic supplements, or products in general, should be target specific, and only made upon successful testing (Sanders, 2009).

Although foods can never be guaranteed 100% safe, the level of harm to the general population of healthy individuals consuming probiotics must be low. For probiotic drugs, however, safety also includes a balance of side effects and potential benefits. Due to the distinction in standards of safety between probiotic foods and drugs, susceptibility of the target population determines the proper terminology. The use of the term *probiotic drug* is advised by Sanders 2009, to be reserved for a probiotic being administered to an unhealthy population to cure, treat, or prevent disease. Although considered safe, probiotics could potentially cause side effects in vulnerable individuals such as systemic infections, harmful metabolic activities, excessive immune stimulation, and gene transfer(Philippe Marteau, 2001; Philippe Marteau & Seksik, 2004).

The most commonly used bacteria in probiotics; *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, and *S. thermophilus* all have excellent safety records. These species of probiotic bacteria hold status as GRAS (generally recognized as safe). This safety covers all age groups as well as vulnerable individuals with compromised immune systems (Del Piano et al., 2006).

2.3.1.1.2 Manufacturing

Manufactures must follow a strict process for probiotics prior to products reaching the market. Figure 5 demonstrates the flow of this process beginning with the identification of strains. Probiotic strains are identified by genus, species, and strain using phenotypic and genotypic methods. Once recognized, strains are input into the international culture collection. Strains are then evaluated for safety and characterized for function. Potential pathogenicity is tested beginning with the historical epidemiology of the strain. Assessment of probiotic strains also includes the potential to cause inadvertent disease by way of toxicity, accidental systemic exposure, and/or administration to vulnerable individuals (Sutton, 2008). The safety evaluation and characterization of function are conducted via in vitro and animal studies. Upon successful completion of these tests strains then move on to human studies. Human studies are conducted using randomized, placebo-controlled, double blind designs. Tests are repeated in order to confirm results, and ensure the effectiveness of probiotics compared to customary treatment. Probiotic foods are then manufactured. Labels for these products must include ingredients (genus, species, strain), minimum number of viable bacteria at the end of the product shelf life, storage conditions, and company contact information.

According to the Dietary Supplement Health and Education Act (DSHEA) manufacturers are responsible for ensuring that dietary supplements manufactured or distributed are safe. Moreover any claims made regarding the supplements must be backed by substantial evidence to prove they are not false or misleading (Venugopalan, Shriner, & Wong-Beringer, 2010).

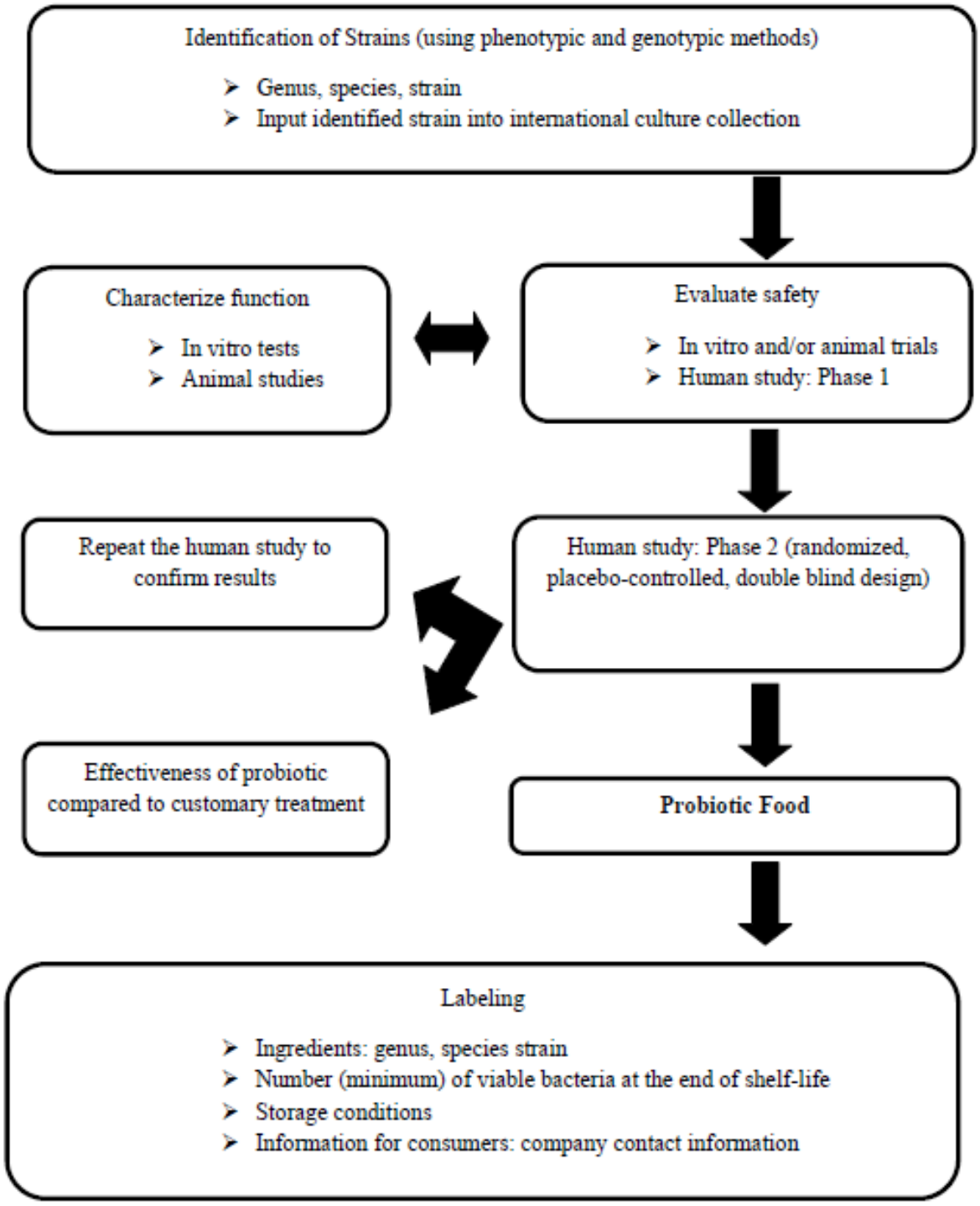

Figure 5 Preparations taken for labeling probiotic supplements.

2.3.2 Health benefits of probiotics

In recent years there has been a dramatic rise in the interest of probiotics due to significant benefits imparted on human health. Scientific evidence supporting the health claims of probiotics stem from in vivo and in vitro studies. Legitimate research groups conduct these studies and publish the findings in peer-reviewed journals (Gorbach, 2002; Vasiljevic & Shah, 2008). Specific health benefits are contributed by specific strains. Table 1 provides a list of probiotic stains and their associated health benefit.

Table 1

Health benefits and associated probiotic strains.

Probiotic Strains and associated health benefits	
Health Benefit	Probiotic Strain(s)
Immune system stimulation	L. acidophilus, L. casei, L. planetarum, L. delbruekii
Anti-tumor activity	L. acidophilus, L. casei, L. planetarum, L. delbruekii, L. bulgaricus, L. helveticus
Protection against acute diarrhea	L. bulgaricus, L. acidophilus, L. bifidus, L. casei
Lactose tolerance improvement	L. bulgaricus, L. bifidus, L. acidophilus, L. sporogenes
Lowered blood cholesterol levels	L. acidophilus, L. sporogenes, L. bifidus
Improved nutrient absorption and toxin elimination	L. planetarum
Protection against antibiotic associated diarrhea	L. acidophilus, L. bulgaricus
Improves Inflammatory Bowel Syndrome (IBS)	L. sporogenes
Protects against vaginal yeast infections	L. fermenti, L. acidophilus, L. bifidus

Some of the most important functional effects, backed up by scientific evidence, have been summarized by Salminen et al. (1996), and include aspects such as immune modulation and strengthening the gut mucosal barrier, due to: (1) gut microflora modification, (2) adherence to the intestinal mucosa with capacity to prevent pathogen adherence or pathogen activation, (3) modification of dietary proteins by the intestinal microflora, (4) modification of bacterial enzyme capacity especially of those suggested to be related to tumor induction, and (5) influence on gut mucosal permeability."(Holzapfel et al., 1998) While many of these benefits are established and well documented, some show great potential in animal models, and need claims validated by human studies (Vasiljevic & Shah, 2008).

One way in which probiotic bacteria are believed to improve human health is by altering the composition of the normal intestinal microflora. By displacing potentially harmful bacteria such as clostridia and coliforms, and replacing them with good bacteria such as lactobacilli and/or bifidobacteria host health is positively impacted (Ouwehand et al., 2002). In the same scenario, increasing the number of good bacteria is also beneficial to host health. Competition for attachment sites in the gastrointestinal tract is another benefit probiotic bacteria provide human hosts. Many strains of bacteria have been found to inhibit adhesion and displace pathogenic bacteria such as *Salmonella*, *Escherichia coli*, *Listeria monocytogens*, *Staphylococcus aureus*, and *Clostridium difficile* (Collado, Meriluoto, & Salminen, 2007). Investigation of other probiotic bacteria has led to findings supporting reduction of atopic eczema, vaginal infections, immune enhancement, rheumatoid arthritis, and liver cirrhosis (Gueimonde, Kalliomäki, Isolauri, & Salminen, 2006; E. Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001).

A specific health issue in which probiotics have been found advantageous is lactose intolerance. As infants all humans are born with the enzyme lactase (β-galactosidase) which

hydrolyses lactose to glucose and galactose in order to be absorbed in the small intestine (Adams & Moss, 1999; Sanders, 2000). About two-thirds of the world‘s population suffers from lactose intolerance. These individuals do not possess this lactase (β-galactosidase) enzyme, and any ingested lactose cannot be digested which leads to the gut microflora attacking the lactose. This microbial attack occurs in the colon and produces abdominal discomfort, flatulence, and diarrhea. However, when lactose intolerant individuals consume fermented milk products the effects do not occur or are less severe. Researchers have found this to be due to the presence of lactase in the probiotic products with incorporated lactic acid bacteria. The lactase is lysed by bile when the bacteria reach the intestinal lumen, and acts on the lactose that has been ingested. Thus relieving lactose intolerance symptoms (Tuohy et al., 2003).

Researchers observed significant decreases in small bowel lesions (associated with low dose aspirin use) in a study of *Lactobacillus casei* as a treatment option. This inquiry and others like it have led researchers to believe that daily consumption of probiotics can potentially aid in gastrointestinal health against medications like aspirin. Amplified probiotic bacteria count in the gut microbiota better equip individuals when medical drugs are administered on a daily basis. The drugs will not diminish as many positive bacteria. Table 2 lists many commonly studied probiotics and their associated health benefits.

2.3.2.1 Immunity

Probiotic testing has revealed promising results in the ability of the bacteria to enhance immune responsiveness. The probiotic bacteria enhance immune responsiveness by supplementing more beneficial bacteria to the preexisting microbiota of the host. The microbiota, which is an active member of the intestinal defense, is charged with the responsibility of maintaining immune responses, and sensitivity to potentially pathogenic bacteria. A LAB strain,

*Bifidobacterium lactis*HN019, was recently found to enhance nonspecific immune functions. –Namely leucocyte (lymphocytes and phagocytes) proliferation, enhance phagocyte production and proinflammatory cytokine production." (Tuohy et al., 2003) Studies have also shown *B. lactis* HN019 to increase peripheral blood leucocytes and natural killer cells in healthy volunteers including the elderly; these attributes were observed to be active in the tumor and viral destruction (Gill, Cross, Rutherfurd, & Gopal, 2001).

Dietary *Lactobacillus rhamnosus* HN001 has been investigated and proven in human studies to enhance the natural immunity in consumers (Gill et al., 2001). Researchers also suggested the use of the probiotic supplement in optimizing immune response in the elderly. *L. rhamnosus* GG was also proven in its ability to improve immune response by way of preventing atopic disease in high-risk children (Kalliomäki et al., 2008).

Other therapeutic applications of probiotics were observed by Rosenfeldt et al. who demonstrated *L. rhamnosus*19070-2 and *Lactobacillus reuteri* DSM 122460 benefit in the treatment of atopic dermatitis in children (Tuohy et al., 2003).

2.3.2.1.1 Probiotics augment gut barrier mechanisms

The intestinal barrier has a variety of components working in conjunction to protect the body from infection as well as absorb key nutrients. As previously discussed the physical components of the barrier such as the crypt depth, villi height and thickness of mucus are vital in barrier function. The size as well as the surface area of villi determine the amount of nutrients that are able to be absorbed (Yang et al., 2009). Larger surface area, allows larger amounts of nutrients to be absorbed. Similarly, larger crypt depths are associated with improved digestive efficacy (Guan, 2000). The crypts in the intestines are believed to facilitate digestion and

absorption. Larger crypt depth means increased crypt cell number, thus increasing digestive efficacy (Guan, 2000).

The importance of the microbial component of the barrier is seen in the absence of gut microbiota, during which antigen transport is increased. Thus confirming the role of the gut microflora in the defense barrier function (Tiihonen et al., 2010). Probiotic bacteria further enhance this barrier function by increasing production of intestinal mucus, effecting tight junction proteins, heightening the response of mucosal immunoglobulin A, introducing cellular heat-shock proteins, as well as increasing stimulation of defensin production (Wallace et al., 2011).

2.3.2.1.2 Functional Enzymes produced by Probiotics

Probiotic bacteria like *Bifidobacteria* have been known to stimulate the production of enzymes such assecretory immunoglobulin A (sigA) in vitro; an enzyme influential in intestinal barrier function. This enzyme inhibits invasion by pathogenic microbes and is an important component of the intestinal immune system (Hanson, 1998). sIgA is produced by immunoglobulin A plasma cells and forms the protective layer over the intestinal membrane surface. *Lactobacillus GG* has also been found to increase numbers of sIgA and immunoglobulin-secreting cells in the intestinal mucosa (Kalliomäki et al., 2008).

2.3.2.2 Cancer

The fight against cancer is a continual battle that has seen major advancements in the past decade. New treatments and possible cures are constantly being searched for. Researchers have found that probiotics help prevent the return of cancers like bladder and colorectal cancers (Elliott, Summers, & Weinstock, 2005).

Despite the fact that cancer can manifest in any individual, at any time, prevalent cases do arise in the elderly population. Reports on colorectal cancer (CRC) specifically, indicate that three-quarters of all incidences are random and the chance of getting this disease increases with age (Tuohy et al., 2003). Although specific species have not been targeted as responsible, many microorganisms in the gastrointestinal tract are capable of producing carcinogens and tumor promoters from dietary content. This excludes *Bifidobacteria* and lactobacilli which do not produce toxic or carcinogenic metabolites. A few species of the gut microorganisms that have been identified as stimulating enzyme activity leading to the conversion of dietary products into toxic or carcinogenic products such as β-glucuronidase, β-glycosidase, azoreductase, nitroreductase, IO ‗hydratase-dehydrogenase' and nitrate/nitrite reductase, are clostridia and bacteroides (Tuohy et al., 2003).

The consumption of probiotic bacteria acts to reduce the count of the potentially pathogenic bacteria in the gut that produce toxins and carcinogens. In humans this reduced risk of developing CRC has been proven, and human epidemiological studies have also suggested that probiotics in the form of products such as yogurt, may reduce the risk of large adenomas in the colon as well(Burns & Rowland, 2000).

Dietary factors are major contributors to the risk of cancers; diets high in fat and red, processed, meat are associated with colon cancer (Bingham, 1999). Dietary factors that are associated with reduced risk of cancer include high consumption of fruits, vegetables, whole grain cereals, calcium, and fish (Bingham, 1999; Rafter & Glinghammar, 1998). Changes in the metabolic activity and composition of the gut microbiota can potentially mediate the effect of diet on the carcinogenic process. Bacteria of the microbiota could have roles in the initiation of

colon cancer through production of carcinogens, cocarcinogens, or procarcinogens (Guarner & Malagelada, 2003).

2.3.2.3 Obesity

In general, the long standing cause of obesity has been considered to be eating in excess, and in some cases genetic predisposition accompanied by a lack of ample physical activity. Mounting new evidence suggests the gut microbiota plays a role in the mechanism of the disease of obesity. Research has associated gut microbiota with intestinal permeability, systemic quantity of adipose tissue and body weight. The original link between gut microflora and weight gain was a result of studies observing dietary fiber intake. In these studies decreasing dietary fiber intake resulted in excess body weight and diabetes; the hypothesized cause of this result was a change in gut microbiota due to an alteration in nutrient supply and digestion. However, consumption of a high fat diet has been found to decrease the total number of bacteria in the gut as well as promote the growth of gram-negative bacteria.

Four bacterial mechanisms have been identified to result in excess bodily energy gain: (1) microbiota increase energy bioavailability by transforming increased proportions of non-digestible food into biochemically absorbable nutrients; (2) the influence of intrinsic bacterial metabolism to generate and raise systemic levels of SCFAs to activate triglyceride synthesis; (3) high fat diets can result in a responsive bacterial metabolism resulting in pathology (such as microbial conversion of choline to methylamines leading to a choline deficient state, which induces liver disease); and (4) the ability of the microbiome in regulating gut gene expression to favor an obese state. Obese humans also demonstrate an alteration of the *Firmicutes* to *Bacteroidetes* ratio that can be altered by weight loss." (Ley et al., 2005; Ley, Turnbaugh, Klein, & Gordon, 2006)

2.3.3 Lactobacillus and Bifidobacterium

Lactobacillus and *Bifidobacterium* are members of a group of bacteria known as lactic acid bacteria (LAB), and are from the phylum *Firmicutes* and *Actinobacteria* respectively. Lactic acid bacteria (LAB) encompass gram positive, non-spore-forming, catalase-negative bacterial species (Otieno, 2011). This group also includes *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus* (Mombelli & Gismondo, 2000). Most probiotics share an optimum growth temperature of 37°C and optimum pH for initial growth is 6.5-7.0; although strains such as *L. casei* prefer temperatures of 30 °C (Von Wright & Axelsson, 2011). Bifidobacteria are taxonomically distant from lactic acid bacteria, but are grouped together because of the shared metabolic end product, lactic acid; in addition they commonly share habitats, for example the intestinal tract and dairy products (Ouwehand et al., 2002).

Lactobacilli are facultative anaerobes, gram positive, non-spore forming rods, they are usually non-motile, catalase negative, and do not reduce nitrate. The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* (Garrity, Bell, & Lilburn, 2004). The population of *Lactobacillus* in the gastrointestinal tract includes a variety of species and subspecies. Six species in particular are commonly isolated from humans: *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, and *L. brevis* (Mikelsaar, Mändar, & Sepp, 1998). Lactobacilli strains are versatile and capable to adapting well because of the diversity in the strains. Fermentation process, bacteriocin, and hydrogen peroxide production vary among the strains.

Bifidobacteria are anaerobes (although there some species capable of tolerating oxygen), also gram positive, non-spore forming rods, but possess a characteristic "Y" or club-shape morphology. Up to 25% of the microorganisms in the gut are bifidobacteria. Nine species that

have been isolated from humans are *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. angulatum*, *B. cantenulatum*, *B. dentium*, and *B pseudocatenulatum* (Ballongue, Salminen, & Wright, 1993).

—These bacteria produce lactic acid, acetic acid, and propionic acid, which lower the intestinal pH and suppress the growth of various pathogenic bacteria, thereby reestablishing the balance of the gut flora." (Alvarez-Olmos & Oberhelman, 2001; Doron & Gorbach, 2006).

2.3.3.1 Role of lactobacilli and Bifidobacteria in human health

The health promoting benefits of lactobacilli and bifidobacteria have been evident since the early 1900s when the first reports were made. French scientist Tissier recommended treating infantile diarrhea with large doses of bifidobacteria, and Nobel laureate Ilya Metchnikoff suggested longevous effects from use of lactic acid bacteria of sour dairy products (Tissier, 1907).

Lactobacillus and *Bifidobacterium* are the two most common bacterial genera used in probiotic products. Both bacteria can be found naturally in the gut microflora. The genera are known to release short chain fatty acids which provide an energy source for the intestinal membrane, promote intestinal epithelium repair, as well as improve the local blood supply (Yang et al., 2009). Research thus far has provided evidence of the clinical effects certain probiotic strains confer, such as *Lactobacillus rhamnosus GG*, *L plantarum* 299v, *L casei*, and *L. johnsonii*La1; these strains aid in the treatment and/or prevention of intestinal origin (Hamilton-Miller, 2004).

The value of lactic acid bacteria to human health revolves primarily around prevention and treatment of gastrointestinal diseases. Examples of gastrointestinal diseases include irritable bowel syndrome (IBS), infectious diarrhea, and inflammatory bowel disease (IBD). Irritable

bowel syndrome comes with an assortment of functional problems in the gut including, constipation, intestinal pain, bloating, and diarrhea. An estimated 10% of the western population has experienced symptoms of IBS (Ouwehand et al., 2002).Although the cause of IBS remains unclear, species specific lactic acid bacteria have shown to improve symptoms (Ouwehand, Kirjavainen, Shortt, & Salminen, 1999).

Infectious diarrhea is a gastrointestinal problem that has caused major concerns due to the mortality in children. The source of diarrhea cases are often speculated, some believe the disease to be caused by over population of pathogens in the gut with organisms like *Clostridium difficile* and *Klebsiellaoxytoca*, during antibiotic treatment, thus altering the balance of the microflora (Tuohy et al., 2003). Of the patients that receive antibiotics, 20% report cases of diarrhea (P Marteau, Seksik, & Jian, 2002). ~~A~~ recent meta-analyses has shown convincingly that prophylactic consumption of lactic acid bacteria reduce the risk of antibiotic associated diarrhea, *Clostridium difficile* associated diarrhea (Hempel et al., 2012), infectious diarrhea(Ritchie & Romanuk, 2012), and necrotizing enterocolitis (AlFaleh, Anabrees, Bassler, & Al-Kharfi, 2011)."

Although the use of lactic acid bacteria as a treatment of chronic diarrhea remain inconclusive, indications of benefit in children have been found (Bernaola Aponte, Bada Mancilla, Carreazo Pariasca, & Rojas Galarza, 2010). In cases of infantile diarrhea, often caused by rotavirus, *Lactobacillus rhamnosus* GG has maintained a good track record of reducing the duration of the diarrhea by about 50% (E. e. a. Isolauri, 1999). Although a mechanism has yet to be clearly explained, possibilities of how probiotic bacteria stains alleviate diarrhea include increasing mucosal integrity, and stimulating the immune response. In stimulating the immune response, it would be key to target antirotavirus-specific immunoglobulin (Ig) A. Combinations

of bacteria have also been used as in the case of *Bifidobacterium bifidum* and *Streptococcuss thermophilus*, and have shown their ability to reduce incidence of diarrhea caused by rotavirus (Saavedra, Bauman, Perman, Yolken, & Oung, 1994).

The gastrointestinal disease inflammatory bowel disease (IBD) is a group of disorders with unknown cause. During IBD gut microflora is imbalanced and there are communication issues which result in the immune system not recognizing the gut microbiota. Characteristically patients suffer from chronic or reoccurring mucosal inflammation. There are two forms of IBD, Crohn‘s disease and ulcerative colitis (UC). Lactic acid bacteria are being studied for their ability to reduce the rate of relapse in UC (Sang et al., 2010). The mechanism by which probiotic bacteria are believed to relieve symptoms and prevent recurrence is through regulation of the inflammatory response or alteration of the composition of the gut microbiota (Tuohy et al., 2003). One case of relief gained from probiotic bacteria is the nonpathogenic strain *E. coli* Nissle 1917, proven more effective in preventing relapse in Crohn‘s disease in patients versus a placebo (Rembacken, Snelling, Hawkey, Chalmers, & Axon, 1999). *S. boulardii* has also shown some success in relieving the symptoms of active Crohn‘s disease by reducing stool frequency and disease activity, as well as reducing the risk of relapse(Guslandi, Mezzi, Sorghi, & Testoni, 2000).

The benefits of the consumption of probiotic bacteria are not limited to gastrointestinal problems. Researchers have found that consumption of lactic acid bacteria reduces the infection rate and duration of symptoms in the common cold (Hao et al 2011). Colds are usually cause by viruses that escaped the immune system.

2.3.4 Application of probiotics

The term *probiotic* is an umbrella term that encompasses foods, drugs, designer probiotics, and direct-fed microbials (Sanders, 2009). Probiotic foods categorize food ingredients, dietary supplements, and food itself. Probiotic drugs categorize supplements used for their potential to prevent, treat, or cure disease. Designer probiotics categorize genetically modified probiotics. Direct-fed microbials categorize probiotics intended for animal use. Probiotic products can contain a single strain of a microorganism or a combination of several species (Williams, 2010). Among the strains most commonly used, and incorporated into products as probiotics include *L.* acidophilus and *L.* casei, due to their known health benefits.

Although lactic acid bacteria like *Lactobacillus* spp. and *Bifidobacterium* spp. are the prevalent species used in dairy related probiotic products, other, non-lactic bacteria are also used. These non-lactic strains are typically used in lyophilized or encapsulated „pharmaceutical‘ forms (Holzapfel et al., 1998). Members of the non-lactic acid bacterial group include Bacillus and the yeast *Saccharomyces* (Mombelli & Gismondo, 2000).

Of the commercially available probiotic products, many are not associated with scientific studies to prove the legitimacy of the health claim offered. It is important to research the products before consuming them. A few examples of products available that are tied to human studies documenting efficacy include: (capsules) Culturelle, Fem-Dophilus, and Align; (drinkable yogurt) Danimals; (yogurt) Activia and Yo-Plus; (powder) VSL #3 and Florastor; (fermented milk) DanActive, and BioK+CL1285 (Douglas & Sanders, 2008).

2.3.4.1 Applications for the Elderly

Mounting evidence supporting the importance of gut microbiota homeostasis to human health has led to the development of medical/ nutritional applications of probiotics targeted

toward the elderly population (Biagi et al., 2012). Incorporating probiotics into the daily diet of the elderly, or any individual seeking a healthier gastrointestinal system is a key aspect to longevity. This integration of probiotics into the diet can take place by way of food products as well as dietary supplements.

2.3.4.2 Dairy Products

One of the most common and historically documented medium for probiotic bacteria are dairy products. Due to the natural properties of dairy products and the refrigerated temperatures at which they are stored these products make suitable vehicles for probiotic bacteria(Song et al., 2012). Dairy products containing probiotics commonly found on the market today include yogurt, cheese, and sour and fresh milk. The probiotic microorganisms are adequately delivered to humans because of the role dairy products play; providing an apt environment conducive of growth and viability (Gardiner et al., 1999; Phillips, Kailasapathy, & Tran, 2006; Ross, Fitzgerald, Collins, & Stanton, 2002; Saarela, Virkajärvi, Alakomi, Sigvart-Mattila, & Mättö, 2006).

2.3.4.3 Fruits and vegetables

Emerging new interest has arisen in the use of non-dairy products as vectors of probiotic bacteria. The shift away from the traditional use of dairy goods derives from issues such as lactose intolerance and high levels of cholesterol in currently available dairy food. Among the requests from consumers as to new forms of probiotic products are vegetarian friendly products (Heenan, Adams, Hosken, & Fleet, 2004). As a result a wider array of probiotic products are being developed like fruits (Lavermicocca, 2006), vegetables (Yoon, Woodams, & Hang, 2006), legumes (Heenan et al., 2004) and cereal products (Helland, Wicklund, & Narvhus, 2004).

Delivering the probiotic bacteria to humans via fermented vegetables is possible, and this substrate has been tested in a variety of forms as a potential vehicle for probiotics.

Table 2

Nontraditional vectors of probiotics.

Product	Bacterial strains
1. Vegetable derived	
1.1 Tomato juice	*L. acidophilus*
	L. plantarum
	L. casei
	L. delbrueckii
1.2 Carrot juice	*Bifidobacterium strains*
2. Fruit derived	
2.1 Banana puree	*L. acidophilus*
2.2 Pomegranate	*L. plantarum*
	L. delbruekii
	L. paracasei
	L. acidophilus
2.3 Orange juice	*L. plantarum*
2.4 Noni juice	*B. longum*
	L. plantarum
2.5 Cashew apple juice	*L. casei*
3. Fruit and Vegetable mediums supporting various probiotic supplements	
3.1 Apple juice	*L. acidophilus*
3.2 Beet juice	*L. acidophilus*
	L. plantarum
3.3 Cabbage juice	*L. plantarum*
	L. delbruekii
3. 4 Cranberry juice	*L. rhamnosus*
3.5 Fermented banana	*L. acidophilus*
3.6 Fermented banana pulp	*L. acidophilus*
3.7 Ginger juice	*L. rhamnosus*
	L. paracasei
	L. plantarum
3.8 Grape juice	*L. reuteri*
3.9 Green coconut water	*L. plantarum*
3.10 Passion fruit juice	*L. acidophilus*
3.11 Peanut milk	*Bifidobacterium strains*
3.12 Pineapple juice	*L. casei*
3.13 Probiotic banana puree	*L. acidophlius*

**Products available in the local market, in Greensboro, NC.*

2.4 Probiotic Supplements

There are a large number of probiotic products on the market today, and this number is steadily increasing. Consumer interest in function food, including those containing probiotics, has risen in the last 20 years (Song et al., 2012). Despite the predominant presence of probiotic food products available to consumers, probiotic supplements are becoming more popular. The *Nutrition Business Journal 2011* reported that consumers spent 626 million dollars on probiotic supplements in 2010 (Coulston & Boushey, 2013).

Probiotic dietary supplements are commercially available in the form of capsules, gummies, liquids, powders, and tablets. Capsules are produced by manufacturers when a drug cannot be compacted into a tablet; this form of the product also aids in swallowing problems. Capsules are produced in soft and hard forms; 1) hard capsules are more commonly seen, they are comprised of two halves fitted together and usually filled with a powder, 2) soft capsules are comprised of a single piece, and are appropriate for oils etc. The technology for creating products like these probiotic containing capsules has been developed to protect the bacteria from damage during transit through the gastrointestinal tract.

Probiotics are also available in the convenience of liquids, ranging from on-the-go single serving sizes to larger bottles for traditional dispensing. One benefit of the liquid probiotics is the coating effect it can have on the gastrointestinal epithelium. The bacteria are applied directly to the wall lining. This form of probiotic supplement does require refrigeration due to the presence of active, live microorganisms. Those liquid supplements containing freeze-dried bacteria do not require this refrigeration step.

Powders are another method of administering probiotics. These products cater more towards individuals with difficulties swallowing capsules and tablets. The powder supplements

can be incorporated into foods and/or beverages. Consumers must be mindful of the temperature of the food or drink item they add the probiotic powder to, the supplement should not be added to hot foods or drinks. It is important that these products be kept dry, and stored in a dark location; moisture and oxygen can be destructive to probiotics.

A broader selection of probiotic supplements to choose from, in regards to forms, benefits consumers and might increase the likelihood of purchase. The varieties of supplements available provide improved convenience of quick and easy administration to confer the same immune enhancing effects. Throughout history probiotics have been predominantly included in dairy products; however with the increase in supplementation, consumers now have the ability to choose between the two products to reap the health promoting benefits of probiotic bacteria.

The elderly population benefits from the convenience of multiple probiotic supplements as well because the assortment of forms allows them to select a product that caters to their mastication or swallowing abilities. Elderly with weak swallowing abilities are now able to choose a liquid or powder form of a probiotic supplement for increased ease in administration.

The increase in probiotic supplementation means a rise in the number of products available on the market. Probiotics, as previously mentioned encompasses many smaller subgroups of products including probiotic food, probiotic drugs, specialty probiotics and probiotic feed (for animals). The intended use of a probiotic agent determines its classification. Probiotic drugs and dietary supplements have differing regulatory requirements to abide by. A drug, defined by the Food and Drug Administration (FDA) is an article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease (Del Piano et al., 2006). Probiotics intended for use as a drug, must go through the regulatory process as a drug. Probiotics intended for use as a dietary supplement, however is considered a ―food" and is

regulated by the FDA‘s Center for Food Safety and Applied Nutrition (Philippe Marteau & Seksik, 2004). This exclusivity in definition is necessary, and in particular in reference to this study, due to the diversity among individuals being classified as elderly. This study is targeting elderly individuals; this classification encompasses both healthy and unhealthy individuals. Some consumers will have preexisting conditions that the probiotics might be able to reconcile in addition to maintaining the microbial balance.

With the rise in probiotic supplements available commercially, it is vital for consumers to research these products before making any general assumptions regarding probiotic use. Currently there is no legal definition for the term *probiotic*, and products on the market labeled ―probiotic" may not be equip with the distinguished characteristics of stability, content, or health effects (Sanders, 2009). Researchers are adamantly testing the potential impact of probiotic strains on a growing array of conditions and subjects. ―A given probiotic, tested in different clinical situations, might exert a beneficial effect, show no effect, or result in an adverse effect. However, a negative or adverse effect in certain situations does not negate probiotic status. Such results do, however, stress the need to be specific about the benefits that are documented for each probiotic and the situations in which use is considered to pose an undue risk." (Sanders, 2009)

Consumers must also remember that probiotic supplements are intended to complement the diet, and therefore should not be used as a replacement for probiotic microorganisms provided through food consumption. Supplements alone are not sufficient, it is recommended for individuals to balance their supplement and dairy probiotic intake.

2.4.1Viability of Probiotic cells in commercially available supplements

Viability of probiotic bacteria in commercially available supplements is crucial given that these microorganisms must survive the shelf life of the product and the harsh environment of

sections of the gastrointestinal tract, including an acidic stomach and enzyme and bile salt filled small intestine (Vasiljevic & Shah, 2008). In order for probiotic supplements or products to exert the health promoting effects claimed on the label, bacterial cells must remain viable up to human consumption. Probiotic products, such as yogurt, often claim to contain bacterial counts of up to several million live cells. Some researchers suggest that the minimum number of microorganisms required to confer positive health effects are 8 log CFU/mL (Shah, 2000).This large number of functional bacteria present during consumption ensures a sufficient amount of the probiotic microorganisms reach the large intestine. The sensitivity of these microorganisms to environmental conditions such as water activity, temperature, acidity, and redox potential (presence of oxygen) can lead to the probiotic cells dying or being present in numbers lower than advertised, at time of consumption; factors such as processing, storage, and time passed before products are eaten(Siuta-Cruce, 2001; Vasiljevic & Shah, 2008).

Despite the importance of characteristics such as population count and viability in respect to probiotics in commercially available products, very few studies have been conducted substantiating the presence and quantity of probiotic bacteria claimed in many of the products on the market. In 2006, Ibrahim and Carr conducted a study investigating the viability of bifidobacteria in commercial yogurt products purchased in Greensboro, North Carolina and found that only 44 out of the 58 commercial yogurt products (76%) tested contained viable cultures (S. Ibrahim & Carr, 2006). Another aspect of the study observed the viability of probiotic cells in the yogurt over time. In all tested samples bifidobacteria populations are nearly undetectable by the fourth week, leading to the conclusion that consumers should wait no longer than three weeks to consume probiotic containing yogurt. Similar results were found in the earlier study by Coeuret et al. 2004, in which the quantity of *Lactobacillus* in probiotic feed and

food products was examined (Coeuret, Gueguen, & Vernoux, 2004). Coeuret's research found that in four of the products that claimed to contain probiotic bacteria the specified strains were not detected. Additionally, five products mislabeled the number of bacteria present, and three mislabeled the species of lactobacilli present. Investigation of probiotic supplement viability has yielded results congruent to the probiotic food counterparts. In 2001 Temmerman et al. tested 30 dried probiotic supplements and found 11 contained no viable bacteria, 18 contained additional species not identified on the label, and only 7contained all species claimed (Temmerman, Pot, Huys, & Swings, 2003). These studies highlight the poor standard of probiotic supplements and food products currently available for consumers. This issue is global, similar reports of low bacterial counts (zero in some cases), contamination, and unhelpful of misleading labeling of this type of product on general sale in the USA (Alcid, Troke, Andszewski, & John, 1994; Gilliland & Speck, 1977; Hughes & Hillier, 1990), Austria (Maurer, 1992; Shah, 2000), Italy (Canganella et al., 1997; Hoa et al., 2000), and UK (Hamilton-Miller & Shah, 2002; Hamilton-Miller, Shah, & Winkler, 1999).

Without reliable analysis of the microbial ingredients contained in probiotic products companies cannot be held accountable for the claims they make on probiotic product labels. Consumers are susceptible to mislabeling and consuming products lacking vital desired ingredients. Additionally, there is major health concerns when results show products containing species of bacteria not claimed on the label. Similar to validating studies conducted to support the health benefits of the commonly used bacterial strains, the same accountability should be applied to these probiotic food and supplement products. The need for current research is apparent.

Table 3

Classification of commercial probiotic supplements available in local stores.

Classification	Example of Commercial Product	Probiotic strains
1. Capsule		
	1.2 Digestive Advantage	*Bacillus coagulansgbi*
	1.3 Udo‘s Choice: Adult‘s Probiotic	*L. casei* *L. rhamnosus* *L. plantarum* *L. acidophilus* *L. bulgaricus* *B. bifidum* *Streptococcus thermophilus*
	1.3 Ultimate Flora 50 Billion	*L. acidophilus* *L. plantarum* *L. rhamnosus* *L. salivarius* *L. bulgaricus* *L. lactis* *L. casei* *B. bifidum* *B. longum* *B. breve*
2. Liquid		
	2.1 Probiotic Acidophilus Original	*L. acidophilus* *L. bulgaris* *L. thermophulis*
	2.2 Keybiotics	*L. plantarum* *L. rhamnosus* *L. acidophilus* *L. casei* *L. salivarius* *L. bulgaricus* *B. longum* *B. bifidum* *B. lactis* *Streptococcus thermophilus*
	2.3 Udo‘s Choice Probiotic (infant)	*L. casei* *L. rhamnosus* *L. acidophilus* *B. bifidum* *B. infantis*
3. Powder		
	3.1 Primadophilus Reuteri	*L. acidophilus* *L. rhamnosus* *L. reuteri* *L. casei* *B. infantis* *B. longum*
	3.2 Jarro- Dophilus + FOS	*L. rhamnosus*

		L. acidophilus *L. plantarum* *L. casei* *B. longum* *B. lactis*
	3.3 Multidophilus Powder	*L. acidophilus* *L. bulgaricus* *B. bifidum*
4. Tablet		
	4.1 Twinlab: Time Release Probiotics	*L. acidophilus* *L. reuteri* *L. plantarum* *L. fermentum* *B. bifidum*
	4.2 Flora Smart: Advanced Probiotic	*L. acidophilus* *L. rhamnosus* *L. salivarius* *L. bulgaricus* *L. plantarum* *L. casei* *B. bifidum* *B. breve* *B. longum*
	4.3 Life Flora	*L. paracasei* *B. longum* *Streptococcus thermophilus*

2.4.1 Required Characteristics for Probiotics in supplements

The proven health and wellness benefits of probiotics have sparked rising interest from consumers and the food industry within the past several years. Additional beneficial effects of probiotics have been recognized in food preservation, specifically extension of shelf-life and enhanced food safety (Gyawali & Ibrahim, 2012; Song et al., 2012). It is important that probiotic bacteria possess two main characteristics, stability and functionality, in order to have practical applications in probiotic supplements (Figure 3). Stability of probiotics is vital in maintaining a high number of viable bacteria during processing and shelf-life. This is referred to as good technological properties. Functionality of probiotics is related to their tolerance to low acid and bile salts and their antimicrobial, adhesive, and enzyme activity. When incorporating probiotic bacteria into supplements, other technological properties to consider are autolytic activity, impact on sensory properties, and phage resistance. It is imperative to consider both characteristics before selecting potential probiotics for use in supplements. It is equally important to note that stability (cell viability) is not always enough to guarantee the probiotic functionality.

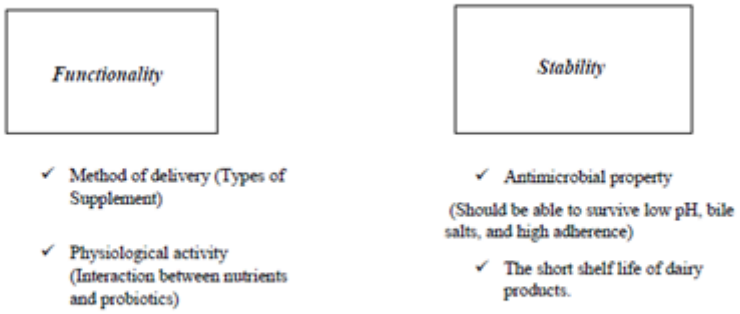

Figure 6 Requirement characteristics for probiotics in supplements.

2.5 Composition of a longevous gut

In a study conducted in 2010 researchers investigated the gut microflora of centenarians of Bama County in the Guangxi Zhuang Autonomous Region in China, famous for the longevity of its population. The 2000 population census in China documented 76 centenarians living in Bama County; the ratio was found to be 30.98 compared to the average ratio in Guangxi province 4.44 centenarians per 1x 10^5 people (Zou, 2002). Researchers gathered bacterial species from three groups of participants, the first group M the mean age was 98 years, group S the mean age was 70 years, and group C the mean age was 82 years (Zhao et al., 2011).

Researchers found that the guts of many of the centenarian participants contained larger numbers of Bifidobacteria in comparison to the young adults; notable due to elderly populations typically possessing fewer Bifidobacteria (Suau et al., 1999). There were significant age related differences in the bacterial species including *Clostridium coccoides- Eubacterium rectal*, *Bacteroides-Prevotella*, and *Clostridium perfringens* all of which were found in the longevous participants (Zhao et al., 2011). *Bacteroides* is an important group due to its role in the digestion of polysaccharides in the cross-feeding network of the microbiota; they are also able to use an array of carbon sources. The high levels of *Bacteroides-Prevotella* indicate a high level of amlolytic activity in the colon of longevous people in Bama." (Zhao et al., 2011)

2.6 Message to the public regarding medication

"Improving the level of health among older people can—and must—be one of the aims of modern gerontology, in order to decrease the rate of hospitalization, the administration of drugs and, consequently, the healthcare cost of the ageing population." (Biagi et al., 2012) The aspiration of this study is not to deter elderly individuals from taking their medication, but to inform them that it is vital to replace the bacteria they lose due to their consumption of the

medication. Maintaining a healthy gut is easily achieved through the incorporation of probiotic products in the diet. Probiotics consumed daily will significantly increase the number of good bacteria in the gut microbiota. By increasing this microbiota composition positively elderly hosts have a larger number of bacteria when consuming medical drugs. Therefore when the drugs do disrupt the microbiota there is still a large probiotic population remaining. Individuals consuming probiotic products sparingly and inconsistently are not equip with such large numbers of good bacteria in the gut. Therefore when the drugs wipe the good bacteria out of the gut microflora the individual might suffer from more gastrointestinal issues.

When searching for probiotic supplements to take consumers should be mindful of the labeling of these products. Labels of probiotic supplements should include: notification of the presence of live bacteria, the exact nature of the bacteria, identification of species present specifically numbers of each species (this should be explained in terms understandable by consumers, yet microbiologically accurate), minimum amount of bacteria require to confer health effects (either in terms of numbers of bacteria or servings), and accurate content at the time of purchase (not simply at some stage of manufacture). Consumers need to inquire about probiotic supplements with a specific benefit in mind in order to find an appropriate probiotic to cater to that need.

2.7 Justification

Numerous studies have shown the impact of probiotics on human health, and have demonstrated that Bifidobacterium and *Lactobacillus* strains can positively impact health. It is proven that probiotic bacteria are involved in immune modulation and strengthening the gut mucosal barrier (Ouwehand et al., 2002). Mechanisms by which these microorganisms accomplish this include gut microflora modification, intestinal mucosa adhesion and facilitation

of the prevention pathogen attachment or pathogen activation, modification of dietary proteins by the intestinal microflora, alteration of bacterial enzyme function, and influence of gut mucosal permeability(Ouwehand et al., 2002). Probiotics benefit individuals of all ages, and age specific probiotic choices are available. The health conferring microorganisms are commercially available to consumers in many products including probiotics supplements. These supplements come in forms such as capsules, liquids, powders, and tablets. The elderly population is often targeted to consume probiotics by the researchers due to the natural decrease of "good" bacteria in their gut microbiota with increasing age; as well as the increase incident of elderly taking medication.

Advanced age is typically accompanied by a decrease in health. As health issues arise in the elderly population, medications are vital for treatment. Interactions between medications and functions of the body have been well studied. There are also many reported adverse effects associated with the drugs commonly prescribed to elderly patients; negative impact on the gastrointestinal tract physically and alteration of the microbial balance are two of significance. The most common adverse effect of non-steroidal anti-inflammatory drugs is damage to the mucosa of the gastrointestinal tract (Bjarnason, 2009). Other side effects include gastric pain, heartburn, nausea, vomiting, bleeding, perforation, and ulceration (Zeino et al. 2010). A major medication of concern is aspirin, a non-steroidal anti-inflammatory drug or NSAID. NSAID drugs are linked to gastric and duodenal damage (Bjarnason & Takeuchi, 2009; Laine, 1996; Soll et al., 1991). The more common locations effected by adverse reaction of drugs are the small and large intestine, which represent 20 to 40 percent of drug side effects (Zeino et al., 2010).

Due to the interaction of medical drugs and functions of the body, there might be a similar interaction of medical drugs and naturally occurring probiotic bacteria of the gut

microbiota. There is limited research in this area; therefore with this justification further study must be conducted. This project is aimed to study the effect of drugs commonly taken by the elderly population, on viability and functionality of probiotics strains found in commercially available probiotic supplements.

2.8 Objectives

The specific aims of this investigation are:

1) To examine the viability of probiotics in commercial dietary probiotic supplements available in local stores.

2) To determine the impact of aspirin and caffeine on the functionality of a probiotic supplement. In this project, functionality was determined by performing: (a) β- galactosidase activity, (b) bile resistance, (c) reducing power, and (d) protein expression.

CHAPTER 3

Materials and Methods

3.1 Objective 1

This objective will examine the viability of probiotics in commercial probiotic supplements.

3.1.1 Materials

3.1.1.1 Probiotic supplements

Ten probiotic supplements were purchased from a local health store in Greensboro North Carolina. Each of these supplements claimed to include various strains of bifidobacteria (*B. longum*, *B. bifidum*, *B. lactis*, *B. breve, B. infantis*) and lactobacilli (*L. acidophilus*, *L. rhamnosus*, *L. reuteri, L. gasseri, L. plantarum, L. casei, L. brevis, L. salivarius, L. paracasei*) (seen in the table below).

Table 4

Composition of commercial probiotic supplements (by strain).

Supplement Number	Commercial Name	Probiotic strains	Form
1.	Culturelle	L. GG	Veggie Capsule
2.	(Natren) Megadophilus	L. acidophilus	Veggie Capsule
3.	(Solaray) Multidophilus	L. acidophilus L. bulgaricus B. bifidum	Veggie Capsule
4.	(Klaire Labs) Ther- biotic Factor 4	B. bifidum	Veggie Capsule
5.	(Nature‘s Way) Primadophilus Reuteri	L. rhamnosus L. acidophilus L. reuteri (HA- 188)	Veggie Capsule
6.	(Pure Encapsulations) Lactobacillus Acidophilus	L. acidophilus (LA-5)	Capsule
7.	(Bluebonnet) Acidophilus Plus FOS	L. acidophilus	Veggie Capsule
8.	(Life Extension) Bifido GI Balance	B. longum (BB536)	Veggie Capsule
9.	Kyo- Dolphilus 9	L. gasseri B L. gasseri M L. rhamnosus B. bifidum B. longum M B. infantis B. breve B. lactis	Capsule
10.	(Garden of Life) Primal Defense: HSO Probiotic Formula	L. plantarum L. rhamnosus L. casei L. brevis L. salivarius L. acidophilus L. paracasei B. lactis B. bifidum B. breve B. longum Bacillus subtilis	Veggie Capsule

3.1.1.2 Chemicals and Media

Three growth media were used to cultivate the probiotic bacteria listed on the commercial supplement labels. MRS agar (deMan, Rogosa& Sharpe) was used for all *Lactobacillus* and *Bifidobacterium* strains, and was prepared following the manufactures recipe. Modified BIM 25 was used for *Bifidobacterium* strains. The medium contained the following ingredients (gram per liter): MRS broth 55; Agar 20; Nalididixic acid 0.02; Polymyxin B sulphate 0.0085; Kananycinsulphate 0.05; Iodoacetic acid 0.025; 2,3,5-triphenyltetrazolium chloride 0.025; cysteine-hydrochloride 0.5; lithium chloride 1.5; beef extract 1.0; and Tween 20 5mL (S. A. IBRAHIM & SALAMEH, 2001). Reinforced Clostridium Agar (RCA) with bromocresol green and clindamycin (RCABC) was prepared following the methods of Darukaradhya et al. 2006 (Darukaradhya, Phillips, & Kailasapathy, 2006).

Table 5

Commercial probiotics supplements and corresponding selective and differential media.

Supplement Number	Probiotic strains	Selective and Differential Media
1.	L. GG	MRS
2.	L. acidophilus	MRS RCA
3.	L. acidophilus L. bulgaricus B. bifidum	MRS BIM 25 RCA
4.	B. bifidum B. longum B. lactis B. breve	MRS BIM 25
5.	L. rhamnosus L. acidophilus L. reuteri (HA- 188)	MRS RCA
6.	L. acidophilus (LA-5)	RCA
7.	L. acidophilus L. bulgaricus B. bifidum B. longum	MRS BIM 25 RCA
8.	B. longum (BB536)	MRS BIM 25
9.	L. gasseri B L. gasseri M L. rhamnosus B. bifidum B. longum M B. infantis B. breve B. lactis	MRS BIM 25 RCA
10.	L. plantarum L. rhamnosus L. casei L. brevis L. salivarius L. acidophilus L. paracasei B. lactis B. bifidum B. breve B. longum Bacillus subtilis	MRS BIM 25 RCA

3.1.2 Methods

3.1.2.1 Sample Preparation

Samples were prepared by adding two capsules of each supplement into individual tubes of fresh MRS broth, and then mixed for 15 to 30 s using a vortex. The ten probiotic cultures were incubated for 12-14 h at 37°C, for the recovery of the cells. Overnight cultures were then used to test for viability and functionality of each probiotic supplement.

3.1.2.2 Viability of bacterial cells

Probiotic cultures were serially diluted in 9 ml of sterile 0.1% peptone water, and then appropriate dilutions (100 µl) were transferred onto the respective agar. Appropriate dilutions were surface plated in duplicate on Lactobacilli MRS (Difco, Detroit, MI, USA) for enumeration of *Lactobacillus* strains. Modified BIM-25 was used for the enumeration of bifidobacteria(S. Ibrahim & Carr, 2006). Reinforced Clostridium Agar with bromocresol green and clindamycin (RCABC), which is a non-selective media used for the growth of *Lactobacillus acidophilus* spp., and Reinforced Clostridium Agar with aniline blue and dicloxacillin (RCAAD), which is differential for *Bifidobacterium* spp. was also be used (Darukaradhya et al., 2006). The plates will be incubated for 72 h at 37°C. Plates containing 25-250 colonies will be counted to calculate bacterial populations.

3.1.2.3 β- Galactosidase Activity

The activity of β-Galactosidase was quantified using the o-nitrophenyl-β-D-galactoside (ONPG) assay as described by Miller (J. Miller, 1993).Cultures were first grown to mid log phase, and an initial O.D. was measured. The cultures were incubated at 37°C until an O.D. of 0.7-0.9 (nm) was observed. A 100 µl aliquot of starter culture was washed, and 900 µl of Z buffer (composed of 0.06M Na_2HPO_4; 0.04M NaH_2PO_4; 0.01M KCl; 0.001M $MgSO_4*7H_2O$)

was added to the pelleted bacterial cells and washed a second time. Chloroform was then added at 10µL per tube and samples were mixed by vortex for 10 s. The mixture was incubated at 37 °C on a shaker with caps removed for 30 min. After incubation, 200 µl of β-ONPG(4 mg/ml in 0.1 M phosphate buffer) was added to each tube and vortex in order to start the reaction. The reaction was stopped by adding 0.5 mL of 1N Na_2CO_3 (10.6g/100ml) after the expected yellow color had developed. The time taken for the color to be developed in each tube was recorded. Optical density was recorded at OD_{420} and OD_{550}. Unit of *β*-gal produced was calculated as follows:

$$\text{Unit of } \beta\text{-gal} = \frac{1000\ (OD_{420} - 1.75 * OD_{550})}{t * v * OD_{610nm}}$$

Units of β-gal were calculated as described by Miller (S. Ibrahim & O'Sullivan, 2000; J. Miller, 1993).

3.2 Objective 2

The objective of this investigation was to determine the interaction between one commercial probiotic supplement, and commonly administered medical drugs of elderly people. Additionally, investigation of the interaction between the supplement and caffeine conducted.

3.2.1 Population

The probiotic supplement 5, obtained from the local market will be tested in this investigation (Table 4). The supplement was initially be cultured in MRS broth in anaerobic culture tubes at 37°C for18 h.

3.2.2 Determination of β- galactosidase activity

The activity of β-Galactosidase was quantified using the o-nitrophenyl-β-D-galactoside (ONPG) assay as described by Miller (J. Miller, 1993). Cultures were first grown to mid log phase, and an initial O.D. was measured. The cultures were incubated at 37°C until an O.D. of

0.7-0.9 (nm)was observed. A 100 µl aliquot of starter culture was washed, and 900 µl of Z buffer (composed of 0.06M Na_2HPO_4; 0.04M NaH_2PO_4; 0.01M KCl; 0.001M $MgSO_4*7H_2O$) was added to the pelleted bacterial cells and washed a second time. Chloroform was then added at 10µL per tube and samples were mixed by vortex for 10 seconds. The mixture was incubated at 37 °C on a shaker with caps removed for 30 minutes. After incubation, 200 µl of β-ONPG (4 mg/ml in 0.1 M phosphate buffer) was added to each tube and vortex in order to start the reaction. The reaction was stopped by adding 0.5 mL of 1N Na_2CO_3 (10.6g/100ml) after the expected yellow color had developed. The time taken for the color to be developed in each tube was recorded. Optical density was recorded at OD_{420} and OD_{550}. Unit of *β*-gal produced was calculated as follows:

$$\text{Unit of } \beta\text{-gal} = \frac{1000\,(OD_{420} - 1.75 * OD_{550})}{t * v * OD_{610nm}}$$

Units of β-gal were calculated as described by Miller (S. Ibrahim & O'Sullivan, 2000; J. Miller, 1993).

3.2.3 Bile Resistance

The effects of bile on the growth of LAB cells will be conducted by a method modified from those of Gilliand and Walker and Yu and Tsen (Gilliland & Walker, 1990; Yu & Tsen, 1993). After drug treatment, the surviving LAB cells were collected by centrifugation (7000 rpm, 5 min) and washed once with PBS (pH 7.2). The cells will be resuspended in 10 ml MRS broth with or without 0.3% (w/v) ox gall bile (Sigma; Louisiana, USA) and incubated at 37ºC for 48 h under anaerobic conditions. Bile tolerance of the LAB cells was determined by comparing the viable LAB counts on MRS agar. Each assayed will also be performed in duplicate (Lin, Hwang, Chen, & Tsen, 2006).

3.2.4 Reducing Power Assay

The reducing power of probiotic supplements was determined according to the method reported by Mau et al. 2004. Supplement 5 (1.0 ml) was mixed with 2.5 ml sodium phosphate buffer (pH = 7.4, 0.02 M) and2.5 ml potassium ferricyanide (1.0%, w/v). This mixture was then incubated at 50 °C for 20 min. After, 2.5 ml of trichloroacetic acid (10.0%, w/v) was added to the mixture to terminate the reaction. The mixture was centrifuged at 200g for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml ferric chloride (0.1%, w/v). The absorbance was measured at 700 nm. A higher absorbance of the reaction mixture indicates a higher reducing power.

3.2.5 SDS Gel

Cell extraction procedure was performed as described by Thermo Scientific: B-PER Bacterial Protein Extraction Reagent. Bacterial cells were pelleted by centrifugation at 5000 × g for 10 min. B-PER Reagent was added at 4mL per gram of cell pellet. The suspension was pipetted up and down until it is homogeneous. When homogeneity was reached the sample was incubated for 10-15 min. at room temperature. The lysate was centrifuged at 15,000 × g for 5 minutes to separate soluble proteins from the insoluble proteins.

BCA was performed as described by Thermo Scientific: Pierce BCA Protein Assay Kit. First standards and working reagent were prepared. Diluted Albumin (BSA) Standards were prepared using the table provided by Thermo Scientific. The contents of one Albumin Standard (BSA) were diluted into several clean vials, preferably using the same diluent as the sample(s). Each 1mL ampule of 2mg/mL Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in the table. There will be sufficient volume for three replications of each diluted standard. The BCA Working Reagent (WR) required the use of the following formula to determine the total volume of WR required.

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required.

Once the standards and working reagent were prepared 0.1mL of each standard and unknown sample replicate was pipetted into an appropriately labeled test tube. WR (2 mL) was added to each tube and was mixed by vortex. Tubes were incubated at 37°C for 30 minutes. With the spectrophotometer set to 562nm, the instrument was zeroed using a curette filled only with water. The absorbances of all the samples were measured within 10 minutes.

The average 562nm absorbance measurement was subtracted from the blank standard replicates from the 562nm absorbance measurement of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 562nm measurement for each BSA standard vs. its concentration in μg/mL. This standard curve was used to determine the protein concentration of each unknown sample.

SDS Page. The polyacrylamide gel was prepared according to standard protocol. Samples were loaded, and the gel was run at 25 mA in 1x SDS Running Buffer. The gel was then stained and placed in a plastic container. The gel was then covered with isopropanol fixing solution and shaken at room temperature. For 0.75 mm-thick gels, shake 10 to 15 min; for 1.5 mm thick gels, shake 30 to 60 min. After appropriate shaking time the fixing solution was poured off. The gel was then covered with staining solution and shaken at room temperature for 2 hours. The staining solution was poured off and the gel was washed with 10% acetic acid.

CHAPTER 4

Results

4.1 Objective 1

In this study we used commercial probiotic supplements available in the market to determine the viability of bacterial cells. These supplements were divided into three groups based on the type of bacterial population (*Lactobacillus* spp., *L. acidophilus*, *Bifidobacterium* spp.). Table 6 shows the initial population of probiotic cultures found in ten commercial dietary supplements. The initial bacterial populations of group 1, *Lactobacillus* spp., ranged from 5.39 to 9.39 log CFU/ml. Supplement 8 exhibited the lowest number at 5.39 ± 0.00 log CFU/ml. Supplement 1 exhibited the highest number at 9.39 ± 0.00 log CFU/ml. The initial bacterial populations of group 2, *L. acidophilus*, ranged from 5.63 to 8.81 log CFU/ml. Supplement 10 exhibited the lowest number at 5.63 ± 0.21 log CFU/ml. Supplement 2 exhibited the highest number at 8.81 ± 0.06 log CFU/ml. In group 3, *Bifidobacterium* spp., the initial bacterial populations ranged from 4.38 to 7.86 log CFU/ml. Supplement 8 exhibited the lowest number at 4.38 ± 0.00 log CFU/ml. Supplement 9 exhibited the highest number at 7.86 log CFU/ml. The initial population count indicates the presence of probiotic bacteria in all tested supplements as claimed on the label.

Table 6 also shows the changes in the viability of bacterial cells present in the supplements after 4 weeks in refrigeration storage (4°C). Viability post storage period in group 1 ranged from 5.27 to 9.10 log CFU/ml. Supplement 10 exhibited the lowest presence at 5.27 ±0.14 log CFU/ml, whereas supplement 1 exhibited the highest presence at 9.10 ± 0.04 log CFU/ml. Viability post storage period in group 2 ranged from 4.34 to 7.89 log CFU/ml. Supplement 10 exhibited the lowest number at 4.34 ± 0.16 log CFU/ml, and supplement 9

exhibited the highest number at 7.89 ± 0.26 log CFU/ml. viability post storage period in group 3 ranged from 4.08 to 7.55 log CFU/ml. Supplement 10 exhibited the lowest population count at 4.08 ± 0.09 log CFU/ml. Supplement 9 exhibited the highest population count at 7.55 ± 0.19.

A comparison of viability pre- and post storage period reveals a reduction in bacterial population count. The maximum reduction in group 1 was found to be 3 log CFU/ml (Supplement 6), followed by 1 log CFU/ml (Supplement 2, 7, 10). All other supplements exhibited reductions ≤ 1 log CFU/ml (Supplements 3 and 9). The maximum reduction in group 2 was found to be 2 log CFU/ml (Supplement 6), followed by 1 log CFU/ml (Supplement 2). All other supplements exhibited reductions ≤ 1 log CFU/ml (Supplements 1, 3, 5, 7, 9, and 10).The maximum reduction in group 3 was found to be ≤ 1 log CFU/ml (Supplements 5, 7, 9, and 10). One supplement, however, did demonstrate ≤1 log CFU/ml increase (Supplement 3).

A few supplements were able to maintain their initial population count after the storage period (Supplements 1, 4, 5, 78, and 10). Post storage viability indicates an increased likelihood of supplement capability to exert health-promoting effects as claimed on the label, up to human consumption. Supplements 1, 5, and 7 each exhibited population counts that are maintained and met the required 8 log CFU/mL minimum. Supplements 4, 8 and 10, however, maintained their initial population count but do not meet the suggested minimum 8 log CFU/mL of microorganisms required to confer positive health effects. Based on the label description supplements 4, and 8 should contain 10+ and 2 billion CFUs respectively. Supplement 10 did not claim a CFU amount on the label. This large number of functional bacteria present during consumption ensures a sufficient amount of the probiotic microorganisms reach the large intestine.

Table 6

Bacterial population in different types of supplements during refrigeration storage (4° C) for 4 weeks.

	Bacterial population (Log CFU/mL)					
Supplements	***Lactobacillus* spp.**		***L. acidophilus***		***Bifidobacterium* spp.**	
	Storage period (weeks)					
	0	**4**	**0**	**4**	**0**	**4**
1	9.39±0.00	9.10±0.04	NP	NP	NP	NP
2	9.12±0.02	7.39±0.00	8.81±0.06	6.89±0.70	NP	NP
3	8.54±0.00	7.96±0.11	7.00±0.30	6.32±0.02	6.87±0.53	7.25±0.02
4	7.76±0.08	7.79±0.02	NP	NP	4.94±0.07	4.49±0.07
5	9.2±0.00	8.59±0.00	6.47±0.00	6.39±0.00	NP	NP
6	8.61±0.00	6.39±0.00	8.07±0.00	4.39±0.00	NP	NP
7	8.80±0.00	8.77±0.00	6.39±0.00	5.07±0.00	6.69±0.00	6.47±0.00
8	5.39±0.00	5.39±0.00	NP	NP	4.38±0.00	4.38±0.00
9	8.44±0.34	8.41±0.38	8.24±0.50	7.98±0.26	7.86±0.06	7.55±0.19
10	5.86±0.24	5.27±0.14	5.63±0.21	4.34±0.16	4.89±0.33	4.08±0.09

Values are means of duplicate samples; ± indicates standard deviation from the mean.
NP: not present; the target strain was not present in the supplement therefore was not plated.

4.1.2 β- Galactosidase (β-gal) Activity

Table 4.2 shows the *β* –gal activity of the ten commercial probiotic supplements both in the presence of glucose (uninduced) and lactose (induced). The mechanism of this color change can be seen in Figure 4.1 below. It involves the breakdown of ONPG (colorless) into galactose (colorless) and o-Nitrophenol (yellow). The bright yellow color signifies the breakdown of lactose; increasing intensity of the yellow color indicates increasing β-gal activity (Figures 4.2 and 4.3). The activity of *β*-gal in the uninduced group, control, ranged between 26and 860 Miller unit/mL, and activity in the induced group ranged from 160to 1,120 Miller unit/mL. The average β- gal activity of the control samples was 170 Miller units. The induction of probiotic

supplements with lactose increased the average β- gal activity. Lactose acted as a carbohydrate source on the induction of β-gal activity. Notable increases from uninduced to induced enzyme activity include: Supplement 5, 50 to 1,120 Miller units; Supplement 6, 28 to 1,068 Miller units; and Supplement 8, 26 to 1,065 Miller units. Two supplements exhibited minor increases, but maintained relatively high β-gal activity levels. Supplement 3, 860 to 885 Miller units; and Supplement 4, 700 to 715 Miller units. Supplements 5 exhibited the strongest enzyme activity at 1,120 Miller units. Supplement 10 exhibited the lowest enzyme activity at 1.45 Miller units. Supplements exhibiting the lowest enzyme activity and lowest increase from uninduced to induced include: Supplement 1, 0.80 to 15 Miller units; Supplement 7, 12 to 160 Miller units; and Supplement 10, 0 to 1.45 Miller units.

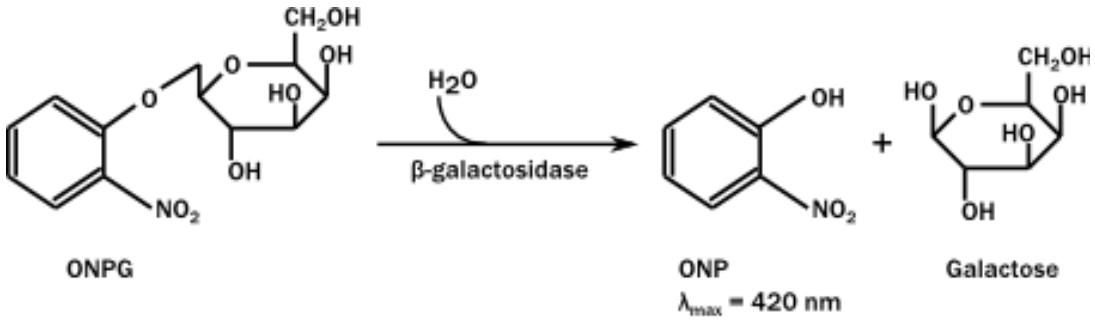

Figure 7 Mechanism of β-galactosidase.

Table 7

β -galactosidase activity in commercial probiotic supplements.

β- Galactosidase activity (Miller units)		
Supplements	*Uninduced*	*Induced*
Supplement 1	0	15
Supplement 2	0	775
Supplement 3	860	885
Supplement 4	700	715
Supplement 5	50	1,120
Supplement 6	28	1,068
Supplement 7	12	160
Supplement 8	26	1,065
Supplement 9	5	6
Supplement 10	0	1

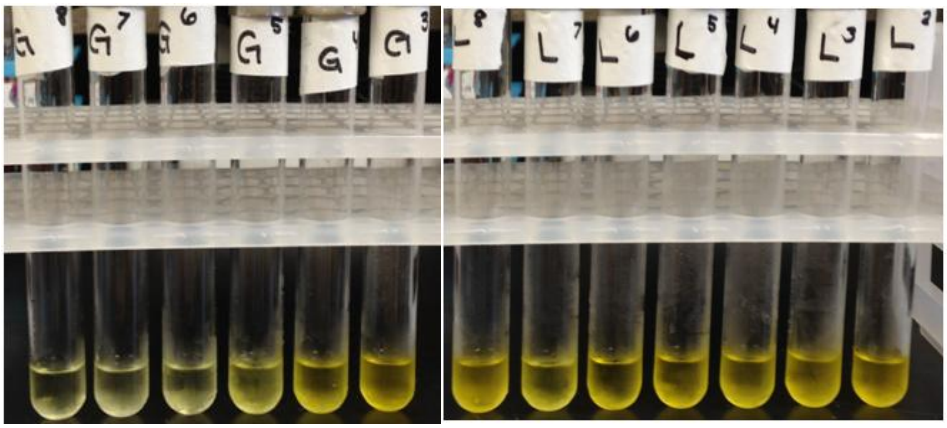

Figure 8 (left) β -gal activity in the presence of glucose (uninduced). (right) β -gal activity in the presence of lactose (induced).

4.2 Objective 2

Since Supplement 5 demonstrated viability and maintained viability during refrigerated storage, it was chosen for additional experiments in objective 2. In this objective we attempt to understand the impact of aspirin and caffeine on the functionality of a probiotic supplement. In this project, functionality was determined by performing: (a) β- galactosidase activity, (b) bile resistance, (c) reducing power, and (d) protein expression.

4.2.1 Exposure to aspirin and caffeine

In the experiment an aspirin stock composed of 1 gram of aspirin and 10 mL of Dimethyl sulfoxide (DMSO) was prepared to determine the impact of aspirin on the supplement. After a 12 h growth period at 37°C the supplement was serially diluted in 9 mL of peptone water. Then 100µl of aspirin stock was added to each dilution and incubated for 2 h at 37°C. The exposure of Supplement 5 to aspirin resulted in a population decrease from 8.95±0.14 to 2.20±3.10 log CFU/mL. When bacterial cells exposed to caffeine there was a slight decrease to 8.72±0.04 log CFU/mL.

4.2.2 β- galactosidase (β- gal) activity

The effect of aspirin and caffeine on the β-gal activity of a probiotic supplement (Supplement 5) was determined. The levels of β-gal activity varied dependent on the exposure to caffeine or aspirin. After 2 h of incubation, enzyme activity of supplement 5 can be seen in Table 4.3 Supplement 5 exhibited an increase to 1090 Miller units compared to the control at 500 Miller units after being exposed to caffeine. Supplement 5 treated with aspirin showed a significant decrease in activity, declining to 62 Miller units.

Additionally, we determined the effect of aspirin on supplement 5 at progressively lower inoculums. The initial β-gal was 697.5 Miller units, while the first inoculum level demonstrated

an increase to 1629.5 Miller units, each subsequent dilution factor decreased in enzyme activity. These results are consistent with research that stresses the importance of probiotic consumption in order to replenish or sustain the level of beneficial bacteria in the microbiota. The higher inoculum levels did not exhibit significant enzyme activity decreases after exposure to aspirin compared to lower inoculum levels which demonstrated weaker enzyme activity post aspirin exposure.

The increase of β-galactosidase after exposure of caffeine can be contributed to the hydrolysis of the cell membrane by caffeine. Caffeine allows the enzyme to be released in the media, whereas aspirin inhibits protein synthesis.

Table 8

(a) Supplement β- gal activity post aspirin and caffeine exposure. (b) β – gal activity in decreasing inoculum levels.

(a) β- Galactosidase activity (Miller units)

Treatment	*Uninduced*	*Induced*
Control	100 ± 84	500 ± 42
Caffeine	162 ± 32	1090 ± 287
Aspirin	51 ± 18	62 ± 79

(b) β-Galactosidase activity in decreasing inoculum levels (Miller units)

Dilution Factor	*Uninduced*	*Induced*
Control	162.5	697.5
-1	200	1629.5
-2	87.5	319.5
-3	6	190.5

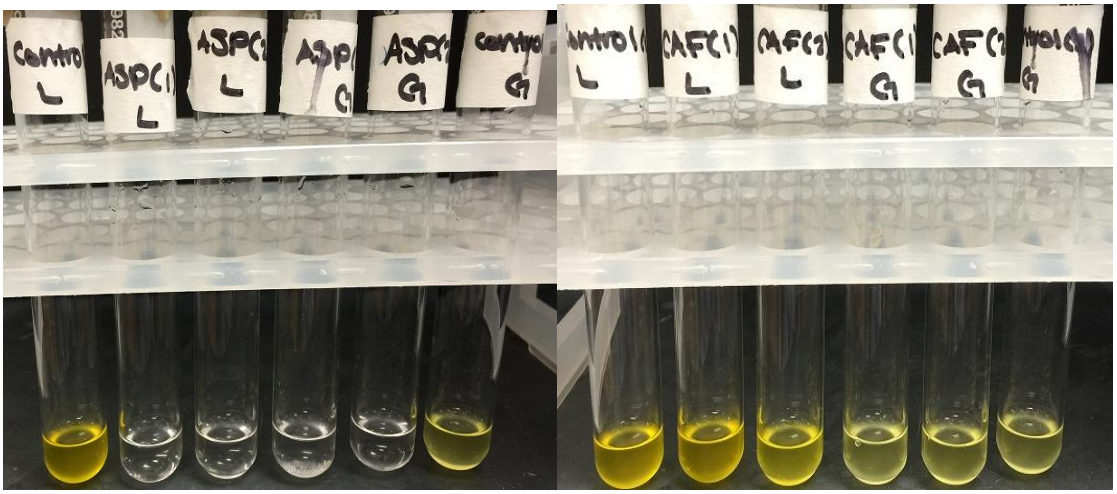

Figure 9 (left) β- gal activity after exposure to aspirin and (right) after exposure to caffeine.

Table 9

β-gal activity post exposure to 3% bile.

	β- Galactosidase activity (Miller units)	
Treatment	***Induced***	***Uninduced***
Control	486 ± 122	52 ± 60
Bile	780 ± 290	327 ± 10

We also evaluated the effect of bile (3% w/v) on the β-gal activity of supplement 5. An incubation period of 2 h resulted in an increase of supplement enzyme activity as seen in Table 4.4. Supplement 5 exhibited an increase to 780 Miller units compared to the control at 486 Miller units after being exposed to bile (3% w/v). Similar studies of the effect of bile on β-gal activity also found the activity to be greater if bile was included in the medium. Zarate et al. 2000 observed the β-gal activity enhancement of *P. acidipropionici* in the presence of bile salts due to the permeabilization of the cells during the first hour of exposure (Zárate, Chaia, González, &

Oliver, 2000). These effects have also been demonstrated in lactobacilli species (De Valdez et al., 1997; Noh & Gilliland, 1993).

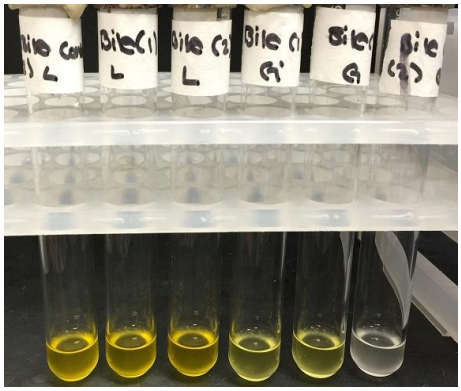

Figure 10 β-gal activity after exposure to bile (3%).

4.2.3 Bile resistance

The survival of Supplement 5 in the presence of bile (3% w/v) was determined. The initial population was 9.14 ± 0.09 log CFU/mL. Post exposure the population slightly decreased to 8.99 ± 0.04 log CFU/mL.

Following survival, bile resistance was investigated. After drug treatment, the surviving LAB cells from supplement 5 were resuspended in 9 mL MRS broth with or without 3% (w/v) bile. Table 4.5 shows the bile resistance of the probiotic supplement after exposure to aspirin. The decreasing dilution factors represent the decrease in bacteria population in the presence of consistent amounts of aspirin (100 µl). Resistance to bile is an important property for probiotic strains.

Table 10

Bile resistance of probiotic supplement after exposure to aspirin.

	Bile Resistance (log CFU/mL)	
	Bile (3% w/v) Exposure	***No Bile Exposure***
Control	7.88 ± 0.26	7.98 ± 0.55
Treatment (aspirin)	5.63 ± 0.15	5.48 ± 0.28

4.2.4 Reducing power

Antioxidant properties were assayed in terms of antioxidant activity (AOA) by reducing power. The behavior of strains found in Supplement 5 in was determined by measuring changes in the absorbance at 700 nm for cultures exposed to caffeine and aspirin. The reducing power of supplement 5 was 0.39.

Figure 11 Reducing power to probiotic supplement. (Left: blank; right: supplement)

4.2.5 Protein expression

The effect of aspirin and caffeine on the protein expression of supplement 5 was examined. First protein concentration was established. Protein concentration detects total protein. Figure 12 shows protein concentration of supplement 5. There was a decrease in protein concentration from 836.21 µg/ml (control) to 547.59 µg/ml after exposure to aspirin. Similarly, when the supplement was exposed to caffeine, protein concentration decreased from 836.21µg/ml (control) to 652.13 µg/ml.

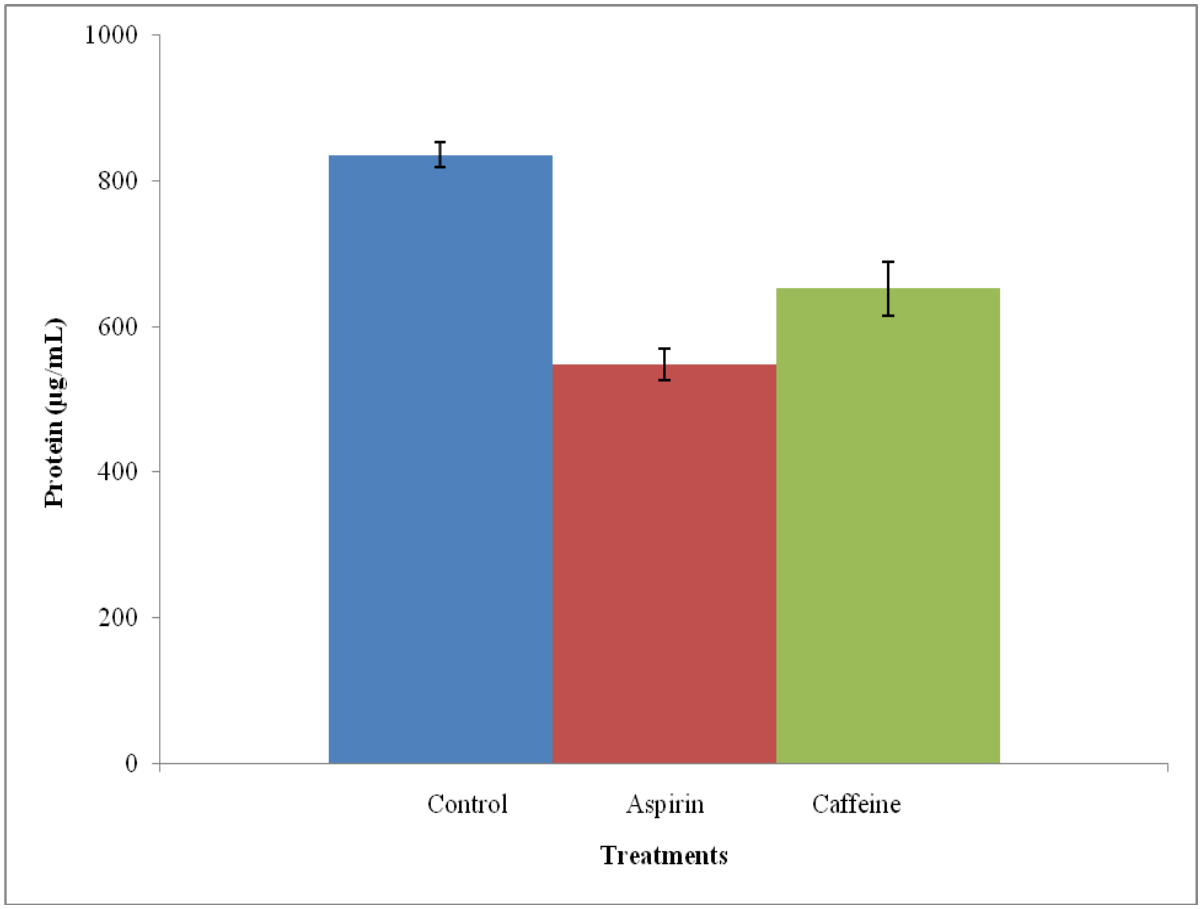

Figure 12 Protein concentration of supplement exposed to aspirin and caffeine.

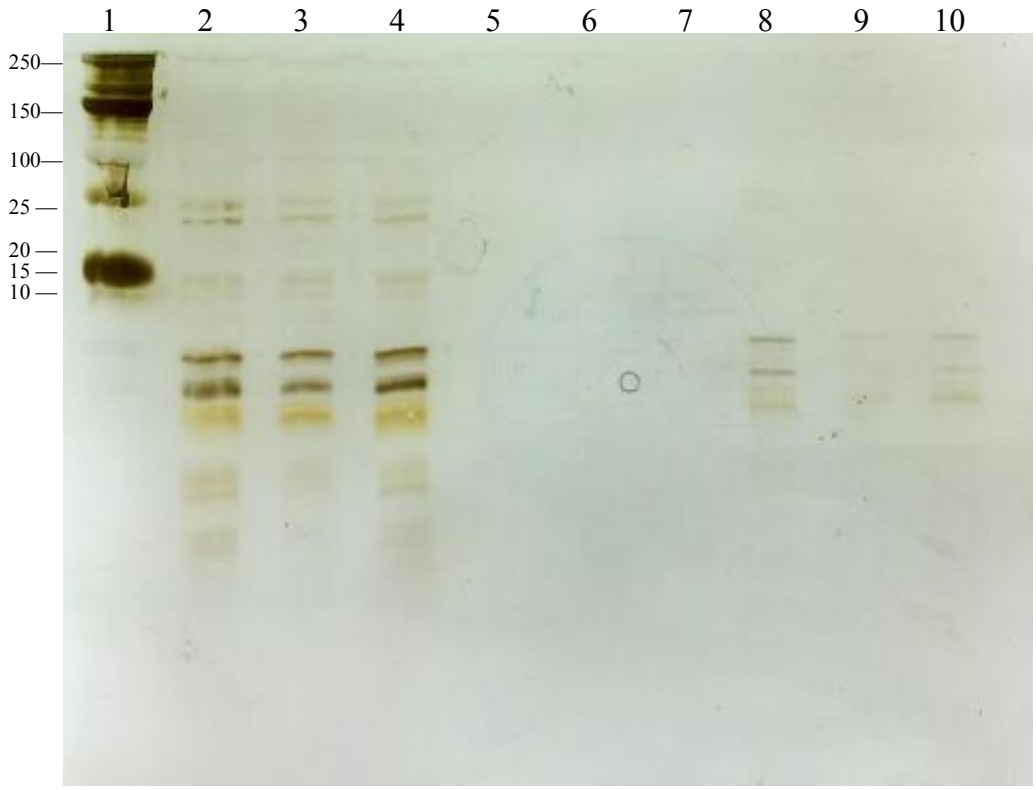

Figure 13 Protein expression (Silver stain)

The result of total protein was confirmed with a gel. From left to right, Figure 13 shows lanes containing the 1) marker, 2) control cells, 3) aspirin treated cells, 4) caffeine treated cells, 5) control supernant, 6) aspirin treated supernant, 7) caffeine treated supernant, 8) control mix, 9) aspirin treated mix, and 10) caffeine treated mix. For explanatory purposes, these samples were grouped into 3 categories. Samples 2 to 4 were grouped as I, characterized by the protein source being the pelleted bacterial cells. Samples 5 to 7 were grouped as II, characterized by the protein source being the supernant. Samples 8 to 10 were grouped as III, characterized by the protein source being a mixture of the supernant and pelleted cells.

Protein expression of Supplement 5 (Figure 13), was markedly affected when treated with aspirin in comparison to treatment with caffeine and the control. Aspirin was found to inhibit individual proteins. Observations indicate that in group I, 9 groups are present in sample 2, 7 groups are present in sample 3, and 8 groups are present in sample 4. In group I the lower profile is absent in both aspirin and caffeine treatments. Group III shows 6 groups are present in the sample 8, 3 groups are present in sample 9, and 3 groups are present in sample 10. The number of observable bands was lower than in untreated samples.

In terms of observable total bands as individuals from group I, sample 2 has 15 bands, sample 3 has 8 bands, and sample 4 has 10 bands. From group III, sample 8 has 6 bands, sample 9 has 3 bands, and sample 10 has 3 observable total bands.

The molecular weight of the probiotic bacteria are lower than 250 kD, which was to be expected. The effect of aspirin and caffeine were different; aspirin had effect on the lower bands and caffeine affected the lowest band the most. Proteins were only detected in samples containing cells; it was these proteins that treatment affected. The supernant demonstrated no protein presence.

CHAPTER 5

Discussion and Future Research

In this project, we have two objectives which were to determine: (a) the viability of probiotics in commercial probiotic supplements in laboratory medium by surface plating and measuring β- galactosidase activity, and (b) the interaction between probiotic supplements and commonly administered medical drugs of elderly people, and commonly consumed caffeine.

The first experiment of Objective 1, we determined bacterial populations by counting CFU/ml after 24 hr incubation at 37°C. Ten commercial dietary supplements were surface plated on selective and/or differential media based on their probiotic content. Viability was demonstrated via presence upon surface plating on respective media, thus confirming the presence of bacterial populations claimed on the supplement labels. Declines in population were observed in a few supplements after the storage period of four weeks. Our results indicate that storage, even as directed by labels, impacts the viability of probiotics in commercial dietary supplements. Ibrahim and Carr (2006) reported, similarly, that probiotic cultures in commercial yogurt brands were not viable after 3-4 weeks of storage at 4°C.Increasing knowledge on the effects storage has on probiotic products is a crucial aspect of ensuring health benefits are conferred. Functionality and health benefits of probiotic supplements may vary widely.

The second experiment of Objective 1 measured β-gal activity of the supplements. This enzyme activity is important in the breakdown of lactose, the main sugar in milk. Milk contains 4.8% lactose (Chandan, 1997). Many humans suffer from lactose intolerance, the inability to digest lactose into its constituents, glucose and galactose, due to low levels of lactase enzyme in the brush border of the duodenum (Rusynyk & Still, 2001). When undigested lactose remains in

the gut of a lactose intolerant individual a range of symptoms can arise. These symptoms typically occur within two hours of lactose ingestion and include abdominal pain, bloating, borborygmi (stomach growling), diarrhea, nausea, and vomiting (van Griethuysen-Dilber, Flaschel, & Renken, 1988). *β*–gal catalyzes the hydrolysis of lactose by breaking the β 1–4 linkage between glucose and galactose. Lactose is believed to trigger β- galactosidase activity by the lac operon mechanism (Reznikoff & Miller, 1980).

Our results were consistent with previous studies in which the presence of lactose in media enhanced β-gal activity when compared to the presence of glucose. Akolkar *et al.* (2005) and Hsu *et al.* (2005) found that the presence of lactose in the media led to β-gal activity enhancement for bifidobacteria and *L. acidophilus* specifically, which also supports the results found in our study. We also examined the effect of bile on *β*–gal activity and found an increase in enzyme activity when bile was included in the medium. This finding is consistent with that of Zarate et al. 2000 who contributed this enzyme activity enhancement to the permeabilization of probiotic strains by bile. This allowed more substrate to enter the cells to be hydrolyzed by *β*–galactosidase (Zárate et al., 2000).

Overall lactose intolerant individuals can be well treated by dietary modification and education one properly diagnosed with the condition (S. A. Ibrahim & Gyawali, 2013). Milk and other dairy products can remain in the diet of lactose maldigesters without them experiencing symptoms through this dietary modification. The addition of probiotics like supplement 5 could be an effective means of alleviating lactose intolerance.

Objective 2 was conducted using supplement 5 which demonstrated a high viability and maintained viability during refrigerated storage during Objective 1. In this objective we attempted to understand the impact of aspirin and caffeine on the functionality of a probiotic

supplement; functionality was determined by performing: (a) β- galactosidase activity, (b) bile resistance, (c) reducing power, and (d) protein expression. The first experiment of the second objective determined the impact of aspirin and caffeine exposure on the supplement. We used both caffeine and aspirin due to caffeine‘s use as an adjuvant to the analgesic actions of aspirin and paracetamol, and because of the high consumption of caffeine containing products such as coffee and tea (Gokulakrishnan et al. 2004). Caffeine is a purine alkaloid which acts as a central nervous system stimulant and also has negative withdrawal effects (Gokulakrishnan et al. 2004). Coffee and tea plants are the major sources of natural caffeine. Average consumption of caffeine in humans ranges from 80-400 mg/ (person day) (Gokulakrishnan et al. 2004).

Exposure to aspirin was found to decrease bacterial population approximately 6.75 log CFU/ml, and exposure to caffeine decreased population approximately 0.23 log CFU/ml. Our results are supported by research that has also demonstrated the negative impact of aspirin on survival rate of probiotic bacteria. Thus indicating the possible negative role of aspirin, in certain doses, on the microbiota.

The second experiment of Objective 2 determined the effect of aspirin and caffeine on the β-gal activity of supplement 5. Aspirin exposure caused a decrease in enzyme activity while caffeine caused a significant increase. This increase in enzyme activity is believed to be contributed to caffeine hydrolyzing the cell membrane, allowing for the nutritive media to enter the cell.

The third experiment of Objective 2 investigated the survival of supplement 5 in the presence of bile (3% w/v) and bile resistance. Supplement 5 demonstrated the ability to survive in the presence of bile by exhibiting a slight decrease to 8.99 ± 0.04 from 9.14 ± 0.09 the control. The survival of supplement 5 meets the criteria for probiotic bacteria.

The fourth experiment of Objective 2 examined the reducing power of supplement 5. Reducing power is often used to evaluate the ability of an antioxidant to donate an electron. Although the reducing power of supplement 5 was found to be relatively low, the supplement does prove to have some antioxidant properties.

The fifth experiment of Objective 2 determined the protein expression of supplement 5. We found BPER useful for lysing bacteria, specifically probiotic bacteria. Our BCA results showed that treatment, with caffeine and aspirin, reduced the total protein concentration of bacteria. SDS Page using silver stain detected major protein bands. Treatment changed the number and intensity of low molecular bands. There were unique target proteins affected by caffeine and aspirin respectively. Overall, treatment with caffeine and aspirin both affected concentration of the target protein.

In conclusion, during the protein expression experiment we identified a useful approach using BPER and SDS Page (12% gel and silver stain) to evaluate probiotic quality that can impact efficacy. Upon completion of the SDS page researchers concluded that the use of 12% gels successful, and in terms of staining the gel a Silver stain was a better approach than the Coomassie stain due to its sensitivity for detection of bacterial proteins. The results of both the Silver and Coomassie stains can be found above (figures 13 and 14 respectively). Both stains were allowed to set overnight.

Overall, the BCA (total protein), cell counts, and protein profile corroborate each other. The protein profile indicates distinct proteins may be the target of aspirin and caffeine. The prominent bands ~75-37 kD are not as susceptible as the lower weight band. Future studies could investigate and identify these proteins and find what factors make them resistant to the effects of aspirin and caffeine. In addition to affecting the viability of bacteria, treatment also affects the

concentration and type of protein in probiotic bacteria. Further studies on the significance of these proteins in probiotic health promoting functions are needed.

Table 5.1 provides a list of additional ingredients found in the probiotic supplements investigated during this study. Many of these ingredients possess prebiotic properties that are believed to boost the health promoting attributes of the probiotic cultures in the supplements. A predominant ingredient in the dietary supplements is inulin (chicory root extract). This soluble dietary fiber is not digested or absorbed in the stomach. Inulin passes through to the large intestine where it ferments and is used by the microbiota as a fuel source. Ingredient content could have an effect on the β- gal activity in the supplements.

Table 11

β-gal activity of probiotic supplements (Miller units) and additional ingredients (excluding probiotic strains).

Supplements	***Induced***	***Uninduced***	***Ingredients***
Supplement 1	15	0	Inulin (chicory root extract) Hypromellose Vegetable magnesium stearate Silica Titanium dioxide (color)
Supplement 2	775	0	Vitamin C Cellulose powder Dehydrated potato powder Organic garbanzo bean (chick-pea) extract Vegetable capsule (hypromellose) L-leucine.
Supplement 3	885	860	Vegetable cellulose capsule Cellulose Stearic acid Magnesium stearate Maltodextrin
Supplement 4	715	700	Inulin (derived from chicory root) Vegetarian capsule (hydroxypropylmethl-cellulose, water) InTactic proprietary polysaccharide complex L-leucine
Supplement 5	1,120	50	Vitamin C (ascorbic acid) NutraFlorascFOS (short-chain fructooligosaccharides) Plant-derived capsule Aqueous enteric-coating (modified cellulose, sodium alginate, stearic acid, fractionate [non-hydrogenated] coconut oil, Oleic acid) Magnesium stearate
Supplement 6	1,070	28	Rice starch Vegetarian capsule (cellulose, water)
Supplement 7	160	12	FOS (fructooligosaccharides) Kosher vegetable capsules Vegetable cellulose Vegetable magnesium stearate
Supplement 8	1,065	26	Microcrystalline cellulose Vegetable cellulose

			Tapioca starch Ascorbylpalmitate Silica
Supplement 9	6	5	Gelatin Starch (potato, tapioca and corn) Silica
Supplement 10	1.45	0	Calcium carbonate Sodium croscarmellose Silica Magnesium stearate (vegetable source) Rice maltodextrin Vegetable coating Bacterial proteases

Based on the results of this study we found that storage of probiotic supplements effects viability and believe that there is a need to address increasing the knowledge on the effects of storage on probiotic products to ensure health benefits are conferred to consumers. We also found that aspirin can reduce the bacterial population of probiotic supplements. However recent studies have found that the dosage of 81 mg of aspirin may not decrease the microbiota population, but change it. Future work should be directed toward the dosage of aspirin and the functionality of the microbiota in order to understand how these two components may work together in promoting heart health. The change in the functionality of the microflora could be a key element in how the microbiota and aspirin work together in heart attack prevention.

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