Examination of Probiotics Labeling: Analyzing the Accuracy of Labeling on Probiotic Products

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Molecular Microbiology and Immunology and the Honors Program

by

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We recommend that the thesis prepared under our supervision by

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entitled

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Abstract

Probiotics are defined as live microorganisms that provide effective benefits to the host by improving the properties of the microbial world of the organism (Nieuwbor, Burgwel, & Classen, 2016). Different strains of probiotic organisms are in use to work through several interrelated mechanisms to promote health at the molecular level (Killian, 2012). As the usage of probiotics constantly expands and more consumers are demanding more probiotic intakes, the market is expanding with the production of probiotics. Consumers are buying probiotics thinking that probiotics are great investment to enhance their overall health. According to the National Health Statistics Reports, Americans spent billions of dollars on natural products supplements annually and that value is increasing around the globe (Nahin, Barnes, & Stussman, 2016). The purpose of the project is to examine the accuracy of probiotic product labeling by comparing the information on the label to the experimental data. The four probiotics products that were used in the research are Ultimate 10 Probiotics, Digestive Advantages Probiotics, Digestive Probiotics, and Women’s Care Ultimate Flora Probiotics. These products were randomly selected with no prior knowledge of the products nor preferences. The analysis showed that there are similarities between what is on the probiotic labeling in comparison to what the experimental data present with limitations. Further research is recommended in the future to gain more knowledge about the probiotics world.
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**Introduction**

Probiotics are defined as live microorganisms that provide effective benefits to the host by improving the properties of the microbial world of the organism (Nieuwbor, Burgwel, & Classen, 2016). Though they are naturally occurring, probiotic supplements are commercially available for purchase and consumption. Although people often think that microorganisms are harmful “germs,” probiotics have shown valuable innovative potential in helping people digest their food better, while also improving the functions of multiple major organs and systems in the body (“Probiotics: In Depth”, 2018). Being the first person to introduce the term “probiotics”, Vergin was “studying the detrimental effects of antibiotics and other microbial substances on the gut microbial population” (Pandey, Naik, & Vakil, 2015, p. 7577). Different strains of probiotic organisms are in use to work through several interrelated mechanisms to promote health at the molecular level (Killian, 2012). These mechanisms include repelling against dangerous foreign organisms, reducing the risk of infections or toxin-mediated diseases, regulating immune responses while enhancing healthy reactions, and improving the ability to act as a barrier to the entry of further potentially dangerous microorganisms and chemicals (Killian, 2012). As the usage of probiotics constantly expands and more consumers are demanding more probiotic intakes, the market is expanding with the production of probiotics.

According to the *National Health Statistics Reports*, Americans spent billions of dollars on natural products supplements and that value is continuing to rise (Nahin, Barnes, & Stussman, 2016). Among those sold, probiotic dietary supplements have been known to be in the top selling products related to enhancing health. As the highest retail value of probiotics, the U.S. spends an estimated of $41 billion in 2015 with a growth rate of 37% of people consuming or purchasing probiotic by the year 2020 (Feldman, 2016). As of 2015, 70% of the probiotic market is yogurt
and sour milk products while only 30% are pure probiotic supplements (Feldman, 2016). Half way around the world, the European’s consumption of probiotics in food and dietary supplements has a consumer market value at more than €1.4 billion and an annual growth of sales set at approximately 7.5% with about 10% coming from probiotic’s sales in the next five years (Saxelin, 2008). In 2002, their marketplace had a significant growth in sales by allowing free promotion of digestive health claims in probiotic yogurt (Feldman, 2016). As the number continues to increase, probiotics contribute to the economic growth of the American nation.

A question that is still being debated around the globe is whether or not the probiotics does provide what it has in the product itself. “Although probiotics provide great benefits to the overall health of the host and brings in a huge amount of revenue for the nation, it is very unclear on whether or not the microorganisms and their effects on humans are accurately represented on the label of the probiotic products. In the health care field, physicians are required to have the medicines they prescribe or recommend [to their patients to be] tested, shown to have clinical effects, and be produced in reliable, reproducible produce formulations… [but] the current research-funding environments has not [been] conducive to sufficiently adequate testing of many probiotic strains in clinical practice…” (Reid, Jass, Sebulsky, & McCormick, 2003, p. 659). Due to the lack of significant evidence, a great amount of probiotic research is still being developed because there are still questions that microbiologists need to answer in order to have a settled agreement on the accuracy of probiotic products and their labeling. Consumers may look at the probiotics and trust the phases that are enlisted on products such as “number one in healthcare,” “daily support supplement,” and “healthier digestion and more energy” and assume they are true without the need to question. No matter how much the contents on the labels look like they are
true, the question remains: do probiotics really provide what the label claims, or are they just words trying to advertise their product and promote sales?

The objective of this project is to examine the accuracy of probiotic products labeling by comparing the information about the different microorganisms listed on the label versus the experimental data collection about the microorganisms. In order to count the number of colony forming units (CFUs) in the probiotic samples, a serial dilution method was used to count the colonies more precisely. After diluting to a ratio that would be easy to count the colony, the samples were placed onto agar plates to be incubated for a day or two to allow colony growth. Another goal was to analyze the different bacteria that are inside the supplements in comparison to the information provided on the label. To perform such a task, two different methods of staining were performed to examine the morphology of the bacterial characteristics and to identify the type of bacteria in the original content of the probiotic samples.

Overall, the experimental results provide an analysis of probiotic labeling and reveal that probiotics does provide correct information with some flaws and misrepresentations in the production of these supplements. These flaws are critical to consumer understanding because people who buy probiotic are basically being lied to if consumers do not receive what they pay for. Although probiotic research shows health benefits, there are also drawbacks. Due to the advancement of modern technologies and the science field, certain products can be created through genetic modification that may seem normal on the outside, but it can cause dangerous effects to different parts of the human body without immediate symptoms. For example, research suggests that although probiotics are beneficial to the digestive system, they may be harmful to the brain (Holzer, Hassan, Jain, Reichmann, & Farzi, 2016). The harmful effects may not be written on the products’ labels. Therefore, it is important to know exactly what is in the probiotic products for
the benefit for the consumer’s overall health. This particular study aims to identify whether the labels accurately represent their respective products.

**Literature Review**

**Probiotics/Microbiome**

Probiotics have been defined in different perspectives depending on one’s own “understanding of the mechanisms of actions [and] their effects on health and the well-being of individuals” (Salminen, Ouwehand, Benno, & Lee, 1999, p. 107). The term came from the Greek language meaning “for life” (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gytopoulou, Skarmoutsou, & Fakiri, 2013). In recent years, the most common used definition for the term probiotics is the “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host” (Salminen, Ouwehand, Benno, & Lee, 1999, p 109). Probiotics are made of small microscopic organisms that perform specific tasks that impact the individual’s health and body.

The collection of microbes or microorganisms that create a “mini-ecosystem” is known as the microbiome. The “human microbiome is made up of communities of symbiotic, commercial and pathogenic microorganisms all of which call the body [their] home” (“Microbiome 101: Understanding Gut Microbiota”). The microbiome is built upon the foundation of its natural exposure to the microorganisms right at the moment of birth via passing through the birth canal (“Microbiome 101: Understanding Gut Microbiota”) and throughout the rest of the journey called life. A child being born through caesarean section will encounter different microbes in comparison to those individuals that goes through the birth canal (Callaway, 2016). In the microbial world, there are thousands of microorganisms that either benefit and/or harm the host’s body that they
invade in which the place is called its home. What is impressive about probiotics is the number of microbial species in the human body that influence probiotic properties, which allow the microbes to perform specific tasks once they are inside of the host. Such tasks like boosting the immune responses or killing the harmful microorganisms depends on the interactions of the microorganisms and the host’s body. As the person begins to explore and get in contact with his or her environment as well as with other people, the composition of their microbiome is being affected (“Microbiome 101: Understanding Gut Microbiota”). Microbiologists have tried to connect the web between the human microbiome and the processes in the body in which they discovered that having an imbalance in the microbial communities can lead to countless health and diseases-related issues (“Microbiome 101: Understanding Gut Microbiota”). A person must be able to balance their microbiome to ensure the microbes can perform specific tasks in order to protect the body from foreign objects and to keep the human strong and healthy.

**Potential Benefits**

The communities of microorganisms carry out a variety of functions that are important for the well-being as well as for survival purposes of the host (“Microbiome 101: Understanding Gut Microbiota”). Studies of probiotics have shown valuable innovative potential in enhancing the medical needs of humanity (Reid, Jass, Sebulsky, & McCormick, 2003). Whenever people get sick or need an extra boost for their health, probiotics are taken in order to keep the natural balance of organisms (microflora) in the human body. With a variety of mechanisms, the purpose of probiotics is to “promote barrier integrity, [while] preventing antigens and pathogens from entering the mucosal tissues” into the body (Zhang, Li, Gan, Zhou, Xu, & Li, 2015, p. 7494) as well as “rang[ing] from bacteriocin [to] short change fatty acid production, lowering of gut pH, and
nutrients competition to stimulation of mucosal function and immunomodulation (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gytopoulou, Skarmoutsou, & Fakiri, 2013, p. 3).

One of the age categories that has been studied in the youth category and the benefits that probiotics have in children. From several studies, there is evidence that supports the usage of “probiotics with documented efficacy for the treatment” of the intestinal tract infections (Wanke & Szajewska, 2014, p. 9). The human intestinal tract carries about one to two kilograms of microbes where infectious diseases can occur; but, the majority of microbes can protect individuals from pathogens by colonization resistance, strengthening immunity, and enhancing the digestion process (Lee, 2014). Intestinal infections are common in newborns due to their underdeveloped immune system to prevent undesired bacterial colonization (Reid, Jass, Sebulsky, & McCormick, 2003); plus, it takes months before a child can fully fight off infections (Simon, Hollander, & McMichael, 2015) since new production of antibodies in the body required time. A human trail showed the most impressive indication that probiotics could benefit newborns with 2.5 x 10^8 live Lactobacillus acidophilus and 2.5 x 10^8 live Bifidobacterim infantis, in which the stains showed a “60% reduction in necrotizing enterocolitis and overall mortality” in newborns (Reid, Jass, Sebulsky, & McCormick, 2003). As a major health problem that reduces the quality of life due to stress and psychiatric disturbances, intestinal inflammation affected disease prognosis and negative responses to treatment (Holzer, Hassan, Jain, Reichmann, & Farzi, 2016). In 2011, a study was conducted to analyze the effects that butyrate has on intestinal diseases along with probiotics. The results showed that the compound has multiple possible effects that decrease the chance of reaching the late stage of intestinal disease (Canani, Costanzo, Leone, Pedata, Meli, & Calignano, 2011). As the targeted probiotics received more attention in treatment for disorders in the gastrointestinal tract, probiotic research revealed that probiotics had a significant negative impact
on brain function and behavior (Holzer, Hassan, Jain, Reichmann, & Farzi, 2016). In order words, probiotics that were beneficial for the gastrointestinal tract have negative effects in other parts of the body, such as the brain, which is something to take into consideration when developing these probiotics products.

Probiotic treatment for diarrhea in young children is also supported as a “documented efficacy for the treatment” (Wanke & Szajewska, 2014, p. 9). Diarrhea is defined by the increase in stool frequency, liquidity, or volume (Sweetser, 2012). When left untreated, diarrhea is a major cause of morbidity with approximately 5% of the “US population experiencing chronic diarrhea as defined by liquid stools lasting longer than 4 weeks” (Sweetser, 2012, p. 596). According to an article, treatment and prevention of infectious diarrhea are the most widely accepted health benefit of probiotics microorganisms (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013). Although several potential mechanisms were proposed for how Lactobacilli species reduce the duration of diarrhea, there is no actual evidence that gives support to the theory (Reid, Jass, Sebulsky, & McCormick, 2003). As a significant cause of mortality in developing countries, 16.5 million children under the age of 5 years are affected by diarrhea per year in the USA (McFarland, 2010). As more people from all ages are buying probiotics to improve their health status and to get rid of the ‘bad’ microbes in the body, probiotics has the potential to expand its course to other dimensions such as in clinical practices (Reid, Jass, Sebulsky, & McCormick, 2003).

Clinical Practices/Research Perspective

There has been an increasing number of claims that support the usage of probiotics to provide health benefits to the human body in the medical practices (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013). Several strains of
Probiotics have been tested and show a 78% significant effects for pediatric diarrhea, which suggests that the usage of several types of probiotic strains for pediatric diarrhea is supported with clinical evidence (McFarland, 2010). In 2014, a study was performed by Monika Wanke and Hanna Szajewska that show evidences on the efficiency of using probiotics to prevent diarrhea in children. While doing their research, they focused on the bacteria \textit{Lactobacillus rhamnousus} (LGG) strain to understand whether or not the bacteria has the capability in reducing the risk of diarrhea. Their studies provided evidence that supports the usage of LGG in diarrhea reduction and prevention (Wanke, & Szajewska, 2014). Generally, there has been both scientific and clinical studies on selective probiotics that demonstrated beneficial effects to reducing the risk of a certain disease and/or to increase the overall health of the individual (Lee, 2014).

In contrast, a significant number of studies suggest that probiotics have not been fully analyzed to conclude that the components in a probiotics supplement do what the microorganisms are supposed to do in the human body. There are also uncertainties about probiotics, such as safety usage ("Probiotics: In Dept", 2018). In 2003, the products that were used by many health care professionals to treat their patients contained \textit{Lactobacilli}, \textit{Bifidobacterium}, and other possible probiotics; but the advantages and disadvantages of these microorganisms were not fully evaluated (Reid, Jass, Sebulsky, & McCormick, 2003). Therefore, there was no evidence in knowing whether or not the treatments were efficient and effective. Since there is still a lack of support for probiotics usage and their capabilities, probiotics research continues to explore all the different type of probiotics products and to see if there is a probability that some manufacturers are not paying attention to the details of the ingredients or other factors that are in probiotic products but not mentioned on the labels and/or kept as secrets. Because there is not a standard set of evidence of how beneficial probiotics are, probiotics research has created a trend of experimental designs that
analyze the role and the impacts of taking supplements and the interactions the microbes had with the individual’s body and health (“Probiotics: In Depth”, 2018).

With the rise in the number of medical requests from patients, probiotics has reached high demands in a variety of clinical settings as the people involved in medicine are trying different methods to enhance health status. Such tasks in choosing the appropriate probiotic for the treatment can be challenging because of the small chance of matching the correct probiotic strain with the appropriate indication and amount (McFarland, 2010). Laboratory studies have opened up possibilities of probiotic supplementation to bring a new era of disease prevention and treatment (Lee, 2014) to be tested and to have each health-related property within the probiotics to be strain specific (Kechagia, Baseless, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013). According to an article written by M.E Sanders (2008), she described that the human microbiome is often the source where probiotic microbiota are isolated from to create the probiotic product but the microorganisms are not considered components of probiotics until the strains are fully isolated and characterized for content, stability, and health effect. The microorganisms that are considered to be true in probiotics belong to the *Lactobacillus* species and *Bifidobacterium* species; yet, there are some lactic acid and non-lactic acid bacteria that are adopted into probiotics products as well (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013). The activities of probiotics activities are strictly strain related; therefore, “strain identification is recommended in order to establish their suitability and performance for industrial application” with desirable properties (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013).

In research, strains are important to analyze because the different types can provide information about the properties and the characteristics of that specific probiotic species as well as
the species’ capabilities to perform tasks once inside the human’s body (Sanders, 2008). Certain species- or genus- specific contribution to the body, physically and physiological effects, can be found by a full and deep analysis of the strain of the microorganism (Sanders, 2008). The key approach in probiotics research is innovation, which is defined as the intentional introduction and application within a role, group, or organization, or ideas, processes, products procedure, new to relevant unit of adoption, designed to significantly benefit the individual, the group, or wider society (Nieuwbor, Burgwel, & Classen, 2016). Microbiologists try to understand the main barriers of innovation through interviews and questionnaires in terms of probiotics (Nieuwbor, Burgwel, & Classen, 2016), but there are no quality conclusions made due to the lack of sufficient data. M. van den Nieuwboer, al (2016), also set out an experiment to analyze how the microorganisms found in probiotics create beneficial effects to the human body. However, the clinical trial revealed a “lack of biomarkers to demonstrate effectiveness and…. still unclear how to demonstrate the effect of probiotics [particularly] in the healthy population” (Nieuwboer, Burgwel, & Classen, 2016, pp. 12-13). The lack of verifiable information, as found by the researchers, can degrade the innovation process in which knowledge cannot be built and the regulation of making these probiotics are not be effective.

**Marketplace Status**

“Probiotics represents an expanding research area” (Reid, Jass, Sebulsky, & McCormick, 2003, p. 658) which can range from one field to another such as medicine and the economy. For many years, medical microbiologists have been analyzing the clinical practices of probiotics and the efficiency of the products while ecologists are examining the impacts of probiotics at the economy and political level based on the number of sold probiotics products in the marketplace.
Though probiotics generally have great impacts in the human’s health and contribute considerably to a nation economy, one of the biggest evaluations in probiotics research is the accuracy in labeling on the products that are given in clinical practices and/or sold in stores and pharmacies. From previous research, many probiotic products have been discovered not to have proper identification and documentation, unacceptable manufacture practices and labels, and some inefficient clinically results (Reid, Jass, Sebulsky, & McCormick, 2003). An interesting study was based on the European’s perspective of probiotics being consumed in foods. In their view, “the market for food applications of probiotics is clearly larger than that for probiotics sold in capsules, sachets, and the pharmaceutical forms” (Saxelin, 2008, p. S76) is due to the consumptions of fermented daily products made from probiotic bacteria. With the lack of information, the idea questioned many nutritionists and microbiologists and made them become interested in analyzing the microbiota effects and to evaluate the accuracy of the labels of probiotics products (Saxelin, 2008) that are located in fermented daily products that are bought in stores. At this state, it is unclear on whether or not probiotic foods should be sold in grocery stores since there is limited evidence that shows whether the supplements can do what is claimed on the product’s labels.

With the increased amount of attention to the quality of probiotics products, it is possible that these products have improved (Weese & Martin, 2011) and created an impact on the nation’s income. Probiotic are sold everywhere around the globe to enhance the quality of life. At the same time, the profits that are being made from selling probiotics products benefit the economic as the incoming go beyond the average earning per year. Specially sold in foods-related products, a wide range of products contain probiotics strains and that number is still growing (Kechagia, Basoulis, Konstantopoulou, Dimitridadi, Gyftopoulou, Skarmoutsou, & Fakiri, 2013). The main products in the marketplace are dairy-based ones, which include fermented milks and cheese, ice cream,
Buttermilk, and yogurts. In the United States, probiotics is growing rapidly as it is evidently shown by the increase interest of industry, consumes, and researchers (Sanders, 2008).

To date, research have showed discrepancies between the stated and actual contents in commercial probiotic products (Sanders, 2008). Due to the high demands of probiotic products, there could be “mistakes” made along the path with either the manufacture and/or the handling process that may change the quality and quantity of the desired results in which are labeled on the products. A study was done by J. Scott Weese in 2003. All 44 probiotics were purchased at different location to avoid selection bias with 21 designated for human use and the remaining for veterinary use (Wesse, 2003). After performing some tests, the results indicated that the number of colony form units (CFUs) labeled on the container in comparison to the experimental data were quite variable, ranging from 1.3 million to 22 billion per gram (g), milliliter (ml), or capsule potentially leads to negative impacts in the human body. Another study was also done by J. Scott Weese with the company of Hayley Matin in 2011. Due to the increased attention of probiotics and other commercial nutraceuticals quality, two individuals worked together to test for the accuracy of the product’s label (Weese & Martin, 2011) on some probiotics. Based on the data they collected, the viable growth ranged 0 to 2x10^9 CFU / g. There was no growth claimed of the expected bacteria species in contrast to what the description on the product’s label presented. Through the findings of numerous product deficiencies such as inadequate description, grammatical errors, and not having precise bacterial numbers, efficacy studies have questioned the accuracy of probiotic labeling (Weese & Martin, 2011).

Since the term probiotics does not have a legal definition, it gives people who create these probiotics the opportunity to avoid meeting fundamental criteria that are enlisted in the scientific definition of the term “probiotics” (Sanders, 2008); therefore, there is a chance of inaccurate
labeling and information given to the public. According to federal law, it is critical that all prescribed drugs and any related medical products have correct labeling with different sections (description, clinical pharmacology, indications and usage, contraindications, warnings, precautions, drug abuse and dependence, dosage and administration, effective date to expiratory, etc.) on the container that explain everything about that specific product (“Federal Register: Department of Health and Services”, 2006). Along with the sections, there is a specific formatting that must be follow and labels must be clear of spelling and grammatical errors on the labeling (“Federal Register: Department of Health and Services”, 2006). In the absence of a legal definition for the term “probiotic” while trying to prevent inaccurate information being passed out, it is necessary for industry participants adopt to the scientific definition of the term to ensure the proper factory processes with correct content on the labels (Sanders, 2008) and that the products are processed in the recommended method(s) by the federal government of health and service.

Research Question

Over a lifetime, each person develops a populated microbiome that has specific microbiota that somehow contribute to the health of that specific individual (Cho and Blaser, 2012). Because every person has a different microbial system and there is a possible chance of changes within the strains of the microorganisms each year, it is necessary to take the first step in establishing standards and guidelines to ensure that probiotic products have 100% accuracy on their labels and efficiency before being sold in stores. Probiotics boost the systems in the human body, so they are needed in daily lives; but, the rate of which probiotics are being marketed must not go to the level in which probiotics are being sold to make money. As mentioned before, probiotic labeling should have the correct number of organisms that are present to the strain level and the number of live organisms, no misspellings, and accurate content such as a guarantee expiratory date/time (Weese,
2003). Currently, there is still a lack of evidence and support indicating that probiotic labeling has great accuracy. Therefore, it is imperative to continue the analysis and evaluation of label claims on probiotic products to eliminate mistakes that may be made. Such inaccuracies in labeling may lead to undesirable results being inflicted upon individual(s) who consume probiotic-related food and/or erroneously thinking that they were enhancing their body’s health.

**Methodology**

The purpose of the experiment was to analyze the accuracy of commercial probiotics labeling by performing several methods to gather experimental data that were used to compare to what the information on the probiotics provides. In this specific experiment, four different probiotic products were used to evaluate the information on the labels of probiotics products. The four probiotics products were Ultimate 10 Probiotics, Digestive Advantages Probiotics, Digestive Probiotics, and Women’s Care Ultimate Flora Probiotics (pictures of each of the probiotic products are in the appendix). These products were randomly selected with no prior knowledge nor preferences. As mentioned in previous section, probiotics products contain microorganisms. Table 1 described the type of microorganisms that each capsule contains. Background research on the microorganisms was done to know if the bacteria is gram-positive or gram-negative, whether or not there are endospores in the capsule, and what shape is the bacteria (rod versus cocci). The research information would be useful when it comes to analyze and compare the morphology of the bacteria based on the stains to what is expected.
TABLE 1: Probiotic Supplement Information Each probiotic supplement contains different types of microorganisms that perform specific tasks in the body. The microorganism can either be gram-positive or gram-negative, have endospores or none at all, and be either a rod shape or cocci shape.

<table>
<thead>
<tr>
<th>Product</th>
<th>Species</th>
<th>Gram Stain</th>
<th>Endospores?</th>
<th>Rods/Cocci?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate 10 Probiotics</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus casei</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus acidophilus</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus plantanum</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus bulgaris</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus salivarius</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Bifidobacterum bifidum</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Bifidobacterum longum</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Bifidobacterum breve</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus thermophilus</em></td>
<td>+</td>
<td>No</td>
<td>Cocci</td>
</tr>
<tr>
<td>Digestive Advantages Probiotics</td>
<td><em>Bacillus coagulans</em></td>
<td>+</td>
<td>Yes</td>
<td>Rod</td>
</tr>
<tr>
<td>Digestive Probiotics</td>
<td><em>Bifidobacterium lactis</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus plantarum</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td>Women’s Care Ultimate Flora Probiotics</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus gasseri</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus brevis</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus casei</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus paracasei</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus plantarum</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus salivarius</em></td>
<td>+</td>
<td>No</td>
<td>Rod</td>
</tr>
</tbody>
</table>

Through serial dilution and two types of staining methods, the data can provide information in regard to if the bacteria that are enlisted on the labels match the morphology of the experimental data for each probiotic product.
**Experiment One: Serial Dilution and Growing Bacteria**

A serial dilution is a series of sequential steps that reduces the density of cells cultures to a more usable concentration by a specific amount. With this method, the calculation of colonies growth was more reasonable to obtain. To perform the task, there are steps to follow as well as calculations to have accurate data analysis. Illustration 1 showed a basic image of how each probiotic supplement was diluted and the setup for the serial dilution test is shown in Figure 1. The steps in how the probiotic supplement was diluted and the setup was the same for all the probiotics products.

![Illustration 1: Serial Dilution Steps](image)

**ILLUSTRATION 1: Serial Dilution Steps** A supplement was dissolved in 99 mL of solution test tube. One milliliter of the solution is added to another test tube filled with 99 mL of solution. This step is repeated two times. On the last dilution, there 1 mL of the solution is added into 9 mL of solution. 0.1 mL of solution from the $10^5$ dilution was plated. 1 mL of solution from the $10^6$ dilution was plated. From the $10^7$ test tubes, 0.1 mL and 1 mL of the solutions was plated onto separate plates. Each of the dilution plates was plated three times.
FIGURE 1: Serial Dilution (A) The picture shows the setup for serial dilution. The containers that have the orange cap are the pure neutral and acidic solutions. The ones with the black caps are the diluted solutions. There are also 24 plates (12 TSA and 12 Lactobacillus agar plates). The microscopic slides were used to prepare the sample for staining. The test tubes were used for dissolving the supplement in the two different solutions. (B) The picture shows a picture of me preforming the serial dilution process.

Because the research is performed in-vitro, two types of solutions were used to analyze the interaction between the supplement and the stomach acid / small intestine. Neutral pH solution (~pH 7) was made with saline and sodium hydroxide to represent the liquid in the small intestine while acidic pH solution (~pH 2.5) with saline and hydrochloride acid represent the stomach acid. In order to start the process of dilution, each supplement was put into a container filled with 99 mL of solution to be completely dissolved with some parts of the capsule parts still floating around. The next few steps involved diluting the concentration by 100-fold into 99 mL of solution and the last container of only 9 mL of solution to obtain a certain concentration to plate the sample. To have accurate results, a range of dilutions ($10^5$ - $10^8$) was created to have efficient data. Each dilution was plated onto two different types of agar plates, Typtic Soy Agar (TSA) plates and Lactobacillus agar plates. Different types of agar plates were used is to give the bacteria the chance to grow depending on what nutrients they need. The TSA plate is usually the general type that microbiologists used to grow bacteria colonies while the Lactobacillus agar plate provide nutrients to certain bacteria, mainly Lactobacillus species. To have a reasonable value for the colony count,
each dilution was plated three times on two separate types of agar plates in which a total of 24 plates were used per supplement (96 plates in total) which is shown in Figure 2.

![Figure 2: Plates with Colony Growth](image)

**FIGURE 2: Plates with Colony Growth** All 96 plates were colonized with the prepared mix of the saline solution and components from the probiotic products.

Once the samples were evenly spread onto the plates by using aseptic techniques (refers to the appendix), the plates were placed in a 37°C incubation for one to two days. If no growth were shown, the plates were placed back into the incubation for another day or two. Once colony growth was observed on the plates, the plates were analyzed for any abnormal growth. From each plate, isolated colonies were counted and recorded.

Looking at the colonies with the naked eye does not show any characteristics of the bacteria. In order to visualize the “live” or vegetative microorganisms, simple staining such as gram stain and endospore stain were performed to analyze the morphology of the cells through the microscope which is shown in Figure 3.
Experiment Two: Gram Stain

Gram staining is a technique used to recognize the differences between two large groups of bacteria based on their different cell wall components (Bruckner, 2016). The method distinguishes between gram-positive (violet color due to a thick layer of peptidoglycan cell wall) and gram-negative (red color due to a thin peptidoglycan cell wall). How gram stain works depends on the thickness of the peptidoglycan layer in the cell membrane (Bruckner, 2016). The gram staining method shows the shape of the microorganism as well as the thickness of the peptidoglycan layer in the cell wall. To conduct this test, the materials that are necessary include microscope slides and a microscope, cultures of the organisms, distilled water and immersion oil, methanol, lens paper/cleaner, and reagents (crystal violet, grams iodine, 50/50 ethanol/acetone, and safranin). [Check appendix for reagent reference.] Each supplement was taken out of its capsule and dissolved in 99 mL of each solution in separate test tubes before starting the method. The first step was to put a droplet of culture sample onto the microscope slide and allow the smear to air-dry, then to add a droplet of methanol on the culture to “fix” the culture and allow it to air-dry. The next step was to place the
slide on the staining tray and gently flood the smear with crystal violet and let it stand for one minute. After one minute, I tilted the slide slightly and gently rinsed it with tap water or distilled water using a wash bottle. I gently flooded the smear with grams iodine and let it stand for one minute. Again, tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The next step requires fast reaction. Decolorize using 50/50 ethanol/acetone (tilt the slide slightly and apply the alcohol drop for 5 to 10 seconds until the alcohol runs almost clear; be careful not to over-decolorize). Immediately rinse with water. Finally, gently flood the smear with safranin and let it stand for one minute. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle and allow it to air-dry. Using the light-microscope, view the smear using a light-microscope under oil immersion. [Directions on how to use the microscope is in the appendix.]

**Experiment Three: Control Test with Samples**

Performing any scientific experiments can have limitations as well as data not making any sense. To confirm the data that was collected from gram-staining, a control test was performed with each supplement. Two control samples are required to represent gram-positive and gram-negative. In this experiment, the controls were *Escherichia coli* (*E.coli*) and *Staphylococcus epidermidis* (*S. epidermidis*). The *S. epidermidis* is a gram-positive cocci and *E. coli* is a gram-negative rod. The bacteria were prepared and grown on plates two days prior to the test. On the day of the test, the components from each of the supplement was taken out of its capsule and dissolved in 3 mL of distilled water. Once the supplements completely dissolved, each sample was placed on a microscopic slide by using aseptic technique, which is shown in Figure 3. The two controls were placed on each side of the sample. Illustration 2 showed how the solutions were
placed on the microscopic slide. After the samples were prepared, gram staining method is as followed (check experiment two for the procedure) and slides were viewed under the microscope.

ILLUSTRATION 2: Microscopy Slide for Control Test The supplement sample was placed on the center of the microscopic slide. *Staphylococcus epidermidis* was placed on the left side and *Escherichia coli* was placed on the right side.

FIGURE 4: Using Aseptic Technique A picture of me using aseptic technique to inoculate samples onto microscopic slides to perform the control test.
Experiment Four: Endospore Stain

The endospore stain is a differential stain that is used to detect the presence and location of endospores in bacterial cells. Being able to concentrate and coat their protoplasm allows them to survive the harmful environmental conditions they experience in their unusual habitat and to have the spores resist staining. As for the results, there are two possible colors: pink rods (cultures are young) and green ovals (cultures are too old). Ideally, it is one best interest to see the green oval bodies of the endospores surrounded by the pink vegetative bacterial cell.

To perform this test, each supplement was prepared by taking the components out of its capsule and dissolving the sample in 99 mL of each solution (neutral and acidic) in separate test tubes. First step was to make a smear of the specimen by using aseptic technique and methanol fix the sample. After the methanol was dry, I flooded the smear with 5% malachite green and allow the stain to react at least 30 minutes (can go longer) at room temperature. More stain can be added if the slide begins to dry out. Then, I rinsed the slide with water and flooded the specimen with 0.25% safranin for 1 to 5 minutes. After about five minutes, I rinsed the slide with water and allow the slide to air-dry. Lastly, I viewed the microscopic slide underneath the microscope and recorded the observations.

Results

Experiment One: Serial Dilution and Growing Bacteria

The serial dilution method provided efficient information about the probiotic supplements that are useful in probiotic research. In the first part, each supplement was timed to see how long the compounds would take to dissolve in neutralized saline and acidic saline. Table 2 shows the amount of time it took for each supplement to dissolve in the solutions.
TABLE 2: Time of Probiotic Supplement Dissolvent  Each supplement was taken into account in the estimated time in how long the probiotic components would take to break out of its capsule and be completely dissolved between neutralized saline and acidic saline.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Neutralized Saline (Capsule Broken)</th>
<th>Neutralized Saline (Complete dissolved)</th>
<th>Acidic Saline (Capsule Broken)</th>
<th>Acidic Saline (Complete dissolved)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate 10 Probiotics</td>
<td>40 minutes</td>
<td>85 minutes</td>
<td>35 minutes</td>
<td>75 minutes</td>
</tr>
<tr>
<td>Digestive Advantages Probiotics</td>
<td>80 minutes</td>
<td>102 minutes</td>
<td>80 minutes</td>
<td>101 minutes</td>
</tr>
<tr>
<td>Digestive Probiotics</td>
<td>22 minutes</td>
<td>137 minutes</td>
<td>27 minutes</td>
<td>137 minutes</td>
</tr>
<tr>
<td>Women’s Care Ultimate Flora Probiotics</td>
<td>45 minutes</td>
<td>85 minutes</td>
<td>45 minutes</td>
<td>135 minutes</td>
</tr>
</tbody>
</table>

The time frames show what products dissolve when, which indicates when/how much would be absorbed by the solution of the stomach acid and small intestines. Table 2 shows that it takes longer for the product to dissolve than what was expected. Based on biology, the stomach breaks everything down before the components enter the small intestine. Table 2 indicates that probiotics products do not follow the expected path of the digestive system. Depending on the metabolism of the individual, the amount of time for the food to pass through the stomach into the small intestine varies. Food generally stays in the stomach for about 20 to 30 minutes before entering the small intestine, where the food stays for a couple of hours. For example, the Women Care Ultimate Flora Probiotics would have its capsule broken after about 45 minutes. Since it takes about 30 minutes at the most where food stays in the stomach, the result suggests that the capsule will not be broken in the stomach acid but rather in the small intestine where there is neutral...
solution as well as the components dissolving and being process / delivery to its designated location(s) in the body.

The final step of this experiment is counting the colonies. Table 3 shows a summarized version of the colony count. For the raw data of each supplement, check out the appendix.

**TABLE 3: Colony Count Data** The data presents how many isolated colonies were taken estimated with the naked eye for each probiotic product. The experimental values are located in the central column for each type of agar plate. The expected values of colony count are on the third column.

<table>
<thead>
<tr>
<th>Probiotic Supplement</th>
<th>Dilution with averages and type of plates (acidic versus neutral solution)</th>
<th>Expected with dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ultimate 10 Probiotics</strong></td>
<td>Tryptic Soy Agar plate 10^4 - 0 / 14 10^4 - 0 / 10 10^4 - 0 / 1 10^3 - 0 / 1 10^3 - 0 / 0.123 Original - 0 / 100</td>
<td>10^9 - 6 10^7 - 30 10^6 - 300 10^5 - 3000 Original - 2 x 10^10</td>
</tr>
<tr>
<td><strong>Digestive Advantages Probiotics</strong></td>
<td>Tryptic Soy Agar plate 10^4 - 0 / 1 10^4 - 4 / 10 10^4 - 20 / 0 Original - too many to count / too many to count</td>
<td>10^9 - 6 10^7 - 30 10^6 - 300 10^5 - 3000 Original - 2 x 10^10</td>
</tr>
<tr>
<td><strong>Digestive Probiotics</strong></td>
<td>Tryptic Soy Agar plate 10^4 - 0 / 1 10^4 - 4 / 10 10^4 - 20 / 0 Original - too many to count / too many to count</td>
<td>10^9 - 6 10^7 - 30 10^6 - 300 10^5 - 3000 Original - 2 x 10^10</td>
</tr>
<tr>
<td><strong>Women's Care Ultimate Flora Probiotics</strong></td>
<td>Tryptic Soy Agar plate 10^4 - 0 / 1 10^4 - 4 / 10 10^4 - 20 / 0 Original - too many to count / too many to count</td>
<td>10^9 - 6 10^7 - 30 10^6 - 300 10^5 - 3000 Original - 2 x 10^10</td>
</tr>
</tbody>
</table>

Based on the data, the amount of colony growth is either lower or higher / too many to count than what is expected. By looking at the data, there is a significant amount of plates that had zero colony growth, specifically the ones that were prepared in the acidic solution and plated on the Tryptic Soy Agar plates. There is a significant larger amount of growth on the *Lactobacillus* agar plates than the Tryptic Soy Agar plates. Between the solutions, the components that are were dissolved in neutralized pH had a larger amount of growth than the ones that were dissolved in acidic pH. The data shows that there is a possible chance that there are dead cells presented in the solutions.
because there should be similar number of cells in each of the diluted plates whether the cells were alive or dead.

Experiment Two: Gram Stain

The gram stain method is one of the techniques that allows the visualization of the bacteria in order to analyze the morphology and the type of wall the bacterial cell may have in order to identify the type of bacteria that are present on the slide. Figure 5 to 8 show the gram staining results of each of the probiotics that were examined from the two types of solutions that the supplement components were dissolved in.

**FIGURE 5: Ultimate 10 Probiotics Gram Stain** (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

**FIGURE 6: Digestive Advantages Probiotics Gram Stain** (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.
FIGURE 7: Digestive Probiotics Gram Stain (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

FIGURE 8: Women’s Care Ultimate Flora Probiotics Gram Stain (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

The gram-stain results show mostly dark purple with a few areas having pink color cells in the neutralized ones but a lot of pink / red stain than purple in the acid ones. From experiment one, it was predicted that there could be possible dead cells on the plates that could explain why there was not much growth in about 1/3 of the plates that have the sample. Since gram positive is represented by purple, the gram-stain matches with what was expected from Table 1 for the samples that was prepared in neutralized solution. As for the acidic ones, something might have occurred that have caused the value of colony growth to varies between zero to a few to too many
Whether the cells were alive or dead after the interaction with the solution provide crucial information that suggested that bacterial cells does not have a great survival rate in the acidic versus the neutralized solution. At the same time, the idea that the cells were alive before the components were dissolved in the solution is still debatable. With deep analysis at what the data present, there are still more to discover.

**Experiment Three: Control Test with Samples**

The control test was performed to confirm the results that was collected from the gram-staining experiment. Table 4 showed the results between the experimental design mainly presented and what was expected.

**TABLE 4: Control Test Result** Each probiotic sample was tested with the two control plates to confirm the gram-stain test. The results showed that two of the four products were different from what was expected while the other two products came out to what was expected of them.

<table>
<thead>
<tr>
<th>Probiotics</th>
<th>Experimental Gram-stain</th>
<th>Expected Gram-stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate 10 Probiotics</td>
<td>Gram-positive</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Digestive Advantages Probiotics</td>
<td>Gram-negative</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>Digestive Probiotics</td>
<td>Gram-negative</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>Women's Care Ultimate Flora Probiotics</td>
<td>Gram-positive</td>
<td>Gram-positive</td>
</tr>
</tbody>
</table>

By performing the control test, each of the samples had purple and pink stains but the main color was listed in Table 4 in the experimental gram-stain column. The control test supported that there are some cells that are gram-positive and others that are gram-negative on the microscopic slides. The data also supported the results from experiment two and the idea that there are live and dead cells in the samples after the probiotics are dissolved in the solutions.
Experiment Four: Endospore Stain

During the bacterial research, it was found that Digestive Advantages Probiotics has endospores bacteria in the supplement. To test for the existence of endospores, an endospore strain was performed because any endospores present will be shown in the stain. Figure 9 to 12 showed the results of the endospore stain test.

**FIGURE 9: Ultimate 10 Probiotic Endospores Stain** (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

**FIGURE 10: Digestive Advantage Probiotics Endospores Stain** (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.
FIGURE 11: Digestive Probiotics Endospores Stain (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

FIGURE 12: Women’s Care Flora Probiotics Endospores Stain (A) The picture shows the result of the supplement when it was dissolved in acidic saline. (B) The picture shows the result of the supplement when it was dissolved in neutralized saline.

As expected, only one of the four probiotics products showed the endospores which is represented by the dark green dots in Figure 10. All the cells that were present are stained in pink but the color does not mean anything besides the idea that the cells exist on the prepared slides. The data that is presented from this experiment suggests that the bacteria labeling on the probiotics products is accurate since the endospores stain does show existence of endospores in the Digestive Advantage Probiotics endospore stain. The other three probiotic products did not show any existence of
endospores. Overall, the endospores stain results 100% supported Table 1 that shows what was expected from the tests.

**Discussion**

**Analysis and Interpretation of the Data**

Based on the probiotics research and the experimental data from each probiotic product, the information between the probiotic’s labeling and the data does not have a 100% match but there was a significant amount of similarities that supported the idea that the labels are accurate with limitations.

Based on the products that were used in the thesis, the data from Table 2 revealed that the probiotics components are not released from its capsule until they are actually in the small intestine because it takes more than 30 minutes for the capsule to be broken and to completely dissolve. The information is very important for probiotics research because it suggested that probiotics absorption in the body actually takes place in the small intestine instead of inside the stomach. It also means that those people who believed probiotics capsules are generally broken down in the stomach have false information.

From the serial dilution and colony count experiment, the data show that there is a significant difference between what is expected and what was experimentally observed. The table showed that there is more growth in the *Lactobacillus* agar plates than the Tryptic Soy Agar plates. Between the two solutions that were used to dissolve the solutions in, the cells grew more in the neutralized one than the acidic one. There could be many factors that occurred for the experimental results to happen. The main possibility for the occurrence of the colony growth differences is the idea that there are a certain number of bacteria that were dead once they are plated on the agar
plates, which means that those dead cells could not grow even if nutrients are provided. The chance of them being killed can range from the time the probiotic was manufactured till it was dissolved in the solutions. A possible explanation is that a portion of the cell population within each supplement were killed by the acidic solution as the components were dissolving because there was less growth on the plates that contained the cells that were dissolved in acid saline. This information is important for probiotics research because the data showed that not all billions of bacteria that are noted on the label would actually take effects in the human body and the host will not get all the benefits that are listed on the label since some of the cells would be dead by the time the probiotic components are in the body.

From the gram-stain test, the pictures of the specimen on the microscopic slides show violet and red stains. On most of the slides, the colors of the cells were red/pink which means the bacteria were gram-negative and the bacteria had a thin peptidoglycan wall. There were also purple cells which means the bacteria were gram-positive and the bacteria had a thick peptidoglycan wall. Based on what was predicted from the serial dilution due to the colony count results, the idea that there could be dead cells present in the prepared solutions is supported by what the gram-stain show. The background research from Table 1 presents that the bacteria should be gram positive so it is expected to have purple/violet stains, which is present on certain areas of the slide. There is a lot of areas that have pink stains which means there are a lot of dead cells. Besides the stains, the shape of the bacterial cells is rod-shaped, which is expected based on the background research. The control test showed both gram-positive and gram-negative color stains but Table 4 shows what the majority of the cells stain in.

Out of the four probiotic products, the Digestive Advantage Probiotics supplement is the only probiotic that contains bacterial microorganisms with endospores. From the endospores stain
test, the results also show that the Digestive Advantage Probiotics supplement contain endospores, which are the dark green circular shape in the center of pink vegetative cells (Figure 10). All the other probiotics products showed the lack of green dots in the center of the pink vegetative cells, which means that the bacteria do not form any endospore. The confirmation of endospores in the Digestive Advantage Probiotics suggests that the labeling of the bacteria is accurate.

Further Research

Overall, the experimental data does confirm the background research with a few addition information; but further research is still necessary in order to fully analyze the accuracy of the information on the probiotic labeling. Since there is a possible chance that other outside factors could have cause changes in the components of the probiotic supplement between the manufacturing processes to the time when the supplement is consumed, it is also important to take those into consideration when the data are being analyzed. The probiotics that were used in the experiment are for digestive system; therefore, the results do have limitations. It is crucial to also analyze other probiotics that provide benefits to different types of systems in the human body such as the immune or cardiovascular. It is recommended that future probiotics research should design experiments that look into all types of probiotics that enhance an individual’s health such as for brain development, immunity strength, and heart health as well as those probiotics that help prevent disease-related bacteria. The data would be valuable in providing a great range of probiotics and the accuracy on their labels to the world of knowledge.

Probiotics products play a huge and great role in society, but it is incredible to know that there is information that are still yet to be discover. Generally, scientists would like to have a better understanding of the probiotics world; therefore, more probiotics research designs are important to continue the learning process about probiotics and to explore different levels of probiotics.
Because most consumers relied on the probiotic labels to provide correct information about the product, it is critical that the information on the labels are accurate as possible and provide the maximum benefits to the people who consume probiotic to enhance their overall health.
Appendix

Probiotic Products

Microscope Usage

The field of microbiology requires a special type of technology that allows a person to see very small objects that can’t be seen with the naked eye. Using the microscope can be difficult at first, but it is easy to use once the individual learned how to properly operate one. The main key to keep
in mind is to be careful when handling so they will last for many years. Microscopes are expensive scientific instruments and can break easily if not properly use. The following steps explain the proper use of a microscope after the microscopic slides are prepared. When moving the microscope, always carry it with both hands. Grasp the arm with one hand and place the other hand under the base for support. Turn the revolving nosepiece so that the lowest power objective lens is “clicked” into position. Place the microscope slide on the stage and fasten it with the stage clips. Using the coarse adjustment, lower the objective lens down as far as it will go without touching the slide. Look through the eyepiece and adjust the illuminator (or mirror) and diaphragm for the greatest amount of light. Slowly turn the coarse adjustment so that the objective lens goes up away from the slide). Continue until the image comes into focus. Use the fine adjustment, if available, for fine focusing. Move the microscope slide around so that the image is in the center of the field of view and readjust the mirror, illuminator or diaphragm for the clearest image. One should be able to change to the next objective lenses with only slight focusing adjustment. Use the fine adjustment, if available. If one cannot focus on the specimen, repeat step 4 through 7 with the highest power objective lens in place. DO NOT ALLOW THE LENS TO TOUCH THE SLIDE! The proper way to use a monocular microscope is to look through the eyepiece with one eye and keep the other eye open (this helps avoid eye strain). If the individual has to close one eye when looking into the microscope, it’s ok. Do not touch the glass part of the lenses with the fingers. Use only special lens paper to clean the lenses. When finished, raise the tube, click the low power lens into position and remove the slide. When using the microscope, oil immersion needs to be added until to view the specimen through the 1000x. Generally, the microscope allows a person to see all the unique features of a microorganism and to perform effective and efficiency research. With microscopic skills, different types of stains and morphology analysis can be performed.
Gram Stain Reagents

“The gram stain reagents are used to determine the gram reaction for microorganisms’ identification” (“Gram Stains Reagent”). Each reagent of the gram-stain has a special purpose. The primary stain is called crystal violet, which stains the bacterial cell. The mordant one is the grams iodine and binds the stain with the cell. The 50/50 ethanol/acetone is the decolorizer reagent. It differentiates bacteria by retaining the crystal violet or not within the cell wall. The secondary stain is the safranin, which is the counterstain if the bacteria cannot retain the crystal violet by the cell wall.

Aseptic Techniques

The practice of aseptic technique is crucial to the field of microbiology that involves a variety of procedures to prevent contamination of cultures (“Aseptic Technique and the Transfer of Microorganisms”). These procedures include transferring cultures, inoculating media, isolation of pure cultures, and performing microbiological tests. Through any laboratory work, personal protection equipment (PPE) must be worn at all time to keep the individual safe from harm.

In the research, there were two different aseptic techniques that were used: transferring sample from broth to a plate and transferring sample from broth to microscopic slide. During the serial dilution and colony count procedure, transferring sample from the most diluted broth to a plate required aseptic technique to prevent any contamination and undesirable growth on the plates. To do so, an inoculation loop is sterilized with flame from any heat source before and after each use. After the loop has been sterilized and cooled, the loop is dip into the broth using one hand while the other hand is holding the clean plate. There are many different ways to plate the sample; therefore, check out the article “Aseptic Laboratory Techniques: Plating Methods” (2012) to learn about the different types and how each procedure is performed. During the stain tests, the sample
was transferred from the broth to a microscopic slide. Similar to the aseptic plating technique, the inoculation loop is sterilized with available heat and be cooled before being inserted into the broth to grab some of the liquid. Then, gently smear and spread the sample on the slide. Lastly, sterilized the loop with available heat.

Overall, practicing aseptic techniques is important to prevent any contamination and to receive the most accurate and pure results as possible.

**Colony Count (Raw Data)**

Table 5 to 8 are the raw data for the colony count for each probiotic product. Table 3 is the summarized version for all four probiotic products.
### TABLE 5: Ultimate 10 Probiotics Serial Dilution and Colony Count

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>TSA (AS)</th>
<th>AVG</th>
<th>TSA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-8}$</td>
<td>0, 0, 1</td>
<td>0</td>
<td>13, 17, 11</td>
<td>~14</td>
<td>5</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1, 0, 0</td>
<td>0</td>
<td>115, 115, 91</td>
<td>107</td>
<td>52</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>1, 3, 0</td>
<td>~1</td>
<td>231, 205, 207</td>
<td>~214</td>
<td>520</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>1, 0, 0</td>
<td>0</td>
<td>48, 40, 35</td>
<td>123</td>
<td>5200</td>
</tr>
<tr>
<td>original</td>
<td>0</td>
<td>0</td>
<td>150</td>
<td>150</td>
<td>5.2 x $10^8$</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>LA (AS)</th>
<th>AVG</th>
<th>LA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-8}$</td>
<td>0, 0, 0</td>
<td>0</td>
<td>17, 23, 35</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1, 0, 0</td>
<td>0</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>52</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0, 0, 0</td>
<td>0</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>520</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0, 0, 0</td>
<td>0</td>
<td>56, 65, 165</td>
<td>62</td>
<td>5200</td>
</tr>
<tr>
<td>original</td>
<td>0</td>
<td>0</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>5.2 x $10^8$</td>
</tr>
</tbody>
</table>

**KEY:** LA = Lactobacillus Agar; TSA = Tryptic Soy Agar; AS = acid saline; NS = neutralized saline; AVG = average; TMC = too many count
<table>
<thead>
<tr>
<th>DILUTION</th>
<th>TSA (AS)</th>
<th>AVG</th>
<th>TSA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
</tr>
</thead>
<tbody>
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<tr>
<td>10^-7</td>
<td>1, 4, 6</td>
<td>~4</td>
<td>14, 7, 25</td>
<td>~15</td>
<td>8</td>
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<tr>
<td>10^-6</td>
<td>1, 0, 79</td>
<td>~24</td>
<td>85, 7, 28</td>
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<td>80</td>
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<tr>
<td>10^-5</td>
<td>23, 2, 4</td>
<td>29</td>
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<td>0</td>
<td>800</td>
</tr>
<tr>
<td>original</td>
<td>TMC</td>
<td>TMC</td>
<td>TMC</td>
<td>TMC</td>
<td>8x10^7</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>LA (AS)</th>
<th>AVG</th>
<th>LA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
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</thead>
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<tr>
<td>10^-7</td>
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<td>8</td>
</tr>
<tr>
<td>10^-6</td>
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<td>95</td>
<td>246, 286, 272</td>
<td>268</td>
<td>80</td>
</tr>
<tr>
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<td>20, 23, 3</td>
<td>~15</td>
<td>13, 22, 25</td>
<td>20</td>
<td>800</td>
</tr>
<tr>
<td>original</td>
<td>TMC</td>
<td>TMC</td>
<td>TMC</td>
<td>TMC</td>
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</tr>
</tbody>
</table>

KEY: LA = Lactobacillus Agar; TSA = Tryptic Soy Agar; AS = acid saline; NS = neutralized saline; AVG = average; TMC = too many count
### TABLE 7: Digestive Probiotics Serial Dilution and Colony Count

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>TSA (AS)</th>
<th>AVG</th>
<th>TSA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
</tr>
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<td>$10^{-8}$</td>
<td>756, 29, 28</td>
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<tr>
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<tr>
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<td>2760, 2760, 2760</td>
<td>2760</td>
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<tr>
<td>$10^{-5}$</td>
<td>0, 0, 0</td>
<td>0</td>
<td>839, 984, 1868</td>
<td>~1064</td>
<td>3000</td>
</tr>
<tr>
<td>Original</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>3x10^8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>LA (AS)</th>
<th>AVG</th>
<th>LA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-8}$</td>
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<td>0</td>
<td>2826, 2934, 2652</td>
<td>2804</td>
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<td>$10^{-7}$</td>
<td>TMC (smear)</td>
<td>TMC</td>
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<td>1, 9, 0</td>
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<td>1680, 1560, 1650</td>
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<td>TMC</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>3x10^8</td>
</tr>
</tbody>
</table>

**KEY:** LA = Lactobacillus Agar; TSA = Tryptic Soy Agar; AS = acid saline; NS = neutralized saline; AVG = average; TMC = too many count.
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<tr>
<th>DILUTION</th>
<th>TSA (AS)</th>
<th>AVG</th>
<th>TSA (NS)</th>
<th>AVG</th>
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<td>$10^{-6}$</td>
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<td>2000, 2168</td>
<td>~2165</td>
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<td>740, 540</td>
<td>548</td>
<td>6000</td>
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<td>TMC (smear)</td>
<td>TMC (smear)</td>
<td>TMC (smear)</td>
<td>TMC</td>
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<table>
<thead>
<tr>
<th>DILUTION</th>
<th>LA (AS)</th>
<th>AVG</th>
<th>LA (NS)</th>
<th>AVG</th>
<th>EXPECTED</th>
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<td>~472</td>
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<td>TMC (smear)</td>
<td>TMC</td>
<td>60</td>
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<tr>
<td>$10^{-6}$</td>
<td>TMC (smear)</td>
<td>TMC (smear)</td>
<td>TMC (smear)</td>
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<td>600</td>
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<tr>
<td>$10^{-5}$</td>
<td>2, 3, 4</td>
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<td>1008, 1052</td>
<td>~1032</td>
<td>6000</td>
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<tr>
<td>original</td>
<td>TMC (smear)</td>
<td>TMC (smear)</td>
<td>TMC (smear)</td>
<td>TMC</td>
<td>6x10^8</td>
</tr>
</tbody>
</table>

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References


http://www.microscope-microscope.org/activites/school/microscope-use.htm


https://doi.org/10.1016/S0924-2244(99)00027-8


