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An Overview of the Extracurricular Polysaccaride Production of Probiotic Lactic Acid Bacteria

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AN OVERVIEW OF THE EXTRACELLULAR POLYSACCARIDE PRODUCTION OF PROBIOTIC LACTIC ACID BACTERIA

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The study focuses on a comprehensive review of lactic acid bacteria and their production of extracellular polysaccharide substrates (EPS). Two important factors are addressed in this work including identification of LAB stains found in the commercially sold daily milk and the significant impacts of polysaccharide production. The motivation behind this study is to contribute to growing research of the probiotic benefits of lactic acid bacteria. With microbial EPS production being a recent interest of study among healthcare and food industries sectors, new species of bacteria are extensively exploited for further investigation to improve human health. Being that LAB is the more predominant population of bacteria in dairy milk, this research concentrates LAB byproducts known as extracellular polysaccharides (EPS) among three types of bacteria and evaluating any relationship between broth media viscosity and EPS production.

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LIST OF ABBREVIATIONS

EPS	Extracellular Polysaccharide Substrates
LAB	Lactic Acid Bacteria
CCD	Central Composite Design
RSM	Response Surface Methodology
GI	Gastrointestinal Tract
HePS	Heteropolysaccharides
HoPS	Homopolysaccharides
LTLT	Low Temperature Long Time
HTST	High Temperature Short Time
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
GRAS	Generally Recognized As Safe
SPP	Species Pluralis or Multiple Species
PCR	Polymerase Chain Reaction

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CHAPTER 1

INTRODUCTION

The COVID-19 pandemic occurring during the year 2020 has brought much attention to the topic of human wellness and self-care on a global level. Either if it is the use of hand sanitizers, facial mask, or even nitrile gloves, people everywhere around the world are taking the extra step to protect their bodies from these life-threatening viruses. Furthermore, it is common nowadays to see diets being personally tailored in the hope to boost one's immune system.

Advocates of a strong and effective immune system may often consider particular food options containing beneficial microbes. These organisms are often known as probiotics. It is studied that these microorganisms, as ingested, aid a healthy body by promoting beneficial health effects. One example is lactic acid bacteria (LAB)` which can be found in the gastrointestinal tract to decrease any pathogenic population present (Rafieian-Kopaei, 137). The basis of this study will be heavily revolved around the LAB EPS production and the effect on viscosity of resulting broth media.

LAB is most commonly known for their ability to produce long polysaccharides/ exopolysaccharides chains. These polymers consist of branched and repeating units of sugars in varied ratios have been reported (Pan & Mei, 2010; Wang et al., 2010; Li et al., 2014; Imran et al., 2016). The EPS produced have immense commercial importance

because of the industrially beneficial physico-chemical properties they exhibit and GRAS (generally recognized as safe) status of the LAB from which they are secreted (Surayot et al., 2014). Exopolysaccharide produced by LAB play essential roles in improving the taste, texture, and rheology of fermented food preparations. They also serve as food additives, prebiotics and demonstrate useful physiological effects such as anticarcinogenecity, antitumor, immunomodulating activities and as blood cholesterol-lowering agents in humans (Kim et al., 2010).

Different species of LAB, especially *Lactobacillus plantarum* have been reported to produce EPS (Wang et al., 2010; Imran et al., 2016). *L. plantarum* perform important fermentative roles during Nigerian traditional food preparation and provides positive health impacts which are strain specific. They exhibit an outstanding effect on the flavor and texture of these foods, with specific metabolic and technological properties, such as production of EPS (Adesulu-Dahunsi et al., 2017). Recently, researchers have reported that EPS produced from LAB species have antioxidant activities and are non-toxic. These characteristics are of great importance and may replace the synthetic antioxidants (Li et al., 2013; Zhang et al., 2013; Abdhul et al., 2014). Exopolysaccharides produced by Lactobacillus species improves sourdough properties by aiding water absorption, improving its structure, thereby prolonging shelf life of the fermented foods. Few works have been reported on EPS producing ability of LAB strains isolated from cereals-based fermented food. Included in these are the work of Torres-Rodríguez et al., 2014; Adesulu-Dahunsi et al., 2018; Adesulu-Dahunsi, Jeyaram, & Sanni, 2018).

The study focuses on a comprehensive review over the lactic acid bacteria and their production of EPS. Two important factors are addressed in the work; and these are 1)

identification of LAB stains found in the commercially sold daily milk, 2) significant factors impacting polysaccharide production. With microbial EPS production being a recent interest of study among healthcare and food industries sectors, new species of bacteria are extensively exploited for further investigation to improve human health, and other raw dairy materials.

There is high speculation that the production of proteins and other products will increase the viscosity of their growth media. Through a series of identification and isolation processes, EPS was able to be quantified. In this study, we discovered that an increase of EPS had a positive impact on the increased viscosity of their broth media. This information can be used to study which bacterial species may best be used for harvesting these beneficial probiotics.

CHAPTER 2

LITERATURE REVIEW

Probiotics

Human milk has always had a reputation of being sterile. However, this statement has been challenged and heavily debated over during the past decades. Surprisingly, reports have shown viable bacteria in healthy human milk (Oikonomou, 2). This brings us new and exciting study accentuating analytic approaches of bacteria among human milk.

The role of human milk serves an important role maintaining the gut microbiome. In addition, the bacteria act as a source of probiotic supplementation providing nutritional by-products to human milk such oligosaccharides for developing infant gut (Kordy, 2). This is an extremely vital discussion of research as many industries across many sectors incorporate lactic acid bacteria (LAB) for untapped human wellness and medicine. LAB can be found in a wide spectrum of environments including plants, land and marine animals, fermented food, and mucosal surfaces. They possess unique characteristics that are often utilized by medical and pharmaceutical industries to prevent the growth of microbial pathogens. In addition, certain LAB strains are able to influence both the innate and acquired immune system (Florou, 589). Most interestingly is the use of LAB today as probiotics and their recent exploitation of it as a new source of health benefits.

Probiotics may come in several forms, functions, and structures giving rising to several categories of these microorganisms. The four main categories of use of probiotics

are as: drugs, food, direct- fed microbes, or as genetically modified probiotics (Florou-Paneri, 591). The holistic approach of using probiotic LAB has been on the decrease ever since the rise of antibiotics. Increasing numbers of researchers, nutritionist, and healthcare professionals all strongly believe in the efficiency and health impact of LAB used as an alternative way of promoting health.

The gastrointestinal tract (GI) is protected mainly by the LAB present in the microbiota human flora. The bacteria triggers are able to promote its diverse effect on the host in several ways. The antimicrobial activity of LAB serves to block colonization of pathogenic bacteria. LAB decreases the luminal pH which inhibits bacterial adhesion and entrance to epithelial cells (Florou-Paneri 591). In addition, further antimicrobial compounds such as organic acids, defensins, bacteriocins, and hydrogen peroxide are all produced in response to foreign pathogenic invaders (Florou-Paneri 591). All these interactions taking place with the mucosal epithelial cells ultimately lead to enhanced immune response in the GI tract.

The next line of mechanism used by LAB allows enchantment of mucosal barrio against pathogens by mucus production. This is achieved by modifications of the cytoskeletal and tight junctional protein phosphorylation (Florou-Paneri, 591). Mucosal barrier can be further strengthened by LAB competing with pathogenic strains, such as Salmonella and *E. coli*, for binding sites (Florou-Paneri, 591). This process allows colonization of pathogens to be inhibited. These are few of many ways the mucosal barrier can be bolstered with the supplementation of LAB probiotic.

As mentioned earlier, probiotics are able to have much influence on both the innate and acquired immune system. The final mechanism involves immunomodulation of B-

lymphocytes and antibody production that has been shown to show an increase in IgA secretion and enhancement of response to vaccination (Florou-Paneri, 592). This is important because this allows the buildup and diversification of the host body's immune response reservoir. In this study, our goal will be to isolate probiotic LAB from dairy milk produce and analyze changes on their ability of secrete favorable byproducts.

In the food industry, LAB is essential for the production of fermented dairy products. These gram-positive bacteria are able to withstand technological stresses as well as biological stresses, such as environment of the gastrointestinal tract (Patil, 280). In addition, the byproducts produced by LAB offer heavy influence for the favorable rheological food properties (Patil, 280). For example, some bacterial strains produce exopolysaccharide (EPS) which is important for texture development in yogurt other fermented milks. EPS has recently gained much attention in the food industry due to their useful physico-chemical properties having immense commercial value.

Over the years, consumer demand for natural and beneficial healthy products has increased. The shifting trend from consumption of addictive substances, such as gelatin and starch, for natural bacterial probiotics has been a rising topic in research. The importance of this study is to further contribute to a global collaborative effort to better understand isolation and characterization of the beneficial properties of bacterial probiotics with hopes of fulfilling consumer demand.

LAB probiotic serves to promote health and well-being. Beneficial strains are derived from dairy production systems with LAB identified as the most common groups of microbial probiotic organisms (Colombo, 1). Probiotic bacteria by-products from food fermentation plays a vital role in lactose digestion, prevention of diarrhea, and stimulation

of the immune system (Colombo, 1). This comparative analysis will quantify and compare LAB EPS production cultured in different environmental conditions.

The increased knowledge of LAB and their EPS products have led to findings supporting alternative use of LAB and EPS yielding favorable natural ingredients from animals and plants.

Plant and animal LAB EPS have been increasingly used as an alternative source of nutrition and health benefits. Starter cultures for selected LAB strains are exploited to improve fermentation processes and quality of end products. Furthermore, selection of LAB strains that are known to have high yielding beneficial properties are now used as starter cultures to improve fermentation process and enhancement of by product's quality (Patil, 208).

Recently, EPS has become the fresh, new initiative in medicine exploiting the many benefits of bacterial polysaccharides. Studies have shown that these exopolysaccharides display health benefits making them exceptional candidates with the potential to completely replace synthetic drugs. In this study, we will be investigating the effects of EPS production in dairy milk LAB with media supplementation of metal ions, toxins, and incubation modifications. This will lead to more information regarding the abilities and limitations of these microbial probiotics.

Varying among strains, probiotic bacteria are able to produce a wide variety of EPS groups providing different functions. Morphologically, these polysaccharides, if covalently linked with the bacterial cell surface, may appear in the form as a capsule (Ahmad, 333). On the other hand, EPS can loosely bind to the cell surface in the form of slime allowing

them to be secreted into their surroundings (Ahmad, 333). These unique features were visually observed on MRS agar.

Moreover, another category of EPS includes two major groups of EPS. Depending on their repeating units of sugar, including glucose, galactose, and mannose, EPS can be classified as heteropolysaccharides or homopolysaccharides (Ahmad, 334). Homopolysaccharides contains only one sugar component whereas heteropolysaccharides consist of two or more sugar components.

The structural and chemical composition of EPS allows researchers to identify the type and functionality of LAB exopolysaccharides. The two types of EPS include heteropolysaccharides (HePS) and homopolysaccharides (HoPS) in which heteropolysaccharides are composed of D- galactose, D- glucose, and L- rhamnose and homopolysaccharides are made of either glucan or fructan (Leroy, 1).

Milk Microbiome

The microbiome of dairy milk is unique and driven by a wide range of microorganisms. Many microbes presented in milk provide a source of nutrient contents such as carbohydrates, vitamins, amino acids, and fats. They are often classified by what products they produce along with their biochemical properties such as gram type, sporulating vs non- sporulating, anaerobic vs non-aerobic, to even their morphology of being cocci or rod shaped. LAB is known to be the dominant population in milk including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptocossus*, and *Enterococcus* genera (Florou, 589). These type of cocci or rod-shaped bacteria have been classified as being gram positive, unable to sporulate and either anaerobes or facultative aerobes.

Furthermore, dairy milk microbiome may possess strains of non-LAB genera bacteria including yeasts and molds. Consequently, these forms of organisms may not contribute beneficial health implications seen with LAB bacteria. In many cases, microbial pathogens may contaminate milk leading to severe illnesses and diseases. These detrimental microbes are capable of surviving host immune surveillance and extreme environmental conditions. One prime example is psychrotrophs, a bacterial strain able to withstand cold temperatures. One type of these organisms seen in dairy milk is *Pseudomas* and *Acinetobacter spp* bacteria strains (Florou, 590). With the use of proteins such as lipases and proteases, they are able to proliferate during refrigeration and produces extracellular proteins that negatively impact the quality of milk (Florou, 590). Fortunately, there are processing techniques such as thermization, Low Temperature Long Time (LTLT) pasteurization, High Temperature Short Time (HTST) pasteurization and more, that are generally used for quality assurance treatment for raw milk.

The dairy milk microbiome houses a handful of beneficial microbes as well. These organisms are capable to aiding in digestion or by reducing allergies (Florou, 592). These bacteria are commonly defined as probiotics due to their role in health maintenance of treating diseases. One example is the probiotic bacteria *Lactobacillus reuteri* that is used as dietary supplementation assisting in younger appearance in both aged human and mice subjects (Zannini, 6). Another probiotic present in this microbiome is *Propionibacterium freudenreichii* which plays a crucial role in inducing apoptosis among human colon cancer cell lines by the release of anti-carniogenic metabolites and compounds (Cousin et. al, 1). Dairy's microbiome continues to be studied for a range of diverse microbes that can potentially play major roles in our health and food industries.

Lactic Acid Bacteria

As mentioned, LAB are the predominant microbes present in dairy milk and can also be found naturally in milk, cheese, meat, beverages, and vegetables (Ahmad, 336). LAB is highly utilized in the food industry often utilized as a major application in food fermentation. These bacteria can be further grouped into categories known as Homoefermenters or Hetereofermenters. The former produces lactic acid as their main product of glucose fermentation while Hetereofermenters produce lactic acid, carbon dioxide, acetic acid, and ethanol from glucose fermentation (Ahmad, 334). Through metabolic activities, LAB are able to produce organoleptic products to yield aroma and flavor compounds contributing to the general texture of fermented food (Florou 592). Hence, these microorganisms are studied and used for their fermentative ability in order to enhance food safety and providing much health benefits.

LAB plays a critical role in filtering out undesirable pathogens and other harmful bacteria. The antibacterial attributes of LAB are most commonly against both gram positive and negative bacteria strains such as *Escherichia coli*, *Pseudomas aeruginosa*, and *Staphylococcus aureus* (Colombo, 2). As of today, LAB is gaining much attention medically and environmentally as potential tools for pathogenic treatments. They are able to illicit an immunological cascade inside of a host to help prevent diarrhea, viral infections, to even reducing cholesterol levels in the blood.

Supported metagenomic data have shown that LAB are involved in the human microbiome as well as other animals. They are classified as gram positive, non-sporing bacteria either being microaerophilic or anaerobic microbes with low GC content (<50 mol%) (Florou 589). LAB can also show a wide range of biochemical test results such as

being catalase and cytochrome negative, fastidious, aerotolerant, and acid tolerant organisms (Florou 589). Further grouping of LAB, of course, also includes other genera classification such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, and more.

Extracellular Polysaccharides (EPS)

A diverse group of polysaccharides, also known as glycans, consists of differentiating chemical structures, physical properties and biological functions that serve to play a role as structural or storage purposes. Unlike macromolecules such as DNA, RNA, and proteins, polysaccharide synthesis is not a template-based driven process, but instead, structures are pre-determined primarily by their corresponding polysaccharide enzymes found in the host. Furthermore, polysaccharide structure may either be linear or branched helping with distinguishment among extracellular polysaccharides (EPS) homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

The main difference among HoPS and HePS is that the former mainly consists of only one type of monosaccharide wile HePS presents two or more types of sugar groups. Both HoPS and HePS are labeled as EPS due their unique ability to extracellularly attach to the outer cell wall of forming capsules to eventually be released into the environment (Angelin, 854). This is important due to the yielding of structural diversity that contributes to the ensemble of biological functions. This also renders an array of physiochemical and theological properties that can be exploited for commercial applications of the industrial, food and medical sectors (Angelin, 854). Even more, with the global market being far dominated by polysaccharides derived from plants and algae, bacteria raise questions if

they indeed represent a relatively untapped source of an immense polysaccharide repertoire.

EPS is a new area of study that is heavily studied for food safety and human health. They are described as long-chain, high-molecular-mass polymers that are able to disperse in water to give a thickening or gelling property in food products (15). Today, most biothickeners used by the food industry are polysaccharides derived from plants, such as starch, pectin, and seaweeds, and animal's proteinaceous hydrocolloids gelatin and casein (15). However, these polysaccharides may not always be readily available in terms of quality or desired rheological properties. Furthermore, plant carbohydrates can often be chemically modified in order to improve their structural and food texture properties. In addition, these polysaccharides are strongly regulated and commonly restricted. Hence, microbial EPS are now viewed as an alternative class of biothickeners.

Along with LAB, EPS occur widely among microalgae but less frequently among yeasts and fungi. It has been studied that EPS in their natural environment carry out important roles in protection of microbial cells against desiccation and prevention of phagocytosis and phage attack (15). Other functions also include protection against antibiotics and/or toxic compounds such as toxic metal ions and ethanol, predation by protozoans, and osmotic stress (15).

Microbial EPS used in the food industry includes, but not limited to xanthan, pullulan, dextran, and bacterial alginates (Vu et. al., 2547). These novel examples, although representing only a small fraction of current biopolymers, are market-available polymers that could soon take place of traditional products to improve theological and stability food characteristics. For example, the bacterial species Xanthomonas campestris produces EPS

Xanthan that offers unique rheological properties and is low-cost efficient (Vu et. al., 2547). However, obstacles still remain for researchers and food industry personnel facing microbial EPS use and knowledge of their biosynthesis production, adaptability to bioprocess technology, and the high cost of their recovery (Vu et. al., 2547). Fortunately, bacteria strains that are generally recognized as safe (GRAS) are gradually being discovered. In this study, strains of GRAS lactic acid bacteria EPS production efficiently will be studied undergoing various stress conditions.

CHAPTER 3

DESIGN OF THE STUDY

Methodology Description

The overall approach was based on the modifications of EPS compositions of a *lactobacillus SPP* cultivated with different nutritional supplementation. From the initial cultivation medium, it was determined that the viscosity of the broth will give an indication of the best EPS producer. Then, the culture conditions were modified, and, in each case, EPS was determined and compared with the viscosity.

Next, a seed culture of the LAB was prepared by inoculating the MRS broth with the selected LAB. The inoculum load was stored (Figure 1) and determined by following the McFarland standard method.

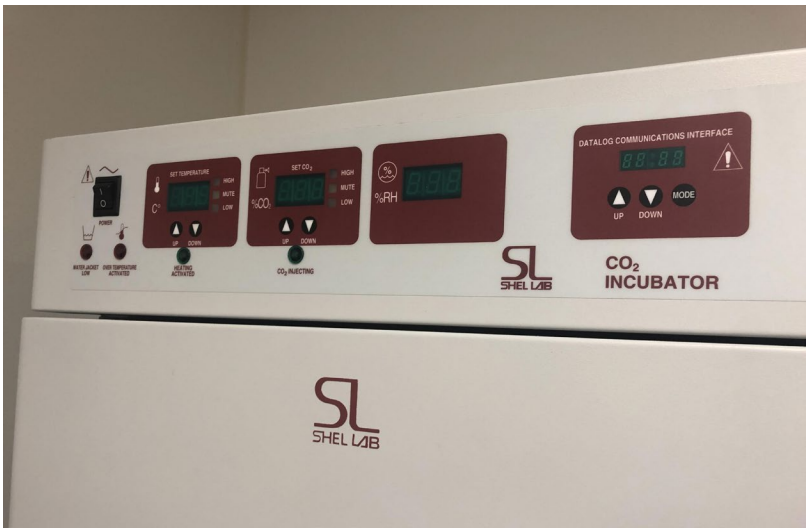

Figure 1: Shel Lab Incubator Used In Experiment

Finally, the viscosity of the broth was determined so that the positive results could be evaluated, and comparison made between this observation and the actual EPS quantification after EPS recovery. This method required precise identification of bacteria species grown in the broth media as well as quantitative measured of the resulting sheath viscosity of the broth media. With appropriate filtration of contaminants and other molecular by-products, biochemical analysis, and isolation techniques, including centrifugation, was used to study LAB EPS.

Itemized below is the outline of the step-by-step approach:

1. Isolate/Screen available LAB for EPS production to select the LAB to be used for the work.
2. Determine the contributions of culture amendments (media composition, incubation temperature and time) on EPS yield. It should be noted that the same inoculum load using an already prepared media was utilized.
3. Characterize EPS formed (sugar composition, EPS structure, etc, enlarged clearly) from the most potent/promising cultural composition/amendments such as the varied sugar substrates, pH etc.
4. Utilize AR-2000 Visco- Rheometer to evaluate viscosity of bacterial broth media.

Media Preparation and Culture Conditions

The media preparation and culture conditions generally followed the sequence delineated below:

- Isolation of exopolysaccharide producing lactic acid bacterial strains from raw milk:

- Preparation of skim milk lactose agar medium by mixing 11g skim milk with sterilized 0.35% yeast extract, 1% lactose, and 1.5% agar 0.012g containing bromocresol blue/purple. Stab LAB isolates on this medium, and use this medium to screen for LAB by incubating plates at 30°C for 48 hrs. TIME LINE: 1 Week
- Selection of the LAB colonies which are good EPS producers and preserve them by storing in MRS-lactose broth containing 50% glycerol at −80˚C. TIME LINE: 1 day
- Identification of LAB by 16S rRNA gene molecularly
- Production of EPS: inoculate the selected LAB strain in 11% skim milk, incubate for 24 hrs. at 30ºC. To the broth medium add 12% trichloroacetic acid and incubate for 24 hrs. TIME LINE: 1 day/OVER A WEEK
- Centrifuging at 7000 rpm at 4°C for 20 min to separate LAB cells, EPS, and other proteins. TIME LINE: 1 day
- Precipitation of EPS from the supernatant by adding three times the volume of the supernatant of cold absolute ethanol and incubate at 4°C for 48 hrs. TIME LINE: 2 days
- Centrifuge, (7000 rpm at 4°C for 20 min) using the precipitate (EPS)

Evaluating the Characteristics of the EPS

The evaluation of the characteristics of the EPS consists of several steps. Each stage comprises of (a) combination of centrikon (Pall, Nanosep centrifugal devices) and size

exclusion high performance liquid chromatography (SE-HPLC) by using several dextran standards injecting at an elution rate of 1 mL/m.

Determination of Reducing Power of EPS

The reducing power of the EPS was determined according to the method of Oyaizu [8]. Various concentrations of EPS (300–1500 μg) in 1 mL of distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%; 2.5 mL) was added to the mixture and centrifuged for 10 min at 3000 rpm. Distilled water (2.5 mL) and 1% ferric chloride (0.5 mL) were added to 2.5 mL of the upper layer. After an incubation of 10 min, the absorbance was read at 700 nm in a spectrophotometer. A higher absorbance indicates the greater reducing power.

Determination of Total Antioxidant Capacity of EPS

The analysis here is one day analysis. It ties with what was designed at the onset. It shows an application of the EPS in a relevant healthcare field. Total antioxidant capacity was prepared by dissolving 1.235 g of ammonium molybdate (4 mM) and 0.9942 g of sodium sulfate (28 mM) and 45 mL of sulfuric acid (0.6 M) into distilled water (250 mL). Various concentrations of EPS (300–1500 μg) were dissolved in 1 mL of total antioxidant capacity. The absorbance was measured at 695 nm after 15 min incubation. Ascorbic acid was used as standard (7).

Rheological Properties of Dissolved Aqueous EPS

Determine the rheological properties of dissolved aqueous EPS. The viscosity of the aqueous purified polysaccharide solution was measured using AR 2000 Visco-Rheometer. The viscosity measurements were performed at 25°C with increasing shear rate. The determination of viscosity was performed in 20 points with a reading duration of 10 sec and shear rate ranging from 1 to 2001/s. The viscosity measurements were performed according to shear rate and stress applied to the samples. The numerical values of the velocity gradient Dr, the modulus of shearing, and flow behavior kinetics were calculated using the Oswald de Waele equation (6).

CHAPTER 4

RESULTS AND DISCUSSION

Lab Isolation and Screening for EPS Production

A total of 50 isolates were obtained from milk. Morphology of the samples were evaluated while I selected for rod and cocci shaped isolates. These isolates were all gram positive and catalase negative.

Table 1: Biochemical Properties of Isolated Lactobacillus Strains

Biochemical Characteristics of Desired LAB Stains					
Isolates	Test				
	Catalase	Oxidase	Indole	Growth at 15ºC	SIM
L. lactis	-	-	-	+	-
L. acidophilus	-	-	-	+	+
S. thermophilus	-	-	+	+	-

After preliminary testing of identification of lactic acid bacteria, further technological isolation tools were used to identify the species of the probiotic bacteria. To further confirm our Lactobacillus and S. thermophilus bacteria, PCR was used using primers provided by Song et al., (2000). Table 1 displays the sequences of primers and their corresponding PCR cycle. This process was done with a Rio Rad T100 PCR Thermal

Cycler. Using agarose gel electrophoresis, PCR products were amplified, stained with ethidium bromide, and observed with UV.

Table 2: PCR of LAB Isolates and Their Specific Primers

Organisms	Primer	PCR Cycle Time	Amplicon Size	Reference
Lactobacillus lactis	F-5' CTAGCGGGTGCGACTTTTGTT 3' R-5' GCGATGCGAATTTCTATTATT3'	95ºC 4min, 95ºC 4min, 62ºC 2min, 60ºC 2min, 70 min 5 min. 15 cycles	85 bp	Song et al. (2000)
Lactobacillus acidophilus	F-5' ACTAACTTGACTGATCTACGA 3' R-5' TTCACTGCTCAAGTAATCATC 3'	95ºC 4min, 95ºC 4min, 62ºC 2min, 60ºC 2min, 70 min 5 min. 15 cycles	110 bp	Song et al. (2000)
Streptococcus thermophilus	F-5' CACTATGCTCAGAATACA3' R-5' CGAACAGCAATGATGATA3'	95ºC 4min, 95ºC 4min, 62ºC 2min, 60ºC 2min, 70 min 5 min. 20 cycles	220	Song et al. (2000)

Identification of Lab 16s rRNA Gene

Using BLAST program (http://www.ncbi.nlm.nih.gov), the 16S rRNA gene (around 1,000 bp) can be sequenced to confirm isolate's identity and relative strains. The three primary samples we were able to identify were *Lactobacillus lactic, Lactobacillus acidophilus,* and *Streptococcus thermophilus*.

EPS Production and Isolation

The strain grown in the dairy milk produced a ropy, thick, and viscous texture used to extract the polysaccharides. After purification, 1.25 g/L of EPS was collected with negligible amount of proteins present. Afterwards, the final purified polysaccharide's morphology and characteristics were further examined.

EPS Estimated Molecular Weight:

Exclusion chromatography was used to analyze molecular size. Four different fractions of weights were found within the range of 55 kDa to 175 kDa. *Lac*. *lactis* produced two fractions of around 100 * 10^4 kDa and 1. 22 * 10^4 Da. Next, *Lac*. *acidophilus* has a molecular weight of around 75 * 10^5 Da. And lastly, *S. thermophilus* had a molecular weight of around 44 * 10^4 Da.

Table 3: EPS Estimated Molecular Weight

SE-HPLC		
Samples	RT (retention time)	Mol. Wt. (kda)
L. lactis	10.3	
L. acidophilus	12.6	75.4904
S. thermophilus	13	44.40432

Determining EPS Reducing Power:

Transformation of Fe3+- Fe2+ was investigated to measure the reducing powers of LAB EPS. Results indicated that reducing activity similar to the antioxidant activity was dose dependent. There was an increasing correlation found between EPS reducing power and concentration.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The dairy milk used in this experiment was from a local supermarket located. Main goal in this experiment was to extrapolate any data that showed any effects of media supplementation on LAB polysaccharide production. Based on the results, further research can be speculated, and which can be done to fully utilize these probiotic bacteria.

A moderate amount of dairy LAB samples was quantified ranging from 5.24 - 10.12 Log CFU/mL. This is considered low amount compared to the studies studying dairy milk LAB. In one study, Dewan and Tamang (2007) obtained close to 60 samples of in fermented Himalayan milk (Source 1). The quantity of samples raised were likely due to environmental issues, human errors, and type of dairy. With the samples, much information regarding lactic acid bacteria is gathered.

In this study, we were able to reveal the composition of LAB among dairy milk products. We were able to perform 16S rRNA gene sequencing techniques to identify differences in species diversity among the samples. This gave us a lead to the foundation of species needed to be further studied and their role in polysaccharide production in dairy.

One keynote was the inclusive similarities in the polysaccharide production among the LAB species. The predominate species *Lb*. *brevis*, *E*. *faecalis*, and *Lac*. *lactis* exhibited similar viscosity characteristics regardless of media supplementation and modification. Also discovered were different genera of LAB consisting of genera *Enterococcus*,

Lactobacillus, and *Streptococcus*. I believe we need to bring more attention to the species of LAB that fall under these genera for further investigation of their polysaccharide production. In our study we focused on media viscosity among *Lac*. *lactis*, *Lb*. *brevis*, and *E*. *faecalis*.

Further investigation also includes prior knowledge of fermentation process of the daily milk samples used to study LAB isolates. Along with media supplementation and should be aware of the details of earlier fermentation processes and see if that plays any role on polysaccharide secretion. In one study by Sakai et al., (2014), a Japanese mustard leaf known as Takanazuke exhibited microbial community that changed in the amount of microflora proportions during fermentation processes. In efforts to maximize full probiotic potential of polysaccharides, it is critical for further research to consider any cellular changes that may have occurred during prior fermentation processes.

This research serves to contribute to the preexisting research to provide further understanding on LAB diversity from dairy products. A predominant species in LAB was able to find and gained with more knowledge on polysaccharide production. With the knowledge gained, it is expected that further research can be performed and with additional applications.

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