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A Modified RCM Medium for the Growth of Lactobacillus Bulgaricus

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Biographical Sketch

Ayowole Caleb Oyeniran earned his Bachelor of Science degree in Food Science and Technology from the Obafemi Awolowo University, Ile-Ife in 2015. After graduation, Ayo went on to undergo the compulsory one-year national youth service program in Akwa Ibom state, Nigeria. In January 2018, he started his Master of Science program in the Food and Nutritional Sciences Program with Food Microbiology and fermentation concentration at the North Carolina Agricultural and Technical State University, Greensboro. As part of his training as a graduate student, he visited several local high schools where he conducted workshop and laboratory exercises to draw the attention of the students and teachers to the importance of dairy foods in the food science curriculum and to the human health. Ayowole worked as a graduate research assistant in dairy fermentation and lactic acid bacteria. He was inducted as a member of the Gamma Sigma Delta NCA&T SU chapter. He has been able to present his findings at different regional and national conferences including Southeastern Regional Meeting of the American Chemical Society (SERMACS) (Augusta, USA, 2018), American Dairy Science Association (Cincinnati, USA, 2019), Association of 1890 Research Directors (Florida, 2019) and Institute of Food Technologists (New Orleans, USA, 2019) where he was a finalist in the Dairy Foods Division Graduate Research Paper Poster Competition. It is his dream to reach out and help the less privileged bright kids in his home country by giving them a platform to transform the world with their ideas.

Dedication

This work is dedicated to Almighty God for wisdom and gift of life. To my lovely parents, Mr. Femi and Mrs. Wunmi Oyeniran for your sacrifices and prayers. To my siblings, Olajire, Temilade and Tolulope for your support and encouragement. Thank you Olajire for always having my back. To Foluke Adelabu, for initiating this journey and encouraging me to pursue my dreams in this part of the world. Finally, to Comfort Onuoha for persisting with me all through this journey. I am grateful for the presence of you all in my life.

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Abstract

In this study, we modified reinforced clostridial medium (RCM) to selectively enumerate and isolate *Lactobacillus bulgaricus*, a probiotic and an important starter culture in the dairy industry. The disparity in the reported carbohydrate fermentation pattern of *L. bulgaricus* was used to develop a growth medium not only selective for *L. bulgaricus* but significantly inhibitory to the growth of other lactic acid bacteria. A recently modified RCM (mRCM) was optimized for this study by the addition of 0.5% fructose, 0.5% dextrose, 1% maltose and 0.25% sodium pyruvate while replacing lactose as a carbohydrate source. The cell recovery and bacterial counts of *L. bulgaricus* in tested products (Pure *L. bulgaricus* strains, Starter Culture, Probiotic Supplements and Yogurt) using our modified RCM with sodium pyruvate (mRCM-PYR) were significantly higher ($P < 0.05$) than in the recently modified RCM and the common de Man, Rogosa and Sharpe (MRS) culture medium. The growth of other lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*) and Bifidobacteria was retarded in this modified medium compared to MRS and mRCM. This result is a significant improvement in the enumeration and differentiation of *L. bulgaricus* in mRCM-PYR compared to the results in MRS and mRCM where the high background growth of similar species interferes with bacterial population counts. Our results thus suggest that mRCM-PYR could be recommended as an alternative and reliable growth medium for the selective enumeration and isolation of *L. bulgaricus* in a mixed culture.

CHAPTER 1

Introduction

Lactobacillus delbrueckii subsp. *bulgaricus* is an important and highly regarded species of lactic acid bacteria due to its application in the production of several dairy products including yogurt, a fermented milk product that is well-known for its health benefits (Zhu et al., 2010). Yogurt starter cultures require a symbiotic blend of *L. bulgaricus* and *Streptococcus thermophilus* in approximately equal amounts in order to obtain desired organoleptic properties that result from the metabolic activity of both micro-organisms during their growth in milk. Consequently, the final quality of yogurt can suffer with regard to texture, acidity and flavor if this proper bacterial balance is not achieved (Lourens-Hattingh & Viljoen, 2001).

L. bulgaricus is a proven probiotic (Mahmood, et al., 2015; Pescuma et al., 2015; Singh, et al., 1979), and, as with any probiotic, confers various health benefits to the host when administered at an effective dose. Consequently, in order for *L. bulgaricus* to play its role as a probiotic, the requisite bacterial population levels are again critical. In order to support the quality of yogurt, many countries have adopted standards for the viable numbers and the ratio of *L. bulgaricus* and *S. thermophiles.* The *Codex Alimentarius*, a collection of internationally recognized standards for food production, and most other national regulatory agencies in the U.S. have established a minimum level of 1×10^7 colony-forming units (CFU)/g of yogurt starter microorganisms. Other countries such as France, Switzerland, Italy and Spain also agreed to follow the Codex Alimentarius*'* recommendation of a minimum level of yogurt starter microorganisms at the time of consumption. However, in the U.K., a minimum requirement of 10 million viable lactobacilli per ml of yogurt at the time of sale was established. (Davis & McLachlan, 1974; Hamann & Marth, 1984; Robinson & Tamime, 1976).

The current standard medium, de Man, Rogosa and Sharpe (MRS) is unreliable as a selective method for enumerating *L. bulgaricus* species in mixed bacterial cultures as it often underestimates true counts due to the presence of high background colonies of similar species. Other media proposed for the selective enumeration of specific lactic acid bacteria (Galat et al., 2016; Ghoddusi & Robinson, 1996; Matalon & Sandine, 1986; Saeed et al., in press, Tabasco et al., 2007; Yamani & Ibrahim, 1996) also struggle to selectively enhance the growth of *L. bulgaricus* in the presence of other lactic acid bacteria. A modified reinforced clostridial medium by Nwamaioha & Ibrahim, 2018 recently showed remarkable results in the differentiation and enumeration of two strains of *L. bulgaricus* in a mixed bacterial culture by giving distinct and large colonies. This culture medium supports the growth of other lactic acid bacteria other than *L. bulgaricus* which still makes the isolation and enumeration steps required for L. bulgaricus very challenging. Nevertheless, preliminary studies have demonstrated that mRCM can be optimized to improve the recovery of stressed bacterial cells and select for a broader range of *L. bulgaricus* strains.

Therefore, the objectives of this study were to:

i. modify RCM for the selective isolation and enumeration of *L. bulgaricus*
ii. examine the effectiveness of this modified RCM in the isolation and enumeration of *Lactobacillus bulgaricus* from a mixed culture compared with other culture media

CHAPTER 2

Literature Review

2.1 Lactobacillus delbrueckii

The lactic acid producing *Lactobacillus delbrueckii* is the type species of the genus Lactobacillus serving as a big umbrella for the subspecies *delbrueckii, bulgaricus, indicus, lactis* and *sunkii* (Adimpong et al., 2013a). *Lactobacillus delbrueckii* is regarded as one of the most important bacteria used in the dairy industry owing to the adaptability of subspecies *bulgaricus* and *lactis* in milk. The subspecies (subsp.) *bulgaricus* has become synonymous with yogurt production while the subsp. *lactis* is used in the production and ripening of cheese. While both subspecies are closely related; expressing a significant level of similar DNA-DNA hybridization (Weiss, Schillinger, & Kandler, 1983), they can be identified based on their ability to use different carbohydrates (Michaylova et al., 2007).

However, this method is not the most effective as results can be inconclusive as they share similar nutritional needs and sugar fermentation can be strain specific. The need for a precise identification is not only essential for body of knowledge but crucial in industrial processes. Molecular fingerprinting techniques, phenotypic and genotypic methods have been used in instances where a detailed and more precise analysis and identification of the subspecies were needed. Giraffa et al., 1998 differentiated *Lactobacillus delbrueckii* subspecies *bulgaricus* and subspecies *lactis* using amplified rDNA restriction analysis. Two PCR methods; the species-specific PCR and randomly amplified polymorphic DNA PCR (RAPD-PCR) were successfully used in differentiating these two closely related species (Torriani, Zapparoli, & Dellaglio, 1999). Although described as an expensive procedure, Gatti et al., 2001 successfully differentiated *L. bulgaricus* from *L. lactis* by analysing cell-wall-associated proteins using the sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Multi-locus sequence typing has been described as the preferred method of differentiating between the various subspecies of *L. delbrueckii* because of its ease, higher resolution and ease of data exchange between laboratories all over the world (Song et al., 2016). A combination of phenotypic and genotypic analyses will however erase any doubt as to the taxonomy of particular subspecies.

2.2 Origin of *Lactobacillus bulgaricus*

Although wide attention was drawn to yogurt by Elie Metchnikoff, a French-Russian biologist and Nobel Prize laureate who attributed the longevity of Bulgarians who were regular consumers of yogurt to the lactobacilli bacteria of yogurt; it was in fact the Bulgarian graduate student Stamen Grigoroff (1905) who first isolated and characterized *L. bulgaricus* from the starter used in producing Kiselo Mlyako (Bulgarian Yogurt). Grigoroff named this bacterium '*Bacillus A'* now recognized as *L. bulgaricus* according to the Bergey's classification of bacteria.

The origin and natural habitat of commercial *L. bulgaricus* strains may not have a definite answer despite its strong Bulgarian ties as countries like China, Mongolia, Russia and Turkey also enjoy a long history of naturally fermented dairy products. A study by Song et al., 2016 highlighted the uniqueness of *L bulgaricus* strains isolated from traditionally fermented milk products from some of the aforementioned countries.

Moreover, it appears *L. bulgaricus* is on a continuing evolutionary journey as it has adapted itself from a plant source to milk-rich environment (van de Guchte et al., 2006). Michaylova et al., 2007 have been able to isolate and characterize *L. bulgaricus* from certain plant species (*Cornus mas*) gotten from four regions in Bulgaria. Yılmaz et al., 2015 also isolated *L. bulgaricus* from raw milk samples collected from different parts of Turkey while *L. bulgaricus* was one of the isolates from raw milk samples obtained from four races of Algerian

goats (Badis et al., 2004). A study by Song et al., 2016 is also a pointer to the diversity of *L. bulgaricus* and to the fact it might not be an exclusive preserve of Bulgaria.

2.3 *L. bulgaricus*'s Metabolic Adaptation to Milk

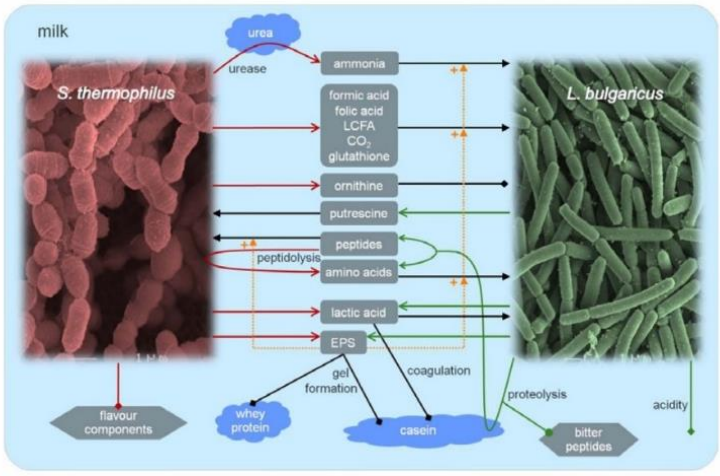
Lactobacillus bulgaricus have been used over the years in protocooperation with *Streptococcus thermophilus* in milk for yogurt and cheese production in which they contribute to texture, flavor development, ripening; all vital to the unique properties of these products. They basically do this by fermenting and depleting lactose (Milk Sugar), reducing redox potential and degrading casein (Olson, 1990). However, the milk environment does not supply the fastidious *L. bulgaricus* enough nutrients necessary for survival to impact properties unique to yogurt and other fermented products. It therefore needs to resort to adaptive mechanisms; a characteristic which is not alien to *L. bulgaricus*. El Kafsi et al., 2014 documented a genomic sequence highlighting the evolutionary adaptation of *L. bulgaricus* to the protein-rich milk environment made possible by doing away with superfluous amino acid biosynthesis functions. The traditional way of transferring samples of yogurt cultures to fresh milk played a role in this adaptation. In milk fermentation, optimal growth and survival of *L. bulgaricus* in milk rely on its ability to metabolize casein and lactose; the major sources of the much-needed amino acids, peptides and carbon. *L. bulgaricus* does this by banking on its efficient proteolytic capacity to compensate for milk's lack of free amino acids and peptides in a process that involves the breaking down of casein which is the most abundant milk protein and main source of amino acids. The proteolytic system of lactic acid bacteria consists of cell-envelope proteinases that hydrolyze caseins to peptides, peptidases that further break down these peptides and transport systems responsible for the translocation of these products across the cytoplasmic membrane (Kunji et al., 1996). In yogurt production, *L. bulgaricus* boasts the greater proteolytic activity in

the protocooperation with *S. thermophilus*; supplying it with amino acids and peptides which otherwise would have inhibited optimal growth. However, proteolytic activity differs among *L. bulgaricus* strains and effect on the growth of *S. thermophilus* may vary.

Another important component of milk is the milk sugar (Lactose) which is the preferred carbon source for yogurt bacteria according to (Chervaux et al., 2000). Lactic acid bacteria generally engage two systems in the metabolism of lactose as: **(a)** a phosphoenolpyruvate (PEP) lactose phosphotransferase system (PTS) involving a phospho-β-galactosidase enzyme found in many species including the lactococci and **(b)** a lactose permease system with a β-galactosidase usually used by *L. bulgaricus* (Leong-Morgenthaler et al., 1991; Postma & Lengeler, 1985; Thompson, 1987). Lactose is transported into the cell by lactose permease and hydrolysed by β-galactosidase into galactose and glucose. While glucose is metabolized via the glycolytic pathway with the production of D-lactic acid, galactose is usually transported out by the permease.

2.4 *L. bulgaricus* – *S. thermophilus* Interaction as Yogurt Starter Cultures

Even though both thermophilic bacteria can ferment milk individually, the symbiotic relationship between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, otherwise known as proto-cooperation, is crucial in the fermentation process and the eventual quality of yogurt. The role of *L. bulgaricus* is however critical that when excluded from the starter cultures, final product quality suffers in terms of texture, acidity and aroma (Lourens-Hattingh & Viljoen, 2001). *L. bulgaricus* and *S. thermophilus* are involved in three major metabolic processes during the fermentation process; conversion of lactose into lactic acid, hydrolysis of caseins into peptides and free amino acids and hydrolysis of milk fat into free fatty acids (Smit, et al., 2005). This results in a better cell growth of the two species, a rapid milk



Representation of the interactions between *L. bulgaricus* and *S. thermophilus* during yogurt fermentation and their effects on product characteristics. The dotted lines indicate that EPS is hypothesized to facilitate the exchange of metabolites by establishing close proximities between the two species the two species → :production or enzymatic activity; →:positive effect of the component; —● : negative effect of the component; —◆ :neutral or yet to be confirmed effect; EPS: Exopolysaccharides; LCFA: Long-Chain Fatty Acids

Figure 1. L. bulgaricus – S. thermophilus interaction in yogurt production

acidification, significant abundance of aroma volatiles and non-volatile metabolites needed for a good organoleptic quality of yogurt (Sieuwerts, 2016). This is illustrated in Figure 1.

Most yogurt cultures usually consist of proteolytic *L. delbrueckii* subsp. *bulgaricus* and nonproteolytic *S. thermophilus* (Courtin & Rul, 2004; Pette & Lolkema, 1950). Yoghurt fermentation usually contains two exponential growth phases separated by a transition phase with lower growth (Courtin & Rul, 2004). During the first exponential phase, almost no growth of *L. delbrueckii* subsp. *bulgaricus* is observed while there is an exponential growth of *S. thermophilus.* This is because *S. thermophilus* is more tolerant to neutral pH and is more effective at taking up free amino acids, peptides and trace elements available in milk than *L. bulgaricus.* The exponential growth of *S. thermophilus* is marked by the production of formic acid and folic acid, important precursors and co-factors that could help with purine biosynthesis in *L. bulgaricus.* Also, oxygen consumption and urea metabolism by *S. thermophilus* produces carbon dioxide to support the growth of the less oxygen tolerant *L. bulgaricus. S. thermophilus* is

also able to supply *L. bulgaricus* with long-chain fatty acids as it has an incomplete pathway to synthesize these compounds.

Available free amino acids and peptides soon get depleted and the transition phase kicks in. *S. thermophilus* suffers a decline in growth during this transition phase as most strains lack extracellular proteases to compensate for depleted amino acids, particularly sulfur and branched-chain amino acids (Sasaki et al., 2014). During this transition phase, growth and protease gene expression (prtB) is initiated in *L. bulgaricus*. This protease hydrolyses casein, increasing the levels of oligopeptides and amino acids needed to support a second exponential growth phase of *S. thermophilus* while also supporting the exponential growth of *L. bulgaricus* (Sieuwerts et al., 2008).

Other metabolic products from this proto-cooperation include lactic acid, acetaldehyde, acetic acid and diacetyl which are responsible for the characteristic flavor of yogurt. Acetaldehyde is suggested to be the major flavor compound as it is largely responsible for the typical aroma of yogurt (Hamdan et al., 1971). Threonine aldolase, the enzyme responsible for the breakdown of threonine to acetaldehyde and glycine has been reported in both *L. bulgaricus* and *S. thermophilus* and this forms the most important biosynthetic pathway of acetaldehyde. However, *L. bulgaricus* could be the major producer of this flavor compound as there is usually a decrease in the aldose activity of *S. thermophilus* when the growth temperature is raised from 30 to 42 °C, the typical temperature used in yogurt production (Lees & Jago, 1976; Routray & Mishra, 2011; Zourari et al., 1992). Overproduction of acetaldehyde might however lead to production of harsh flavors (Lindsay et al., 1965). Although a controversial source of aroma in yogurt, diacetyl is believed to be a major aroma compound and this controversy extends as to the

major producer of diacetyl in yogurt. Rasic & Kurmann, 1978 report *S. thermophilus* as the major producer while others suggest *L. bulgaricus* as the major producer (Dutta et al., 1973).

Despite the discrepancies in the role of diacetyl in the overall aroma expression of yogurt, it is one of the other major aroma compounds (GuerraHernández and others 1995; Beshkova and others 1998). *Streptococcus thermophilus* is reported as exclusively responsible for the production of diacetyl by some researcher (Rasic and Kurmann 1978), but others support *L. bulgaricus* as the major source of production of diacetyl (Dutta and others 1973; Beshkova and others 1998).

Proto-cooperation between *L. bulgaricus* and *S thermophilus* also contributes to the texture of yogurt. Acidification by these bacteria coagulates protein, changing the viscosity of milk in the process. Also, the production of exopolysaccharides (EPS), mainly by *S. thermophilus*, contribute to the texture by forming a matrix with the milk proteins. EPS also protects the starter cultures against unfavorable conditions like high acidity and plays a role in cell aggregation and cell to cell communication (Sieuwerts, 2016; Zannini et al., 2016).

2.4.1 History of Yogurt. Yogurt, a product of milk fermentation is an ancient food that has evolved over the years from just a means of preserving milk to a veritable source of essential nutrients. Its potential in conferring a wide array of health benefits in man (Soustre & Marmonier, 2014) has increasingly made it an important research interest. The origin of yogurt seems to have been lost in history as there are conflicting versions of narratives detailing its root. It is no surprising that different countries of the world have different traditional names for yogurt and yogurt-like products as can be seen in Table 1.

Table 1

Yogurt and yogurt-like products as known in different regions of the world

Traditional Name of Yogurt/Yogurt-Like Products	Country/Region of Origin
Amasi/Maas	South Africa
Nunu	West Africa
Matzoon	Armenia
Mursik	Kenya
Zabadi	Egypt
Nyarmie, fènè, and lait caillé	Ghana, Mali and Burkina Faso
Skyr	Iceland
Salcë kosi	Albania
Kishk, Kushuk, Keshkeh or Kichk	Middle East
kiselo mlyako	Bulgaria
Qatiq/katik	Central Asia
Suzma	Central Asia
Ayran	Turkey
Ymer	Denmark
Piima	Finland
Hangop	Netherlands

Cont.

Matzoon/Matsoni	Armenia/Georgia
Laban	Middle East
Villi	Scandinavia
Tzatziki/Cacik/Tarator	Ottoman Empire
Chal	Central Asia
Mishti Dahi	India
Shrikhand	India
Lassi	India
Mishti Doi	India/Bangladesh
Dadiah/Dadih	Indonesia
Mast-o Khiar	Iran

There is the Persian version of the yogurt origin attributing Abraham's fertility and long life to consumption of yogurt; the preparation of which was revealed by an angel (Rosell, 1932). A view commonly shared by historians is that yogurt was an accidental discovery by the Neolithic herdsmen of Central Asia who pioneered the milking of animals and carried this milk in containers made of animal stomachs and sheep-skins (Weerathilake et al., 2014). The natural enzymes in these containers would later curdle this milk typically making it yogurt. In another twist, some authors suggest it must have emanated from the middle east some 10,000 years (Fisberg & Machado, 2015; Macbean, 2010; A Y Tamime & Deeth, 1980a).

Some of the earliest texts about yogurt were that of the roman author and philosopher, Pliny who lived in the first century A. D. and documented the ancient barbarous nations' knowledge of thickening milk into a substance with a satisfying acidity (Weerathilake et al., 2014). In what rather seems a plausible account, the word 'yogurt' as we know it today is said to have come from the Turks as far back as the 8th century in what appeared at that time as 'yoghurut' making it safe to assume the Turkish nomads in Asia made yogurt (Chandan et al., 2017).

Also corroborating the Turkish root; Fisberg & Machado, 2015 suggest 'yogurt' is rather a derivative of the Turkish word "yog˘urmak which means to thicken, coagulate, or curdle. It is even believed in some quarters that the Turks were also the first to discover the potentials of yogurt as medicine as it was used for treating a variety of conditions such as diarrhea and cramps, and to soothe the discomfort of sunburn.

In 1542, King Francoise I of France introduced this dairy product to Western Europe after having been treated for some severe illness by the country's Turkish allies (Tamime & Robinson, 1999). However, it wasn't until the beginning of the 20th century that yogurt was recognized for its health beneficial effects and sold in pharmacies as a medicine. The first industrialized yogurt production is said to have started in 1919, in Barcelona, Spain, when Isaac Carasso produced yogurt with jams while his son, Daniel Carasso founded Danone in France after fleeing the Nazi occupation (Fisberg & Machado, 2015).

2.4.2 Standardization of Yogurt. Yogurt is generally regarded as a fermented dairy product but there is a need for a clear classification that sets it apart from a diverse group of other fermented dairy products. It is worthy to note that standards and definition vary between countries and food regulatory bodies. Mercosur which is regional trade agreement between some

South American countries (Argentina, Brazil, Paraguay, Uruguay, Bolivia, Chile, Colombia, Ecuador and Peru) makes it mandatory for yogurts to contain live microorganisms under the ‘‘Reglamento tecnico Mercosur de identidad y calidad de leches fermentadas’’. This idea is also shared by France which only recognizes yogurt as milk cultured by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* which must be alive and abundant (at least 10^6 CFU/g) in the finished product. The two thermophilic lactic acid bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are considered as ‘Generally Recognized as Safe’ in the United States and possess the ‘Qualified Presumption of Safety’ status in Europe, because of a long history of safe use in food and an absence of pathogenicity (Corrieu & Béal, 2016). However, in Germany and Spain, yogurt is usually heat-treated after production to kill the bacteria.

The CODEX STAN 243-2003 distinguishes ‘Yogurt’ from ‘Alternate Culture Yogurt’ and ‘Acidophilus Milk’. It defined yogurt as a milk product obtained from the fermentation of milk, which may or may not be limited by composition of milk protein, milk fat and titratable acidity, by the symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* resulting in coagulation due to a drop in pH. In Alternate Culture Yoghurt, cultures of *Streptococcus thermophilus* and any *Lactobacillus species* are used while only *Lactobacillus acidophilus* is used as a starter in ‘Acidophilus Milk’. These starter microorganisms are required to be viable, active and abundant (at least 10^6 cfu/g) in the product to the date of minimum durability. It however added that fermented milk products that are heat treated be labelled as ‘Heat Treated Fermented Milk’ so as not to mislead consumers.

The Food and Drug Administration (FDA) under the Code of Federal Regulations Title 21 describes yogurt as food produced by culturing one or combination of cream, milk, partially skimmed milk or skim milk (which shall be pasteurized or ultra-pasteurized before the addition

of the bacterial culture), with a characterizing bacterial culture that includes *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* The FDA permits heat treatment after culturing is complete; thereby destroying viable microorganisms, to extend the shelf life of the food. Yogurt shall contain not less than 8.25% percent milk solids not fat and a titratable acidity of not less than 0.9%. The FDA differentiates yogurt, Low-fat yogurt and Nonfat yogurt on the basis of milk fat content; 3.25% in yogurt, 0.5-2% in Low-fat, and < 0.5% in Nonfat before bulky flavoring ingredients are added. The United States Department of Agriculture (USDA) use the FDA's guidelines in describing yogurt.

2.5 *L. bulgaricus* - A Probiotic

2.5.1 Probiotics. Elie Metchnikoff can be credited as the progenitor of what has now become a money-spinning industry; probiotics. He theorized that health could be improved, and senility delayed by colonizing the gut with host-friendly bacteria found in yogurt. In a market report published by Allied Market Research, the global market is expected to garner $57.4 billion by 2022, registering a compound annual growth rate (CAGR) of 7.7% during the period 2016-2022. Asia-Pacific was the dominant market and is expected to be the leading contributor in global revenue, due to its high adoption of probiotic based food and beverages.

The definition of probiotics has evolved over the years due to some grey areas regarding the characteristics of a typical probiotic. The internationally endorsed definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). Modulation of host immune system and promotion of host defense are the most commonly supported benefits of the consumption of probiotics. Most probiotics include species of lactobacillus and Bifidobacterium; certain *Escherichia coli* strain Nissle 1917; the yeast *Saccharomyces boulardii*; some enterococci (*Enterococcus faecium* SF68); *Bacillus spp*;

and *clostridium butyricum* (Elmer et al., 1999; Senesi et al., 2001; Takahashi et al., 2004). There might however be possible health risk of eating foods that contain enterococci. A study by Lund & Edlund, 2001 showed that intake of high concentrations of viable *E. faecium,* which may act as a potential recipient of glycopeptide resistance genes, might cause proliferation of resistance genes.

2.5.2 Required Attributes of Probiotics

2.5.2.1 Safety. There are general criteria a microorganism should meet to be classified as a probiotic. Probiotic strains should be safe for human consumption; non-pathogenic and non-toxic. Lactic acid bacteria enjoy a good record of safety and are generally Recognized as Safe (GRAS). Probiotic strains should be assessed for antibiotic-resistance patterns and side effects during human studies. Starter cultures used in fermented food products are not excluded in this safety assessment and the need for this assurance is stressed by the guidelines provided by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/ WHO, 2002). Aside the reported antibiotic resistance reported in lactic acid bacteria, there are concerns about the transfer of antibiotic resistance via gene transfer to pathogens. This could further complicate the growing concerns about the potency of some antibiotics in treating infections (Y. Li et al., 2019; Marshall et al., 2009; Nawaz et al., 2011).

Studies have reported the intrinsic resistance of *Lactobacillus* spp. to different antibiotics; aminoglycosides (gentamicin, kanamycin, streptomycin), nucleic-acid-synthesis inhibitors (ciprofloxacin, metronidazole, pefloxacin), folic-acid-synthesis inhibitors (cotrimoxazole, trimethoprim) and glycopeptides (teicoplanin and vancomycin). Resistance to vancomycin is reported to be the best-characterized natural resistance in lactobacilli (Goldstein et al., 2015; Y. Li et al., 2019).

On the flipside of antibiotic resistance is the susceptibility of lactic acid bacteria to antibiotics. The concern here is the effectiveness of probiotic and starter culture strains when use is combined with antibiotic treatment of infection in consumers. The complexity in the classification of various Lactobacillus species and limited research in this area make it difficult to generalize about their susceptibility to various antibiotics. Most probiotic strains of lactobacilli are reported to be metronidazole and vancomycin resistant. Thus, usage of probiotic strains of lactobacilli can be combined with these treatments. On the other hand, many lactobacilli species are reported to be susceptible to penicillin and ampicillin. Consequently, care must be taken when combining these antibiotics with probiotic strains of lactobacilli. The range and susceptibility of *L. bulgaricus* to various antibiotics as reported by different studies are presented in Table 2.

Table 2

Susceptibility of L. bulgaricus to Antibiotics Using Several Standard Assays[a]

Antibiotics	Range
Penicillin	0.06–0.25
Ampicillin	0.125–0.25
Clindamycin	0.06–0.5
Vancomycin	0.125–0.5
Nitrofurantoin	≤32
Quinupristin/dalfopristin	≤0.5
Fusidate	>4
Sulfamethoxazole	>512
Trimethoprim	>32

Cont.

Daptomycin	>4
Levofloxacin	>4
Erythromycin	0.06–0.5
Nitrofurantoin	≤32
Bacitracin	0.05–2
Chloramphenicol	0.25—16
Mupirocin	≤0.5
Tetracycline	0.125–2
Oxacillin + 2%NaCl	≤0.25
Gentamicin	4–64
Tiamulin	≤0.5
Trimethoprim/sulfamethoxazole	≤0.5/9.5
Streptomycin	≤4
Ciprofloxacin	2-32

a (Karapetkov, Georgieva, Rumyan, & Karaivanova, 2011; Nawaz et al., 2011)

To further ensure safety, potential probiotic strains must be properly identified by internationally accepted methods and named according to the International Code of Nomenclature with strains deposited in an internationally recognized culture collection (Huys et al., 2006). It is recommended to employ a polyphasic method (combination of phenotypic and genetic techniques) to ensure a precise typing and classification. The source of a probiotic is safety related. Potential probiotic source can be from a human origin like human large intestine, small intestine, or a breast milk, animal origin, food source like a raw milk or fermented food. Probiotics meant for human use are preferentially sourced and isolated from a human microflora

and are said to more likely adhere human intestinal wall. However, many food-associated LAB have been isolated from fermented foods and even plant sources (Michaylova et al., 2007a; Zago et al., 2011).

2.5.2.2 Functionality. Adherence and colonization of intestinal epithelium/ tissues are important in the functionality of probiotics as adhesion to the intestinal mucosa will give probiotic cells ample time for temporary colonization, immune modulation and competitive exclusions of pathogens. Also, of importance is acid and bile resistance. The Probiotic strains should be able to produce antimicrobial substances necessary in fighting off potentially pathogenic bacteria. Several metabolic compounds produced by lactic acid bacteria including organic acids, bacteriocins, hydrogen peroxide and diacetyl have antimicrobial activity (Shewale et al., 2014).

2.5.2.3 Probiotic Stability and Viability. Stability and viability of probiotics before and post production are significant factors for manufacturers. The industrial production is often a long and complicated process. Probiotics could easily be affected by high temperatures, oxygen humidity and high-water activity in the culture. Ability to grow quickly to maximum concentration in a simple and fermentation medium, survival in food matrices and during processing, viability and stability (physiologically and genetically) during shelf life when selecting for probiotics in the industry (Shewale et al., 2014; Wedajo, 2015).

2.5.3 *L. bulgaricus* as a Probiotic. Elie Metchnikoff was able to connect the link between regular consumption of lactic acid bacteria in fermented milk products to longevity and good health in certain group of people in Bulgaria. He linked this beneficial effect to the colonization and implantation of the Bulgarian bacillus which is now characterized as *L. bulgaricus.* Elie Metchnikoff is regarded in some quarters as the grandfather of probiotics

because of this profound observation he made at the beginning of the 20th century; a time when the function of the gut flora was completely alien and unknown. He believed aging and diseases were caused by putrefaction of protein in the bowel by intestinal bacteria and that LAB could inhibit the growth of these putrefactive bacteria. Such was his belief in the fact that fermented products could beneficially alter the microflora of the gut and prolong life that he committed to drinking sour milk fermented by lactic acid bacteria everyday till his death (Anukam & Reid, 2007; Hawrelak & Myers, 2004; Kulp & Rettger, 1924). *L. bulgaricus* has got all the attributes to be regarded as a probiotic as proven by several studies highlighted in Table 3.

Table 3

Probiotic strains of Lactobacillus bulgaricus

L. bulgaricus Strain	Probiotic Activity	References
RTF	Antibacterial activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas fragi, Micrococcus flavus*	Singh et al., 1979
7994	Inhibitory effect on *Achromobacter liquefaciens, S. aureus, P. fragi*	Abdel-bar & Harris, 1984
848	Immunomodulatory Function	Dixon, 2002
Tor grypus Isolate	Immune Function	Mohammadian et al., 2019
OLL1073R-1	Reduces risk of catching common cold, anti-influenza virus activity	Y. Yamamoto et al., 2017; Nagai et al., 2011, Makino et al., 2010
KLDS1-0207	Protects against lead toxicity	Fewtrell et al., 2004; Li et al., 2017

Cont.

BB18	Production of Bacteriocin (bulgaricin BB18); Bactericidal against *Helicobacter pylori*	Simova et al., 2006
ATCC 11 842, LBL-23, LBL-12, LBL-22, LBL-6, LBL-10, LBL-13, LBL-83, LBL-42, LBL-9, LBL-11	Inhibitory action against periodontal pathogen; *Aggregatibacter actinomycetemcomitans*	Stamatova et al., 2007
B-30892	Inhibits Clostridium difficile-mediated cytotoxicity on Caco2 cells	Banerjee et al., 2009
Commercial Yogurt Isolate	Inhibitory action against periodontal pathogens; *Porphyromonas gingivalis, A. actinomycetemcomitans*, and *Prevotella nigrescens*	Zhu et al., 2010
Commercial Yogurt Isolate	Bacteriocin production inhibitory against *Vibrio cholerae* and *E. coli*	Tufail et al., 2011
Commercial Yogurt Isolate	Inhibitory effect on *E. coli* O157:H7	Fooladi et al., 2014
NCTC 12197 Tat, DSMZ 20080T	Inhibitory effects on *Salmonella* spp., *Pseudomonas aeruginosa, E. coli, S. aureus*	Nour et al., 2015
TLB06FT	Antibacterial activity against *E. coli, S. aureus, P. aeruginosa, Listeria monocytogenes*	Mahmood et al., 2015
CRL 454	Aids digestion of allergenic β-Lactoglobulin	Pescuma et al., 2015
CRL 871	Production of folate; an alternative to folic acid fortification	Laiño et al., 2015
761N	Free radical scavenging ability; antiviral ability	El-Adawi et al., 2015
GLB	Antimicrobial; Control of *H. pylori*	Boyanova et al., 2017
GB N1 (48)	Hypolipidemic and protective cardiovascular effect	Doncheva et al., 2002
D6R; PTCC 1332	Inhibitory effects on *S. aureus* and *E. coli*	Akpinar et al., 2011; Tebyanian et al., 2017

Cont.

F5R	Inhibitory effects on *Bacillus coagulans, B. cereus, P. fluorescens, K. pneumoniae, L. monocytogenes, S. aureus* and *E. coli*	Akpinar et al., 2011
DSM 20081	Inhibitory effect on *E. coli*	Ravindran et al., 2016, Abedi et al., 2013
DWT1	Inhibitory action on tumor growth	Guha et al., 2019

It is crucial for probiotic strains to be able to colonize the intestine and survive passage through the upper gastrointestinal tract in order confer health benefits (Lick et al., 2001). There are however doubts about the adhesion and survival of *L. bulgaricus* after passage through the human gut primarily because they are not native flora of mammals and coupled with the fact that *L. bulgaricus* does not have enough genes of bile salt hydrolase and cannot synthesize mucin binding proteins, all important in surviving the GI (Klaenhammer et al., 2008). However, regular consumption of yogurt fermented by *L. bulgaricus* may facilitate the colonization of the bacteria in the gut (Elli et al., 2006). Mater et al., 2005 had earlier established the survival of *L. bulgaricus* and *Streptococcus thermophilus* after passage through the human gastrointestinal tract. Thirty-seven of the 39 stool samples retrieved from 13 healthy subjects over a 12-day period of taking yogurt contained viable *L. bulgaricus*. Also, encapsulated mix of *L. bulgaricus* and *S. thermophilus* in chitosan and sodium alginate survived a simulated gastrointestinal tract (Vodnar et al., 2010).

In a study involving 61 elderly volunteers who were randomly assigned to receive either placebo or probiotics, Moro-Garcia et al., 2013 evaluated the immunomodulatory capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* 848; a strain isolated from a region of Bulgaria (Stara Planina) known for the longevity of its population (Dixon, 2002). A positive effect on the

immune system was recorded. Blood samples were taken at the start, at 3 months and at the end of 6 months after which they characterized cell subpopulation, measured cytokines, quantified T cell receptor excision circle (TREC), and determined human β-defensin-2 (hBD-2) concentrations and human cytomegalovirus (CMV). The group which received this probiotic had an increase in the percentage of NK cells; an improvement in the parameters defining the immune risk profile (IRP), and an increase in the T cell subsets that are less differentiated. There was also reduced concentration of pro-inflammatory cytokine Interleukin-8 but an increased antimicrobial peptide hBD-2.

In a similar study, consumption of yogurt fermented by *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1; a polysaccharide-producing lactic acid bacterial strain was effective in reducing the risk of catching common cold in elderly people compared with the intake of milk. *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 has been proven by studies to have better control of the immune system than other lactic acid bacteria. The cell body and the immunostimulatory polysaccharides were identified as responsible for the activation of biological defence mechanisms against pathogens such as viruses (Makino et al., 2010). A recent study by (Yamamoto et al., 2017) corroborated the immunomodulatory effect of *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1. Thirty-seven elderly persons residing in a single nursing had their IgA levels increased after ingesting 112 g of the yogurt every morning for 12 weeks. The IgA plays a critical role in the defense of mucous membranes against foreign antigens and pathogens, directly neutralizing the infectivity of pathogens and their toxins.

There is an increasing awareness among health-conscious consumers as to the content of their foods; which is a driving force for the organic market. Some consumers are willing to go the extra length to stay clear of foods containing chemical preservatives. Despite the fact that

chemical preservatives are generally regarded as safe, the long-term side effects are unknown. Focus is being shifted to Bio-preservation as an alternative. The use of LAB strains as probiotic and as bioprotective culture in fermented products has been widely studied. Bio-preservation involves the use of microorganisms or their antimicrobial metabolites to extend shelf life and enhance safety of foods (Ross et al., 2002). The antimicrobial properties of LAB are linked to competition for nutrients, production of organic acids; majorly lactic and acetic acid as well as propionic, sorbic and benzoic acids, hydrogen peroxide, diacetyl, ethanol and also bacteriocins (Cizeikiene et al., 2013; Reis, et al., 2012). The major inhibitory effect observed in yogurt is due to reduced pH which is a result of lactic acid, a metabolic compound from the starter cultures. This drop in pH alters the environment and make it unfavorable medium for the development of some pathogens and spoilage microorganisms (Bachrouri et al., 2006). Strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* isolated from Turkish homemade yoghurts had inhibitory effects on *Escherichia coli* and *Listeria monocytogenes* in a study by Akpinar et al., 2011.

The use of bacteriocins in the food industry will go a long way in minimizing the use of chemical preservatives and could be used in hurdle technology to produce a more naturally preserved food. Bacteriocins are grouped as ribosomal-synthesized peptides, as biologically active proteins or protein complexes with antimicrobial activity against closely related species. Lactic acid bacteria produce bacteriocins; which are bioactive peptides or proteins, and bacteriocin-like inhibitory substances which are antimicrobial compounds that possess bacteriocin capacities requisites but that have not been characterized for their amino acid sequence (Reis et al., 2012, Cizeikiene et al., 2013).

Some *L. bulgaricus* strains which were isolated from yoghurts had antibacterial effect on *Vibrio. cholerae* and E. coli, because of significant characteristic of bacteriocin production (Maria Tufail et al., 2011). A study by Boyanova et al., 2017 suggested bacteriocin-like inhibitory substance production of GLB strains of *L. bulgaricus* can be valuable probiotics in the control of *Helicobacter pylori* infection. Clinical benefits were reported in Thailand where adding *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* either before or before and after 1-week tailored triple therapy significantly improved eradication rates in *H. pylori infection* treatment (Tongtawee et al., 2015).

Lead (Pb) is a toxic heavy metal which can have devastating effect on human health and remains a public health concern. Major sources of this poisonous metal include gasoline, mining, industrial activity, lead-based paint and diet. *L. bulgaricus* KLDS1.0207 which was isolated from traditional dairy products in Sinkiang Province, China has been evaluated for protective effects against acute lead toxicity in mice. High Pb-binding ability and high resistance to Pb in *L. bulgaricus* KLDS1.0207 offered protective effect to acute Pb toxicity in mice. The results in vivo showed this strain of bulgaricus can relieve renal pathological damage, reduce mortality rate and enhance antioxidant index in the liver and kidney; making it a potential probiotic against lead toxicity (Fewtrell et al., 2004; Li et al., 2017).

Another claimed health benefit linked to probiotics is improvement of lactose metabolism. It is widely agreed that fermented milk product such as yogurt can help with lactose digestion in lactose malabsorbers and therefore can be well tolerated by most lactose-intolerant subjects. Yogurt preparation using the traditional *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are even more effective because of their higher β-galactosidase activity. Lactose intolerance is a β-galactosidase deficiency resulting in the inability to digest lactose into the

monosaccharides glucose and galactose. People with Lactose intolerance develop diarrhea, abdominal discomfort, and flatulence after consumption of milk or milk products. Numerous studies have shown better lactose digestion and consequently less hydrogen exhalation in lactose malabsorbers who consumed yogurt with live cultures rather than milk or pasteurized yogurt (de Vrese et al., 2001; Kechagia et al., 2013).

All these benefits and characteristics are some of the best documented effects of probiotics which consequently provide a strong argument for the effectiveness of *L. bulgaricus* as a probiotic. Also, yogurt remains one of the most important vehicles for delivery of probiotic bacteria.

2.6 Trends in Probiotics

It appears the bar keeps getting raised as the year goes by in probiotics research. Recombinant technology and genetic modification may offer endless possibilities to the benefits we can derive from beneficial microorganisms as they are made to carry specific genes achieving a probiotic effect. Studies have shown that bacteria carrying either the gene for IL-10 or for trefoil factors when used in an animal model or as part of a human trial to improve inflammatory conditions in the colon. Such an approach is said to have the advantages of a long-term delivery, the potential for fewer side effects and use in many other conditions like autoimmune diseases, dental caries, candidiasis and allergies. Benefits may also be obtained by genetically engineering a probiotic bacterium to modulate production of pro-inflammatory cytokines or making a more effective delivery system with enhanced colonization. Other proposed health benefits could involve altering the fatty acid composition of adipose tissue by colonizing the intestine with a bacterium carrying the gene involved in fatty acid metabolism which also carries the additional benefit of inducing apoptosis in cancer cells.

The food and pharmaceutical industries will fancy their chances of taking advantage of the potentials Genetically modified lactic acid bacteria (GM-LAB) offer as regards improving human health and product improvement. Post-fermentation acidification in yogurt during the shelf life period was inhibited by tampering with lactose metabolism in *L. bulgaricus* through a spontaneous insertion sequence (IS) element-mediated deletion of the lacZ gene (Mollet & Delley, 1990).

Genetic mutation has been used to improve carbon dioxide production in some *L. lactis* strains which proved effective in enhancing the quality of Roquefort cheeses (El Attar et al., 2000). Induced mutation with roseoflavin increased vitamin B2 production in *L. lactis* (Sybesma et al., 2004). Monnet et al. 2000 also described the selection of a mutated *L. lactis* strain that overproduced diacetyl, responsible for the butter flavor in many fresh dairy products. Lactic acid bacteria can also be selected for the removal of undesirable compounds from raw food materials. For instance, galactose resulting from lactose breakdown in yogurt is harmful to people suffering from galactosemia and might cause cataract problems. Vaughan et al., 2001 described the selection of spontaneous galactose-fermenting mutants of Streptococcus thermophilus that may assist in removal of undesired galactose from the food matrix.

Texture is another important characteristic of food products, especially in yogurts. There could be a transfer of complete gene clusters coding for exopolysaccharides (EPSs) producing enzymes from one LAB strain to another. These new strains are then able to influence textural properties of fermented products (Germond, et al., 2001). In *S. thermophilus,* the low levels of EPSs was improved by inactivating the phosphoglucomutase gene resulting in an enhanced viscosity of fermented product (Levander & Svensson, 2002). As expected, the genetic-modification is not an idea embraced by so many countries; citing safety as a major concern. The

United States of America is liberal and open minded about the genetically modified organisms (GMOs) but met with strong resistance particularly in Europe as the majority do not support agri-food biotechnology (Gaskell et al., 2005).

The study of bacterial structural molecules offers an alternative to the whole-live-bacteria definition of probiotics. It challenges why probiotic bacteria must be alive to confer health benefits on the host and shifts more focus on the bioactive molecular components responsible for this effect; and even considers the probiotic capability of non-probiotic bacteria. Major advances in this field will create a shift from a generic, cure-all concept of probiotics to a rather specific mode of action (Caselli et al., 2011).

The standard for any food sold with health claims from the addition of probiotics is that it must contain at least 10^6 to 10^7 cfu per ml of viable probiotic bacteria (FAO/WHO, 2001). However, survival and viability of bacteria in fermented dairy products are marred by several factors; acidity, hydrogen peroxide, dissolved oxygen content, storage temperature, species and strains of associative fermented dairy product organisms. Probiotics also need to survive passage through the upper digestive tract in large numbers to effectively benefit the host. Microencapsulation is being taken advantage of to improve the rate of survival of probiotics. Microencapsulation is defined as a technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules designed to release their contents at controlled rates under the influences of specific conditions. In the food industry, microencapsulation finds its use in stabilizing the core material, controlling oxidative reaction, providing sustained or controlled release, masking flavors, colors or odors, extending the shelf life and protecting components against nutritional loss. Food-grade polymers such as alginate, chitosan, carboxymethyl cellulose

(CMC), carrageenan, gelatin and pectin are the main encapsulating materials usually applied using various microencapsulation technologies (Anal & Singh, 2007).

Psychobiotics is another interesting trend; which has to do with bacteria-brain relationship. They are defined as live bacteria, that confer mental health benefits by interacting with the gut bacteria. The scope of psychobiotics is said to cover prebiotics which support the growth of these beneficial microbes. Psychobiotics harness the gut-brain axis to influence stress, mood, anxiety and cognition. They influence mood through the modulation of neural networks associated with emotional attention and also influence psychophysiological markers of anxiety and depression (Sarkar et al., 2016).

In foods; probiotics have found most application in dairy products with yogurts, kefir and cultured drinks standing as notable representatives. Emerging food applications include nutrition bars, breakfast cereal, infant formula, probiotic cheese and ice creams (Cruz, et al., 2009), non-dairy probiotic products (Bansal, et al., 2016; Vijaya Kumar, et al., 2015).

2.7 Viability of *L. bulgaricus*

Probiotics in foods should be alive in adequate amount at the time of consumption to confer health benefits on the host. It is usually recommended that probiotics should be present in foods at a minimum number of 10^7 cfu/g. This is to make up for loss of viability during food processing, transportation and unfavorable storage conditions (Ferdousi et al., 2013). Maintaining viability of probiotics during food processing is a major challenge in the dairy industry and several studies have been geared towards improving their survival chances. The dairy industry has had to contend with loss of viability to cold and heat stress during the production and preservation of starter strains; processes which are critical in meeting the increasing demand for yogurt. Common methods used in starter production and preservation of

starter cultures in the industry are freeze drying, freezing, fluidized bed drying and spray drying. In cases where viability of cells is not in question, these processes cause cell injury which can induce a longer stationary phase in cells making them unfit for direct inoculation of milk for fermentation products (Busta, 1976). Different approaches and techniques have been developed to minimize the adverse effects of these processing conditions on the acidity activity and viability of *L. bulgaricus*.

2.7.1 Viability of *L. bulgaricus* in industrial processing

2.7.1.1 Low-Temperature processing. Freezing and Freeze-drying is a common method used in the preservation of starter cultures in the dairy industry. Despite the many benefits of freeze-dried cultures as can be seen in their ease of transport, storage and use; some strains do not survive the process. The damaging effects of freeze-drying on probiotic cells can be seen in their drop in metabolic activity, membrane damage, alterations in cell morphology which is critical in physiology and characterization of lactic acid bacteria, effectiveness in fermentation and the resultant impact on quality of product (Castro et al., 1997; Li et al., 2015; Streit et al., 2008). The main reasons for this loss of viability have been attributed to ice crystal formation which could puncture the cell membrane, osmotic stress, denaturation and dehydration (Thammavongs et al., 1996). Many studies have tried addressing these problems by tampering with process variables such as pH, temperature and cell harvesting time; rehydration conditions; use of cryoprotective agents; and pre-adaptation of cells to moderate stress before freezing.

A better response to the freeze-thaw cycle was observed in *L. bulgaricus* cells preconditioned by incubating at 30°C for 60 minutes before freezing at -20°c in lactose broth (de Urraza & De Antoni, 1997). Streit et al., 2007 improved cryotolerance in *L. bulgaricus* CFL1 by cultivating cells at an optimal pH of 5.15 for 30 minutes; achieving a better result with H_2SO_4

than with HCl. This treatment improved the stability of the cells during freezing and frozen cold storage although reduced their biomass productivity. The positive effect is largely due to physiological adaptation of cells to moderate acid stress rather than the acidity itself as a pH of 4.7 was not effective in adapting the cells in same study.

Wang et al., 2005 suggested positive results from acidic pH was due to alterations of membrane fluidity which allowed cells to more effectively tolerate cold stress during freezing. Physiological alterations in the fatty acid and proteome composition of cells have also been observed in improved cryotolerance of *L. bulgaricus* (Mansilla et al., 2004; Streit et al., 2008). Rault et al., 2010 stressed the importance of harvesting time in the cryotolerance of *L. bulgaricus* CFL1 even when an optimal pH was used. Cells recovered in the log phase exhibited lower viability, enzymatic activity and cultivability during freezing and frozen storage than cells recovered in the stationary phase.

The role of several media components (sugars, sugar alcohols, salt, antioxidants and amino acids) as protective agents has been identified in the stability of probiotic cells during freeze-drying and storage. Sucrose, trehalose and other carbohydrates are known to protect complete cells, liposomes and isolated biological membranes from the impact of freezing and dehydration through a number of mechanisms. They have been shown to prevent phase transition and the resultant leakage of cell contents upon rehydration by replacing water molecules between lipid headgroups thereby lowering the transition temperature of dry membranes. Sucrose also protect protein function in cells by initiating high viscosity or low mobility through formation of glassy matrix; also replacing water when the hydration shell of proteins is removed by binding to the dried protein (Bell & Hageman, 1996; Carpenter et al., 1992; Castro et al., 1997; Franks et al., 1991; Leslie et al., 1995).

An increase in the growth rate of *L. bulgaricus* was recorded when cryoprotective agents; sucrose, lactose and trehalose were each used in equal dilutions with MRS medium to preadapt cells to freeze-thaw cycles (Panoff et al., 2000). Adding sorbitol and monosodium glutamate to the drying medium increased survival of *L. bulgaricus* during storage at 20°C although both media components had no effect on the viability during freeze-drying in a study by Carvalho et al., 2003. Fonseca et al., 2001 identified Tween 80 and glycerol as important cryoprotective additives in their study of factors contributory to the resistance of *S. thermophilus* and *L. bulgaricus* to freezing and frozen storage. Tween 80 enhanced resistance to freezing but not in frozen storage while the addition of glycerol to the conditioning medium increased acidification activity after freezing and during frozen storage. Polyols like sorbitol, glycerol and mannitol protect cells by maintaining turgor pressure, stabilizing structures of lipid membrane and proteins at low water activity, and preventing damage due to oxidation (de Valdez et al., 1985; Kets et al., 1996; Yoo & Lee, 1993). Bacterial cells of *L. bulgaricus* suspended in buffer solutions containing 5% glutamate and 5% aspartate respectively; had high viability rate during freeze-drying and storage due to an increase in the membrane fluidity (Martos et al., 2007).

In a study evaluating the cryoprotective effects of media components, Fonseca et al., 2003 observed a combination of sodium ascorbate with either betaine or sodium glutamate significantly enhanced the recovery of acidification activity in *L. bulgaricus* cells after freezing and during frozen storage. The reaction between glutamate's amino group and carboxyl group of microorganism's proteins coupled with high water retention capacity have been suggested as the protective mechanism of monosodium glutamate (de Valdez et al., 1985). In same study, Fonseca et al., 2003 also underlined the impact of combining various additives as the effect of certain additives on the acidification activity of cells vary during the freeze-drying process and

frozen. Although glycerol's cryoprotective effect was not as pronounced as other additives during freezing, glycerol and sodium ascorbate were the only additives in the study that had protective effect on the acidification activity of *L. bulgaricus* CFL during storage at -20°C. Adding 2% NaCl to MRS during the late growth phase of *L. bulgaricus* improved the viability of freeze dried cells by influencing glucose metabolism during drying (Li et al., 2015).

2.7.1.2 High-Temperature processing. *L. bulgaricus* and other bacterial cultures also suffer cell injury and death when subjected to high temperatures and dehydration. The resultant effects of cell injury and death are seen in the destruction of functional properties and the quality of fermentation products. Thermotolerance of a microorganism describes its ability to display increased survival after exposure to high temperatures. Just like in cold stress; operating conditions, growth medium and protective media components are methods used to maintain the membrane and functional integrity of *L. bulgaricus* cells exposed to high temperatures. Heat shock, usually a mild heat treatment has been used in microbial cells to induce an increased tolerance to subsequent heat exposures. A possible mechanism is the synthesis of heat shock proteins which regulate the increased thermotolerance. Results from a study by Teixeira et al., 1994 showed that *L. bulgaricus* could be made more tolerant to lethal temperatures by brief exposure to a mild temperature although this outcome was dependent on the growth phase of the cell. Gouesbet et al., 2001 observed that *L. bulgaricus* ATTC11842 incubated at 50 °C for 30 min had a much more increased thermotolerance compared with cells not exposed to this treatment. It was also observed that cells of *L. bulgaricus* in the stationary phase were more resistant than those in the exponential phase when exposed to heat treatment at 65 °C for 10 min. Resistance to high temperatures can also be induced by accumulation of osmolytes like sugar and

salt which are said to improve stability of protein and prevent heat inactivation of enzymes (Abee & Wouters, 1999).

The water-replacement hypothesis further explains that sugars can replace water during drying of microbial cells by forming hydrogen bonds around the polar groups in phospholipid membranes and proteins making them retain their original structure in the absence of water (Crowe et al., 1984). The heat resistance of *L. bulgaricus'* cells in stationary phase was increased when sucrose and/or NaCl were added to MRS as growth medium in a study by Carvalho et al., 2003. The authors used decimal reduction time, D_T value as a yardstick for measuring the thermotolerance of *L. bulgaricus* and observed the D_{57} values of cells in MRS were about half less than in MRS medium containing sucrose and/or NaCl. The highest recovery of *L. bulgaricus* cells was observed in a study by Gomez et al., 2003 when trehalose was used in the drying medium. Trehalose also reduced the lag phase of rehydrated *L. bulgaricus cells* grown in MRS. A significant reduction in lag time; comparable to that of nondehydrated cells was also observed by Tymczyszyn et al., 2007 when *L. bulgaricus* was dried at 70°C in the presence of trehalose or sucrose. Accumulation of sucrose is said to induce thermotolerance and this was the case when sucrose was added to the growth medium which enhanced the survival of *L. bulgaricus* cells when heated in sterile Ringer's solution and when stored in the dried state (Silva et al., 2004; Welsh, 2000).

2.7.2 Viability of *L. bulgaricus* in Foods.

Survival of probiotic bacteria in fermented dairy products stored for an extended period at refrigeration temperature has been reported to be unsatisfactory. The viability of probiotic bacteria in yogurt depends on so many factors; culture condition, type of strain, yogurt ingredients and additives, acidity of the medium, milk solids present, interaction between yogurt

starter organisms, storage temperature and period, nutrient availability, growth promoters and inhibitors, type of package, concentration of sugars, and level of dissolved oxygen (Dave & Shah, 1997; Ibrahim & Carr, 2006; Karlton-Senaye, Tahergorabi, Giddings, & Ibrahim, 2015; Rybka & Kailasapathy, 1995).

2.7.2.1 Oxygen content and redox potential. Oxygen content and redox potential are important factors to be considered in the viability of probiotics especially during extended storage period. *L. bulgaricus* is a facultative anaerobe and does not require strict anaerobic growth conditions. Regarded as one of the least aerotolerant lactic acid bacteria, the presence of oxygen could negatively impact on the physiology and growth of *lactobacillus bulgaricus* (Archibald & Fridovich, 1981). A dramatic decrease in the viability of *L. bulgaricus* in pure and mixed cultures was recorded at 30% dissolved oxygen in a study by Beshkova et al., 2002. Probiotics are affected by oxygen in three major ways; (a) it is directly toxic to some cells, (b) some cultures produce toxic peroxides from the reduction of oxygen, and (c) free radicals produced from the oxidation of components like fat are toxic to probiotic cells. High levels of oxidases; which are responsible for removing oxygen from the intercellular medium have been reported in aero-tolerant species (Roy, 2005). Reduction of oxygen by L. bulgaricus with an NADH oxidase usually leads to an accumulation of hydrogen peroxide which inhibits not only the growth of *L. bulgaricus* but other associative bacteria present (Marty-Teysset et al., 2000). Products of lipid peroxidation have been shown to initiate DNA damage in a model system and in bacteria (Akasaka, 1986; Marnett et al., 1985).

These destructive mechanisms of oxygen stress the need for oxygen levels in packages to be as low as possible to prevent loss of viability in probiotic microorganisms and consequently, the loss of functionality of the product. Different methods and approaches have been attempted

to achieve low oxygen levels during packaging and during storage of probiotic foods. These include the use of antioxidants and oxygen scavengers, vacuum packaging, use of packaging materials with low oxygen permeability, and optimizing the production process in a way to ensure minimum dissolved oxygen enters the product (Dave & Shah, 1997; Korbekandi et al., 2011; Talwalkar et al., 2004). The use of aerotolerant strains of *L. bulgaricus* yogurt production can also reduce the weakening impact of oxygen on the growth and metabolism of this probiotic bacteria. A method developed by Talwalkar et al., 2001 can assist in differentiating oxygen sensitive strains of probiotic bacteria from oxygen tolerant strains. They used modified relative bacterial growth ratio (RBGR) to successfully measure the oxygen tolerance of different strains of several probiotic bacteria. The addition of antioxidant compounds in yogurt have the potential to reduce the growth-inhibitory effects of oxygen exposure during extended storage.

Antioxidant compounds reduce oxygen tension by scavenging reactive oxygen species and nitrogen species; and also sequester metal ions which may initiate free radicals. Glucose oxidase, ascorbic acid and L-cysteine improved the viability of *L. bulgaricus* and were effective in reducing redox potential thus reducing the oxidative stress suffered by this probiotic(Cruz et al., 2012; Dave & Shah, 1997a, 1997b). However, glucose oxidase increased post acidification which also inhibits the growth of probiotic bacteria. The use of antioxidants in combination with vacuum storage with a controlled water activity will be effective in maximizing probiotic viability during storage.

2.7.2.2 Packaging material. A wide range of packaging materials and techniques play an important role in the viability of probiotics. The type and thickness of packaging materials, gas and light permeability of materials, packaging techniques; vacuum, modified, active/intelligent packaging systems, are all important factors in ensuring viability in food products (Korbekandi et

al., 2011). Most of the dairy probiotic and other products on the market are in plastic packages with high oxygen permeability. Thermoformed high impact polystyrene (HIPS) is the most popular material used for spoonable yogurt in the form of small cups or larger tubs using either aluminum foil/plastic laminate or a paper/plastic laminate heat-seal lid or closure. Pigments such as TiO_2 are added to HIPS to enhance appearance of the package and offers some barrier to light (Robertson, 2006b). Rectangular paperboard cartons, glass containers, polypropylene and blow-molded highdensity polyethylene (HDPE) containers are also all in common use. Laminated materials are used for spoonable yogurt products as they require a low water vapor transmission rate (WVTR) to prevent water loss during shelf life, a good oxygen barrier to prevent oxidation and a good light barrier to inhibit light-induced oxidation. The most popular containers for drinking-yogurt products are high density polyethylene (HDPE) bottles sealed with either aluminum foil laminate heat-seal closures or with low density polyethylene (LDPE) snap or screw caps. Laminate cartons with good oxygen, water vapor and light barriers are also frequently used (MacBean, 2009).

Miller et al., 2003 showed permeation through packaging material as well as the production method influence the oxygen level in yogurt. The study indicated the relative permeability of HIPS to oxygen. The thickness of HIPS containers at various points of the package affected the O_2 content and the level of oxygen increased in the package during storage to levels around 12.5 ppm by day 35 of a total of 42 days of storage. It was also observed that using a laminate consisting of HIPS/tie/EVOH/tie-layer/LDPE (where EVOH is ethylene vinyl alcohol copolymer) was more effective in reducing oxygen content in yogurt compared with using only HIPS. Also, adding an O_2 scavenger ($ZerO_2$) to the packaging material further

reduced the O_2 content, particularly for set yogurt over the first few weeks of a 6-week storage trial.

Glass packages have low oxygen permeability which favors the survival of probiotic cultures. The high cost of glass coupled with the associated hazards when handling tilts manufacturers towards marketing food products containing probiotics in plastic packages. The effectiveness of glass bottles in maintaining viability was reported by (Jayamanne & Adams, 2004). The authors fermented and stored buffalo milk in clay pots, plastic cups and glass bottles, and reported Bifidobacteria had the best viability in the glass bottle followed by the plastic packages and the clay pots when stored at 29°C.

2.7.2.3 Storage temperature. Foods serving as vehicles for probiotics should preferably be stored a temperature of 4-5°C as viability of probiotic bacteria varies inversely to storage temperature. In general, increased storage temperatures initiate an increase in the metabolic activities of bacterial cells and in the process cause an increase in their death rate. Mortazavian et al., 2007 in a bid to identify the best storage period, studied the effect of storage temperatures; 2, 5 and 8°C, on the viability of mixed cultures of *L. acidophilus*, *Bifidobacterium lactis*, *S. thermophilus* and *L. bulgaricus* in yogurt over a 20-day refrigerated storage period. Highest viability of *L. acidophilus* was recorded at 2°C while the highest viability of *B. lactis* was recorded at 8°C over a period of 20 days of refrigerated storage. At temperatures 5 and 8°C, *L. bulgaricus* grew faster and increased the amounts of hydrogen peroxide and lactic acid. Accumulation of hydrogen peroxide is not only toxic to *L. bulgaricus* but also other associative probiotic bacteria present. According to Dave & Shah, 1997a, hydrogen peroxide production by *L. bulgaricus* is the most critical factor responsible for loss of viability during refrigerated storage.

2.7.2.4 pH and post-acidification. The pH value and titratable acidity of a food product significantly affect the viability of probiotics during storage (Mortazavian et al., 2010). The use of probiotic species with little or lack of acid tolerance is a major drawback in fermented milk products (Klaver et al. 1993). Food products with low pH provide a difficult growth medium for probiotics to thrive as acid content in fermented milk products shows a direct correlation with the value of redox potential. A very low pH increases the concentration of undissociated organic acids in fermented food products; thus, strengthening the antibacterial effect of organic acids. It also impacts on the metabolic activity of lactic acid bacteria; affecting the expression of various enzymes.

The pH value of 4.5 is preferred in commercial as it prolongs the shelf life of yogurt, impacts a mild flavor and a pleasant product appearance (Hui et al., 2007) while pH below 4.0 contributes to *L. bulgaricus* producing extreme lactic acid especially during refrigerated storage. When the concentration of lactic acid increases, pH levels correspondingly decrease leading to over-acidification or post-production acidification. Consequently, the viability of other probiotic bacteria present in yogurt comes under threat because *L. bulgaricus* is more acid tolerant. The effect of low pH and post acidification on viability can be minimized by using strains of probiotic bacteria that are acid tolerant and strains of *L. bulgaricus* with weak over-acidification property. Bacteria exposed to acidic conditions respond by maintaining a pH homeostasis through a discharge of H+ from cells; a process controlled by the activity of proton-translocating ATPase (H^+-ATPase). The ATPase is a reversible ion translocating pump that catalyses the movement of hydrogen ions across the membrane ensuring the intracellular pH remains neutral. Hence, it has been reported that acid-tolerant bacteria have a higher H^+-ATPase activity than those sensitive to low pH (Kobayashi, Suzuki, & Unemoto, 1986; N. Yamamoto, Masujima, &

Takano, 1996; Yokota, Amachi, Ishii, & Tomita, 1995). However, reduced ATPase activity is preferred when screening for strains of *L. bulgaricus* with weak post acidification property. Ongol et al., 2007 found that the mutational *L. bulgaricus* with reduced proton-translocating ATPase (H+ -ATPase) activity had a significant reduced post-acidification. In another study by Wang et al., 2013, the parent *L. bulgaricus* strain grew and acidified milk faster than strains with reduced H+ -ATPase activity. Other methods targeted at reducing post acidification have also been reported.

Addition of encapsulated probiotic bacteria reduced acid build-up in yogurt during storage and post acidification was better compared to yogurt cultured with traditional yogurt starter. This may not be a practicable solution as the technology of encapsulation and the encapsulated materials do not come at a cheap. The addition of encapsulated probiotic bacteria will increase the cost of producing yogurt.

Thus, there is a need for encapsulation for probiotic bacteria to survive in adverse environment of yogurt. However, the encapsulant materials are natural polysaccharide with high price. Thus, addition of encapsulated probiotic bacteria will add the prime cost of yogurt. The use of high pressure processing has also been reported in minimizing post acidification. de Ancos et al., 2000 used pressures over 200 MPa to prevent post-acidification in low-fat stirred-type yoghurt during chilled storage. Pressurized yogurts also showed higher viscosity and amino acids content than the yogurts used for control. This research was similar to the low acidification in full-fat yogurt achieved by Tanaka & Hatanaka, 1992. However, high pressure might have injured the bacterial cells preventing their replication and subsequent acidification of yogurt during refrigerated storage.

2.7.2.5 Food components. Food ingredients play a significant role in the stability and viability of probiotics. Care must be taken with the choice of ingredients in food products as they can inhibit or support the growth of these beneficial microbes. Food ingredients and additives used in yogurts and other fermented food products include food flavors and colors, fat replacers, amino acids, antioxidants, aroma compounds, different types of sweeteners, salts and preservatives. Ibrahim & Carr, 2006 recorded higher populations of Lactobacilli and Bifidobacteria in yogurt products supplemented with antioxidants, amino acids, casein hydrolysate or peptides during refrigerated storage. However, some of the additives in food products have been identified as inhibitory to the growth of probiotics.

Curing agents such as sodium nitrite, usually used in meat preservation is a challenge for the survival of probiotic bacteria in meat fermentation (Kołożyn-Krajewska & Dolatowski, 2012). Ramchandran & Shah, 2008 reported a decrease in growth when *L. bulgaricus* 1368, *L. casei*, and *L. acidophilus* were grown in versagel®, a whey protein-based fat replacer from Australia; although a significant increase in growth was recorded for *S. thermophilus* and *B. longum*.

Several studies have also focused on gums, fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin and whey protein concentrate (WPC) as ingredients to promote survival and viability of probiotics. Shin et al., 2000 reported an increase in growth when Bifidobacterium Bf-1 and Bf-6 were grown in the presence of FOS, GOS and inulin.

2.8 Developing a Culture Media for *L. bulgaricus*

Lactobacillus bulgaricus is an important starter in commercial yogurt production and some cheese (Italian-type and Swiss-type varieties). It is used as mixed cultures in these

applications and as a result, often pose a challenge when important analyses such as isolation, and enumeration of viable *L. bulgaricus* cells need to be done.

Enumeration of *L. bulgaricus* in yogurt is particularly important as several studies have pointed out and highlighted the probiotic characteristics of *L. bulgaricus*, which further lends credence to the claims of Elie Metchnikoff who attributed the positive health benefits and longevity enjoyed by the native population in certain regions of Bulgaria who were regular consumers of yogurt; to the presence of Bulgarian bacillus now referred to as *Lactobacillus bulgaricus* (Metchnikoff, 1908). Probiotics are to be consumed in adequate amounts to confer various health benefits and it has been argued that a minimum level of 10^6 cfu/mL should be present to enjoy these benefits (Shah, 2000). An effective medium for the enumeration and isolation of *L. bulgaricus* is also vital in ensuring proper ratios of starter cultures in yogurt and cheese production.

A culture medium contains necessary nutrients; growth promoting factors, buffers, energy sources, nitrogen, vitamins and minerals; crucial for the survival, growth and metabolism of microorganisms. The nutritional demands of various bacterial species differ greatly and is a determining factor in the chemical composition of a culture medium. Therefore, an understanding of the metabolism and nutritional requirements of a microbe of interest is important to develop the perfect growth conditions and culture media. Lactic acid bacteria (LAB) are fastidious when it comes to nutritional requirements; the presence or lack of certain nutrients not only control their growth but also vital metabolic and enzymic activities (Hoefnagel et al., 2002; Loubiere et al., 1996; Sanchez & AL, 2008). A number of culture media have been developed to leverage on these nutritional demands to grow and isolate LAB; with the most commonly used being deMan-Rogosa Sharpe agar (MRS) (De Man, Rogosa, & Sharpe, 1960).

However, it is difficult to differentiate various LABs on MRS thereby creating a need for a differential and selective medium.

In addition to nutritional requirements for growth, differential and selective media have been developed based on LAB sensitivity to acidity, inhibitory compounds, metabolism of sugars other than glucose and addition of various dyes (Coeuret et al., 2003; Dave & Shah, 1996; Lee & Lee, 2008; Roy, 2001). Selective media inhibit the growth of other microbes present while supporting the growth of microorganism of interest. Differential media employ color changes resulting from the biochemical activity of microorganisms present to visually create a distinction between them. A number of selective and differential media have been developed to facilitate the use of *L. bulgaricus* for industrial and research purposes.

2.8.1 Biochemical Characteristics of *L. bulgaricus.* *Lactobacillus delbrueckii* subsp. *bulgaricus* is one of the members of the six subspecies of *L. delbrueckii;* which share at least 78% DNA similarity. The other members of this group are *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *Lactis*; with the recently discovered, *L. delbrueckii* subsp. *indicus*, *L. delbrueckii* subsp. *jakobsenii*, and *L. delbrueckii* subsp. *sunkii* (Adimpong et al., 2013b; Dellaglio et al., 2005; Kudo et al., 2012). The biochemical characteristics are shown in Table 4. *L. bulgaricus* is broadly described as rod-shaped, lactic-acid producing, homofermentative with all strains reported being able to ferment glucose and lactose.

Table 4

Biochemical characteristics of L. bulgaricus

Characteristics	Result in *L. bulgaricus*
Motility	Non-motile
Fermentation	Homofermentative

Cont.

Lactic acid Production	D (-) lactic acid
Gram Staining	Gram Positive
Catalase Reaction	Negative
Oxidase Reaction	Negative
Arginine Hydrolysis	Negative
Spore Formation	Non-Spore Forming
Oxygen Utilization	Facultative Anaerobic
Growth Temperature	No growth at 15 ^{0}C, Optimum growth at 45 ^{0}C
Carbohydrate Metabolism	Readily ferments Lactose and Glucose

Variations in the fermentation of other sugars have been reported; making identification by this means only speculative and not conclusive. The characteristics of *L. bulgaricus* are outlined in the table above. *L. bulgaricus* are thermophilic with the ability to grow at a temperature as high as 55 ^{0}C. Aghababaie, Beheshti, & Khanahmadi, 2014 in their study using the response surface methodology reported a maximal growth and acid production at 44 ^{0}C with optimum pH for these attributes at 5.7 and 5.13 respectively. Optimal growth for *L. bulgaricus* was also recorded at the same temperature, 44 ^{0}C at pH 5.8 by Beal, Louvet, & Corrieu, 1989. Rault, Bouix, & Béal, 2009 reported high viability and stable acidification activity in *L. bulgaricus* at pH 5 in contrast to the decrease in viability and fluctuation of activity at pH 6.

2.8.1.1 Carbohydrates metabolism of L. bulgaricus. Carbohydrates, proteins, amino acids and glycerol are important sources of carbon and energy required by microorganisms for optimal growth and functionality. However, lactic acid bacteria do not possess the metabolic system to use proteins, amino acids and glycerol to meet their carbon and energy needs.

Consequently, they ferment carbohydrates and get the needed energy through substrate-level phosphorylation and the adenosine triphosphate enzymes (ATPases) of the cytoplasmic membrane (Nannen & Hutkins, 1991; A Y Tamime & Robinson, 2007). Lactic acid bacteria are classified as homofermenters or heterofermenters depending on the products resulting from hexose fermentation. Heterofermentative LAB (*L. brevis*, *L. fermentum*, *L. reuteri*, *L. rhamnosus*, *L. amylovorus*) metabolize hexoses to produce lactic acid, CO_2, acetic acid and/or ethanol using the pentose-phosphate pathway. Homofermentative LAB (*L. bulgaricus*, *L. acidophilus*, *S. thermophilus*) produce lactic acid as the primary product from the metabolism of hexoses using the glycolytic pathway (Fig. 2) (Kandler, 1983).

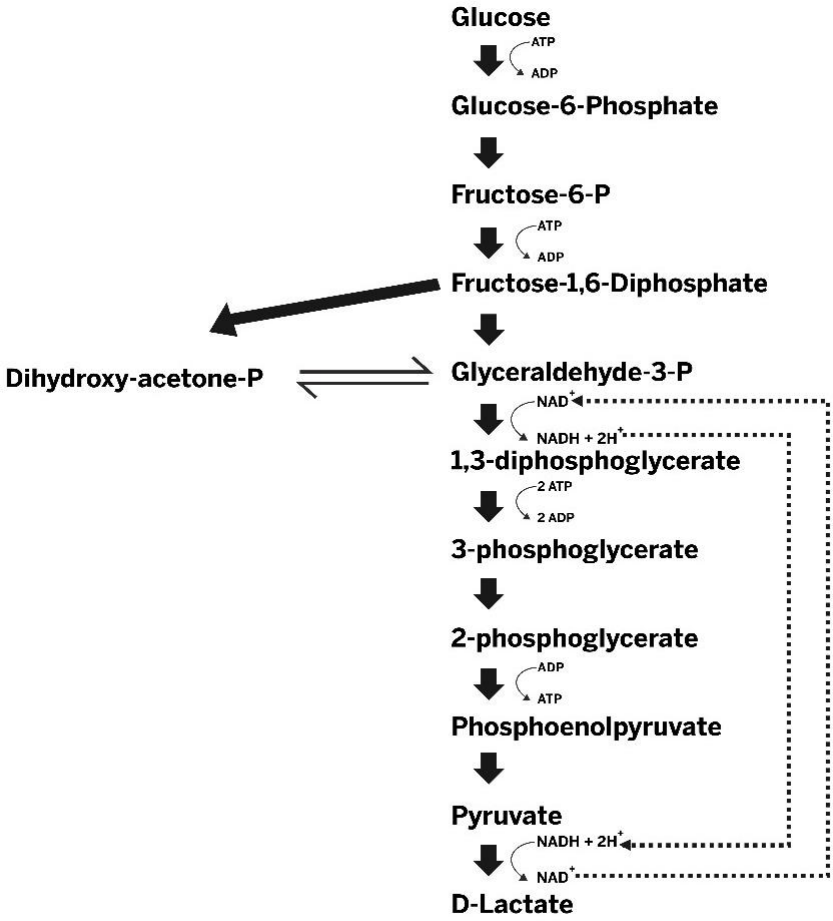


Figure 2. Homolactic Fermentation of *L. bulgaricus* (Glycolysis, Embden-Meyerhof-Parnas pathway)

Information about the metabolism of sugars by *L. bulgaricus* has not been extensively reviewed and updated over the years with available literatures reporting a preferential metabolism of few sugars by *L. bulgaricus* (Michaylova, 2012; Zourari et al., 1992). Amidst the discrepancies reported about the ability of *L. bulgaricus* to use and ferment some sugars, available literatures have all consistently reported *L. bulgaricus* was able to metabolise lactose, glucose and in some cases, galactose (Kulp & Rettger, 1924; Petry et al., 2000; Wheater, 1955). Lactose is fermented by *L. bulgaricus* using a combination of the lactose permease system and the enzyme, β-galactosidase.

The lactose permease system transports which has been described as similar to that in *Escherichia coli*, transports lactose into the cell where it is cleaved by β-galactosidase into non-phosphorylated glucose and galactose (Tamime & Robinson, 2007). The Embden Meyerhof pathway is then used to metabolize glucose to pyruvate; which is converted to lactic acid by the enzyme, lactate dehydrogenase. Lactic acid production is crucial in yogurt production as it helps to initiate the desired gel formation by destabilizing casein micelle (Tamime & Deeth, 1980b; Tamime & Marshall, 1997). Galactose accumulates in yogurt and is not metabolized as much as glucose. Galactose is said to be metabolized by *L. bulgaricus* after the glucose moiety of lactose has been exhausted via the Leloir pathway (Fig. 3) having galactokinase as its first enzyme (Tamime & Robinson, 2007). Studies have been conducted on enhancing the sweetness of yogurt and reducing its galactose content through the use of *S. thermophilus* and *L. bulgaricus* mutants having the metabolic mechanism to ferment galactose (Anbukkarasi et al., 2014; Sørensen et al., 2016). The inconsistencies recorded in *L. bulgaricus'* use of fructose, mannose, maltose amongst other sugars; have been attributed to strain type and composition of growth medium (Chervaux et al., 2000; Zourari et al., 1992).

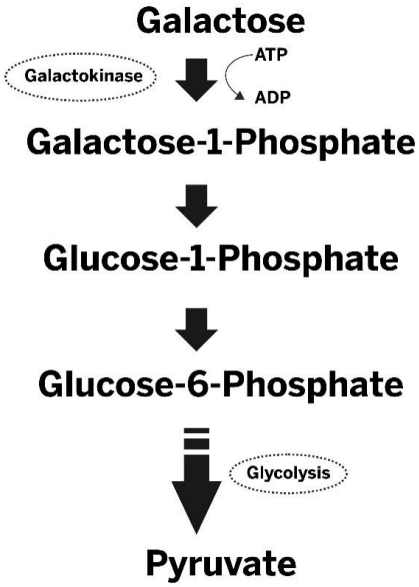


Figure 3. Galactose Metabolism via the Leloir Pathway

Kulp & Rettger, 1924 gave an extensive account of the utilization of different sugars by *L. bulgaricus* while trying to differentiate it from *L. acidophilus*. Maltose which had been suggested by Rahe, 1914 as a differential test to separate *L. bulgaricus* from *L. acidophilus* was not decisive in this case as two strains of the former fermented this sugar. However, none of the *L. bulgaricus* strains used in a study by Wheater, 1955 was able to ferment maltose. A strain of *L. bulgaricus* in the same study by Kulp & Rettger, 1924 was able to produce acid from sucrose while results from trehalose fermentation varied like they had in maltose. Hodge, 1937 had earlier suggested *L. bulgaricus* may be able to ferment maltose and sucrose after an extended period of continuous culturing in the laboratory. None of the *L. bulgaricus* isolates from yogurt was able to ferment maltose and sucrose in a study by Zahoor et al., 2003.

However, in a more recent research, a *L. bulgaricus* isolate by Turgay & Erbilir, 2006 was able to ferment maltose, ribose and trehalose. In another case of disparity in sugar fermentation by *L. bulgaricus*, all the strains in the study by Kulp & Rettger, 1924 were only

able to ferment fructose (levulose) when heated with no acid production recorded when fructose was sterilized by filtration. This was not the case in the study by Wheater, 1955) who recorded fermentation of unheated fructose in 74% of the strains used. Tabasco et al., 2007 were able to selectively grow *L. bulgaricus* in an MRS basal medium devoid of glucose and meat extract while supplementing with 1% fructose.

There is limited information available about the metabolism of oligosaccharides, which contain 2-10 monosaccharide residues by *L. bulgaricus*. Most strains of bifidobacteria are said to be able to ferment oligosaccharides with only a few strains of lactobacilli possessing same ability. Some strains of *L. bulgaricus* isolated from dairy products were able to grow in different MRS media modified with 2% of galactooligosaccharide, glucooligosaccharide or fructooligosaccharide (Ignatova et al., 2009). One of the three strains of *L. bulgaricus* in a study by Kaplan & Hutkins, 2000 was able to ferment a pure form of fructooligosaccharide (FOS); the authors eluted the fructose, glucose and sucrose components usually present in commercial formulation of oligosaccharides.

Pyruvate could be included in growth media despite the ability of LAB to use some hexoses as substrates for this molecule. Pyruvate is a vital source of energy and a key intermediate in the metabolism of lactic acid bacteria; especially in the production of lactic acid by the homofermentative species. Higashio et al., 1977 attributed the stimulatory effect *S. thermophilus* had on the growth of *L. bulgaricus* to the production of formic and pyruvic acids. Improved growth was recorded for *L. rhamnosus* when glucose, the carbon source in the growth media was supplemented with sodium pyruvate (Bajpai-Dikshit et al., 2003; Polak-Berecka et al., 2011).

2.8.1.2 Mineral Requirements. Minerals are required by microbes generally for growth and enzyme activity. Usually supplied in growth media as metal salts, the metal ions affect bacterial growth and metabolism by functioning as activators or cofactors of a variety of enzymes, components of molecules or structural complexes; and as an integral part of membrane transport (Hébert et al., 2004). Boyaval, 1989 in the review of lactic acid bacteria and metal ions highlighted some limitations associated with the effective study of the mineral requirements of a microbe. Chief among these limitations is the contamination of media components and water resulting from traces of metallic ions. Other limitations include; 1) metals replacing each other 2) some metals adsorbing others, 3) some metals interacting differently in the presence of others and 4) organic substances making these metals unavailable for growth by combining with them. The use of deionized water in media makes sure contamination from water is well taken care of.

Some methods suggested to rid culture media of metal ions contaminants come with their own inadequacies. Extraction of complexes chelated to organic compounds with anionic solvents poses an issue when these metal ions tightly bind or are adsorbed to organic materials present in the culture medium. Traces of manganese, magnesium, iron and potassium were removed by growing *L. arabinosus* in a growth medium for 24 hrs. after which the cells were removed and growth medium refortified with required nutrients leaving out the metal to be tested (Bentley et al., 1947; MacLeod & Snell, 1948). Generally, lactic acid bacteria need Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, K^{+} and Na^{+} as either essential or stimulatory factors for transportation of nutrients and enzymic activity (Hayek & Ibrahim, 2013).

Magnesium and manganese ions play crucial roles in the growth of lactic acid bacteria, particularly their enzymic activity. Manganese plays a major biological role in the structure and activation of enzymes such as lactate dehydrogenase, RNA polymerase, glutamine synthetase,

and alkaline phosphatase (Fitzpatrick et al., 2001). Mn^{2+} was found to be essential for growth and exopolysaccharides production in *L. bulgaricus* with best results achieved at a decreased concentration of manganese (Grobben et al., 2000). The role of manganese in growth has also been recorded in other lactic acid bacteria. A conclusive effect of manganese on bacterial growth could not be drawn by MacLeod & Snell, 1947 even though more growth of *S. faecalis* was recorded in a growth medium supplemented with trace amounts of manganese than in a medium lacking this metal ion. A study by Foucaud et al., 1997 showed Mn^{2+} was stimulatory for *Leuconostoc mesenteroides* while Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} were unessential. Magnesium is another essential metal ion; a major divalent cation in living cells that triggers different metabolic processes such as cell division, stabilization of nucleic acid and gram complex synthesis (Henry & Stacey, 1946; Webb, 1948, 1949, 1951a, 1951b). Hébert et al., 2004 showed Mg^{2+} was the only essential oligoelement needed for the growth of *L. delbrueckii* subsp. *lactis*. Mg^{2+} influenced the increase in the survival rates of *S. lactis* resuspended in phosphate buffer with a reduced RNA content also recorded when suspended in a buffer without Mg^{2+} (Thomas & Batt, 1968; Thomas & Batt, 1969). Many enzymes that require Mg^{2+} for activation can make do with Mn^{2+} but manganese cannot substitute for Mg^{2+} in some enzymic reactions mediated by magnesium ions (Nilsson et al., 1942).

It has been reported that calcium ions (Ca^{2+}) play a role in bacterial processes like maintenance of cell structure, cell division, gene expression, substrate uptake, ion transport and motility (Norris et al., 1996; Smith, 1995). Calcium enhanced cell division and also preserved the structural integrity of *L. bulgaricus* cells in a study by Wright & Klaenhammer, 1983. The authors drew attention to the morphological state of bacterial cells. Long filamentous cells are

said to exhibit a clumping phenomenon likened to agglutin phenomenon which makes them unsuitable for fermentation of milk (Lawrence et al., 1976).

The addition of calcium to the growth medium by these authors also prevented bleb formation which heightens structural damage during freezing. Wright & Klaenhammer, 1983 in another study, reported an improved resistance to freezing and freeze-drying in *L. bulgaricus* cells grown in the presence of calcium than cells grown in its absence. Calcium ions enhanced the stability of bacteria cell enveloped proteinases in *L. lactis* by binding to bioactive peptides produced during proteolysis while also enhancing the thermal stability of these proteinases (Espeche Turbay et al., 2009). The elimination of manganese or iron in a chemically defined medium by Chervaux et al., 2000 did not affect the growth of *L. bulgaricus* upon addition of calcium and other micronutrients. The growth-supporting role of calcium in *L. bulgaricus* cells was also corroborated by (Petry et al., 2000).

Iron ions have been reported to have minimal or no effect on the growth of several lactobacilli; making its inclusion as a component of growth media unnecessary (Imbert & Blondeau, 1998; Lawrence et al., 1976). Sodium chloride (NaCl), although stimulatory for growth and acid production in some lactobacilli is required in trace amounts (Irvine & Price, 1961). In a study by Chikthimmah et al., 2001, an increased concentration of NaCl (5%) inhibited the growth of LAB in Lebanon bologna while a concentration of 0-2.5% stimulated a rapid growth of LAB. Salinity was also reported by Nour et al., 2014 to affect the proteolytic activity of *L. bulgaricus* as an increased concentration of sodium chloride decreased this activity. Best results were achieved at concentrations of 0.5% and 0.75%. NaCl concentrations (2-2.5%) have also been reported to enhance the viability of freeze-dried *L. bulgaricus* cells (Carvalho et al., 2003; Crowe et al., 1984).

2.8.1.3 Buffering Agents. Buffers are essential to ensuring the pH in a culture medium remains optimal for the growth and enzyme activity of lactic acid bacteria. Most lactobacillus strains are reported to grow at optimum pH of 5-6, occasioned by a slow growth at a pH as low as 4.4 (Lahtinen et al., 2011). The production of lactic acid resulting from the fermentation of various carbon sources in a growth medium only contributes to the lowering of the pH below the optimal levels which could be inhibitory to the growth of lactic acid bacteria. A suitable buffer should: 1) possess a high buffering capacity in the optimal pH area 2) not adsorb or chelate other medium components, and 3) not be metabolized (Hébert et al., 2004). Hayek & Ibrahim, 2013 identified some of the essential buffering agents used in MRS and M17 growth media. These include sodium acetate, trisodium citrate, di-sodium-glycerophosphate, disodium phosphate, ammonium citrate and dipotassium phosphate. Michael et al., 2015 recorded greater counts of *L. bulgaricus* and *L. acidophilus* in yogurt samples supplemented with sodium acetate compared with yogurts lacking this additive. They attributed this to the buffering ability of sodium acetate.

2.9 Culture Media for the Enumeration of *L. bulgaricus*

Available selective/differential culture media used for the enumeration of *Lactobacillus bulgaricus* in mixed culture are highlighted in Table 5.

Table 5

Selective/differential Media for Lactobacillus bulgaricus in Mixed Culture

Medium Name	Cause of Selectivity/Differentiation	Colony Morphology	References
MRSF	Incubation at 45 °C for 72h, Fructose, Tween 80	Lenticular	Tabasco et al., 2007
MRS 5.2	Anaerobic incubation at 45 °C for 72 h, Low pH 5.2	White and Irregular shape	Dave & Shah, 1996

Cont.

RCPB 5.0	Low pH 5.0, Prussian blue	White with wide dark blue halo	Rybka & Kailasapathy, 1996
mRCM	Aniline blue	Large, irregularly shaped with dark blue centers	Nwamaioha & Ibrahim, 2018
X-Gal	X-gal chromogen, Anaerobic incubation at 47 °C for 72 h, Low pH 5.2 ± 0.2	Green colonies	Galat et al., 2016
BGWA	Bromocresol green	Lightly colored with greenish center, Irregular mass with twisted filament projections of about 2-5 mm in diameter	Yamani & Ibrahim, 1996
HHD	Bromocresol green, Fructose	Large, irregular in shape with flat surface, bright green with an internalized undulating streak	McDonald et al., 1987
TPPY-Prussian Blue	Prussian blue	Shiny white appearance surrounded by a wide royal blue zone	Ghoddusi & Robinson, 1996
TPPY-Eriochrome	Eriochrome black T	Transparent, 4-6 mm in diameter, Undefined shape with irregular edges	Bracquart, 1981
YLA Agar	7% skim milk	Large white colonies surrounded by a cloudy zone	Matalon & Sandine, 1986
Lee's Agar	Bromocresol purple	Yellow, flat, large colonies with irregular edges	Lee et al., 1974

TPPY: Tryptose proteose peptone yeast extract, RCA: Reconstituted clostridial agar, MRS: deMan Rogosa Sharpe, HHD: Homofermentative- Heterofermentative Medium, BGWA: Bromocressol green whey agar, YLA: Yogurt lactic agar, MRS F: deMan Rogosa Sharpe- Fructose, RCA: Reconstituted clostridial agar, RCPB: Reconstituted clostridial- Prussian blue

2.9.1 MRS 5.2 medium. MRS agar could be made selective for *L. bulgaricus* by adjusting the pH of the medium. A number of studies recorded appreciable results with pH 5.2 or pH 4.8. *L. bulgaricus* was enumerated using MRS agar at pH 5.2 (MRS 5.2) under anaerobic conditions at incubation temperature of 45 °C for 72 h with the occasional presence of Bifidobacterium (Dave & Shah, 1996; Lankaputhra & Shah, 1996). Van de Casteele et al., 2006 also reported a lack of selectivity in this growth medium towards *L. acidophilus*, *L. rhamnosus* and Bifidobacteria. Under anaerobic conditions at 43 °C with pH 5.2, only growths of *L. bulgaricus* (white and irregular colonies), *L. rhamnosus* (shiny smooth and white colonies) and *L. acidophilus* (brown and rough colonies) were observed by (Tharmaraj & Shah, 2003); indicating this growth medium could be selective for *L. bulgaricus* in the absence of both *L. rhamnosus* and *L. acidophilus*. They also suggested lowering the pH of MRS to 4.58 under anaerobic incubation at 43 °C could be used for selective enumeration of *L. bulgaricus* in a product.

2.9.2 MRS Fructose agar. Tabasco et al., 2007 recommended MRS fructose (MRSF) for the selective enumeration of *L. bulgaricus* in mixed culture. The authors replaced the glucose in basal MRS medium with 1% fructose while also excluding meat extract. This new medium was supplemented with 0.2% Tween 80, 0.8% casein acid hydrolysate, 0.05% cysteine, and 1.5% agar. Growths of *L. paracasei* spp. *paracasei* and *B. lactis* were inhibited when incubated using this medium at 43 °C for 72h. The addition of 0.2% Tween 80 allowed for a differential

enumeration of *L. bulgaricus* (lenticular colonies) when *L. acidophilus* (cottony-fluffy colonies) was present.

2.9.3 Lee's agar. Lee's agar is a differential medium for yogurt starter bacteria developed by (Lee et al., 1974). *S. thermophilus* had a yellow, smooth, discrete colony with entire edges while *L. bulgaricus* could be identified as yellow, flat, larger colonies with irregular edges. Differentiation in this medium is achieved through the acid-producing activity of the yogurt starters coupled using bromocresol purple as an acid-base indicator. Casein enzymic hydrolysate and yeast extract are the nitrogenous components while calcium carbonate and dipotassium phosphate were used as buffers. *S. thermophilus* can ferment both sugars (Lactose and Sucrose) used in this medium with the production of more acid as opposed to the inability of most strains of *L. bulgaricus* to use sucrose as an energy source thus, producing less acid and with a restricted growth. The versatility of *S. thermophilus* to use both sugars allow it to grow first, producing a creamy and buttery aroma from diacetyl and in the process lowering the redox potential of the medium for lactobacilli to grow. This agar medium is not recommended when populations of *L. bulgaricus* and *S. thermophilus* are disproportionate and are present in large numbers. The best results with this medium are obtained when the proportions of these starter cultures are fairly equal and the total number of colonies on plate does not exceed 250.

2.9.4 Homofermentative - Heterofermentative medium (HHD). This is a culture medium developed by McDonald et al., 1987 for the differential enumeration of homofermentative and heterofermentative lactic acid bacteria. The differential ability of this medium derives from the pH difference established when both divisions of LAB are exposed to a limited fructose as the only source of energy. Other composition of the medium included KH_2PO4, casamino acids, trypticase peptone, phytone peptone, yeast extract, tween 80 with

bromocresol green as the pH indicator. A green color was recorded when HHD broth was fermented by homofermentative LAB while the same broth inoculated with heterofermentative LAB maintained the blue color. Also, the sedimented cells of the homofermentative LAB while the heterofermentative cells were white. By using the HDD agar, the authors were able to identify nine heterofermentative species of LAB and twelve homofermentative species of LAB; none of which was any of yogurt starter cultures. After 3 days of incubating a mixed culture of homofermentative and heterofermentative LAB at 30 ^{0}C, colonies of homofermentative LAB were blue to green while heterofermentative colonies remained white. The authors noted 96% accuracy could be achieved in colony identification in a 1:1 mixture of homofermentative and heterofermentative organisms. However, HHD agar was used for the differential enumeration of mixed cultures of thermophilic LAB and bifidobacteria by Camaschella et al., 1998. As references, the authors used MRS agar 5.4 for *L. bulgaricus*, M17 agar for *S. thermophilus*, MRS Agar Dicloxacillin (MRSD) for bifidobacteria and MRS agar for *L. acidophilus*. Colonies of *L. bulgaricus* were recorded as large, irregular in shape with flat surface, bright green with an internalized undulating streak. The colonies of *S. thermophilus* were of two types; the first set of colonies were small, smooth and transparent while the other set were convex, circular and dark green colored. Colonies of *L. acidophilus* were recorded as large, irregular shape, convex and pyramid shaped surface, light brown with a small central spot of dark green color. The characteristics exhibited by the colonies of bifidobacteria were similar to those of *S. thermophilus* but appeared translucent and convex like a drop of water. Counts on HHD were also recorded as slightly but significantly higher compared with the reference media (MRS, MRS pH 5.4, M17, MRSD).

2.9.5 Tryptose Proteose Peptone Yeast Extract Prussian Blue agar. Ghoddusi & Robinson, 1996 modified tryptose proteose peptone yeast extract agar (TPPY) by adding prussian blue dye. This medium allowed for the differential enumeration of mixed cultures of *L. bulgaricus, S. thermophilus*, *L. acidophilus* and bifidobacteria on one medium. The colonies of *L. bulgaricus* were small with shiny white appearance and surrounded by a wide royal blue zone; *S. thermophilus* produced pale blue colonies with a thin pale blue zone surrounding it, *L. acidophilus* gave large pale blue colonies with a wide royal blue zone around it while the colonies of bifidobacteria were white.

2.9.6 Tryptose Proteose Peptone Yeast Extract Eriochrome agar. Bracquart, 1981 also modified TPPY agar base by adding eriochrome black T for the differential enumeration of *L. bulgaricus* and *S. thermophilus* in yogurt. Other components of this medium included tryptose, proteose peptone, yeast extract, glucose, lactose and tween 80. Visible colonies of yogurt starter cultures were observed after only 24 hours of incubation on this medium. The colonies of *S. thermophilus* appeared as 1-3 mm in diameter, circular or semi- circular and convex with a peculiar white-violet color, usually having darker centers. *L. bulgaricus* produced colonies that were transparent, 4-6 mm in diameter and undefined in shape with irregular edges. The colonies later became granular with 1cm in diameter after 48 hours. This medium proved to be effective for the differential enumeration of both yogurt starters, with an added advantage of a rapid result within 24 hours. This is unlike MRS agar where bacterial growth is slow, and colonies may not be observed until after 48 hours. However, TPPY eriochrome media may not be used in cultures where a great imbalance exists between the two starter cultures because the medium is not selective.

2.9.7 Reinforced Clostridial agar 5.3 (RCA 5.3). Reinforced clostridial agar medium (RCA) was used for the selective enumeration of *L. bulgaricus* by Dave & Shah, 1996. With the pH adjusted to 5.3, microbial growth was well inhibited. Selectivity for *L. bulgaricus* was achieved when incubation was done at 45 ^{0}C for 72 hours. The authors were elusive with the colony description of *L. bulgaricus* on this medium. Although it permitted the growth of bifidobacteria, the colonies were different and could not be mistaken for *L. bulgaricus*. The authors suggested this medium may not be fit for differential enumeration of *L. bulgaricus* as the growth of some strains of *L. acidophilus* was also recorded. Van de Casteele et al., 2006 also reported the growth of *L. acidophilus*, *L. rhamnosus* and bifidobacteria. They noted the possibility of recovering only *L. bulgaricus* on this medium when the highest sample dilution is plated; citing reason as the presence of higher concentrations of yogurt starters in yogurt than other probiotics. Another experiment revealed a low pH could affect cell counts negatively. The recovery of *L. bulgaricus* cells was highest when the pH of RCA was increased to 6.8, followed by results observed on MRS agar.

2.9.8 Reinforced Clostridial Prussian Blue (RCPB) agar. RCPB agar has been reported as effective in the differential enumeration of *L. bulgaricus*, *S. thermophilus* and bifidobacteria in the presence of other probiotics. RCPB was initially used for the differential count of anaerobic flora in human feces by Van der Wiel-Korstanje & Winkler, 1970. RCPB medium consists essentially of the basal RCA-Reinforced Clostridial Agar (Oxoid, Hampshire, UK) modified with 0.03% Prussian Blue dye. Onggo & Fleet, 1993 evaluated the use of RCPB for the isolation and enumeration of lactic acid bacteria from yogurts. Inoculated plates were incubated at 37 ^{0}C for 48 hours with growths of *L. bulgaricus, S. thermophilus* and *B. bifidum* recorded. *L. acidophilus* did not grow on this differential medium. *L. bulgaricus* formed small,

discrete light blue colonies with white centers, about 1mm in diameter surrounded by wide, clear blue zones. The authors were able to recover *L. bulgaricus* from six yogurts on RCPB which otherwise did not grow on MRS agar. Grosso & Fávaro-Trindade, 2004 reported a similar description of *L. bulgaricus* on RCPB in their study. These authors incubated for a longer time (72 hours) at 37 ^{0}C and reported *L. bulgaricus* as easily differentiated on RCPB; colonies were 2-3 mm in diameter, each with small white clearly defined center surrounded by a blue halo. *S. thermophilus* also had its colonies form a white center but less clearly defined than those reported for *L. bulgaricus*, and a blue halo with a diameter of about 1 mm.

Rybka & Kailasapathy, 1996 evaluated the effectiveness of a different variant of RCPB in the enumeration of yogurt bacteria. The pH of this variant was adjusted to 5.0 to improve selectivity for yogurt bacteria. Growth of bifidobacteria was recorded on this medium and was able to select for only *L. bulgaricus* in traditional yogurts (fermented with *L. bulgaricus* and *S. thermophilus*). In mixed cultures of *L. bulgaricus* and Bifidobacteria; *L. bulgaricus* was distinguishable by its white colonies with wide dark blue halo while *B. infantis* and *B. breve* formed white colonies.

2.9.9 Modified reinforced clostridial medium (mRCM). mRCM is a selective medium developed by Nwamaioha & Ibrahim, 2018 for the enumeration of *L.* bulgaricus. The authors modified reinforced clostridial medium by adding 0.025% $CaCl_2$, 0.01% uracil, and 0.2% Tween 80. This modification enhanced the growth of *L. bulgaricus* significantly while inhibiting the growths of Bifidobacteria, *L. rhamnosus* and *L. reuteri*. The addition of aniline blue dye improved the selectivity of *L. bulgaricus* in mixed culture. Colonies of *L. bulgaricus* appeared distinct after 48 hours of incubation at 40 ^{0}C while those on MRS and lactic agar only became

more visible after 72 hours. The colonies of *L. bulgaricus* were easily distinguishable in mixed bacterial culture as it formed large, irregularly shaped colonies with dark blue centers.

2.9.10 Elliker's Lactic agar. Elliker's lactic agar, also known as Lactobacillus gar, is an all-purpose media used for enumerating streptococci and lactobacilli, mostly in dairy procedures. Streptococci and lactobacilli are differentiated based on colony morphology. Components of this medium included tryptone, yeast extract, gelatin, glucose, sucrose, lactose, sodium chloride, sodium acetate and ascorbic acid (Elliker, Anderson, & Hannesson, 1956). This medium was improved on by Barach, 1979; who added disodium phosphate to improve its buffering ability. He reported an improved enumeration of streptococci in his study as compared to the original medium.

2.9.11 Yogurt Lactic agar (YLA). Matalon & Sandine, 1986 also modified Elliker's lactic agar by supplementing with 1% Tween 80 and 50 μg/ml of 2,3,5-triphenyltetrazolium chloride. They reported a small, red *S. thermophilus* colonies and a larger white *L. bulgaricus* colony. The authors tried replacing 2,3,5-triphenyltetrazolium chloride with 7% skim milk and named this medium yogurt lactic agar (YLA). Yogurt lactic agar allowed a good differentiation between rods and cocci; with *L. bulgaricus* appearing as large white colonies surrounded by a cloudy zone and *S. thermophilus* forming smaller white colonies without a surrounding halo.

2.9.12 Hansen yogurt agar (HYA). Hansen yogurt agar used colony morphology to enumerate and identify *L. bulgaricus* and *S. thermophilus*. Components of this media included beef extract, proteose peptone, dextrose, galactose, lactose with a final pH of 6.8 ± 0.2. Hamann & Marth, 1984 used this medium to enumerate the survival of yogurt starters during a storage study. Porubcan & Sellars, 1973 described the colonies of *L. bulgaricus* as diffuse with low mass

(2-10 mm in diameter); colonies of *S. thermophilus* as distinct with high mass (1-3 mm in diameter).

2.9.13 X-gal chromogenic medium. Galat et al., 2016 developed two chromogenic media for the differential and selective enumeration of lactic acid bacteria in fermented milk products. This is achieved through the cleavage of a chromogen by the target bacteria's enzyme which triggers the release of a chromophore responsible for colored colonies observed. One of the media, M2, having X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the chromogenic substrate allowed for an easy identification of *L. bulgaricus* in mixed cultures at 47 ^{0}C under an anaerobic condition. The β-galactosidase activity of *L. bulgaricus* allows it to cleave X-gal to galactose and 5-bromo-4-chloro-3-hydroxyindole which produces a blue coloration after oxidation. The low pH (5.4 ± 0.2) of this medium was explained as the reason green colonies were observed for *L. bulgaricus.* Although *L. rhamnosus* was also able to grow on this media, it produced colorless colonies that were distinct from the colonies of *L. bulgaricus*. Other components of this medium included peptone, yeast extract, beef extract, potassium phosphate dibasic, sodium acetate, ammonium citrate dibasic, magnesium sulfate and manganese sulfate monohydrate. This growth medium has a limited carbon source as no fermentable sugar was used.

2.9.14 Bromocresol Green Whey agar (BGWA). BGWA was developed by Yamani & Ibrahim, 1996 to differentially enumerate *L. bulgaricus* and *S. thermophilus* in commercial yogurt and labneh. The composition of this medium included whey gotten from reconstituted non-fat dry milk (NFDM), yeast extract, potassium phosphate dibasic, bromocresol green dye with the final pH adjusted to 5.7. The authors reported colonies of *L. bulgaricus* as light colored with greenish center and in the form of an irregular mass having twisted filament projections of

about 2-5 mm in diameter. Colonies of *S. thermophilus* were green and lenticular with entire edges, occasionally having white margins (1-1.5mm in diameter). The authors reported BGWA performed better than MRS and M17 when enumerating *L. bulgaricus* and *S. thermophilus* respectively in commercial yogurt and labneh.

CHAPTER 3

Methodology

3.1 Objective 1

3.1.1 Source of *Lactobacillus bulgaricus.* A total of 32 sources of *L. bulgaricus* strains were used in the study (Table 6). Eleven strains of freeze-dried *L. bulgaricus* cultures intended for industrial production of fermented milk products were supplied by Dr. Albert Krastanov, Department of Biotechnology at the University of Food Technologies, Plovdiv, Bulgaria. Six yogurt starter cultures and three probiotic supplements from Europe were selected. All samples were maintained at -20 °C until further use. In addition, twelve yogurt samples were obtained from Europe and stored under refrigeration until further use.

3.1.2 Lactobacilli MRS Agar Medium. A Lactobacilli MRS agar medium was prepared by dissolving 55g of MRS (Neogen Co, Michigan, USA) and 0.5g of L-cysteine in 1 L of deionized distilled water (DDW), and the resultant solution was stirred well until all particles were completely dissolved. Agar powder (15g) was added, and the agar medium was sterilized at 121 ^{0}C for 15 min and then cooled in a water bath.

3.1.3 Modified Reinforced Clostridial Medium (mRCM). Modified RCM (mRCM) was prepared according to the method of Nwamaioha & Ibrahim, 2018 by completely dissolving 10g peptone #3, 10g beef extract, 5g yeast extract, 10g lactose, 5g sodium chloride, 3g sodium acetate, 2g K_2HPO_4, 0.1g uracil, 0.25g calcium chloride, 0.2% Tween 80 and 0.5g L- cysteine in 1 L DDW. This solution was adjusted to a final pH of 6.0 ± 0.2 using 6M HCl prior to the addition of 0.01% aniline blue and 15g agar. This medium was then autoclaved at 121 ^{0}C f or 15 min and cooled in a water bath.

Table 6

Description of Samples Used

No	Product Code	Sample	Source	Bacterial Composition as labeled[1]
1.	S28	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
2.	S6	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
3.	S19	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
4.	S8	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
5.	LB6	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
6.	S1	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
7.	LB9	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
8.	S22	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
9.	S9	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
10.	S7	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
11.	S5	Pure Industrial Strain	Bulgaria	*L. bulgaricus*
12.	ST11	Starter Culture	Bulgaria	*S. thermophilus, L. bulgaricus, L. rhamnosus*
13.	ST12	Starter Culture	Bulgaria	*S. thermophilus, L. bulgaricus*
14.	ST13	Starter Culture	Bulgaria	*S. thermophilus, L. bulgaricus*
15.	ST14	Starter Culture	Bulgaria	*S. thermophilus, L. bulgaricus*
16.	ST15	Starter Culture	Canada	*S. thermophilus, L. bulgaricus, L. acidophilus*
17.	ST16	Starter Culture	Bulgaria	*S. thermophilus, L. bulgaricus*
18.	SP17	Probiotic Supplement	Bulgaria	*S. thermophilus, L. bulgaricus, L. reuteri*
19.	SP18	Probiotic Supplement	Bulgaria	*S. thermophilus, L. bulgaricus*
20.	SP19	Probiotic Supplement	Bulgaria	*S. thermophilus, L. bulgaricus, L. rhamnosus*
21.	SP8	Yogurt	Spain	*S. thermophilus, L. bulgaricus, Bifidobacterium, L. reuteri*
22.	SP9	Yogurt	Spain	Live and active cultures
23.	SP10	Yogurt	Spain	Live and active cultures
24.	E1A	Yogurt	Netherlands	*S. thermophilus, L. bulgaricus, L. acidophilus, Bifidobacterium*
25.	E2Z	Yogurt	Netherlands	Live and active culture
26.	E3D	Yogurt	Spain	Live and active culture
27.	E4P	Yogurt	Bulgaria	Live and active culture
28.	L6N	Yogurt	USA	*S. thermophilus, L. bulgaricus, Bifidobacterium lactis, L. acidophilus, L. rhamnosus,*
29.	BY2	Yogurt	Bulgaria	*L. bulgaricus*, other live culture
30.	BY4	Yogurt	Bulgaria	*L. bulgaricus*, other live culture
31.	BY5	Yogurt	Bulgaria	*L. bulgaricus*, other live culture
32.	BY6	Yogurt	Bulgaria	*L. bulgaricus*, other live culture

[1]*L. = Lactobacillus S. = Streptococcus*

3.1.4 Modified Reinforced Clostridial Medium-Pyruvate (mRCM-PYR). A reinforced clostridial medium was optimized for selectivity and accurate enumeration of *L. bulgaricus* by dissolving 10g peptone #3, 10g beef extract, 5g yeast extract, 5g sodium chloride, 3g sodium acetate, 2g K_2HPO_4, 0.1g uracil, 0.25g calcium chloride, 5g Dextrose, 5g Fructose, 10g Maltose, 2g Sodium Pyruvate, 0.2% Tween 80 and 0.5g L- cysteine in 1 L DDW. This solution was adjusted to a final pH of 6.0 ± 0.2 using 6M HCl before the addition of 0.008% aniline blue and 15g agar. The medium was autoclaved at 121 ^{0}C for 15 min and then cooled in a water bath. All freshly prepared media in this study were poured into sterile petri dishes and stored at 4 ^{0}C until needed. The difference in the carbohydrate sources of mRCM and mRCM-PYR is highlighted in Table 7.

Table 7

Composition of modified reinforced clostridial medium (mRCM); and modified reinforced clostridial medium-Pyruvate (mRCM-PYR)

mRCM	mRCM-PYR
10g Peptone#3	10g Peptone#3
10g Beef Extract	10g Beef Extract
5g Yeast Extract	5g Yeast Extract
5g Sodium Chloride	5g Sodium Chloride
0.25 Calcium Chloride	0.25 Calcium Chloride
3g Sodium Acetate	3g Sodium Acetate
2g Ammonium Phosphate Dibasic	2g Ammonium Phosphate Dibasic
0.5g L- cysteine	0.5g L- cysteine
0.1g Uracil 0.01% Aniline blue	0.1g Uracil 0.008% Aniline blue
0.2% Tween 80	0.2% Tween 80
*10g Lactose	*5g Dextrose
	*5g Fructose
	*10g Maltose
	*2g Sodium Pyruvate

Components listed are needed to prepare 1L of medium broth
*Carbohydrate sources

3.2 Objective 2

3.2.1 Enumeration of *L. bulgaricus* in Pure Industrial Strains, Yogurt Starter Culture and Probiotic Supplements.

For the enumeration of *L. bulgaricus* in these products, 0.1 g of freeze dried samples was measured and transferred into 3ml skim milk with the addition of 10µl Tween 80 and 0.1ml yeast extract in order to enhance the recovery of stressed bacterial cells. This mix was fermented at 44 ºC for 7hrs, at which point the skim milk curdled. The curdled milk was shaken, and 1ml was transferred for serial dilution into a 9ml peptone solution. Aliquots (100 µL) of appropriate dilutions were plated in duplicates onto the three culture media (MRS, modified RCM and optimized RCM) for the enumeration of *L. bulgaricus*. The inoculated plates were incubated for 72 hours at 44 ºC in anaerobe chambers, and colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA, USA).

3.2.2 Enumeration of *L. bulgaricus* in Commercial Yogurt Samples.

Dilutions of yogurt samples were prepared by adding 10g of each sample to a screw-capped bottle containing 90 mL of deionized water and then properly shaking the mixture. This mixture was placed in an incubator at 44 ºC for 15 minutes in order to repair weak and damaged cells. Subsequent dilutions of up to 10^{-7} were made in a 0.1% peptone solution and vortexed well between transfers. Aliquots (100 µL) of appropriate dilutions were then plated in duplicate onto the three culture media (MRS, modified RCM and optimized RCM) for the enumeration of *L. bulgaricus*. The inoculated plates were incubated for 72 hours at 44 ºC in anaerobe chambers and colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA, USA).

3.2.3 Enumeration of *L. bulgaricus* in Traditional Bulgarian Yogurt.

In order to enumerate *L. bulgaricus* in traditional Bulgarian yogurt, 5ml of samples were measured and transferred into 45ml of skim milk with 2ml yeast extract (20%) added to repair damaged and

stressed bacterial cells. This mix was fermented at 44 ºC for 7hrs, at which point the skim milk curdled. The curdled milk was stirred, and 10ml was sampled for enumeration of bacterial cells following the method outlined for the enumeration of *L. bulgaricus* in yogurt samples.

3.2.4 Statistical Analyses. SAS version 9.4 (Cary, NC) was used to analyze the experiment data obtained in this study. One-way ANOVA was used to determine significant differences between the values. Significant differences ($p < 0.05$) between means of bacterial counts (CFU/g) were compared using Tukey's test. Bacterial population counts were converted to $\log_{10}$ transformation prior to analysis.

CHAPTER 4

Results

4.1 Preliminary Study

In preliminary studies, the effect of different carbon sources on the morphology of *L. bulgaricus* colonies was evaluated. We observed that the use of 2% sodium citrate and 1% sodium pyruvate as alternatives to a proteose peptone diluent solution not only produced more prominent and distinguishable colonies in the previously modified reinforced clostridial media (mRCM) by Nwamaioha & Ibrahim, 2018 but also improved the bacterial count (Figures not shown). Our findings also showed that sodium pyruvate retarded the growth of lactic acid bacteria other than *L. bulgaricus* which otherwise grew when 2% sodium citrate and 0.1% proteose peptone were used as diluent media. These observations would suggest that the inclusion of sodium pyruvate as a component in a growth medium could improve the recovery of injured or damaged bacterial cells. Further tests employed various concentrations of individual and combined treatments of fructose, maltose, dextrose, lactose, sodium citrate and sodium pyruvate, as components of mRCM. Initially, a combined treatment of 0.5% dextrose, 0.5% fructose, 1% maltose and 1% sodium pyruvate replacing 1% lactose as a carbon source in mRCM appeared to be the most effective in selecting for only *L. bulgaricus* in the mixed bacterial culture. However, it became evident during the screening that a reduced concentration of sodium pyruvate would be needed in order to be effective in improving the selectivity of the modified medium. A 1% sodium pyruvate concentration in this growth media composition not only retarded other bacterial growth but converted the characteristic rough-edge morphology of the surviving *L. bulgaricus colonies* to a smooth one *(Fig. 4)*. Therefore, a combination of 0.5% Dextrose, 0.5% Fructose, 1% Maltose, 0.2% Sodium Pyruvate as carbon sources in our growth

medium proved to be the most effective in improving the morphology and cell recovery of *L. bulgaricus* colonies.

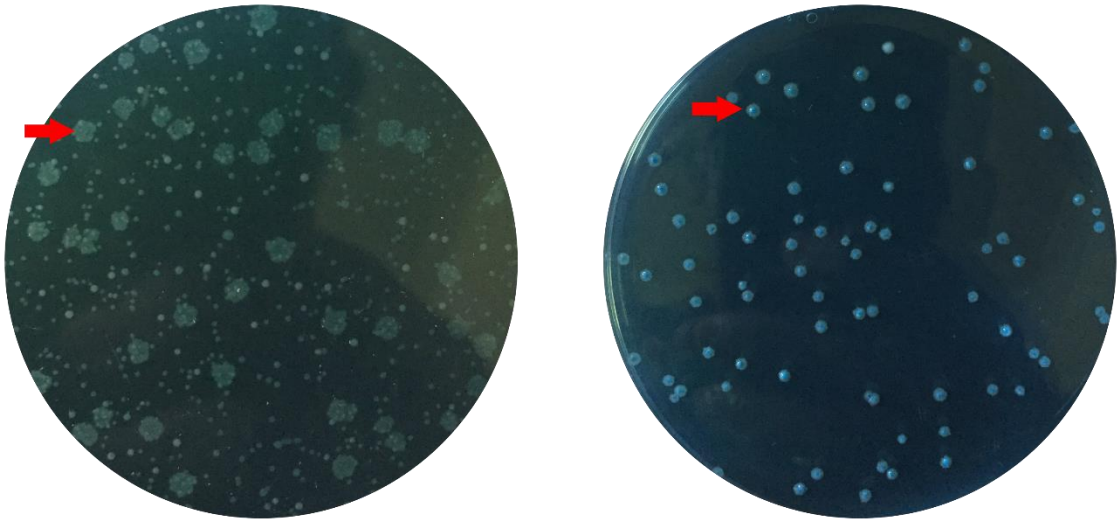


Figure 4. Red arrow shows a rough-edged *L. bulgaricus* colony in mRCM (Left image) morph into a round and smooth colony in mRCM-PYR having a concentration of 1% sodium pyruvate.

4.2 Objective 1

4.2.1 Cell Repair and Recovery. *L. bulgaricus* colonies that were isolated from freeze dried probiotic supplements on MRS, mRCM and mRCM-PYR growth media are shown in Figure 5. The SP18 cell colonies (*L. bulgaricus* encircled in red) appear small and fragile in both MRS and mRCM media suggesting that the cells were stressed or injured; however, this was not the case in mRCM-PYR where the *L. bulgaricus* cells appeared as large colonies. The result for SP19 further highlighted the advantage of the mRCM-PYR growth media over the previously modified RCM media with regard to the repair of damaged or stressed microbial cells. Non-lethal injury to bacterial cells can occur for a number of reasons. Destruction of bacterial cells as a result of exposure to low temperatures in the form of freeze drying, freezing/ thawing and

prolonged storage at low temperatures is common in the industrial production of probiotic supplements and starter cultures.

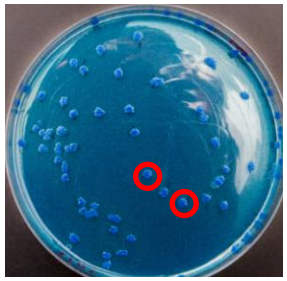
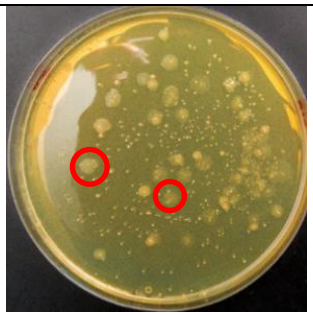
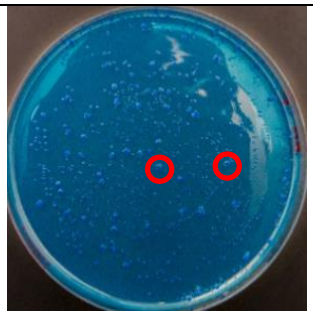
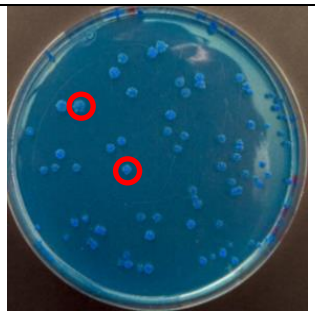
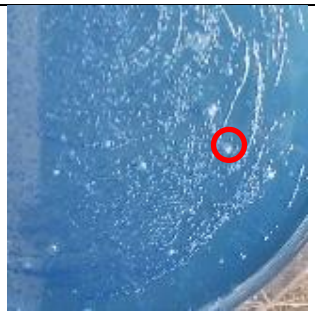
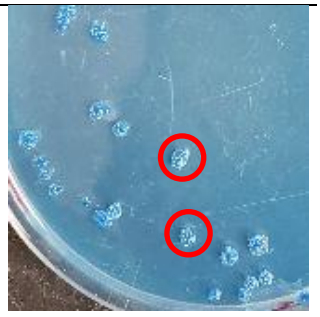
[1]Samples	MRS	mRCM	mRCM-PYR
SP18			
SP19			
ST 11			
ST 14			

Figure 5. Morphology of *L. bulgaricus* colonies (Encircled in Red) on de Man, Rogosa, and Sharpe medium (MRS); modified reinforced clostridial medium (mRCM); and modified reinforced clostridial medium-Pyruvate (mRCM-PYR)
[1]*See Table 5 for sample description*

The cell colonies in mRCM were the smallest while the mRCM-PYR culture media facilitated the optimum growth and repair of *L. bulgaricus* cells resulting in larger colonies. Similar results were noted with other starter cultures that were evaluated for the viability of *L. bulgaricus*. In the previously modified RCM growth media, the colonies of *L. bulgaricus* in samples ST 11 and ST 14 were tiny and very difficult to identify in the presence of other bacteria colonies. In contrast to the modified RCM, our optimized RCM medium produced visible and large colonies for the same samples, and the growth of other bacteria was significantly inhibited (Figure 5).

4.2.2 Selective Growth and Enumeration of Pure *L. bulgaricus* Strains. The bacterial counts of 11 pure *L. bulgaricus* strains are presented Fig. 6 and Table 8. mRCM-PYR performed significantly better than MRS and mRCM ($P < 0.05$) in 6 of the strains (S6, S19, LB9, S9, S7 and S5) as measured by the bacterial populations. There was no significant difference in the bacterial counts recorded in mRCM and mRCM-PYR in 4 *L. bulgaricus* strains (S28, S8, S1 and S22). However, both growth media performed significantly better than MRS ($P < 0.05$) in these strains. The only case of MRS performing noticeably better ($P < 0.05$) than mRCM-PYR and mRCM was with *L. bulgaricus* strain LB6.

Table 8

Bacterial count (mean ± SD; n = 3) of 11 pure Lactobacillus bulgaricus strains expressed as log cfu/g on MRS, mRCM and mRCM-PYR after incubation at 44 °C for 72 h.

Pure Industrial *L. bulgaricus* Strains[1]	Growth Medium		
	MRS	mRCM	mRCM-PYR
S28	7.7 ± 0.14^{a}	8.1 ± 0.28^{ab}	8.6 ± 0.14^{b}
S6	8.68 ± 0.04^{a}	8.83 ± 0.06^{a}	9.3 ± 0.14^{b}
S19	8.79 ± 0.07^{a}	9.46 ± 0.07^{b}	9.85 ± 0.07^{c}
S8	9.63 ± 0.04^{a}	9.825 ± 0.04^{b}	9.9 ± 0^{b}
LB6	9.51 ± 0^{a}	7.48 ± 0^{b}	8.1 ± 0.14^{c}

Cont.

S1	7.7 ± 0.14[a]	8.55 ± 0.07[b]	8.95 ± 0.07[b]
LB9	7.65 ± 0.07[a]	8.17 ± 0.08[b]	8.75 ± 0.07[c]
S22	8.4 ± 0.07[a]	8.75 ± 0.07[ab]	9.1 ± 0.14[b]
S9	7.49 ± 0.07[a]	8.4 ± 0.14[b]	9.05 ± 0.21[c]
S7	7.25 ± 0.07[a]	7.56 ± 0.07[a]	8.1 ± 0.14[b]
S5	7.9 ± 0[a]	7.95 ± 0[a]	8.8 ± 0.14[b]

[a,b,c] Means with different superscripts within a row differ significantly (P < 0.05)
[1]See Table 5 for sample description

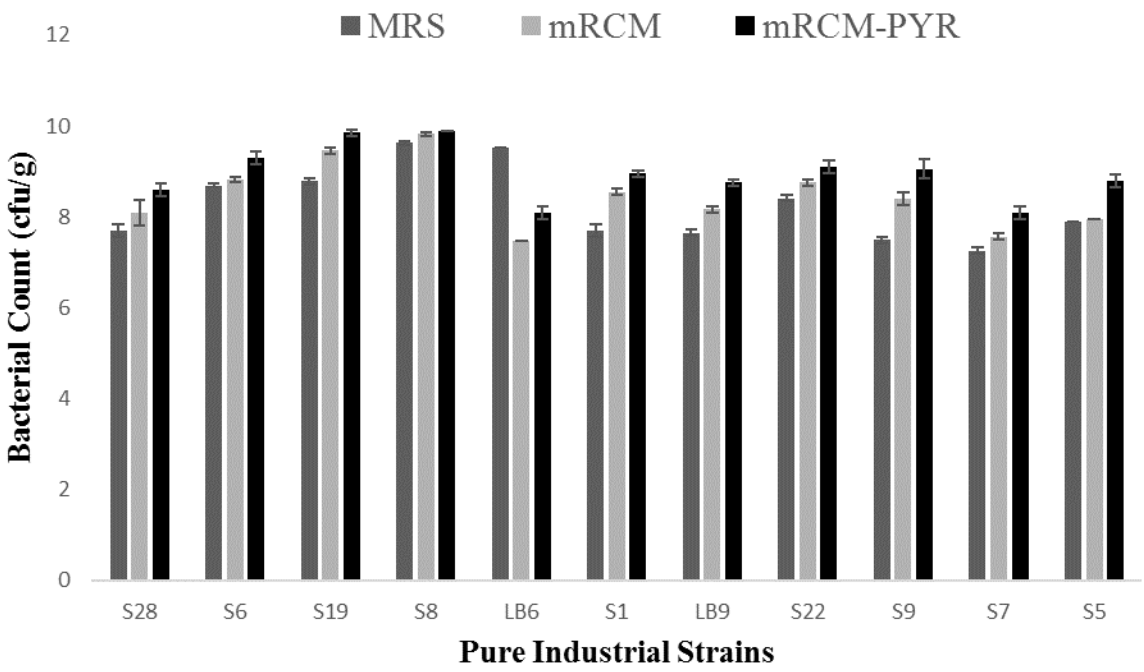
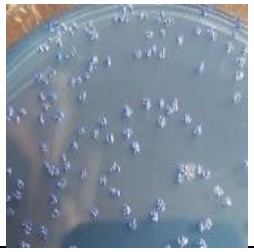
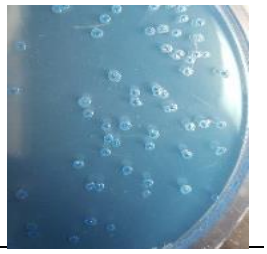
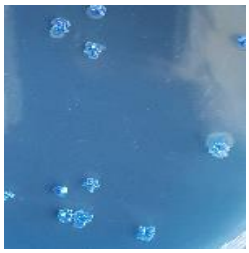
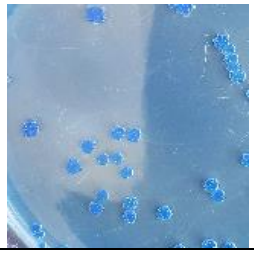
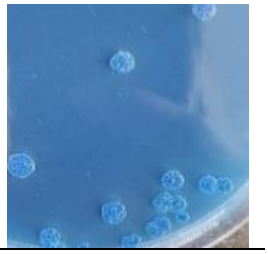
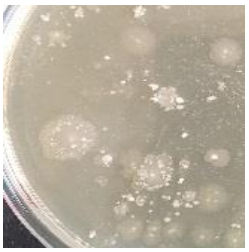
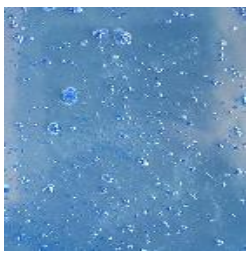
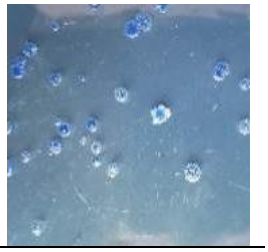
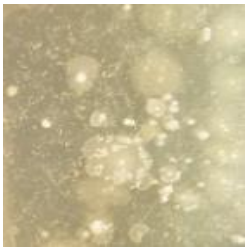
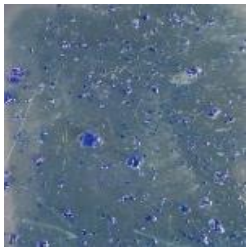
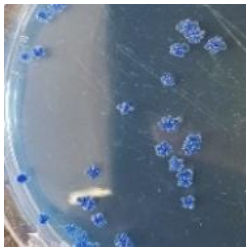
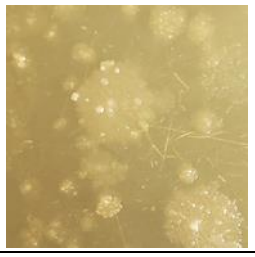
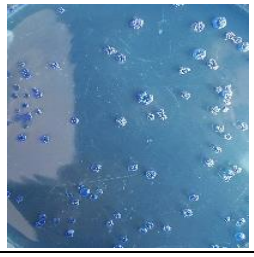
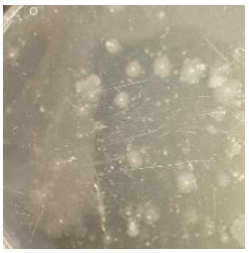
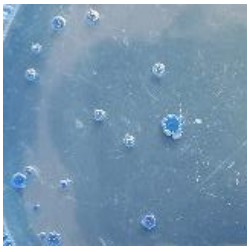
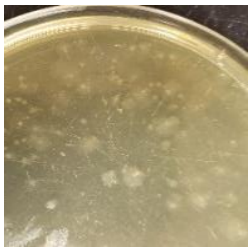
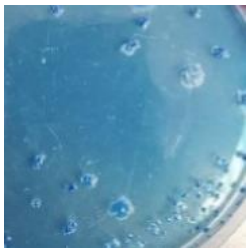
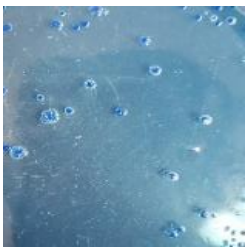


Figure 6 . Bacterial count (mean ± SD; n = 3) of 11 pure *Lactobacillus bulgaricus* strains expressed as log cfu/g on MRS, mRCM and mRCM-PYR after incubation at 44 °C for 72 h. Error bars indicate SD for an experiment performed in duplicate.

The effect of the three media (MRS, mRCM and mRCM-PYR) on the morphology of the 11 pure strains of *L. bulgaricus* colonies is shown in Figure 7.

Sample	MRS	mRCM	mRCM-PYR
S28			
S6			
S19			
S8			
LB6			

Cont.

S1			
LB9			
S22			
S9			
S7			

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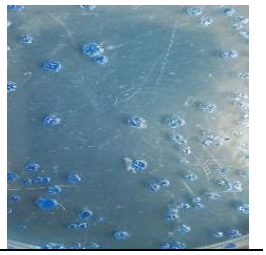
S5			

Figure 7. Morphology of pure *Lactobacillus bulgaricus* on MRS, mRCM and mRCM-PYR after incubation at 44 °C for 72 h.

4.3 Objective 2

4.3.1 Selective Enumeration and Differentiation of *L. bulgaricus* in Mixed Cultures.

The bacterial counts of *L. bulgaricus* recovered from 21 products using MRS, mRCM and mRCM-PYR are presented in Fig. 8 and Table 9. The 21 products consisted of a starter culture, probiotic supplements and yogurt and contained mixed bacterial cultures. The three media had similar effects on the bacterial counts of 4 products (E1A, BY2, BY4 and SP8). There was no significant difference in the bacterial counts in mRCM and mRCM-PYR for 6 products (E2Z, L6N, BY5, ST12, SP17 and SP19). However, both growth media performed significantly better than MRS ($P < 0.05$) in these products. mRCM-PYR performed better than MRS and mRCM ($P < 0.05$) in 10 products (E3D, BY6, SP9, ST11, ST14, SP10, ST13, ST15, ST16 and SP18). The bacterial counts of *L. bulgaricus* in E4P were similar in mRCM-PYR and MRS but considerably higher than those in mRCM $P < 0.05$). The results confirmed the effectiveness of our growth media, mRCM-PYR, as a suitable alternative to MRS and mRCM in the selective enumeration and isolation of *L. bulgaricus* in mixed bacterial cultures.

Table 9

Bacterial count (mean ± SD; n = 3) of Lactobacillus bulgaricus in mixed culture; expressed as log cfu/g on MRS, mRCM and mRCM-PYR after 72 h of incubation at 44 °C

		Growth Medium		
Mixed Culture[1]	Source	MRS	mRCM	mRCM-PYR
E1A	Yogurt	7.33 ± 0.67[a]	7.8 ± 0.14[a]	8.25 ± 0.35[a]
E2Z	Yogurt	6.965 ± 0.23[a]	7.85 ± 0.07[ab]	8.6 ± 0.28[b]
E4P	Yogurt	6.27 ± 0.38[a]	TFTC ± 0[b]	7.05 ± 0.21[a]
E3D	Yogurt	6.915 ± 0.02[a]	7.6 ± 0.14[b]	8.7 ± 0.14[b]
L6N	Yogurt	6.85 ± 0.21[a]	7.8 ± 0.14[b]	8.4 ± 0.14[b]
BY2	Bulgarian Yogurt	6.33 ± 0.75[a]	7.46 ± 0.08[a]	8.2 ± 0.28[a]
BY4	Bulgarian Yogurt	7.62 ± 0.17[a]	7.75 ± 0.35[a]	8.7 ± 0.28[a]
BY5	Bulgarian Yogurt	6.63 ± 0.18[a]	7.6 ± 0.14[ab]	8.45 ± 0.35[b]
BY6	Bulgarian Yogurt	7.04 ± 0.2[a]	[2]TFTC ± 0[b]	7.8 ± 0.14[c]
SP8	Yogurt	7.32 ± 0.07[a]	7.68 ± 0.07[a]	7.8 ± 0.28[a]
SP9	Yogurt	6.6 ± 0.07[a]	7.42 ± 0.06[b]	8.12 ± 0.16[c]
SP10	Yogurt	6.3 ± 0.13[a]	7.1 ± 0.28[a]	8.2 ± 0.28[b]
ST11	Starter Culture	5.97 ± 0.1[a]	7.1 ± 0.14[b]	8.05 ± 0.21[c]
ST12	Starter Culture	7.55 ± 0.07[a]	7.75 ± 0.07[ab]	8.15 ± 0.28[b]
ST13	Starter Culture	6.41 ± 0.07[a]	6.9 ± 0.14[a]	7.9 ± 0.14[b]
ST14	Starter Culture	6.71 ± 0.05[a]	7.4 ± 0.14[b]	8.1 ± 0.14[c]
ST15	Starter Culture	7.15 ± 0.21[a]	7.65 ± 0.21[a]	8.89 ± 0.14[b]
ST16	Starter Culture	6.09 ± 0.13[a]	6.4 ± 0.07[a]	7.37 ± 0.18[b]
SP17	Supplement	7.55 ± 0.07[a]	8.1 ± 0.14[b]	8.6 ± 0.13[b]
SP19	Supplement	7.65 ± 0.21[a]	8.1 ± 0.14[ab]	8.45 ± 0.07[b]
SP18	Supplement	6.8 ± 0.14[a]	7.1 ± 0.14[a]	8.15 ± 0.21[b]

[a,b,c] Means with different superscripts within a row differ significantly ($P < 0.05$)
[1]See Table 5 for sample description
[2]Too few to count

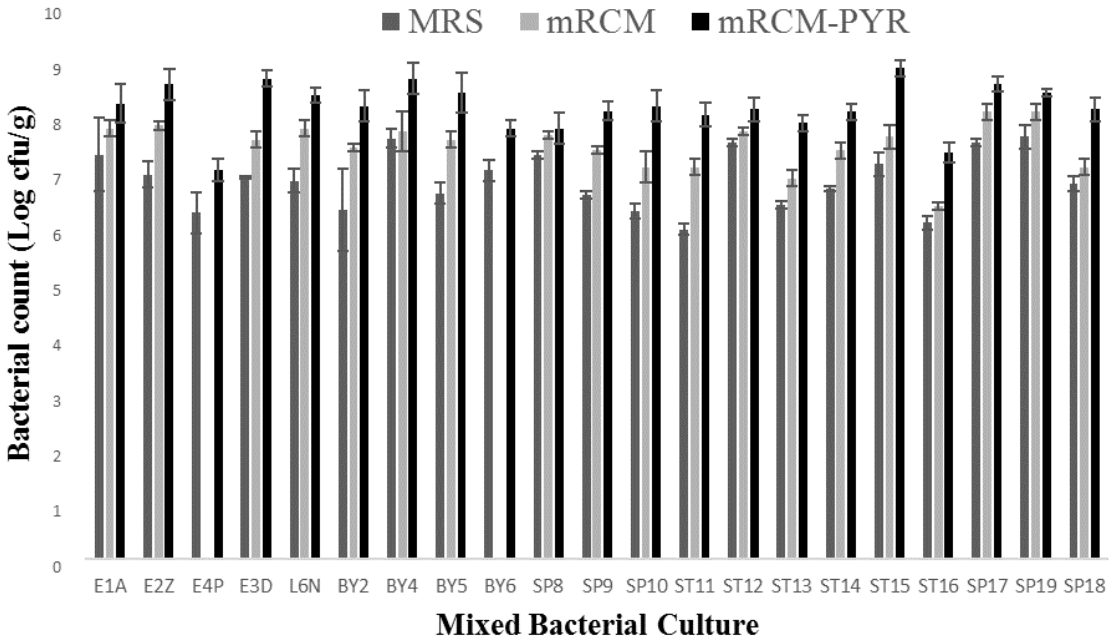
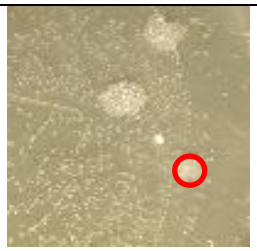


Figure 8. Bacterial count of *Lactobacillus bulgaricus* in mixed culture on MRS, mRCM and mRCM-PYR at 44 °C after 72 h of incubation. Error bars indicate SD for an experiment performed in duplicate.

The morphology of *L. bulgaricus* in mixed cultures is shown in Figure 9. The colonies of *L. bulgaricus* in mRCM-PYR were easy to identify and enumerate as the growth of other lactic acid bacteria was highly retarded. The colonies of *L. bulgaricus* in mRCM-PYR were easy to identify and enumerate as the growth of other lactic acid bacteria was highly retarded.

Sample	MRS	mRCM	mRCM-PYR
ST11			

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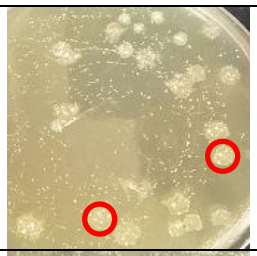
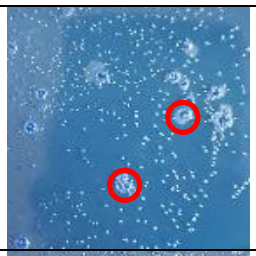
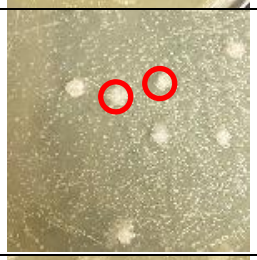
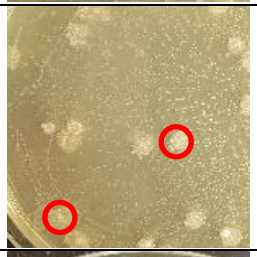
ST12			
ST13			
ST14			
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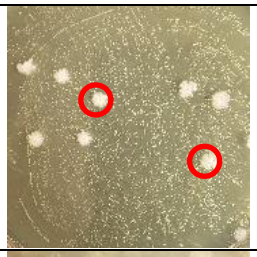
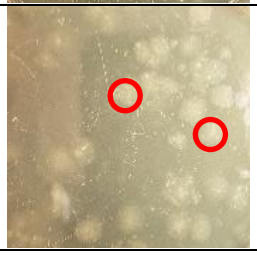
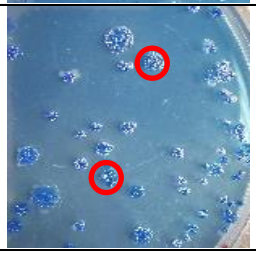
ST16			
SP17			

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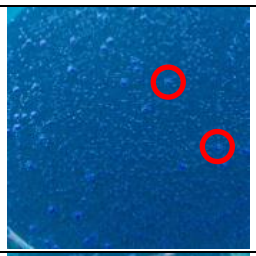
SP18			
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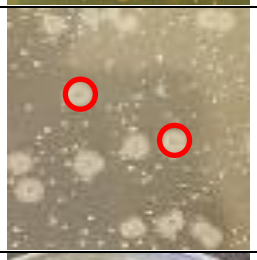
E1A			
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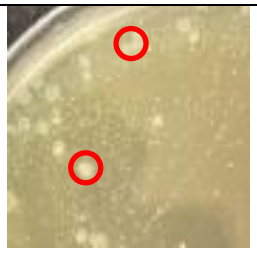
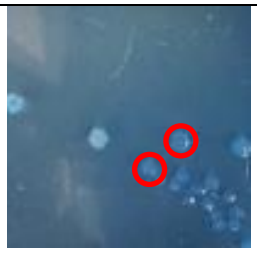
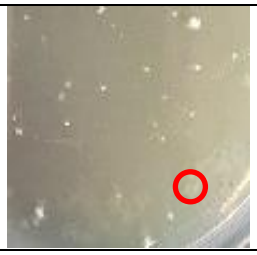
E4P			
E3D			

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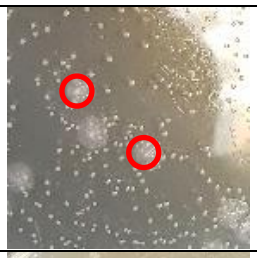
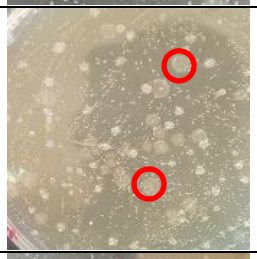
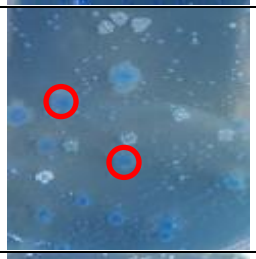
SP8			
SP9			
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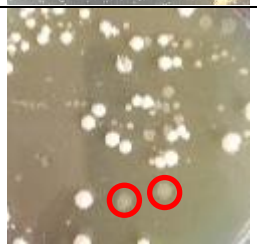
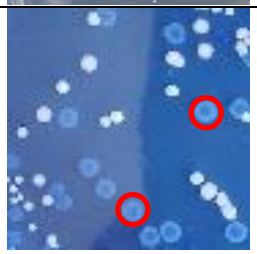
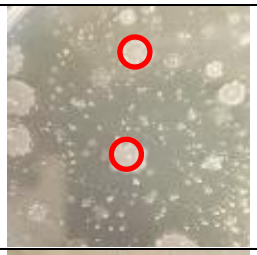
L6N			
BY2			
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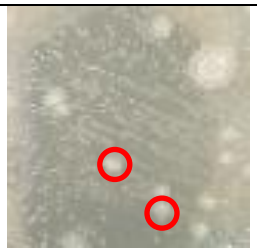
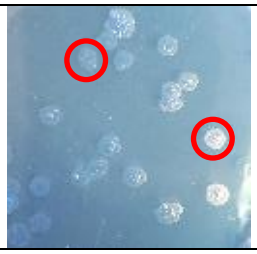
BY5			
BY6			

Figure 9. Morphology of *L. bulgaricus* in mixed culture on MRS, modified reinforced, mRCM and mRCM-PYR after incubation at 44°C for 72h.

4.4 Discussion

A culture medium is comprised of essential nutrients needed for the optimal growth of microbes and is the result of years of research into the diverse metabolic and growth needs of different microbes. Consequently, when a new selective growth medium is developed, it is more challenging to target the growth of a specific microbe as opposed to the non-selective medium that permits the growth of different bacterial species. This is because all bacterial species need basic nutrients such as carbon and nitrogen sources to thrive. The development of a growth selective medium is thus a tedious process and represents a confluence of art and science. If not, how do you optimize a growth medium for a specific bacterial species while making the same medium inhibitory to the same species of the microbe. The selection of the appropriate dye for differential enumeration of the difference strains also considered as another challenge in the development of such selective medium (Ghoddusi & Robinson, 1996; Yamani & Ibrahim, 1996). The microbe of interest in the current study is *L. bulgaricus*. It is an important species of LAB that is popular for the use in dairy production in addition to its increasing relevance as a probiotic. The need for accuracy in the estimation of

L. bulgaricus is crucial to the quality of the dairy product and the efficacy of this microbe when used as a probiotic. The current standard medium, deMan, Rogosa and Sharpe (MRS) is unreliable as a selective method for enumerating *L. bulgaricus* species in mixed bacterial cultures as it often underestimates true bacterial counts due to the presence of high background colonies of similar species. In a previous study by Nwamaioha & Ibrahim, 2018, a growth medium was modified to selectively enhance the isolation and enumeration of *L. bulgaricus* in dairy products. In the present study, we further modified this medium for the superior selectivity and enumeration of *L. bulgaricus* by replacing lactose with 0.5% dextrose, 0.5% fructose, 1% maltose and 0.2% sodium pyruvate. Different carbohydrates that are the primary energy source for bacteria in growth media have been used to selectively enumerate lactic acid bacteria. For example, Tabasco et al., 2007 supplemented MRS with fructose in order to enumerate *L. bulgaricus*. Another justification for the use of different carbon sources is the disparity in the reported carbohydrate use in *L. bulgaricus*. The inconsistencies recorded in *L. bulgaricus'* use of fructose, mannose, maltose among other sugars have been attributed to the strain type and composition of the growth medium (Chervaux et al., 2000; Zourari et al., 1992). mRCM-PYR was developed to optimally enumerate and select for *L. bulgaricus* regardless of the source and strain type. We observed that the combination of carbon sources was responsible for the selectivity and high bacterial counts in mRCM-PYR. However, the substitution or elimination of any of the carbon sources, particularly sodium pyruvate, did not yield the same result. Pyruvate is an essential intermediate in the metabolism of lactic acid bacteria and can be converted to a number of end products such as acetic acid, formic acid and lactic acid. Higashio et al., 1977, attributed the stimulatory effect that *S. thermophilus* had on the growth of *L. bulgaricus* to the

production of formic and pyruvic acids. Polak-Berecka et al., 2010, recorded a better yield of cell biomass when sodium pyruvate was combined with glucose as carbon sources. These results might help to explain the cell repair and recovery observed in this study.

Many of the available standard media in the literature such as MRS (De Man et al., 1960), Lee's Agar (Lee et al., 1974), Yogurt Lactic Agar (Matalon & Sandine, 1986), RCPB (Ghoddusi & Robinson, 1996) and Bromocresol Green Whey Agar (Yamani & Ibrahim, 1996) have not been able to selectively enhance the growth of *L. bulgaricus* in a mixed culture. However, mRCM-PYR performed well in the enumeration of *L. bulgaricus* in a mixed culture while retarding the growth of other lactic acid bacteria. This is an improvement over the previous study by Nwamaioha & Ibrahim, 2018, where a lack of significant differences in bacterial counts coupled with the growth of other lactic acid bacteria was recorded in the tested products.

The conventional methods of identifying closely related lactobacilli species which include carbohydrate fermentation profile, cell morphology, antibiotics sensitivity, salt tolerance and temperature tolerance are not accurate and are time consuming. Few recent studies have suggested to use rapid molecular techniques for the identification of *L. bulgaricus*. However, there are still few challenges technical issues such as the selective of the appropriate primers or optimizing the reaction conditions for the PCR reactions to improve the level of precision. Genotypic methods including 16S rRNA gene sequencing, DNA–DNA hybridization, repetitive element (rep)-PCR, randomly amplified polymorphic DNA (RAPD)-PCR and species-specific PCR techniques have been used to accurately identify lactobacilli strains where biochemical tests produced variable results. Michaylova et al., 2007b used PCR and Pulsed-field gel electrophoresis (PFGE) to verify the identity of

various strains of *L. bulgaricus* while also establishing relationships between the strains. The inadequacy of biochemical tests as methods of identification was further highlighted in the study by Elmacı et al., 2015. The API fermentation test gave false identifications as only 71 of the 152 tested isolates were the same as those of the results obtained from the 16S rRNA method.

New bacterial strains are in constant demand by the dairy industry in response to increasing consumer demands. Consequently, studies are needed not only to isolate these new strains but also to characterize the new strains based on the enzyme activity, secreted proteins, aroma production and stress resistance. The additional exploration of the isolates in this study could lead to the identification of superior probiotic and industrial strains.

CHAPTER 5

Conclusion and Future Research

L. bulgaricus is popular in the dairy industry for the use in the production of yogurt and some cheeses. In addition to the application as a starter culture, *L. bulgaricus* is also marketed as a probiotic. Accurate population counts for this bacterial species are crucial in both applications due to the effect on the final quality of the products. The primary objective of this study was thus to develop a culture medium that is selective for the enumeration and isolation of *L. bulgaricus*, especially in mixed bacterial culture. Carbon sources including 0.5% dextrose, 0.5% fructose, 1% maltose and 0.2% sodium pyruvate in the base RCM growth medium selectively supported the growth of *L. bulgaricus* in a mixed culture. This was evidenced by the absence or inhibited growth of other bacteria species on mRCM-PYR compared to the growth in MRS and mRCM. Preliminary studies identified the significant role of concentrated sodium pyruvate in supporting a growth medium that is selective for *L. bulgaricus* in the presence of other lactic bacteria species. Future research should be targeted at understanding the mechanism responsible for this effect and why selectivity is not recorded when sodium pyruvate is not used in combination with these other carbon sources.

High bacteria counts for *L. bulgaricus* were also recorded in our growth medium compared to the counts in MRS and mRCM. This result again highlights the need to fully understand the effect of sodium pyruvate on the growth of *L. bulgaricus*. Sodium pyruvate could be responsible for the recovery of stressed or injured bacterial cells which otherwise are viable but not culturable on the MRS and mRCM growth media. Future study may thus be warranted in order to establish the possible use of sodium pyruvate as a cryoprotective agent and a component in culturing injured/stressed bacterial cells.

The 32 bacterial species isolated from various sources using mRCM-PYR in this study were identified as *L. bulgaricus* based on their morphological characteristic and the fermentation of the carbon sources used in this medium. This method was not able to differentiate the closely related subspecies of *L. delbrueckii* like *lactis* and *indicus*. It is important that these isolates are subjected to further identification in future study using genotypic techniques like the 16S rRNA gene sequencing, DNA–DNA hybridization, repetitive element (rep)-PCR, randomly amplified polymorphic DNA (RAPD)-PCR and species-specific PCR techniques. The use of these techniques would establish the diversity, genetic evolution across time and the relationship between the different strains of *L. bulgaricus* used in this study. Future study should also investigate and screen *L. bulgaricus* isolates for secreted proteins, aroma production, stress resistance and the enzyme activity. Future study should investigate enzyme activity such as threonine aldolase which is responsible for the production of acetaldehyde as a rapid screening tool to enumerate *L. bulgaricus* in dairy products. These screening procedures could lead to the identification of superior industrial starter strains.

In addition to the increasing consumer demand and awareness about the health benefits of probiotics, the coming years might see consumers discard the use of antibiotics and rather turn to the use of probiotics and yogurt for not only wellness purposes but for the treatment of certain diseases. Our growth medium, mRCM-PYR has the potential of isolating new strains of *L. bulgaricus* that can meet this demand while also enhancing the study of this bacteria.

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