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**YOGURT CULTURES SURVIVE UPON EXPOSURE TO TWO ANTIMICROBIALS
AND *STREPTOCOCCUS THERMOPHILUS* ST-M5 ACQUIRED RESISTANCE TO
BOTH ANTIMICROBIALS**

A Thesis
Submitted to the graduate faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science
In
The Department of Animal Sciences

By
Maria Carolina Vives Habeych
B.S., Universidad de Los Andes, Colombia 2009
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vii
ABSTRACT	viii
CHAPTER 1: INTRODUCTION	1
1.1 Probiotics	1
1.2 Food Antimicrobials	3
1.3. Nitrites	6
1.3.1 Regulations with Nitrites	7
1.4 Sulfites	7
1.4.1 Regulations with Sulfites	8
1.5 Antimicrobial Resistance.....	9
1.5.1 Mutations	11
1.5.2 Plasmid-Mediated Resistance	11
1.5.3 Biofilms and Microbial Response.....	12
CHAPTER 2: MATERIALS AND METHODS.....	14
2.1 Experimental Design	14
2.2 Sample Preparation	14
2.3 Treatments	15
2.4 Preparation of Media:	15
2.4.1 Peptone Water	15
2.4.2 Lactobacilli MRS Broth	15
2.4.3 Lactobacilli MRS Agar	16
2.4.4 <i>Streptococcus thermophilus</i> Agar	16
2.4.5 Modified pH MRS Agar (pH 5.2).....	16
2.5. Analytical Procedure	16
2.5.1 Dilution Method, Growth.....	16
2.5.2 Resistance.....	17
2.6 Statistical Analysis.....	18
CHAPTER 3: RESULTS AND DISCUSSION	19
3.1 Screening	19

3.2 Resistance31
 3.2.1. *Lactobacillus bulgaricus*34
 3.2.2 *Streptococcus thermophilus*39
3.3 Mean Log Reductions for Viable Counts of *Lactobacillus bulgaricus* LB-12 and
Streptococcus thermophilus ST-M546

CHAPTER 4: CONCLUSIONS.....51

REFERENCES.....52

VITA57

LIST OF TABLES

Table 3. 1 Probability > F (Pr > F) of fixed effects for the screening of <i>Lactobacillus bulgaricus</i> LB-12 and <i>Streptococcus thermophilus</i> ST-M5 with different concentrations of Potassium Metabisulfite and Potassium Nitrite individually.....	20
Table 3.2 Probability > t value (Pr > t) of differences of Least Square Means compared to control of <i>Streptococcus thermophilus</i> ST-M5 and <i>Lactobacillus bulgaricus</i> LB-12 at different concentrations of Potassium Metabisulfite and Potassium Nitrite; after 0, 24, 48 and 72 hours of incubation.	20
Table 3.3 Mean Log reduction of the viable counts of cultures treated with different concentrations of Potassium Metabisulfite and Potassium Nitrite obtained by subtracting viable Log CFU/mL counts at 72 hours from those at 0 hours of incubation.	27
Table 3.4 Differences of least square means for growth of <i>Streptococcus thermophilus</i> ST-M5 and <i>Lactobacillus bulgaricus</i> LB-12 at different concentrations of Potassium Metabisulfite and Potassium Nitrite.	27
Table 3.5 Differences of least square means for screening of <i>Streptococcus thermophilus</i> ST-M5 and <i>Lactobacillus bulgaricus</i> LB-12 influenced by different antimicrobial concentrations at different hours of incubation.	28
Table 3.6 Difference of least square means for growth of yogurt bacteria at different antimicrobial concentrations.....	28
Table 3.7 Difference of least square means for growth of yogurt bacteria at different hours of incubation.....	29
Table 3.8 Probability > F (Pr > F) of fixed effects for the resistance of <i>Lactobacillus bulgaricus</i> LB-12 and <i>Streptococcus thermophilus</i> ST-M5 to high doses of Potassium Metabisulfite and Potassium Nitrite.....	32
Table 3.9 Differences of Least Square Means for the two different antimicrobials.	33
Table 3.10 Differences of Least Square Means for <i>Streptococcus thermophilus</i> ST-M5 and <i>Lactobacillus bulgaricus</i> LB-12.....	33

Table 3.11 Differences of Least Square Means for resistance of yogurt bacteria as influenced by higher concentrations of antimicrobial compounds after prior exposure to the low doses of the antimicrobials.33

Table 3.12 Differences of Least Square Means for resistance of yogurt bacteria as influenced by the transfer hour (from a low to a higher dose of antimicrobial).34

Table 3.13 Differences of Least Square Means for the effect of different incubation times.34

Table 3.14 Probability > t value ($Pr > |t|$) of differences of least Square Means compared to control of *Lactobacillus bulgaricus* LB-12 with different treatments of Potassium Metabisulfite and Potassium Nitrite; at different transfer hours and after 0, 24, 48 and 72 hours of incubation.37

Table 3.15 Probability > t value ($Pr > |t|$) of differences of least Square Means compared to control of *Streptococcus thermophilus* ST-M5 with different treatments of Potassium Metabisulfite and Potassium Nitrite; at different transfer hours and after 0, 24, 48 and 72 hours of incubation.43

Table 3.16 Mean Log reduction of the viable counts of *Lactobacillus bulgaricus* LB-12 with different low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite transferred at 24,48 and 72 hours obtained by subtracting viable Log CFU/mL counts at 72 hours from 0 hours of incubation.47

Table 3.17 Mean Log reduction of the viable counts of *Streptococcus thermophilus* ST-M5 with different low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite transferred at 24,48 and 72 hours obtained by subtracting viable Log CFU/mL counts at 72 hours from 0 hours of incubation.47

LIST OF FIGURES

Figure 3.1 Growth of <i>Lactobacillus bulgaricus</i> LB-12 in presence of A. Potassium Metabisulfite and B. Potassium Nitrite.....	21
Figure 3.2 Growth of <i>Streptococcus thermophilus</i> ST-M5 in presence of A. Potassium Metabisulfite and B. Potassium Nitrite.....	24
Figure 3.3 Growth <i>Lactobacillus bulgaricus</i> LB-12 transferred at A. 24 hours, B 48 hours and C. 72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 100ppm.	36
Figure 3.4 Growth of <i>Lactobacillus bulgaricus</i> LB-12 transferred at A. 24 hours, B. 48 hours and C.72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 1,000ppm.	40
Figure 3.5 Growth of <i>Streptococcus thermophilus</i> ST-M5 transferred at A. 24 hours, B. 48 C.72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 100ppm.	42
Figure 3.6 Growth of <i>Streptococcus thermophilus</i> ST-M5 transferred at A. 24 hours, B. 48 hours and C. 72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 1,000ppm.	45

ABSTRACT

Different antimicrobials are added in the manufacturing of dairy products such as flavored yogurts and processed cheese. Potassium Metabisulfite (PM) and Potassium Nitrite (PN) have been reported to have antimicrobial effect on pathogenic microorganisms such as *Clostridium botulinum* and *Listeria monocytogenes*. Yogurt is known for its health benefits, due to the presence of cultured bacteria. PM and PN are not commonly used in the dairy industry hence it would be interesting to study their influence in yogurt culture bacteria. The objectives of this study were: 1. to elucidate the influence of PM and PN at various concentrations, on the growth of yogurt culture and 2. to determine the possible acquisition of resistance after prior exposure to low doses of these antimicrobials. For the first objective different concentrations of PM and PN (100, 1,000, 10,000, 100,000 and 1,000,000ppm) were separately added to broth previously inoculated with *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5. Control samples did not receive any antimicrobial. Growth was determined by plating at 0, 24, 48 and 72 hours of incubation. For the second objective, treatments consisted of separately exposing cultures to 100 and 1,000ppm of both antimicrobials and after 24, 48 and 72 hours, transferring them into 10,000 and 100,000ppm, of both antimicrobials. Growth was measured at 0, 24, 48 and 72 hours of incubation. Data were analyzed using Proc Mixed and Repeated Measures model of the Statistical Analysis System SAS®. Differences of Least Square Means were used to determine significant differences. Neither PM nor PN had an antimicrobial effect on yogurt culture *Lactobacillus bulgaricus* LB-12 or *Streptococcus thermophilus* ST-M5. Both antimicrobials at 1,000,000ppm significantly increased counts of *Streptococcus thermophilus* ST-M5 6 log CFU/mL compared to control. Prior exposure of *Streptococcus thermophilus* ST-M5 to both

antimicrobials at 100 and 1,000ppm for 72 hours showed resistance to 10,000 and 100,000ppm of both PM and PN with a significant increase of 6 log CFU/mL compared to control. Commercial applications of this study would be to incorporate PM and PN in yogurt manufacture for inhibition of spoilage and pathogenic bacteria to ensure good preservation of the product and improved shelf life.

CHAPTER 1: INTRODUCTION

1.1 Probiotics

In the food industry great efforts are made daily on the preservation of the different food products in order to achieve longer and more stable shelf life of each one of the product. Products containing probiotic cultures in their composition may require greater care in what respects the treatment they obtain during the storage. In this particular case, antimicrobials become a crucial alternative to overcome many spoilage possibilities caused by pathogenic or non-pathogenic microorganisms other than the probiotics contained in the product.

Yogurt is one of the most common fermented milk products known for many years and has taken much attention in few years with the introduction of probiotics in dairy products. Lactic acid produced by the fermentation of lactose acts on milk protein to give yogurt its most characteristic texture and flavor (Ashraf and Shah 2011). Yogurt has a long story of recognition as a dietary product with many wanted characteristics. It is made from the symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Ashraf and Shah 2011).

Probiotics were defined by the FAO/WHO Expert Consultation Group as live microorganisms that are introduced orally in the gastrointestinal tract and confer a health benefit to the host's health when administered in adequate amounts (Pennacchia et al. 2006) (Singh et al. 2011). This definition of probiotics states that there are certain requirements for these organisms as they must be alive, not have gone through pasteurization, and must be present in high numbers, generally more than one billion per daily ingested dose (Gorbach 2002). Probiotics can be bacteria, molds and some yeasts. In the human gastro intestinal tract

microbiota, a great diversity of bacterial populations can be encountered (Liévin et al.2000); the most commonly known probiotics are bacteria, being the lactic acid bacteria the most popular ones. Some of these are *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bifidobacterium* species and *Escherichia coli* can be listed as the most common ones (Singh et al. 2011). In order for a particular bacterial strain to be considered a probiotic it has to meet up with certain requirements that have been established; it should be capable of exerting beneficial effects on the host animal, be nonpathogenic and non-toxic, be present as viable cells in large numbers, be capable of surviving in gastrointestinal conditions (low pH, organic acids and bile) (Singh et al. 2011).

Probiotics have been attributed functions that are of great importance for the host's health like aiding in the colon functioning and may reduce colon cancer risk, as mechanisms of defense against different pathogens (Gibson and Wang 1994), favoring calcium absorption, help alleviate lactose intolerance symptoms, improving mucosal barrier functioning amongst many other beneficial effects (Singh et al. 2011) . The microorganisms that constitute this microbiota are unevenly distributed throughout the digestive tract. With their metabolic activities, these microorganisms have shown to play a major role in the use of diverse nutrients ingested with food, also significantly affecting the development and performance of the immune system (Aureli et al. 2011). As time has gone by, probiotics have acquired a remarkable importance as to what concerns their functionality in the human health and also in the food industry.

The particular characteristic possessed by lactic acid bacteria of inhibiting the growth of diverse Gram-negative and Gram-positive bacteria is what makes them outstanding from the

rest of the bacterial populations. Many research projects have dedicated their time to study this inhibition mechanisms, leading to conclusions that show that these bacteria produce several substances such as organic acids (ex. lactic acid) which has shown great inhibitory capacity against gram negative and gram positive bacteria (Tajkarim and Ibrahim 2011). The antimicrobial activity of lactic acid has shown to come from the infiltration into microbial cells leading to an intracellular stress on membrane disruptions and the accumulation of toxic anions (Tajkarim and Ibrahim 2011). Hydrogen peroxide is one other metabolite that these bacteria may exert, having fast action rate against a wide spectrum of microorganisms including bacteria, fungi, virus, mycobacteria and bacterial spores, with the defect that its time of action cannot be stated as long lasting (Luck and Jager 1997). Bacteriocins which are protein like compounds produced by these microorganisms, have shown to be active against many know pathogens (Fernandez et.al 2003) which may contribute to a more strong immune system and act as a barrier of defense against possible infections or illnesses.

Probiotics have shown their great potential to function as antimicrobial sources against many of the gastrointestinal pathogens through the production of substances like the ones mentioned recently, other characteristics such as competitive exclusion of pathogen binding and modulation of the host's immune system are also very important for the action that probiotics may have against other microorganisms that might be considered as a threat for the host's health (Marianelli et al.2010).

1.2 Food Antimicrobials

Food antimicrobials are classified as “preservatives”. The Code of Federal Regulations 21 CFR 101.22 (a)(5) define chemical preservatives as “any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars,

vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties”. The additives used to prevent biological deterioration are denominated “antimicrobials”. The 21 CFR 170.3(o) (2) defines these as “substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors”. The main function of food antimicrobials has been established as prolongation of shelf life and to preserve quality in food products through the inhibition of spoilage microorganisms (Davidson et al. 2005).

The demand for foods that are minimally processed, easily prepared and ready-to-eat fresh represent major challenges for food safety and quality around the world (Appendini and Hotchkiss 2002). For long periods of time, chemicals have been added to preserve foods for later consumption. Some of these chemical food preservatives have been in use for many years, such as salts, nitrites and sulfites. Although many improvements are constantly made in packaging and processing systems to preserve foods, antimicrobial preservatives play a significant role in protecting the food supply (Davidson et al. 2002).

Food products today must comply with certain minimal characteristics that consumers expect them to have; to be available all year round, free of foodborne pathogens and any kind of contaminants and to have a long and stable shelf life.

Research has been done on these antimicrobial substances in order to understand their mode of action and their main compositional characteristics. They have been classified either as traditional or naturally occurring (Davidson 2001). For the different food products that may contain antimicrobials, the selection of the agent to use must be careful and may depend on

several factors, in order to ensure that the most adequate one will be used and that no alterations will be produced in the food product. First, the target pathogen must be identified followed by the evaluation of the possible preservation systems that are going to be used according to the food product that is being dealt with (Davidson et al.2005). It is important to evaluate the spectrum of a certain antimicrobial compound by following the growth of different organisms in the presence of different concentrations of the antimicrobial compound (Davidson et al. 2005). Probably the best way to determine what type of antimicrobial should be used would be by having an accurate knowledge of its mechanism of action and target within the microbial cell (Davidson et al. 2005). The selection of the proper antimicrobial depends on several factors; the spectrum of its antimicrobial activity, the chemical properties that the antimicrobial has, the physicochemical properties, the composition of the food product to which it is going to be applied to and finally the type of preservation method that will be used with this particular product microorganisms (Davidson et al. 2005). The mechanisms of action of food antimicrobials are generally classified as reaction with the cell membrane, permeability changes, interference with uptake and transport, inactivation of essential enzymes, interference with genetic mechanisms and inhibition of protein synthesis (Davidson et al. 2005).

One more important aspect to have present at the moment of choosing the antimicrobial agent to be used in any particular food product are its toxicological characteristics. It is very important that an additive for the use as an antimicrobial agent be safe for human consumption, and it does not represent any health risk in any aspect (Davidson et al. 2005).

1.3. Nitrites

Nitrites have been used as food preservatives in a variety of products in the industry. In many different countries sodium nitrite has been used in the form of a curing salt. It has to be used in determined quantities due to its probable toxicity to human health (Burden 1961).

Antimicrobial action in nitrites is based on the production of nitrous acid which will interact directly with the amino acid groups of the microorganism cell (especially in bacterial cells) causing an inhibitory effect (Quastel and Woolridge 1927). The rate of action of the nitrites may be increased as the pH of the medium in which it is acting is lowered (Castellani and Niven 1955). Nitrites have shown effective inhibitory action towards bacterium genera such as *Clostridium botulinum* and *Listeria monocytogenes* (McClure et al. 1991). Their main mechanism of action is based on the blocking of sulfhydryl sites within the bacterial cells and inhibition of active transport (Davidson et al. 2005). Nitrite interferes with the conservation of energy by the inhibition of the oxygen uptake, oxidative phosphorylation, and proton dependent active transport. It acts as an uncoupler, which causes a collapse of the proton gradient (Yarbrough et al. 1980).

Different research studies have shown that nitrites delay spoilage of food products. Tarr (1941) demonstrated the importance of pH on the efficacy of action of nitrite. At pH 7.0, little or no inhibition was observed, while at pH 5.7 and 6.0, complete or strong microbial inhibition was presented.

It has been used in several food products, but mostly with meats like; cured meats, canned cured meats, vacuum-packaged and fermented meats, bacon, cheese and seafood.

1.3.1 Regulations with Nitrites

Current USDA regulations that control the use of nitrites in meat and poultry products are specified in 9 CFR 317.17 and 424.21-23 of the federal regulations (Code of Federal Regulations, 2002a) (Davidson et al. 2005). The Food and Drugs Administration (FDA) 172.160 and 181.33 state, that potassium nitrite can be used in concentration of up to 200 parts per million (ppm) in different food products but most commonly in cured meats.

1.4 Sulfites

Sulfites have been used as food ingredients for long periods of time. Among their various purposes in the food industry such as antioxidants, control of enzymatic and non-enzymatic browning reactions; one of their main purposes is to serve as antimicrobial agents (Taylor et al. 1986). In their various forms as salts, dissolved in water or as a gas, they are used in fermentations, on fruit and in other industries to prevent the activity or growth of different microorganisms (Davidson et al. 2005).

Its main inhibitory action has been showed on bacterial and yeast strains of importance in the food industry. Examples are *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Lactobacillus casei*, *Escherichia coli* and *Saccharomyces cerevisiae* (Rehm et al. 1961, Rehm and Wittman 1962) amongst many others, having as their main antimicrobial action, the inhibition of enzyme-catalyzed reactions (Luck and Jager 1997). Amongst the mechanisms of action of sulfites that have been studied and discussed are the blockage of transport, inhibition of glycolysis, nutrient destruction and the inhibition of the general metabolism of the microbe. Sulfites are used in many different food products but their main use as an antimicrobial agent is in beverages and fruits (Davidson et al. 2005).

Among the main uses of sulfites, the preservation of the color and odor of meats has been shown to be improved by sulfite treatment and, even though it slows down and prevents the growth of surface bacteria, which is an important characteristic, the main effect discovered in meat appears to be its antioxidant properties (Roberts and McWeeney, 1972).

1.4.1 Regulations with Sulfites

Sulfites are generally recognized as safe (GRAS) by the FDA 182.3617, when they are used in quantities that go accordingly with good manufacturing practices. They have been allowed in fruit juices and concentrates, in dehydrated fruits and vegetables, as well as wine (Davidson et al. 2005). The FDA (1986b) made the labeling of any product that contains 10mg/ml or more of sulfite concentration is mandatory (Davidson et al. 2005). In many countries, sulfites are used for the inhibition of the growth of different microorganisms on fresh meat and different meat products (Kidney, 1974).

Sulfite or metabisulfite that is added in sausages is very effective when affecting the growth of molds, yeasts and salmonellae during the food product's storage in refrigeration or at room temperature (Ingram et al. 1956, Banks and Board 1982).

In this particular case it is important to note the inhibitory action that both nitrites and sulfites may have towards different members of the lactic acid bacteria group. This may possibly represent a problem when having a food product that contains probiotic strains and a food antimicrobial that might be in the nitrites or sulfites group. Sulfites can cause a portion of lactic acid bacteria populations to enter a viable but non-culturable state. In past research studies that have been done, the cell could not grow on nutrient agar, but showed metabolic activity through the hydrolysis of fluorescent esters and where able to be count,

using a direct epifluorescence microscopy (Davidson et al. 2005). The most important factor about a probiotic product is that its culture population lasts throughout the shelf life of the product without decreasing considerably so that it cannot be considered a probiotic.

1.5 Antimicrobial Resistance

A bacterial strain can be said to be resistant if it can survive and multiply itself in the presence of an antimicrobial agent that would normally inhibit or kill this particular kind of microorganism (Michigan State University 2011). Antimicrobial resistance is just one of the many adaptation characteristics that strong bacterial populations may have or acquire; enabling them to compete and survive amongst their microbial neighbors in whatever environment they are (Michigan State University 2011).

With the use of antimicrobials in the food industry it has become of great interest to study the possible resistance mechanisms that specific groups of bacteria may have towards these. In this particular case the resistance of probiotic bacteria, members of the lactic acid group is to be approached with more emphasis. This opens the possibility to develop probiotic food products that will behave adequately in the presence of antimicrobial agents without negatively affecting the survival of the probiotic bacteria and thus ensure an aggregated protection to these products against pathogenic bacteria, and extending the products shelf life.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) classified antimicrobial resistance into three main categories: 1) Intrinsic or natural resistance inherent to a bacterial species, 2) acquired

resistance caused by the mutation of indigenous genes and 3) acquired resistance due to acquisition of exogenous resistance genes (FEEDAP 2008).

Intrinsic resistance is the natural ability of a certain bacterial species to resist the activity of a particular antimicrobial compound through its inherent structural or functional characteristics, which will result in the tolerance of a particular antimicrobial (Michigan State University 2011). This tolerance can be due to several possible reasons; the lack of affinity of the antimicrobial with the bacterial target, inaccessibility of the antimicrobial to the bacterial cell, extrusion of the antimicrobial by chromosomally encoded active exporters and finally by natural production of enzymes that will result in the inactivation of the compound (Michigan State University 2011).

Acquired resistance, can happen when a particular microorganism obtains the capability to resist the activity of a microbial compound to which it had been previously vulnerable. It can also result from the mutation of genes involved in normal physiological processes and cellular structures from the acquirement of foreign resistance genes or from a combination of the two mechanisms (Michigan State University 2011).

To survive in the presence of an antimicrobial compound the microorganism must be able to disturb the essential steps that the antimicrobial agent requires to complete its action. There are several mechanisms that have been studied that independently or together may work to achieve this resistance, 1) by preventing the antimicrobial compound from reaching its target by the reduction of its ability to penetrate the microbial cell, 2) the removal of the antimicrobial compound from the cell by using general or specific efflux pumps, 3) inactivation of the antimicrobial compound by modifying it or degrading it and finally 4)

modifying the antimicrobial compound target within the microbe cell (Michigan State University 2011).

The presence of resistance genes in many of the lactic acid bacteria members and the transfer of plasmids and conjugative transposons to and from the lactic acid bacteria have been reported as reviewed by Teuber et al.(1999) (Danielsen and Wind 2003).

1.5.1 Mutations

A mutation is an unexpected and spontaneous change in the DNA sequence of the gene that may result in the change of a specific characteristic that it codes for. A change in a base pair may lead to a change in one or more of the amino acids for which it codes, that can alter the enzyme and cell structure that will lead to changes in the affinity of the antimicrobial compound (Michigan State University 2011). One of the main forms of bacterial resistance to antimicrobials is always related with changes caused by mutations in the cellular target sites (Davidson et al. 2005). These mutations will cause the target site of action of the antimicrobial to become insensitive to the inhibitor, but still able to perform its normal functions (Russell and Chopra, 1996).

1.5.2 Plasmid-Mediated Resistance

It is the process of interchanging genetic material between bacterial populations. Much of the antimicrobial resistance genes are contained within plasmids, transposons or integrons that will act as vectors which will transfer the genes to other members of the bacterial population (Michigan State University 2011). The main mechanisms of horizontal transfer in bacteria that are in natural environments are believed to be conjugation and transduction. In conjugation, plasmids play a very important role in the dissemination of antimicrobial

resistance (Ouba et al. 2008). The continuous use of Lactic acid bacteria (LAB) and probiotics has shown their nature to acquire antimicrobial resistance genes (Cataloluk and Gogebakan. 2004).

1.5.3 Biofilms and Microbial Response

Several studies have shown that the limitation of nutrients and reduced growth rates can change the susceptibility of bacteria to antimicrobials (Brown and Williams 1985). Biofilms are consortia of bacteria and possibly other microorganisms contained in an extensive mucoexopolysaccharide polymer or glycocalyx (Lewis 2001). Biofilms are produced as a consequence of an association of microorganisms with solid surfaces (Morton et al. 1998). Several possibilities are considered in the resistance of biofilms: reduced access of antimicrobial agents to the cells within a biofilm, chemical interaction between the biofilm and the molecules of the antimicrobial, modulation of the environment, production of degradative enzymes, genetic exchange between cells and quorum sensing (Spoering and Lewis, 2001). However, microorganisms that are removed from the biofilm and are re-cultured have shown to generally be equally insusceptible than other cells (Davidson et al. 2005).

It is important to highlight the importance of the behavior of both the antimicrobial of choice in the determined product and the probiotic cultures present on it. These have to maintain their viability throughout the disposed shelf life of the product without being affected by the action of the antimicrobial. This may be of great importance in products such as canned foods or dairy products such as yogurts that contain these probiotic cultures. Potassium Metabisulfite and Potassium Nitrite are used in the food processing industry. The influence of these two antimicrobials on yogurt culture bacteria is not known, these two antimicrobial

compounds are not normally used in the dairy industry, this would make interesting a study of these bacteria in their capacity to grow in the presence of these antimicrobials, and when exposed to low doses of these antimicrobials can yogurt bacteria acquire resistance to them.

This study had two objectives. The first objective was to elucidate the influence of potassium nitrite and potassium metabisulfite at various concentrations, on the growth of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The second objective was to determine the possible acquisition of resistance after prior exposure to low doses of these antimicrobials individually.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Design

For the first objective that is the screening study, treatments consisted of 5 different concentrations of Potassium Metabisulfite and Potassium Nitrite (100, 1000, 10,000, 100,000, and 1,000,000ppm) separately added to MRS broth previously inoculated separately with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5. Sterile peptone water (0.1% wt/v) was separately inoculated with 1% (v/v) *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 previously exposed to the different antimicrobial concentrations. The control did not have any concentration of either antimicrobial. Growth was determined by plating at 0, 24, 48 and 72 hours of incubation for both microorganisms. For the second objective that is the resistance study, treatments consisted of separately exposing cultures to 100 and 1,000ppm of both antimicrobials and after 24,48 and 72 hours, transferring them separately into 10,000 and 100,000ppm, of both antimicrobials. Growth was measured at 0, 24, 48 and 72 hours of incubation. This was conducted separately for each bacterial strain for each antimicrobial.

2.2 Sample Preparation

Control and samples treated with Potassium Metabisulfite (Acros Organics, New Jersey, USA) and Potassium Nitrite (MP Biomedicals, LLC, Solon, OH) tested for growth analyses were set by inoculating 1ml of fresh pure frozen cultures of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 (F-DVS LA-K, Chr. Hansen's Laboratory, Milwaukee, WI, USA) carefully thawed, into 99mL of autoclaved MRS broth (Difco™, Dickinson and company, Sparks, MD). Each one of the samples was treated with different concentrations of both antimicrobials individually.

2.3 Treatments

The effect of Potassium Metabisulfite (Acros Organics, New Jersey, USA) and Potassium Nitrite (MP Biomedicals, LLC, Solon, OH) at various concentrations was determined according to the method proposed by Jamuna et al. (2005) with modifications. Potassium Metabisulfite (Acros Organics, New Jersey, USA) at (100, 1,000, 10,000, 100,000 and 1,000,000ppm) and Potassium Nitrite at (MP Biomedicals, LLC, Solon, OH) (100, 1,000, 10,000, 100,000 and 1,000,000 ppm), were added by separate to previously prepared and autoclaved MRS broth (Difco™, Dickinson and company, Sparks, MD). After both the antimicrobial and the MRS broth were homogenized, the mixture was inoculated with *Lactobacillus bulgaricus* LB-12. The inoculated broth containing the antimicrobial compound was taken for anaerobic incubation at 43°C for 48hours. This same procedure was repeated for *Streptococcus thermophilus* ST-M5 and was taken to aerobic incubation at 37°C for 24 hours (Dave and Shah., 1996). For each one of the treatments a control was made, were the broth without any antimicrobial was inoculated with each one of the cultures and incubated under the same conditions.

2.4 Preparation of Media:

2.4.1 Peptone Water

Peptone and water (0.1%) was prepared by dissolving 1g of peptone medium (Bacto™ Peptone, Difco, Dickinson and company, Sparks, MD) in 1L of distilled water, then it was autoclaved in 99mL portions at 121°C for 15 minutes.

2.4.2 Lactobacilli MRS Broth

The MRS broth used was prepared according to the instructions given by the manufacturer (Difco™, Dickinson and company, Sparks, MD).

2.4.3 Lactobacilli MRS Agar

The MRS agar used was prepared according to the instructions given by the manufacturer (Difco™, Dickinson and company, Sparks, MD).

2.4.4 *Streptococcus thermophilus* Agar

The ST Agar was prepared in the following manner:

10g of tryptone, 10g sucrose, 5g yeast extract and 2g of K₂HPO₄ are dissolved in 1L of distilled water. The pH of mixture was adjusted to 6.8 ± 0.1 ; after this 6mL of 0.5% bromocresol purple and 12g of agar were added to the mixture. The medium was then autoclaved at 121°C for 15 minutes (Dave and Shah 1996).

2.4.5 Modified pH MRS Agar (pH 5.2)

The MRS agar was prepared according to the instructions given by the manufacturer (Difco™, Dickinson and company, Sparks, MD). Following this the pH was adjusted to a pH 5.2 using HCL 1N (Dave and Shah 1996).

2.5. Analytical Procedure

2.5.1 Dilution Method, Growth

The growth effect of both probiotic strains; *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 with the different concentrations of both antimicrobials was evaluated according to the method proposed by Lin and Young (2000) and the solution method proposed by Michigan State University (2011), with some modifications. The control and the samples containing each one of the antimicrobials were inoculated (10% [v/v]) in MRS broth (Difco™, Dickinson and company, Sparks, MD). The growth of both probiotic strains was determined in time intervals of 24 hours for a total of 72 hours, starting at 0

hours. During this 72 hour period each one of these cultures were incubated in their corresponding temperatures, 43°C for *Lactobacillus bulgaricus* LB-12 and 37 °C for *Streptococcus thermophilus* ST-M5. 1ml of that inoculated broth was serially diluted in peptone water (0.1%wt/v) and then was pour plated. The *Lactobacillus bulgaricus* LB-12 was plated using Lactobacilli MRS agar with pH 5.2 (Dave and Shah., 1996), and the *Streptococcus thermophilus* ST-M5 was plated using the *Streptococcus thermophilus* agar (Dave and Shah., 1996).

2.5.2 Resistance

The method used to test for microbial resistance to the antimicrobial compound was the dilution method proposed by Michigan State University (2011) with some modifications.

To study the possibility of an acquired resistance to the two antimicrobials and both probiotic strains *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 were subjected to exposure of higher concentrations of potassium metabisulfite (Acros Organics, New Jersey, USA) and potassium nitrite (MP Biomedicals, LLC, Solon, OH) and were tested for growth during 0, 24, 48 and 72 hours, after being exposed to the lower concentrations of these during previous 0, 24, 48 and 72 hours of incubation.

After following the methodology described in the growth procedure above, the *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 grown at 100ppm and 1000ppm of potassium metabisulfite (Acros Organics, New Jersey, USA) and potassium nitrite (MP Biomedicals, LLC, Solon, OH) were transferred after the first 24 hours and re-inoculated (10% [v/v]) in MRS broth (Difco™, Dickinson and company, Sparks, MD) with 10.000ppm and 100.000ppm of potassium metabisulfite (Acros Organics, New Jersey, USA)

and potassium nitrite (MP Biomedicals, LLC, Solon, OH) respectively and were subjected to the same growth protocol established previously. This transfer was also done after 48 and 72 hours of incubation with the lower concentrations of both antimicrobials.

2.6 Statistical Analysis

The data were analyzed using Proc Mixed and a Repeated Measures model of the Statistical Analysis System, SAS® 9.3. Differences of Least Square Means were used to determine significant differences $P < 0.05$ for main effects (Antimicrobial concentration, Time of incubation, Type of antimicrobial) for the screening study and (Type of antimicrobial, type of microorganism, time of transfer and growth time) for the resistance study. Significant differences were determined at $\alpha = 0.05$, significant differences ($P < 0.05$) among the main effects were analyzed using Tukey's adjustment for the screening study and Kenward-Roger approximation for the resistance study.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Screening

The growth of both microorganisms *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12, as influenced by the addition of different concentrations of Potassium Metabisulfite and Potassium Nitrite separately at different times of incubation (0, 24, 48 and 72 hours), are shown in Figures 1 and 2.

There was a significant ($P < 0.0001$) interaction between antimicrobial*microorganism*time (Table 1). The growth of both *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 was significantly influenced ($P < 0.0001$) by the various concentrations of antimicrobials at different time intervals (0, 24, 48, 72 hours) (Table 1). The interaction between antimicrobial*time was significant ($P = 0.0349$). There was also a significant interaction between antimicrobial*concentration ($P = 0.0131$) (Table 1).

Figures 1A and 1B show the growth of *Lactobacillus bulgaricus* LB-12 treated with Potassium Metabisulfite and Potassium Nitrite. The general effect of treatments involving Potassium Metabisulfite was a decrease in viable counts of *L.bulgaricus* between hours 0 and 72 (Figure 1A). At 24 hours there was a significant difference in counts ($P < 0.0001$) between the treatment 1,000,000 ppm of Potassium Metabisulfite and the *L.bulgaricus* control (Table 2). At 48 hours there was a significant difference in counts ($P = 0.0245$) between 1,000,000 ppm of Potassium Metabisulfite and the *L.bulgaricus* control. Viable counts at 48 hours with 1,000,000 ppm of Potassium Metabisulfite reached non detectable levels which represented a decrease in counts compared to the *L.bulgaricus* control which had viable counts between 1-2 log CFU/mL at this time (Figures 1A and 1B)(Table 2).

Table 3. 1 Probability > F (Pr > F) of fixed effects for the screening of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 with different concentrations of Potassium Metabisulfite and Potassium Nitrite individually.

Effect	Pr > F
Microorganism	< 0.0001
Antimicrobial	0.7111
Concentration	< 0.0001
Time	< 0.0001
Antimicrobial*Concentration	0.0131
Antimicrobial*Time	0.0349
Concentration*Time	< 0.0001
Antimicrobial*Concentration*Time	0.8771
Antimicrobial*Microorganism*Time	< 0.0001
Antimicrobial*Microorganism*Concentration*Time	0.0312

Table 3.2 Probability > t value (Pr > |t|) of differences of Least Square Means compared to control of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 at different concentrations of Potassium Metabisulfite and Potassium Nitrite; after 0, 24, 48 and 72 hours of incubation.

Antimicrobial Concentration (ppm)	<i>L.bulgaricus</i>								<i>S.thermophilus</i>							
	Potassium Metabisulfite				Potassium Nitrite				Potassium Metabisulfite				Potassium Nitrite			
	Time (Hours)															
	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
100	0.4872	0.3468	0.1293	0.0097	0.3058	0.5852	0.8956	0.8561	0.1444	0.4869	0.6902	0.4817	0.2878	0.1216	0.0210	0.0384
1,000	0.8616	0.1589	0.1456	0.0212	0.2535	0.7777	0.7986	0.9100	0.7443	0.1195	0.0075	0.3628	0.3875	0.5525	0.0164	0.0029
10,000	0.6958	0.1693	0.3123	0.8996	0.6930	0.5775	0.0219	0.8651	0.3798	0.0370	0.3402	0.2539	0.2473	0.5200	0.1410	0.4491
100,000	0.7966	0.2270	0.4009	0.9112	0.0857	0.9484	0.7264	0.8734	0.2872	0.0132	0.0126	< 0.0001	0.2401	0.8709	0.3574	0.0353
1,000,000	0.8335	< 0.0001	0.0245	0.8945	0.8851	0.8705	0.0196	0.8755	0.7201	0.9205	0.0257	< 0.0001	0.6684	0.0172	< 0.0001	< 0.0001

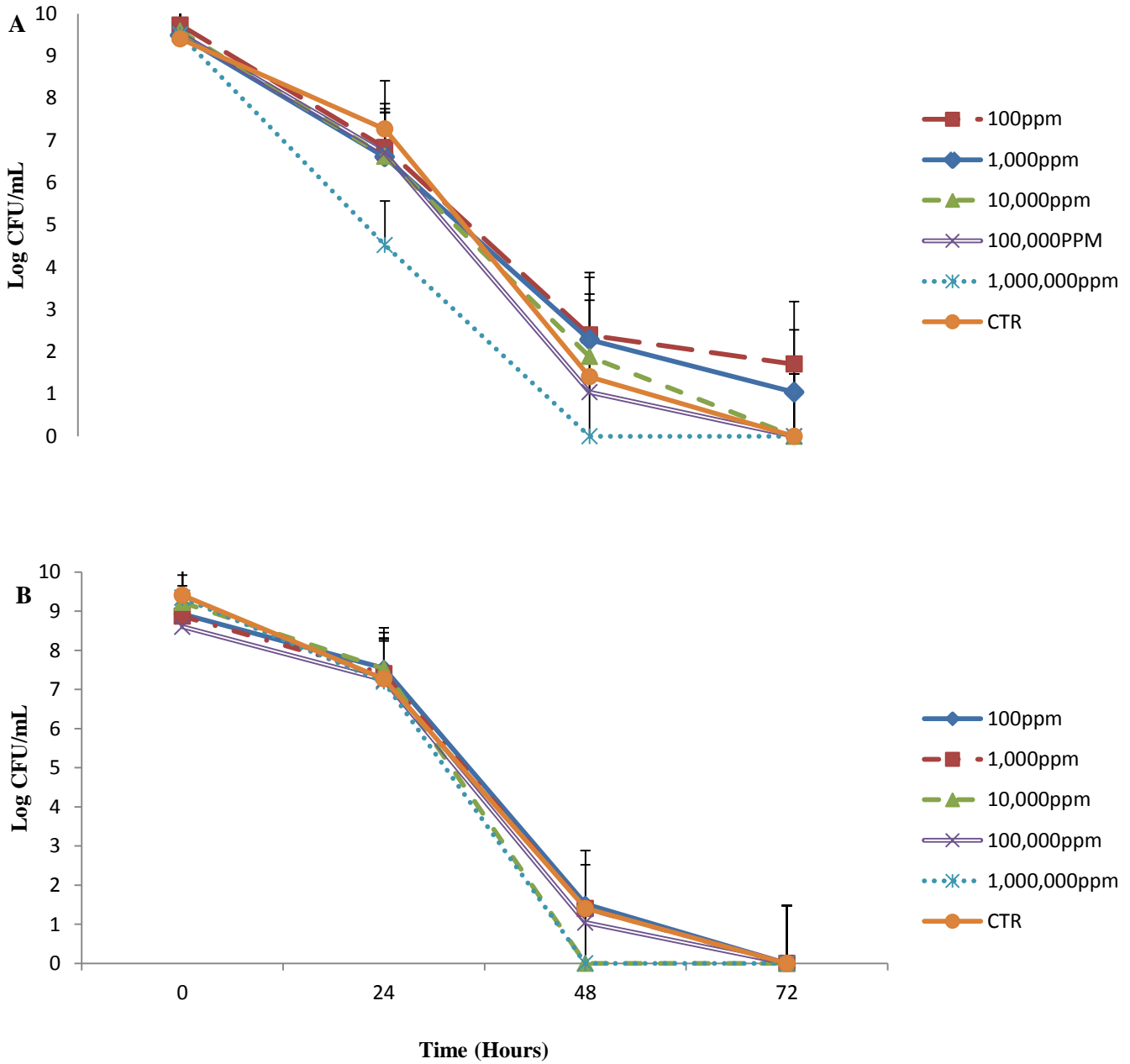


Figure 3.1 Growth of *Lactobacillus bulgaricus* LB-12 in presence of A. Potassium Metabisulfite and B. Potassium Nitrite.

At 72 hours there was a significant difference ($P < 0.05$) between the growth of *L. bulgaricus* with the treatments involving 100 and 1,000 ppm of Potassium Metabisulfite and the

L.bulgaricus control (Table 2). The viable counts at 100 and 1,000ppm were significantly higher than those of the control. There was a greater decrease in counts for the *L.bulgaricus* control, which showed no growth at 72 hours, while 100 and 1,000 ppm had viable counts between 2 and 3 log CFU/mL (Figure 1A). The effect that Potassium Metabisulfite had on *Lactobacillus bulgaricus* LB-12 at 100, 1,000, 10,000, 100,000 and 1,000,000 ppm could not be called antimicrobial. No antimicrobial activity was observed against *L.bulgaricus* when treated with all concentrations of Potassium Metabisulfite. (Table 2) (Fig 1A).

The effect that different concentrations of Potassium Nitrite had on *Lactobacillus bulgaricus* LB-12 is shown in Figure 1B. There was a decrease in viable counts for this bacterium between hours 0 and 72 of incubation (Figure 1B). At 0 and 24 hours of incubation there were no significant ($P>0.05$) differences in counts between the treatments and the *L.bulgaricus* control (Table 2) (Figure 1B).

At 48 hours there were significant differences ($P<0.05$) in counts between 10,000 and 1,000,000 ppm of Potassium Nitrite and the *L.bulgaricus* control (Table 2) (Figure 1B). At 48 hours viable counts of *L.bulgaricus* at 10,000ppm and 1,000,000ppm reached non detectable levels, while viable counts for the control were between 1-2 log CFU/mL (Figure 1B). At 72 hours; there were no significant ($P>0.05$) differences in counts between any of the concentrations of Potassium Nitrite and the *L.bulgaricus* control (Table 2). Effects of Potassium Nitrite on the growth of *Lactobacillus bulgaricus* LB-12 cannot be said to be inhibiting or antimicrobial, as growth of the yogurt bacterium, in comparison to that of the control, was not significantly different (Table 2). The effect on the growth of *Streptococcus thermophilus* ST-M5 with different concentrations of Potassium Metabisulfite is shown in Figure 2A. At 24 hours of incubation significant differences in counts were observed

between 10,000 and 100,000 ppm of Potassium Metabisulfite with the *S.thermophilus* control (Table 2) (Figure 2A). At 24 hours viable counts for *S.thermophilus* treated with 10,000 and 100,000ppm of Potassium Metabisulfite were lower than those of the control (Figure 2A). At 48 hours there were significant differences ($P<0.05$) in counts between *S.thermophilus* treated with 1,000, 100,000 and 1,000,000 ppm of Potassium Metabisulfite and the *S.thermophilus* control (Table 2) (Figure 2A). Growth curves for treatments with Potassium Metabisulfite show a general decrease in viable counts of *S.thermophilus* between 48 and 72 hours except for *S.thermophilus* treated with 1,000,000 ppm of Potassium Metabisulfite, where there was an increase in viable counts (Figure 2A).

At 72 hours of incubation there were significant differences in counts between the control and *S.thermophilus* treated with 100,000 and 1,000,000 ppm of Potassium Metabisulfite (Table 2). This effect was similar to that of a prebiotic since there were positive effects had on the growth of *Streptococcus thermophilus* ST-M5 treated at these concentrations of Potassium Metabisulfite. At 72 hours viable counts for 1,000,000ppm were 5 log CFU/mL higher than the *S.thermophilus* control (Figure 2A).

There was no observed antimicrobial effect on this bacterium with any of the concentrations of Potassium Metabisulfite. For the case of 1,000,000ppm there were positive effects on the growth of *S.thermophilus* which leads to the possibility that in the interaction between the bacterium and Potassium Metabisulfite there are metabolites produced that promote the growth of the bacterium.

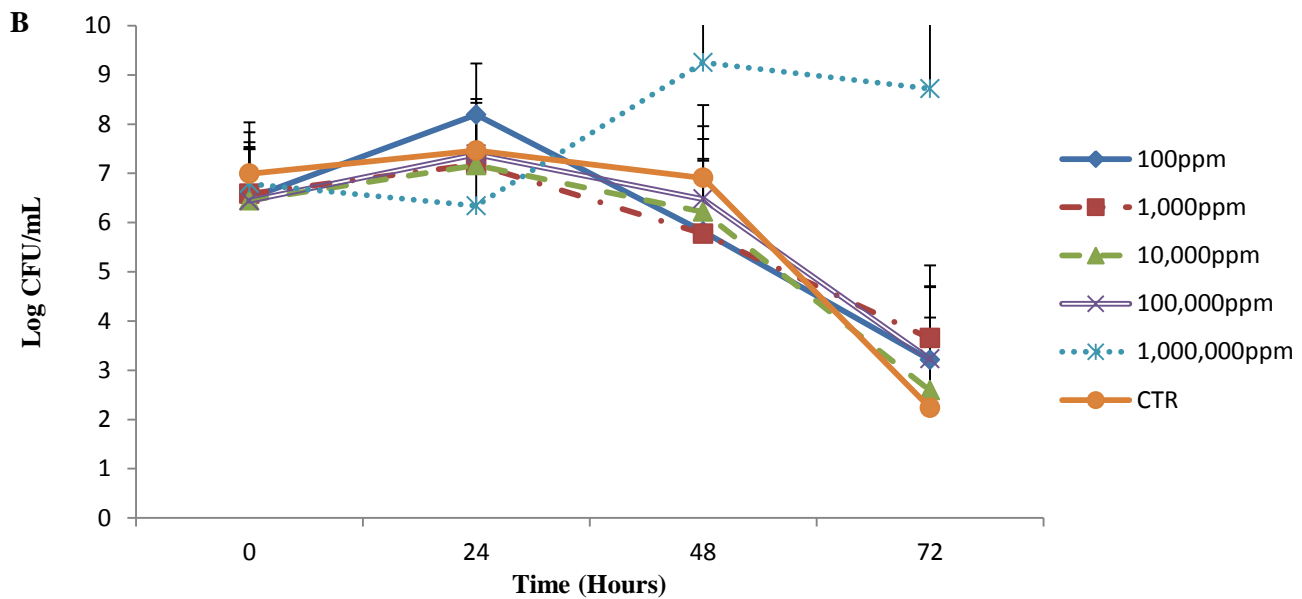
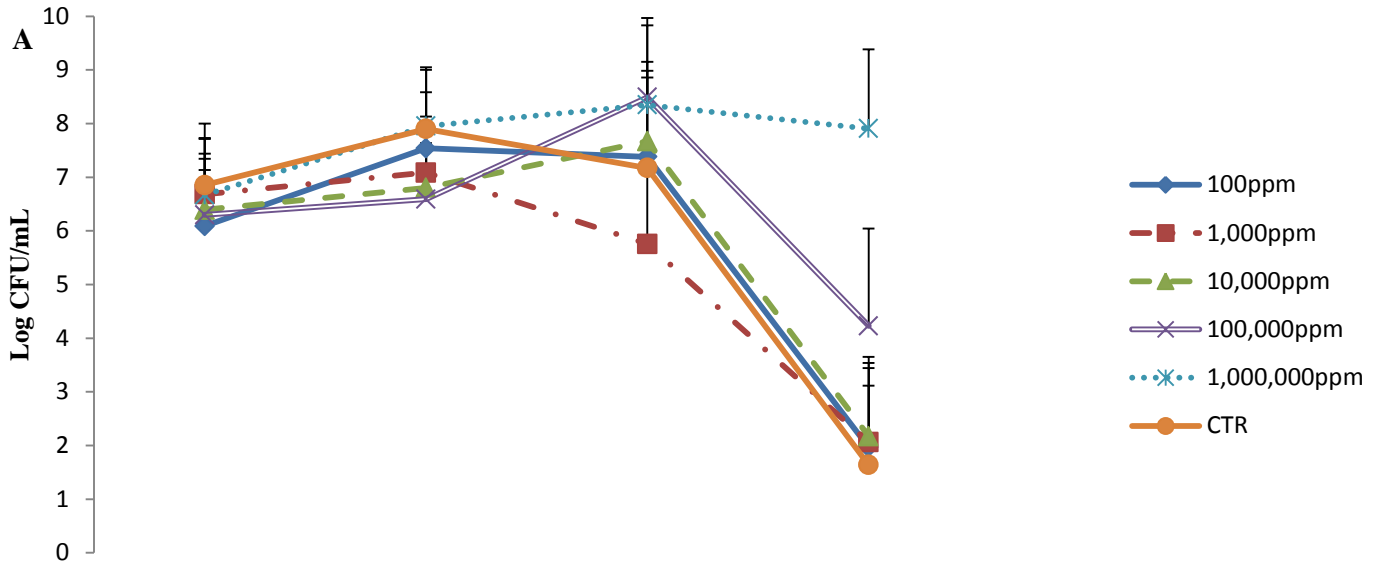


Figure 3.2 Growth of *Streptococcus thermophilus* ST-M5 in presence of A. Potassium Metabisulfite and B. Potassium Nitrite.

Effects of Potassium Nitrite at 100, 1,000, 10,000, 100,000 and 1,000,000 ppm on the growth of *Streptococcus thermophilus* ST-M5 are shown on Figure 2B. At 24 hours the *S.thermophilus* control counts were significantly higher ($P=0.0172$) than those of

S.thermophilus with 1,000,000ppm (Table 2) (Figure 2B). At 48 hours control counts were significantly higher ($P<0.05$) than 100 and 1,000ppm but were significantly lower than 1,000,000ppm (Table 2) (Figure 2B). On the other hand, counts of *S.thermophilus* treated with 1,000,000 ppm of Potassium Nitrite showed higher counts (9-10 log CFU/mL) than those of the control (Figure 2B). At 72 hours of incubation the *S.thermophilus* control counts were significantly lower ($P<0.05$) compared to cultures treated with 100, 1,000, 100,000 and 1,000,000 ppm of Potassium Nitrite (Table 2) (Figure 2B).

Viable counts of *S.thermophilus* at 72 hours for 1,000,000ppm were 5 log CFU/mL higher than the control (Figure 2B). As with Potassium Metabisulfite, the effects of Potassium Nitrite on *Streptococcus thermophilus* ST-M5 cannot be said to be antimicrobial and the use of 1,000,000ppm has effects similar to a prebiotic due to the increases in population that this yogurt bacteria has at this concentration of Potassium Nitrite.

Mean log reductions of the viable counts of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 subjected to various concentrations of Potassium Metabisulfite and Potassium Nitrite were obtained by subtracting counts at 72 hours of incubation from 0 hours (Table 3). In Table 3 a high number indicated high bacterial reduction and a low number indicated low bacterial reduction. The lower the number, more growth was obtained. For *Streptococcus thermophilus* ST-M5 the lowest reduction was observed for 1,000,000 ppm for both antimicrobials (Table 3). The positive effect that both compounds had on the growth of *Streptococcus thermophilus* ST-M5 eliminates the possibility of calling the effect antimicrobial. For *Lactobacillus bulgaricus* LB-12 log reductions were not as low as for *Streptococcus thermophilus* ST-M5, as they were all in the range of 8-10 log CFU/mL (Table 3). In this case the action of Potassium Metabisulfite and

Potassium Nitrite did not have an inhibition effect on the bacterium, but when comparing between cultures, positive effects were greater for *Streptococcus thermophilus* ST-M5 indicating that the growth of this bacterium was stimulated more by these two antimicrobials than *Lactobacillus bulgaricus* LB-12.

For the interaction between the type of antimicrobial and the concentration of the antimicrobial (Table 4) there was a significant difference between the 1,000,000 ppm of Potassium Metabisulfite and the control. Also for Potassium Metabisulfite there was a significant difference between 1,000,000ppm and 100, 1,000 and 10,000ppm (Table 4). For Potassium Nitrite there were significant differences between 1,000,000 ppm and the control, 100, 1,000, 10,000 and 100,000 ppm of this compound (Table 4).

For the interactions that involved the time (hours) of incubation of the bacterial strains and the different concentrations of antimicrobial (Table 5), at 48 hours of incubation there were significant differences between 1,000 ppm and 1,000,000 ppm. At 72 hours of incubation there was a significant difference between the 1,000,000 ppm concentration of antimicrobials with 100, 1,000, 10,000, 100,000ppm and the control (Table 5). The concentrations used of both Potassium Metabisulfite and Potassium Nitrite had a significant effect ($P < 0.05$) on the growth of both *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 (Table 1). For the different concentrations of antimicrobial used, there were significant differences between 1,000,000ppm and the control, 100, 1,000, 10,000 and 100,000ppm (Table6).

Table 3.3 Mean Log reduction of the viable counts of cultures treated with different concentrations of Potassium Metabisulfite and Potassium Nitrite obtained by subtracting viable Log CFU/mL counts at 72 hours from those at 0 hours of incubation.

Antimicrobial Concentration (ppm)	<i>S.thermophilus</i>		<i>L.bulgaricus</i>	
	Decrease in Log CFU/mL		Decrease in Log CFU/mL	
	Potassium Metabisulfite	Potassium Nitrite	Potassium Metabisulfite	Potassium Nitrite
	Log CFU/mL			
100	4.12	3.28	8.02	8.93
1,000	4.62	2.94	8.45	8.87
10,000	4.22	3.86	9.59	9.22
100,000	2.07	3.21	9.53	8.61
1,000,000	-1.24	-1.93	9.50	9.34
Control	5.21	5.21	9.41	9.41

Table 3.4 Differences of least square means for growth of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 at different concentrations of Potassium Metabisulfite and Potassium Nitrite.

	Potassium Metabisulfite	Potassium Nitrite
Antimicrobial Concentration (ppm)	LS Mean Diff	LS Mean Diff
100	14.0669 ^{ABCa}	14.1592 ^{Aa}
1,000	13.0944 ^{ABa}	14.0732 ^{Aa}
10,000	14.129 ^{ABCa}	13.8096 ^{Aa}
100,000	15.7022 ^{ABCDa}	14.126 ^{Aa}
1,000,000	17.6003 ^{Db}	18.8791 ^{Bb}
Control	14.2265 ^{Aa}	14.2237 ^{Aa}

^{ABCD} LSM means with the same letter within the column are not significantly different

^{abcd} LSM means with the same letter within the row are not significantly different

Table 3.5 Differences of least square means for screening of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 influenced by different antimicrobial concentrations at different hours of incubation.

	0 hours	24 hours	48 hours	72 hours
Antimicrobial Concentration (ppm)	LS mean Diff	LS mean Diff	LS mean Diff	LS mean Diff
100	17.9829 ^{ABC}	17.316 ^{ABC}	15.1944 ^{ABC}	5.959 ^E
1,000	18.207 ^{ABC}	16.2799 ^{ABC}	13.2723 ^{CD}	6.5759 ^E
10,000	18,2213 ^{AB}	16.1874 ^{ABC}	15.9884 ^{ABC}	5.4799 ^E
100,000	17.7661 ^{ABC}	16.0726 ^{ABC}	17.2324 ^{ABC}	8.4162 ^{DE}
1,000,000	18.593 ^{AB}	14.9719 ^{BC}	20.2596 ^A	19.1343 ^{AB}
Control	19.122 ^A	17.171 ^{ABC}	16.2842 ^{ABC}	4.4584 ^E

ABCD LSMeans Diff with the same letter within the column are not significantly different

Table 3.6 Difference of least square means for growth of yogurt bacteria at different antimicrobial concentrations

Antimicrobial Concentration (ppm)	LS Mean Diff
100	14.1131 ^B
1,000	13.5838 ^B
10,000	13.9693 ^B
100,000	14.8675 ^B
1,000,000	18.2397 ^A
Control	14.2623 ^B

The effect of time (0, 24, 48 and 72 hours) of incubation of bacterial strains was significant ($P < 0.0001$) (Table 1). There were significant differences between 0 hour with 24, 48 and 72 hours of incubation (Table 7). There were significant differences between 48 and 72 hours of incubation (Table 7).

Cultures of *Streptococcus thermophilus* ST-M5 treated with 1,000,000ppm showed higher viable counts compared to the *S.thermophilus* control. Growths of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 were not negatively influenced by either Potassium Metabisulfite or Potassium Nitrite.

Table 3.7 Difference of least square means for growth of yogurt bacteria at different hours of incubation

Hour	LS Mean Diff
0	18.3169 ^A
24	16.3707 ^B
48	16.3352 ^B
72	8.3344 ^C

In the case of the 1,000,000 ppm concentration for both Potassium Metabisulfite and Potassium Nitrite, the increase in population suggests that, there is no antimicrobial effect; on the contrary, at this concentration, both compounds, like prebiotics, promoted the growth of the microorganism.

These two antimicrobial compounds have been reported to have a kill effect on diverse pathogenic and spoilage bacteria. Potassium Metabisulfite has shown effectiveness towards microorganisms such as *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Lactobacillus casei*, *Escherichia coli* and *Saccharomyces cerevisiae* (Rehm et al. 1961, Rehm and Wittman 1962). Potassium Nitrite has had a kill effect and has been used to control the growth of *Clostridium botulinum* and *Listeria monocytogenes* (McClure et al. 1991) both of which cause food borne diseases.

Different research projects have studied the action and tolerance of lactic acid bacteria towards nitrites. Fornaud et al. (1964, 1966) showed that some dairy cultures of *Lactobacillus* possess a nitrite reductase enzyme system which reduces nitrite to nitrous oxide, nitrogen dioxide or nitrogen gas under anaerobic conditions (Dodds and Collins-Thompson 1984). Dodds and Collins-Thompson 1984 report that lactic acid bacteria can reduce nitrite both chemically and enzymatically. These reports from other studies support the results obtained showing that Potassium Nitrite does not have inhibition activity towards

Lactobacillus bulgaricus LB-12 and *Streptococcus thermophilus* ST-M5 which belong to the lactic acid bacteria group. The division of lactic acid bacteria into homo- and heterofermentative strains is based on their metabolic pathways (Buyze et al. 1957). Homofermentative strains lack glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase and metabolize glucose to lactate via the Embden-Meyerhof-Parnas (EMP) pathway (Dodds and Collins-Thompson 1984). Based on this statement Dodds and Collins-Thompson, 1984 found that the majority of homofermentative strains in their study did not show a significant decrease in growth rate or cell yield in the presence of certain concentrations of nitrite. Being both *Lactobacillus bulgaricus* and *Streptococcus thermophilus* homofermentative lactic acid bacteria, this serves as a possible explanation to the tolerance that both yogurt cultures had towards Potassium Nitrite.

As for Potassium Metabisulfite, Corte et al. 2012 reported that relative abundances of SO₂, bisulfite and sulfite are regulated by the pH. They state that Potassium Metabisulfite requires low pH values (lower than pH 3.7) to have efficacy in its antimicrobial action against microorganisms. Lactic acid produced by lactic acid bacteria has pH values between 4-5 and yogurt pH also lies between these same ranges of pH. In their study Corte et al. 2012 reported that Potassium Metabisulfite caused complete cell inhibition of yeasts at pH 2, whereas at pH values from 2 to 6, mortality decreased gradually and then was stabilized at higher pH values. They report that Potassium Metabisulfite has strong biocide effect up to pH 3, which is the last pH value which indices the presence of molecular SO₂. This statement about the antimicrobial action on Potassium Metabisulfite and its dependency on low pH values, clarifies the tolerance of both yogurt cultures to the different concentrations of this antimicrobial.

These results, and the fact that neither Potassium Metabisulfate nor Potassium Nitrite inhibited the growth of *Streptococcus thermophilus* ST-M5 or *Lactobacillus bulgaricus* LB-12, make the idea of adding these antimicrobials into a yogurt or fermented dairy product a viable food safety option as the health beneficial culture counts will not be decreased by antimicrobial action. This can be a new alternative of a preservation method for these types of products ensuring that certain spoilage and pathogenic bacteria will not negatively affect the quality of the product. Further studies on the influence of both antimicrobial compounds on quality characteristics of dairy products have to be made in the future.

3.2 Resistance

Influences of fixed effects are shown in Table 8. There was a significant interaction ($P=0.0015$) effect for Antimicrobial Type*Microorganism Type*Low Concentration to High Concentration*Transfer Hour*Incubation Time for both yogurt cultures (Table 8). There was a significant influence ($P<0.0001$) of the type of antimicrobials (Table 8). The type of microorganism was also significant ($P<0.0001$) for the level of resistance to the compounds (Table 8). The treatments applied, which consisted of the transfer of the cultures from 100 and 1,000 ppm of Potassium Metabisulfite and Potassium Nitrite 10,000 and 100,000 ppm, were also significant ($P = 0.0084$) for the growth of both yogurt bacteria (Table 8). The transfer hour into 10,000 and 100,000ppm of each antimicrobial individually and the incubation times in which the viable population counts were measured, were also significant ($P<0.0001$) for *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12 (Table 8).

Table 3.8 Probability > F (Pr > F) of fixed effects for the resistance of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 to high doses of Potassium Metabisulfite and Potassium Nitrite.

Effect	Pr > F
Antimicrobial Type	< 0.0001
Microorganism Type	< 0.0001
Low Concentration to High Concentration	0.0084
Transfer Hour	< 0.0001
Incubation Time	< 0.0001
Antimicrobial Type*Incubation Time	< 0.0001
Microorganism Type*Incubation Time	< 0.0001
Low Concentration to High Concentration*Incubation Time	< 0.0001
Transfer Hour*Incubation Time	< 0.0001
Antimicrobial Type*Microorganism Type	0.0115
Antimicrobial Type*Low Concentration to High Concentration	0.8751
Microorganism Type*Low Concentration to High Concentration	0.1063
Antimicrobial Type*Transfer Hour	< 0.0001
Microorganism Type*Transfer Hour	< 0.0001
Low Concentration to High Concentration*Transfer Hour	0.0053
Antimicrobial Type*Microorganism Type*Incubation Time	< 0.0001
Antimicrobial Type*Low Concentration to High Concentration*Incubation Time	< 0.0001
Antimicrobial Type*Transfer Hour*Incubation Time	< 0.0001
Microorganism Type*Low Concentration to High Concentration*Incubation Time	0.0176
Microorganism Type*Transfer Hour*Incubation Time	< 0.0001
Low Concentration to High Concentration*Transfer Hour*Incubation Time	< 0.0001
Antimicrobial Type*Microorganism Type*Low Concentration to High Concentration	0.1488
Antimicrobial Type*Microorganism Type*Transfer Hour	< 0.0001
Antimicrobial Type*Low Concentration to High Concentration*Transfer Hour	0.1613
Microorganism Type*Low Concentration to High Concentration*Transfer Hour	0.1435
Antimicrobial Type*Microorganism Type*Low Concentration to High Concentration*Transfer Hour	< 0.0001
Antimicrobial Type*Microorganism Type*Low Concentration to High Concentration*Incubation Time	< 0.0001
Antimicrobial type*Microorganism Type*Transfer Hour*Incubation Time	< 0.0001
Antimicrobial Type*Low Concentration to High Concentration*Transfer Hour*Incubation Time	< 0.0001
Microorganism Type*Low Concentration to High Concentration*Transfer Hour*Incubation Time	< 0.0001
Antimicrobial Type*Microorganism Type*Low Concentration to High Concentration*Transfer Hour*Incubation Time	0.0015

There was a significant difference ($P < 0.05$) between the two antimicrobials, Potassium Metabisulfite and Potassium nitrite (Table 9).

The effects observed on *Streptococcus thermophilus* ST-M5, following transfers from lower concentrations to higher concentrations of Potassium Metabisulfite and Potassium Nitrite, were significantly different compared to *Lactobacillus bulgaricus* LB-12 (Table 10).

There was a significant difference ($P < 0.05$) between cultures initially exposed to at 100 ppm and transferred into 10,000ppm, with those initially exposed to 1,000ppm of Potassium Metabisulfite and potassium Nitrite (Table 11).

Table 3.9 Differences of Least Square Means for the two different antimicrobials.

Antimicrobial Type	LS Mean Diff
Potassium Metabisulfite	15.2459 ^A
Potassium Nitrite	9.7438 ^B

Table 3.10 Differences of Least Square Means for *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12.

Microorganism Type	LS Mean Diff
<i>Streptococcus thermophilus</i>	16.1578 ^A
<i>Lactobacillus bulgaricus</i>	8.8318 ^B

Table 3.11 Differences of Least Square Means for resistance of yogurt bacteria as influenced by higher concentrations of antimicrobial compounds after prior exposure to the low doses of the antimicrobials.

Low Concentration to High Concentration	LS Mean Diff
100ppm to 10,000ppm	13.0878 ^A
100ppm to 100,000ppm	12.6424 ^{AB}
1,000 to 10,000ppm	12.2001 ^B
1,000 to 100,000ppm	12.049 ^B

There were significant ($P<0.05$) differences for the time periods (24, 48 and 72 hours) at which both cultures were transferred from the lower doses (100 and 1,000ppm) into the higher doses (10,000 and 100,000 ppm) of these antimicrobials (Table 12). The growth at different incubation times (0, 24, 48 and 72 hours) were all significantly different from each other (Table 13).

Table 3.12 Differences of Least Square Means for resistance of yogurt bacteria as influenced by the transfer hour (from a low to a higher dose of antimicrobial).

Transfer Hour	LS Mean Diff
24	13.6231 ^A
48	12.5837 ^B
72	11.2777 ^C

Table 3.13 Differences of Least Square Means for the effect of different incubation times.

Incubation Time	LS Mean Diff
0	8.2498 ^D
24	16.7176 ^A
48	14.7693 ^B
72	10.2427 ^C

3.2.1. *Lactobacillus bulgaricus*

- **Growth of cultures exposed to 100ppm of potassium metabisulfite and potassium nitrite and transferred to 10,000 and 100,000 ppm**

At 0 hours of incubation there was a significant ($P<0.01$) difference in viable counts between all the *L.bulgaricus* transfers and the control (Fig 3A) (Table 14). Initial counts for all low concentration to high concentration transfers were lower than those of the *L.bulgaricus*

control (Figure 3A). At 48 hours transfers involving Potassium Nitrite had significantly ($P<0.01$) higher counts than the control (Table 14)(Figure 3A). At 72 hours of incubation, there was no significant difference in counts between both antimicrobial transfers and the control (Fig 3A) (Table 14). This indicated that the effect that both Potassium Metabisulfite and Potassium Nitrite had on *L.bulgaricus* was not antimicrobial, as they have no significant differences with the control. There was no inhibition of the growth of *L.bulgaricus* at any of the incubation periods of study.

The growth of *Lactobacillus bulgaricus* LB-12, as influenced by the different low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite at 48 hours of transfer is shown in Figure 3B. Viable counts for *L.bulgaricus* decreased over time from 24 to 72 hours. At 0 hours there is a significant ($P<0.01$) difference in counts between Potassium Metabisulfite and Potassium nitrite transfers and the *L.bulgaricus* control (Table 14). At 48 hours of incubation viable counts of *L.bulgaricus* transferred into Potassium Metabisulfite 100ppm to 10,000ppm and 100ppm to 100,000ppm and Potassium Nitrite (100ppm to 10,000ppm) individually were significantly higher than counts of the control (Figure 3B) (Table 14).

There was no significant ($P>0.05$) difference between low concentration to high concentration transfers and the control at 72 hours of incubation (Table 14) (Figure 3B). There was no inhibition effect for *L.bulgaricus* when transferred from 100 ppm into 10,000 and 100,000ppm of both antimicrobials individually.

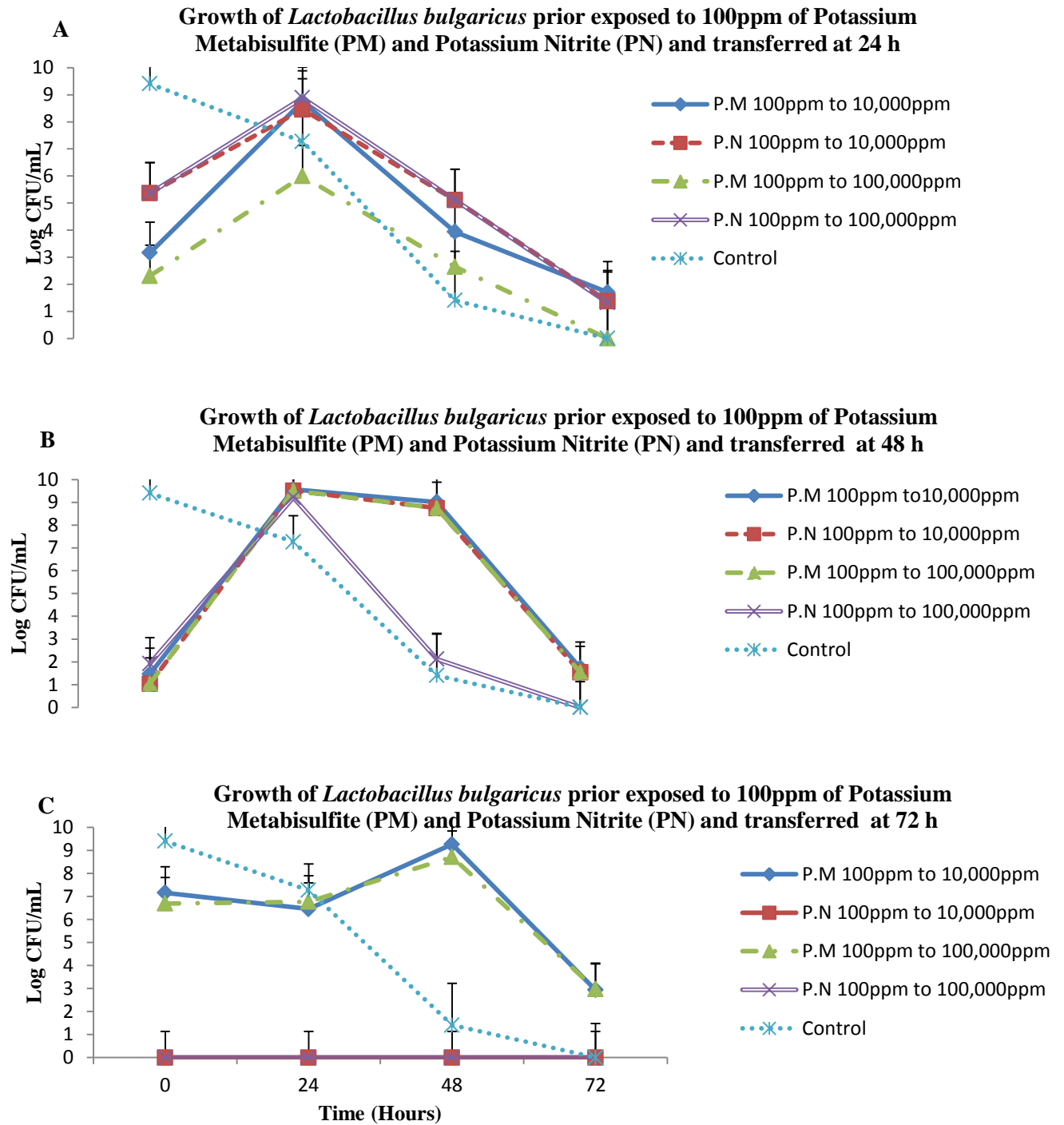


Figure 3.3 Growth *Lactobacillus bulgaricus* LB-12 transferred at A. 24 hours, B 48 hours and C. 72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 100ppm.

Table 3.14 Probability > t value ($Pr > |t|$) of differences of least Square Means compared to control of *Lactobacillus bulgaricus* LB-12 with different treatments of Potassium Metabisulfite and Potassium Nitrite; at different transfer hours and after 0, 24, 48 and 72 hours of incubation.

		<i>Lactobacillus bulgaricus</i>																							
		Transfer Hour																							
		24						48						72											
		Potassium Metabisulfite			Potassium Nitrite			Potassium Metabisulfite			Potassium Nitrite			Potassium Metabisulfite			Potassium Nitrite								
		Incubation Time																							
Low Concentration to High Concentration	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	
100ppm to 10,000ppm	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	<0.01	>0.05	>0.05	
100ppm to 100,000ppm	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	<0.01	>0.05	>0.05	
1,000ppm to 10,000ppm	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	>0.05	
1,000ppm to 100,000ppm	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	>0.05	

The growth of *Lactobacillus bulgaricus* LB-12, transferred at 72 hours from low to high concentrations of Potassium Metabisulfite or Potassium Nitrite individually, are shown in Figure 3C. During hours 0, 24, 48 and 72 *L.bulgaricus* counts for both transfers involving Potassium Nitrite were in the non-detectable ranges (Table 14) (Figure 3C). At 72 hours of incubation viable counts of *L.bulgaricus* for all low concentration to high concentration transfers of both Potassium Metabisulfite and Potassium Nitrite were not significantly different ($P>0.05$) from the control (Table 14) (Figure 3C). Potassium Metabisulfite had no inhibitory effect on *L.bulgaricus* growth. There was an inhibition effect of Potassium Nitrite on *L.bulgaricus* when transferred at 72 hours since there was no growth at any of the incubation periods.

- **Growth of cultures exposed to 1,000ppm of potassium metabisulfite and potassium nitrite and transferred to 10,000 and 100,000 ppm**

Growth of *Lactobacillus bulgaricus* LB-12, as influenced by the different low concentration to high concentration transfers at 24 hours, is shown in figure 4A. At 0 hours viable counts of *L.bulgaricus* transferred to higher concentrations of Potassium Metabisulfite were significantly ($P<0.01$) lower compared to the control (Table 14) (Figure 4A). At 72 hours, viable counts of *L.bulgaricus* decreased to 0-2 log CFU/mL for all low concentration to high concentration transfers and the control. There were no indications of resistance towards the higher concentrations of both antimicrobials after prior exposure to 1,000ppm since there were no significant differences in counts with the *L.bulgaricus* control (Table 14).

Figure 4B shows growth of *Lactobacillus bulgaricus* LB-12 when transferred at 48 hours. At 0 hours there was a significant ($P<0.01$) difference in *L.bulgaricus* counts between all low concentration to high concentration transfers and the control (Table 14). At 72 hours, there

were no significant differences ($P>0.05$) in *L.bulgaricus* counts between any of the low concentration to high concentration transfers and the control. Both Potassium Metabisulfite and Potassium Nitrite showed no antimicrobial effects on the growth of *Lactobacillus bulgaricus* LB-12 when transferred at 48 hours. Viable counts were not significantly different to those of the control which also indicated there was no inhibition effect.

Effects when transferred at 72 hours from 1,000ppm into (10,000ppm and 100,000ppm) of Potassium Metabisulfite and Potassium Nitrite are shown in Figure 4C. *L.bulgaricus* treated with Potassium Nitrite did not grow at any stage of the 72 hours of growth (Figure 4C). This led to the assumption that the antimicrobial had an inhibiting effect on the *Lactobacillus bulgaricus* LB-12 culture when transferred at 72 hours. At 0 hours there were significant differences ($P<0.01$) in initial *L.bulgaricus* counts between all of the low concentration to high concentration transfers and the control (Table 14). At 72 hours there was a significant ($P<0.01$) increase in counts of *L.bulgaricus* exposed to Potassium Metabisulfite compared to the control (Table 14). These higher counts show there was resistance from *Lactobacillus bulgaricus* LB-12 towards the higher concentrations of Potassium Metabisulfite when transferred at 72 hours. The action of Potassium Metabisulfite had positive effects on the growth of *L.bulgaricus* which can be compared to the action of a prebiotic.

3.2.2 *Streptococcus thermophilus*

- **Growth of cultures exposed to 100ppm of potassium metabisulfite and potassium nitrite and transferred to 10,000 and 100,000 ppm**

Growth of *Streptococcus thermophilus* ST-M5 when transferred at 24 hours from 100ppm into 10,000ppm and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite individually is shown in Figure 5A.

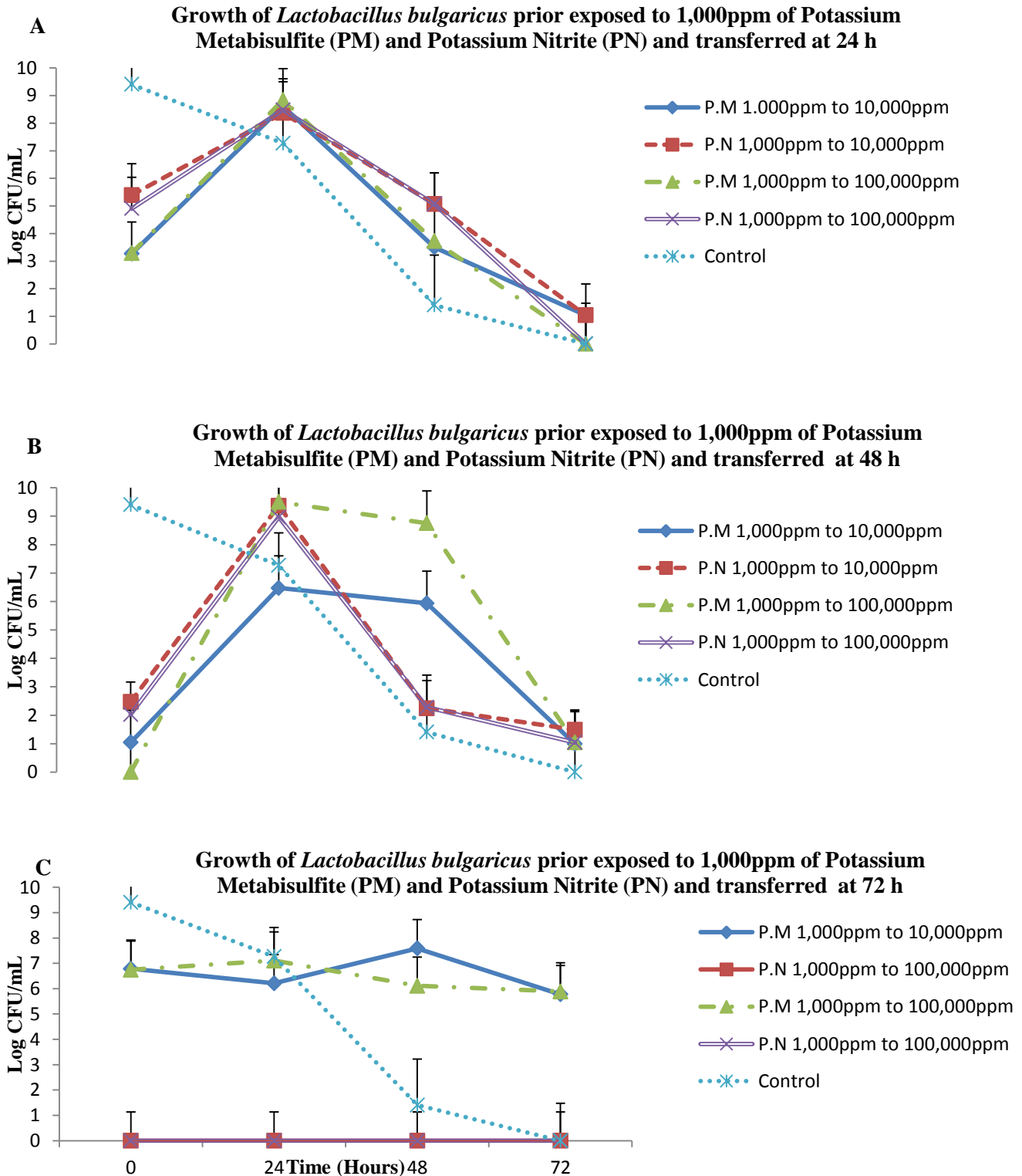


Figure 3.4 Growth of *Lactobacillus bulgaricus* LB-12 transferred at A. 24 hours, B. 48 hours and C.72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 1,000ppm.

At 72 hours of incubation counts of *S.thermophilus* were between 7-10 log CFU/mL for cultures treated with Potassium Metabisulfite and Potassium Nitrite. The control culture was significantly ($P<0.01$) lower than all low concentration to high concentration transfers at 72 hours of incubation (Table 15) (Figure 5A). There was resistance of *S.thermophilus* to both antimicrobial compounds. There was no antimicrobial or inhibition action towards *Streptococcus thermophilus* ST-M5 at 72 hours.

Growth of this yogurt culture when transferred at 48 hours is shown in Figure 5B. At 0 and 24 hours there were significant differences ($P<0.01$) between initial viable counts of *S.thermophilus* transferred to higher doses of Potassium Nitrite and the control (Table 15). At 72 hours there was a significant difference ($P<0.01$) between Potassium Metabisulfite treated *S.thermophilus* and the control (Table 15) (Figure5B). This difference in viable counts between Potassium Metabisulfite treatments and the control made the acquisition of resistance a possible explanation to this behavior. There was no resistance of *S.thermophilus* to Potassium Nitrite since there were no significant differences with the control at 72 hours.

Growth of *Streptococcus thermophilus* ST-M5 transferred at 72 hours from 100ppm into 10,000ppm and 100,000ppm is shown in Figure 5C. *S.thermophilus* transferred to higher doses of Potassium Nitrite had a sharp increase in viable counts between 0 and 24 hours and remained constant at hours 24, 48 and 72. There was a significant ($P<0.01$) difference in counts at 0 hours between low concentration to high concentration transfers of *S.thermophilus* into higher doses of Potassium Nitrite and the control (Table 15). At 72 hours there was a significant ($P<0.01$) difference in counts between the control and all Potassium Metabisulfite and Potassium Nitrite low concentration to high concentration transfers (Table 15) (Figure 5C).

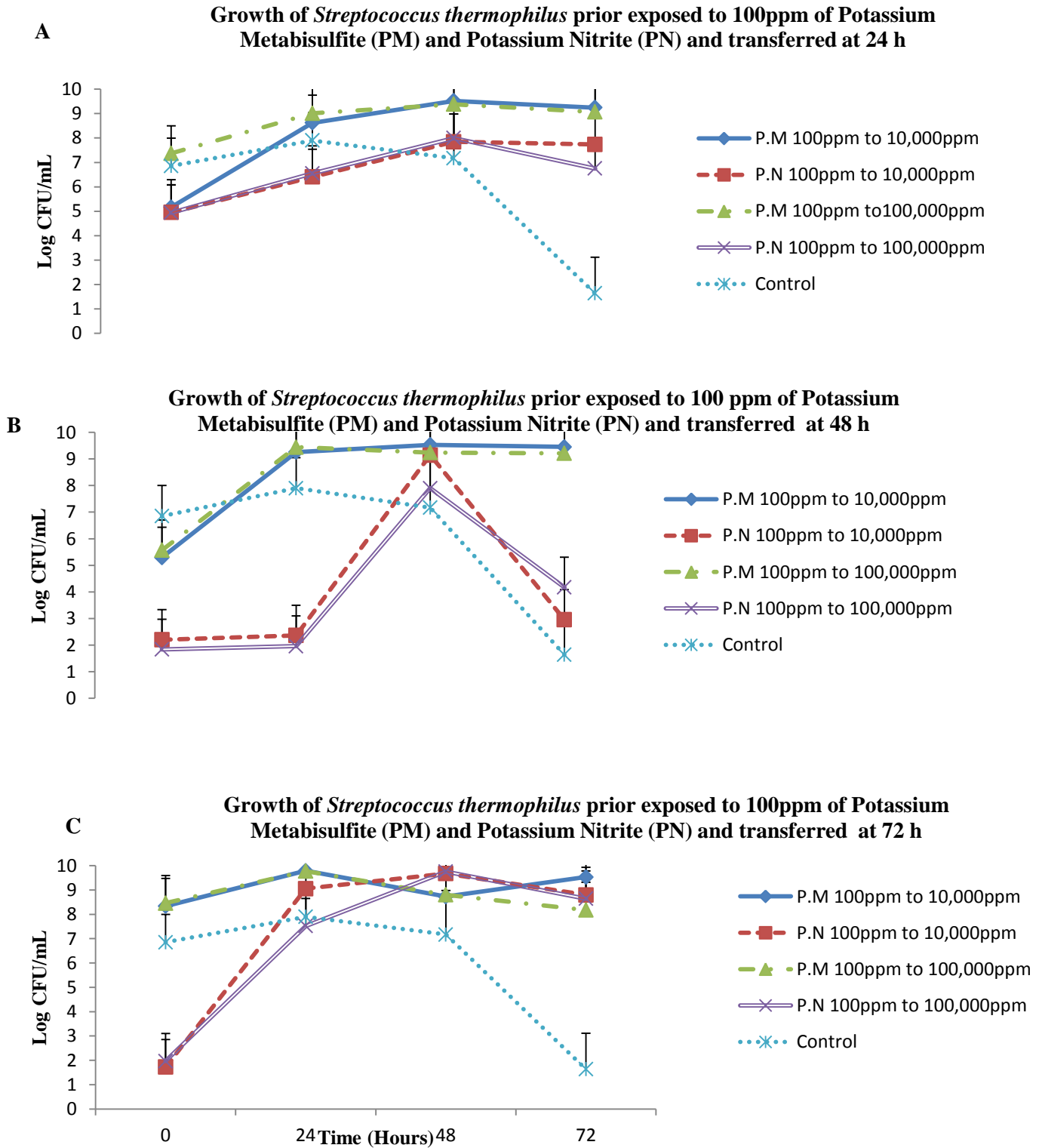


Figure 3.5 Growth of *Streptococcus thermophilus* ST-M5 transferred at A. 24 hours, B. 48 C.72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 100ppm.

Table 3.15 Probability > t value (Pr > |t|) of differences of least Square Means compared to control of *Streptococcus thermophilus* ST-M5 with different treatments of Potassium Metabisulfite and Potassium Nitrite; at different transfer hours and after 0, 24, 48 and 72 hours of incubation.

		<i>Streptococcus thermophilus</i>																											
		Transfer Hour																											
		24						48						72															
		Potassium Metabisulfite				Potassium Nitrite				Potassium Metabisulfite				Potassium Nitrite				Potassium Metabisulfite				Potassium Nitrite							
		Incubation Time																											
Low Concentration to High Concentration		0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72				
100ppm to 10,000ppm		>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	<0.01	>0.05	>0.05	<0.01
100ppm to 100,000ppm		>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	<0.01	>0.05	>0.05	<0.01
1,000ppm to 10,000ppm		>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	>0.05	>0.05	>0.05	>0.05	<0.01	<0.01
1,000ppm to 100,000ppm		>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	>0.05	>0.05	<0.01	<0.01	>0.05	<0.001

The effects of both Potassium Nitrite and Potassium Metabisulfite could not be called antimicrobial or inhibiting, as there are considerable higher counts compared to the control at 72 hours. The 6 log increase in viable counts compared to the control shows there was resistance to the higher concentrations of both antimicrobials and a positive effect that promoted the growth of the yogurt bacteria.

- **Growth of cultures exposed to 1,000ppm of Potassium Metabisulfite and Potassium Nitrite and transferred to 10,000 and 100,000 ppm**

Growth of *Streptococcus thermophilus* ST-M5 transferred at 24 hours to 10,000ppm and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite is shown in Figure 6A. Comparing time periods 0 and 72 hours, the growth curve for the *S.thermophilus* control decreased while for the low concentration to high concentration *S.thermophilus* transfers increased over 72 hours (Figure 6A). This difference in growth effects between the control and the low concentration to high concentration transfers suggested resistance of *S.thermophilus* to the higher concentrations of both antimicrobials (Figure 6A). There was no antimicrobial or inhibiting effect of Potassium Metabisulfite and Potassium Nitrite on *Streptococcus thermophilus* ST-M5.

Growth of *Streptococcus thermophilus* ST-M5 when transferred at 48 hours are shown in Figure 6B. At 72 hours there were no significant ($P>0.05$) difference in *S.thermophilus* counts between treatments involving Potassium Nitrite and the control (Table 15) (Figure 6B). Potassium Metabisulfite treated *S.thermophilus* and the control had significant ($P<0.01$) differences in counts at 72 hours of incubation (Table 15). There was resistance towards Potassium Metabisulfite as counts were considerably higher compared to the control at 72 hours.

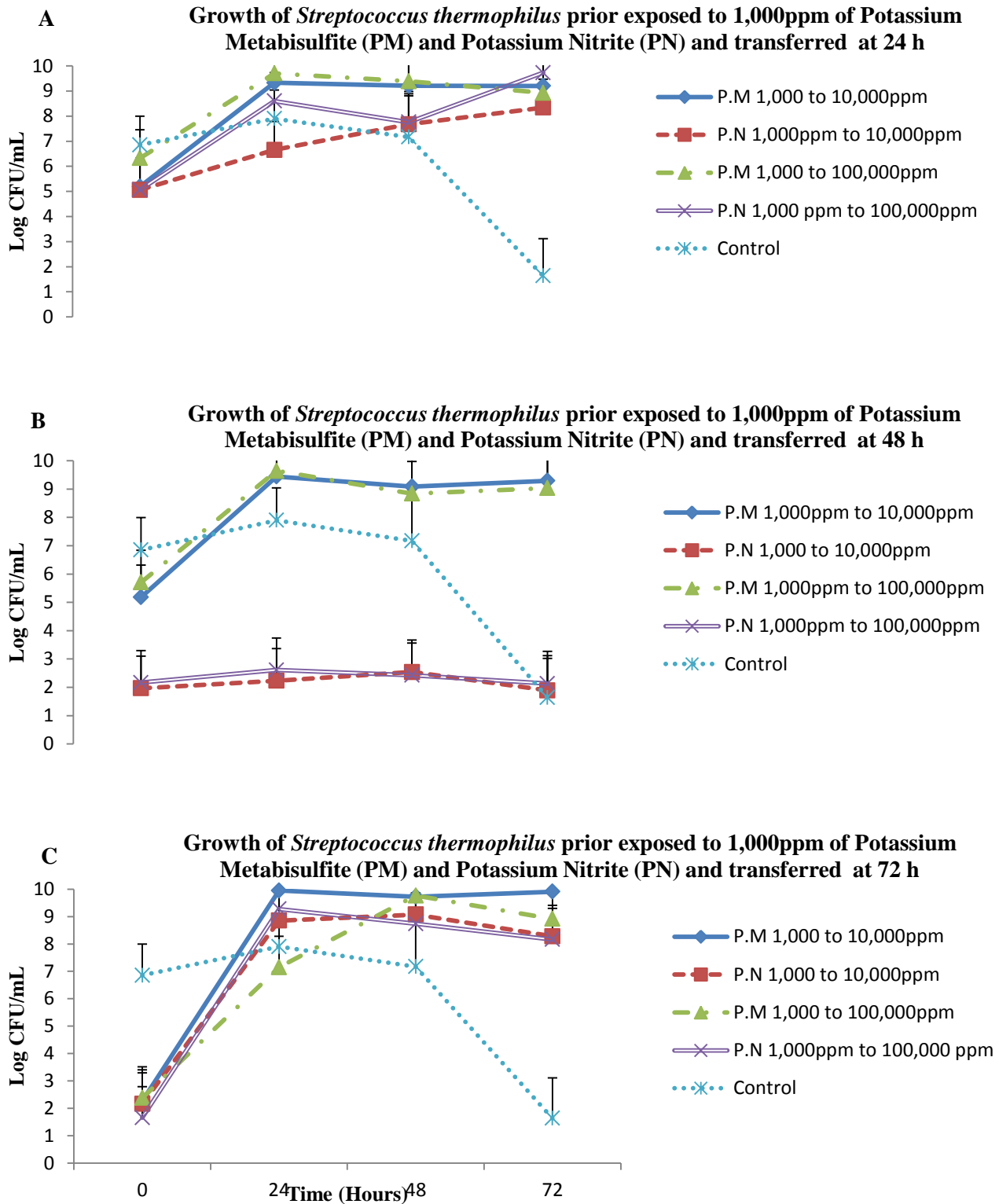


Figure 3. 6 Growth of *Streptococcus thermophilus* ST-M5 transferred at A. 24 hours, B. 48 hours and C. 72 hours of incubation into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite after prior exposure to 1,000ppm.

Growth effects of *Streptococcus thermophilus* ST-M5 transferred at 72 hours into 10,000ppm and 100,000ppm of both Potassium Metabisulfite and Potassium Nitrite are shown in Figure 6C.

At 0 hours there were significant differences ($P<0.01$) between all low concentration to high concentration transfers and the control (Table 15) (Figure 6C). At 72 hours there were significant ($P<0.01$) increases in *S.thermophilus* counts for all low concentration to high concentration transfers compared to the control (Table 15) (Figure 6C). There was resistance towards both antimicrobial agents. There were no inhibiting or antimicrobial effects on *Streptococcus thermophilus* ST-M5 with any of the antimicrobials used. The positive effects of both Potassium Metabisulfite and Potassium Nitrite can be considered to be similar to those of prebiotic compounds. The interaction between *Streptococcus thermophilus* ST-M5 and these antimicrobials is resulting in the possible production of metabolites that the yogurt bacterium is using as growth promoting factors.

3.3 Mean Log Reductions for Viable Counts of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5

Mean log reductions of the viable counts of *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 subjected to various low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite were obtained by subtracting counts at 24, 48 and 72 hours of incubation from 0 hours separately (Tables 16 and 17). This was done for each of the transfer hours. In Tables 16 and 17 a positive number indicates a decrease, while a negative number indicates an increase. A high positive number indicates a high bacterial decrease while a low negative number indicates a low bacterial decrease.

Table 3.16 Mean Log reduction of the viable counts of *Lactobacillus bulgaricus* LB-12 with different low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite transferred at 24,48 and 72 hours obtained by subtracting viable Log CFU/mL counts at 72 hours from 0 hours of incubation.

	<i>L.bulgaricus</i>					
	Transfer Hour					
	24		48		72	
Low Concentration to High Concentration	Potassium Metabisulfite	Potassium Nitrite	Potassium Metabisulfite	Potassium Nitrite	Potassium Metabisulfite	Potassium Nitrite
	Log CFU/mL					
100ppm to 10,000ppm	1.46	3.99	-0.27	-0.24	4.22	0
100ppm to 100,000 ppm	2.31	4.04	-0.51	1.92	3.72	0
1,000ppm to 10,000ppm	2.23	4.35	0.45	0.98	1.01	0
1,000ppm to 100,000ppm	3.29	4.91	-1.04	0.99	0.86	0
Control	9.41	9.41	9.41	9.41	9.41	9.41

Table 3.17 Mean Log reduction of the viable counts of *Streptococcus thermophilus* ST-M5 with different low concentration to high concentration transfers of Potassium Metabisulfite and Potassium Nitrite transferred at 24,48 and 72 hours obtained by subtracting viable Log CFU/mL counts at 72 hours from 0 hours of incubation.

	<i>S.thermophilus</i>					
	Transfer Hour					
	24		48		72	
Low Concentration to High Concentration	Potassium Metabisulfite	Potassium Nitrite	Potassium Metabisulfite	Potassium Nitrite	Potassium Metabisulfite	Potassium Nitrite
	Log CFU/mL					
100ppm to 10,000ppm	-4,08	-2,79	-4,16	-0,76	-1,19	-7,07
100ppm to 100,000 ppm	-1,7	-1,81	-3,64	-2,33	0,28	-6,67
1,000ppm to 10,000ppm	-4,01	-3,28	-4,11	0,07	-7,64	-6,11
1,000ppm to 100,000ppm	-2,6	-4,67	-4,11	0,03	-6,54	-6,5
Control	5.22	5.22	5.22	5.22	5.22	5.22

Mean log reductions for *Lactobacillus bulgaricus* LB-12, at 24 hours of transfer are shown in (Table 16). At 48 hours of transfer, mean log reduction values at 72 hours of incubation for Potassium Metabisulfite treated cultures were lower compared to those of the control and Potassium Nitrite treated cultures (Table 16) indicating an increase in counts with Potassium Metabisulfite. There was no antimicrobial or inhibition effect from the antimicrobial agents, as mean log reductions were less than the control.

Mean log reductions for *Streptococcus thermophilus* ST-M5 are shown in Table 17. At 24, 48 and 72 hours of transfer, all mean log reduction values at 72 hours of incubation for both Potassium Metabisulfite and Potassium Nitrite were negative or fairly close to 0, indicating an increase in counts, while for the control were positive indicating a decrease in counts (Table 17). These differences in counts with the control and the treatments indicate how there was a resistance for 10,000 and 100,000ppm of the antimicrobials. This also suggests a probable prebiotic effect that the compounds had on the *Streptococcus thermophilus* ST-M5 cultures.

Results suggest that Potassium Metabisulfite and Potassium Nitrite are not antimicrobial for both yogurt cultures. There was resistance of *Streptococcus thermophilus* ST-M5 towards these antimicrobials as there were significant increases in counts compared to the control at 72 hours of incubation. This significant increase in counts not only shows that there was resistance, but also that both antimicrobials in fact promoted the growth of this bacterium.

Dodds and Collins-Thompson (1984) say that amongst the bacteria which have been reported to be resistant to nitrite are the lactic acid bacteria. Shank et al. (1962) found that *Lactobacillus sp.* was resistant to nitric oxide and nitrogen oxide which are the main

components of action of Potassium Nitrite. The resistance of lactic acid bacteria to nitrite may be linked to nitrite reductase systems since the reduction of nitrite by microorganisms has been postulated to be a detoxification mechanism (Coleman et al. 1987, Nishimura 1980, Kaspar 1982 in Dodds and Collins-Thompson 1984). In their findings Dodds and Collins-Thompson (1984) report that homofermentative strains of lactic acid bacteria were more resistant nitrite than heterofermentative strains being both *Lactobacillus bulgaricus* and *Streptococcus thermophilus* homofermentative bacteria. These different statements are of good support to the results obtained in this research study where both *S.thermophilus* showed resistance towards the higher doses of Potassium Nitrite.

In the case of Potassium Metabisulfite, there are no research studies that report the resistance of lactic acid bacteria towards this compound. Anyhow, there are certain factors about *Streptococcus thermophilus* that can be of possible explanation to the resistance results obtained in this research study.

Giraffa and Bergere (1987) in Cerning et al. 1988 reported the production of exocellular polysaccharides (EPS) by *Streptococcus thermophilus* in skim milk cultures. Marshall (1987) in Cerning et al. 1988 stated that *S.thermophilus* elaborates a polymer similar to that produced by *L.bulgaricus*. Exocellular polysaccharides or EPSs can form an adherent cohesive layer and are called capsular polysaccharides (Ruas-Madiedo et al. 2002). Bacterial EPSs probably have a protective function in the natural environment, eg. Against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds and osmotic stress (Ruas-Madiedo et al. 2002). In their research study Ruas-Madiedo et al. 2002 report that the ecological function of EPSs produced by lactic acid bacteria is not clearly defined and it is probably complex, but that seems to be related with

cell adhesion and cell protection in different environments. These statements suggest this as the possible resistance mechanism that *Streptococcus thermophilus* is using in order to tolerate and protect itself from the higher concentrations of Potassium Metabisulfite and Potassium Nitrite possibly enabling the antimicrobials to reach their target of action within the cell or producing a chemical reaction between the EPSs and the antimicrobials which break down these compound and produce substances that the bacterium used as nutrients to grow in greater amounts.

At 72 hours of incubation the microbial counts of the control for both *Lactobacillus bulgaricus* LB-12 and *Streptococcus thermophilus* ST-M5 decrease due to the production of acid and waste substances that eventually inhibit the growth of the bacteria and reduce the numbers of the bacterial population. For the case of *Streptococcus thermophilus* ST-M5 when transferred into 10,000 and 100,000ppm of Potassium Metabisulfite and Potassium Nitrite the significant increase in counts suggests that the metabolism of the bacterium with both antimicrobials is producing substances that the yogurt culture is using as nutrients which promote the growth and increase in viable counts.

These findings support the idea of adding these antimicrobials into a yogurt or a fermented dairy product as a viable option for the preservation of these products without negatively affecting the cultures.

CHAPTER 4: CONCLUSIONS

Potassium Metabisulfite and Potassium Nitrite have been reported to have antimicrobial and inhibition effects on pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* and spoilage bacteria such as *Pseudomonas fluorescens*. Results obtained in the present study, show that neither Potassium Metabisulfite nor Potassium Nitrite had an antimicrobial effect against yogurt culture, *Lactobacillus bulgaricus* LB-12 or *Streptococcus thermophilus* ST-M5. Use of both antimicrobials at 1,000,000ppm, significantly increased counts of *Streptococcus thermophilus* ST-M5 by 6 log CFU/mL compared to the control which suggests that not only is there no antimicrobial effect but also that at this concentration both Potassium Metabisulfite and Potassium Nitrite promoted the growth of the yogurt bacteria, behavior comparable to that of a prebiotic. Prior exposure of *Streptococcus thermophilus* ST-M5 to Potassium Metabisulfite and Potassium Nitrite at 100 and 1,000ppm for 72 hours showed resistance to 10,000 and 100,000ppm of both Potassium Metabisulfite and Potassium Nitrite having increased 6 log CFU/mL counts compared to the control at 72 hours of incubation.

A commercial product application of this study would be to incorporate Potassium Metabisulfite and Potassium Nitrite in yogurt manufacture for inhibition of spoilage and pathogenic bacteria to ensure good preservation of the product and improved shelf life.

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VITA

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