Lincoln University
Te Whare Wānaka o Aoraki
CHRISTCHURCH•NEW ZEALAND

# Lincoln University Digital Dissertation

## Copyright Statement

# ANTIMICROBIAL PROPERTIES OF NEW GEL IN YOGURT

A Dissertation

submitted in partial fulfilment

of the requirements for the Degree of

Masters of Science in Food Innovation

at

Lincoln University

by

Natasha Fernandez

---

Lincoln University

2020

Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Masters of Science in Food Innovation.

# Antimicrobial Properties of New Gel in Yogurt

by

Natasha Fernandez

A new gel for yogurt, created using the combination of Chemical X and lactic acid has been studied for its antimicrobial properties. Previous unpublished study of Chemical X with citric acid was found to have excellent gelling abilities and antimicrobial properties when used as an edible coating for meat. The current study explored the antimicrobial properties of the new gel and its components (Chemical X and lactic acid) in yogurt system- particularly, the influence on culture bacteria and contaminating microorganisms like *E. coli*. The first aspect of the study dealt with assessing the effect on *Lactobacillus delbrueckii subsp. bulgaricus*, which is one of the primary bacteria present in the process of yogurt formation, along with *Streptococcus thermophilus*. The counts were enumerated over shelf life on MRS agar at day 4, day 8, day 11 and day 15 of refrigerated storage. Another aspect of the study focused on determining the antimicrobial capabilities of the yogurts containing the new gel and its components, against pathogenic contaminants such as *Escherichia coli.* For this purpose, *E. coli* was inoculated into the samples before incubation and its enumeration was done on TBX agar at day 5 of storage. The results indicated that the new gel does not adversely affect the growth of *L. bulgaricus* over shelf life and maintains a stable count of the culture bacteria till the end of storage period. The gel component Chemical X was also found be better supportive of *L. bulgaricus* growth when compared with yogurt having gelatin as the gelling agent. It was also found that *E. coli* grew only in the milk control without any culture or gels, while all the other samples prevented its growth. The better antimicrobial property of yogurt containing the gel was supported by the counts of *L. bulgaricus*, which showed significantly different counts from the milk sample that permitted *E. coli* growth. The results provide proof of superior antimicrobial properties of the new gel and its gel components in yogurt.

**Keywords:** Yogurt, *Lactobacillus bulgaricus, Escherichia coli, Streptococcus thermophilus*, gel, antimicrobial property, gelatin.

# Acknowledgements

I am grateful to Dr. Ravi Gooneratne, Course co-ordinator, Department of Agriculture and Life Sciences for choosing me to do FOOD660 Research Dissertation.

Next I take this privilege to extent my gratitude to Dr. Maneesha Mohan, lecturer, Department of Agriculture and Life Sciences, for supervising me all throughout my dissertation work.

I also take this opportunity to thank Dr. Venkata Chelikani, for co-guiding me in my work.

Thanks to Ms. Letitia Stipkovitis, lab technician for her assistance and all the other lecturers and technicians for their expert advice and encouragement.

Also, thanks to Ms. Shwetha, who assisted me in my dissertation work.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1

# Introduction

The importance of yogurt, a product formed by the fermentation of milk due to the activity of certain lactic acid bacteria (namely*, Lactobacillus delbrueckii subsp bulgaricus* and *Streptococcus salivarius ssp. thermophilus*), is now recognized by the scientific community, regulatory bodies, and common people in most of the countries. Often realised as a part of balanced and healthy diet, yogurt has found its way into almost all the homes. Different formulations and varieties of yogurts have been created over the years to enhance the health benefits and sensory appeal for the consumers. The health benefits include improvement of bone health, reducing the incidence of chronic diseases, aiding digestion; probiotic yogurt can even enhance the gut microbiota and boost the immune system. Yogurt also supplies with important nutrients like proteins, fats, vitamins like B2 and B12, calcium, magnesium, and potassium. Due to the above mentioned reasons, yogurt production across the globe has been drastically increasing in the past years. With a figure of 27.7 million metric tonnes, a 1.2 fold rise in the production rate of yogurt has been reported in the year 2015, when compared to the rates of 2010(Chen, Lu, Lin, Yao, & Xiong, 2016).

Stabilizers or gelling agents are often added to yogurt to prevent the issues of syneresis and to maintain the texture of yogurt. Many gelling agents such as pectins, starches, gelatin, guar gum, carrageenans, and locust bean gum have been studied and used as gelling agents of yogurt (Priyashantha, Quintáns, Baixauli, & Vidanarachchi, 2019). Commercially, gelatin is used as the major stabilizer for the manufacture of yogurt. Gelatin is a product of animal collagen obtained from fish, pig or cow. In yogurt, gelatin is added at the ratio 0.3- 0.5 % in order to prevent syneresis of the same (Baziwane, & He, 2003). In the global economy, the projected rise of the gelatin industry is expected to reach about 5 billion USD, by the year 2025. However, due to its source, gelatin is a product of concern for people following certain religion(s)/beliefs. Moreover, gelatin has been found to cause allergic reactions and has posed questions regarding safety for the general population (Mullins, James, Platts-Mills, & Commins, 2012). Hence, there is a need to find non-animal origin alternatives to gelatin as a stabilizer or food gel, which led to the discovery of the use of Chemical X. Chemical X with citric acid, has been found to have promising abilities as a gelling agent, with added advantages of antimicrobial activities and extension of shelf life when used as an edible coating in meat (unpublished).

Previous research done regarding the use of Chemical X in combination with lactic acid, as a novel gel base for yogurt preparation has shown that the textural strength of yogurt can be enhanced by the use of this new gel (unpublished). Further, the yogurt formed by the gel, was also observed to exhibit

higher viscosity measurements in comparison to yogurt made using gelatin as the gelling agent (unpublished). However, whether this substance has any effect on the lactic acid bacteria used for the fermentation process, was not previously studied. The lactic acid bacteria is crucial to maintaining the pH and acidity needed for the yogurt formation, and for the development of flavour and taste characteristic of yogurts (Bintsis, 2018). High viable counts of the Lactic acid bacteria (LAB) are crucial for a good quality yogurt. LAB have been found to have antimicrobial properties against pathogens that affect yogurt due to phenomenon like competitive inhibition, production of metabolites like hydrogen peroxide and high amounts of lactic acid (Mirzaei, Lashani, & Davoodabadi, 2018). Hence, higher viable counts are required to maintain good antimicrobial qualities of yogurt. In this context, the present research aims to prove that the new gel does not negatively impact the growth of the culture bacteria. The antimicrobial effect of new gel and Chemical X against *E. coli* was also assessed. *E. coli* is a Gram negative bacteria which is capable of contaminating fermented milk products like yogurt and cause food poisoning (Morgan et al., 1993, Mead et al., 1999). The ability of yogurt bacteria to inhibit their growth is important to maintaining the quality of yogurt and hence the current study also focuses on assessing the effect of yogurt made using new gel and its gel components against such pathogenic contaminants.

# Chapter 2

# Review of Literature

## 2.1 Yogurt and its relevance

Yogurt is stated to be a type of fermented milk product with a semi-solid structure that results from the coagulation of milk proteins, resulting from the lowering of pH due to the action of specific bacterial starter cultures, namely, *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, which should be abundant as well as viable at the time of consumption. Furthermore, the product may or may not contain additional substances such as skim milk powder, caseinates, whey concentrates or certain probiotics (Sfakianakis, & Tzia, 2014; WHO/FAO, 2010 as cited in Gómez-Gallego, Gueimonde, & Salminen, 2018). Based on the manufacturing processes involved, the different types of yogurts consumed are set yogurt, stirred yogurt and the recent frozen yogurt and drinking yogurt (Sfakianakis, & Tzia, 2014). Although in some countries 'yogurt' is restricted only to those fermented milk products produced by the symbiotic association of *L. bulgaricus* and *S. thermophilus*, in other countries, adjunct cultures such as *Lactobacillus acidophilus , Bifidobacterium spp., Lactobacillus reuteri , Lactobacillus casei, Lactobacillus rhamnosus* GG , *Lactobacillus gasser*i and *Lactobacillus johnsonii* LA1 are used (Chandan, & Nauth, 2012).

The origin of yogurt is thought to be from ancient Middle Eastern countries and its production since then, has rapidly increased in commercial point of view after its perceived health benefits. These include gastrointestinal benefits, reducing the symptoms of lactose-intolerance, rotavirus and antibiotic therapy, control of vaginitis, improving the immunity along with protection from certain symptoms caused by food borne microbes, and also possible prevention of cancer (Chandan & Kilara, 2008). Apart from the health benefits, yogurt is accepted by consumers for its sensory properties which include texture, flavour and colour. The flavour is described as strong acidic taste with a hint of green apple, having its own characteristic aroma (Sodini, Remeuf, Haddad, & Corrieu, 2004; Salvador, & Fiszman, 2004). Current New Zealand standards of yogurt state that regular yogurt should contain a minimum of 3.25% fat. No fat yogurt must contain less than 0.5% of fat, while the fat content of low fat yogurt should fall between 0.5- 2%. Regardless of fat content, the non-fat milk solids should be at least 8.25%, with a titratable acidity of ≥ 0.9, and pH ≤ 4.5. Optional ingredients that could be included in yogurts, as per these standards, are buttermilk, skimmed milk powder, whey proteins, caseins, whey protein concentrates, caseinates milk powder, water soluble proteins, and sugars in sweetened yogurts (Codex Standard, 1975, as cited in Kilara, 2007; Food Standards Australia New Zealand, 2016).

## 2.2 Microbial aspects of yogurt

The microbial populations added to milk in order to cause the fermentation of milk into yogurt is known as starter culture of yogurt. Often, the causative agents of the fermentation process are homofermentative bacteria *L. bulgaricus*and *S. thermophilus.* They maintain a complex interaction throughout to ensure the stability of yogurt qualities through its shelf life and also inhibits the growth of other microbes in the same.

### 2.2.1 Yogurt Starter Culture

Starter cultures are microbial cultures, which when added to milk, would aid in the fermentation of milk, by converting the lactose to lactic acid. This increase in lactic acid would cause a drop in pH, thereby causing the milk to clot, leading to the formation of a soft gel structure, which is the characteristic of fermented milk products such as yogurt. Other than the traditional *L. bulgaricus*and *S. thermophilus* species used as starter cultures for yogurt, many other bacterial strains have also been introduced for the same purpose of fermented milk product formation. *Lactococcus lactis* is a milk fermenting species which has been widely used for the production of cheese, buttermilk and other fermented milk products, by virtue of its ability to produce various antimicrobial compounds such as nisins, diacetin and lactococcin (Kim, & Lee, 2016). Some microbial strains are added to food products like yogurt, as adjunct cultures because the yogurt bacteria alone cannot survive the gastric passage and colonize the gut. Since they confer certain health benefits to the host, they are called probiotics. The probiotic bacteria most often used include *Lactobacillus casei*, *Lactobacillus acidophilus, Lactobacillus helveticus ,Bifidobacterium bifidus, Bifidobacterium longum, Bifidobacterium lactics* (Granato, Branco, Cruz, Faria, & Shan 2010). For instance, *Bifidobacterium lactics*, in conjunction with *Lactococcus lactics*, is discovered to be an effective starter culture of yogurts, with a hypocholesterolemic property (Nishiyama et al., 2018). Some strains belonging to *Lactococcus* and *Leuconostoc*, such as *Lactococcus lactis* and *Leuconostoc citrovorum*, are often incorporated into yogurts as adjunct cultures, in order to provide the yogurt with its characteristic butter-like flavour (Chen, Zhao, Hao, Yu, Tian, & Zhao, 2017). The concentrations of probiotics usually range from $10^7$-$10^8$ CFU/g or mL, unless they participate along with the traditional starter strains in the fermentation process. However, if they do participate, their levels can go upto $10^8$-$10^9$ CFU/g or mL (Vinderola, Binetti, Burns, & Reinheimer, 2011). The standard microbiological count of culture bacteria in fermented milk products should be at least $10^6$ CFU/ mL according to New Zealand standards for fermented milk products (Food Standards Australia New Zealand, 2016).

### 2.2.2 Fermentation

The main mechanism behind the formation of yogurt is fermentation, whereby the starter culture causes the conversion of milk to the yogurt curd through some biochemical changes, which in turn changes the textural properties and imparts distinct flavour to the same (Cheng, 2010). Microbial fermentation refers to the process of breaking down sugars and proteins into a large array of organic compounds, mediated by microorganisms. Fermented milk is obtained by fermenting milk, the composition of which may or may not be modified by suitable microorganisms, including the possible coagulation of milk (Codex Standard, 2003 as cited in Kilara, 2017). The need to prolong the shelf life of milk, rather than discard it, lead to the production of fermented milk products (Tamime, & Robinson, 1999). In the case of milk fermentation, the two main genuses of bacteria initiating the process are *Lactobacilli* and *Streptococci* (Leroy, & De Vuyst, 2004). For yogurts, the fermentation process can be achieved by *S. thermophilus* species in combination with any *Lactobacillus* species. Lactase enzyme is produced by both *L. bulgaricus* and *S. thermophilus*, but the latter having thrice as more lactase production than *L. bulgaricus*, under appropriate stimulation like sonication (Kilara, & Shahani, 1976). The lactose in milk is initially hydrolysed into glucose and galactose using this lactase enzyme. Subsequently, the glucose is converted to lactic acid via Embden Meyerhof-Parnas pathway or EMP pathway (Garvie, 1984). This conversion of lactose to lactic acid results in the creation of characteristic acidic taste of yogurt. The variations in the substrate, starter cultures and environmental conditions can result in either of the two categories of fermentative pathways- homofermentative pathway or heterofermentative pathway, which in turn can yield different metabolic products of lactose metabolism (Mayo, Aleksandrzak-Piekarczyk, Fernández, Kowalczyk, Álvarez-Martín, & Bardowski, 2010). Whereas the homofermentative pathways yield only lactic acid, the heterofermentative pathways results in the production of other alternatives such as carbon dioxide, ethanol or acetic acid along with lactic acid (Holzapfel, & Wood, 2014, as cited in Chen, Zhao, Hao, Yu, Tian, & Zhao, 2017). The acidification created as a result of fermentation action causes the caseinate particles to destabilize at pH around 5.3- 5.2, resulting in the complete precipitation at pH of 4.7- 4.6. The cross-linking of such protein chains yields in better stability of the three-dimensional network of yogurt gel (De Brabandere, & De Baerdemaeker, 1999; Lorenzen, Neve, Mautner, & Schlimme, 2002). Lactic Acid Bacteria (LAB) also produces exopolysaccharides (EPS) and/or capsular polysaccharides, often excreted into the surrounding medium, and these polysaccharides are discovered to play an important part in modifying the sensorial and textural properties of yogurt (Girard, & Schaffer-Lequart, 2007; Behare, Singh, Kumar, Prajapati, & Singh, 2009).

### 2.2.3 Interactions between Yogurt Bacteria

The kind of starter cultures has also been found to have an effect on the flavour of yogurt and it has been suggested that mixed cultures, impart a greater level of flavour than individual strains alone, due

to their mutual stimulation and associative growth (Tamime, & Robinson, 1999; Sieuwerts, De Bok, Hugenholtz, & van Hylckama Vlieg, 2008). Both *L. bulgaricus* and *S. thermophilus* are thermophilic LAB, capable of growing on their own. However, together these species form a type of symbiotic association called 'proto-cooperation', which has a beneficial effect by enhancing their growth, as well as the production of lactic acid and aromatic compounds (Settachaimongkon et al., 2014). The pH of the product is lowered due to the increase in the levels of lactic acid, thereby making the fermented product unsuitable for the growth of microorganisms, and spoilage that occurs as a result of it (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007). This interaction takes place by means of the exchange of several growth stimulating metabolites, that mutually benefits the microbes (Sieuwerts, De Bok, Hugenholtz, & van Hylckama Vlieg, 2008). *S. thermophilus* is discovered to grow rapidly at 40 to $45^0$C and grows best in milk pasteurized at low temperatures. During the initial stages of fermentation, *S. thermophilus* undergoes exponential growth due to its ability to tolerate more neutral pH, and its effectiveness in using trace elements and amino acids, much better than *L. bulgaricus*. Oxygen is used up by *S. theromphilus* and converted to carbon dioxide, which benefits *L. bulgaricus*, as the latter is less aero-tolerant. During this stage, *S. thermophilus* also produces metabolites such as formic acid, pyruvic acid, ornithine, folic acid, long chain fatty acids which positively influence the growth of *L. bulgaricus*. The lactic acid produced by *S. thermophilus* also reduces the pH to an optimum level suitable for the growth of *L. bulgaricus*. As a result of the growth of *L. bulgaricus*, free amino acids (mainly valine), peptides and putrescine is supplied, which consequently promotes the second growth of *S. thermophilus* (Smid, & Lacroix, 2013). *L. bulgaricus* also accounts for increase in lactic acid during storage period as it produces lactic acid through a process called post-acidification. It is also understood that among the two LAB strains, *L. bulgaricus* has more proteolytic activity, capable of hydrolysing caseins, while *S. thermophilus* is found to exhibit limited proteinase activity (Shihata, & Shah, 2000).

On the other hand, growth-detrimental interactions between some strains of *L. bulgaricus* and *S. thermophilus* has also been reported in some studies. Studies have also brought forth to light that certain strains of *L. bulgaricus* cannot be used for yogurt production as they maintain a clear antagonistic relation with *S. thermophilus* via competition or amensalism. Amensalism is exhibited when some *S. thermophilus* strains that produce peptide bacteriocins inhibits certain strains of *L. bulgaricus* (Teusink et al., 2006; Sieuwerts, De Bok, Hugenholtz, & van Hylckama Vlieg, 2008). Competition for limiting nutrients such as nitrogen, free amino acids and small peptides available in milk is often seen between the culture bacteria during the later stages of fermentation. Mostly in yogurt the *S. thermophilus* is a better competitor than *L. bulgaricus* due to the greater nutrients requirement of *L. bulgaricus* (Peirera, & Luchese, 1988; Sieuwerts, De Bok, Hugenholtz, & van Hylckama Vlieg, 2008).

## 2.3 Factors Affecting Microbial Qualities of Yogurt

The viability of yogurt cultures and the probiotic bacteria added to it, depends on a number of factors such as the culture conditions, interactions between the microbes present, pH, incubation temperature, fermentation time, concentration of sugars, chemical composition of the medium involved in fermentation, concentrations of lactic acid and acetic acid, storage temperature, amount of milk solids present, hydrogen peroxide content, and dissolved oxygen content (Rybka, & Kailasapathy, 1995). Chandan & O'rell (2006) claims through their studies that, yogurt can maintain its qualities upto 4-7 weeks, if the viable count of individual bacteria at the end of the said time period is ≥6 log CFU/g.

### 2.3.1 Physical Parameters

Physical conditions of yogurt, such as the pH, incubation temperature, acidity, storage temperature, presence of oxygen, are essential in determining the microbiological quality of yogurt (Shah, 2000). A pH of ≤ 4.7 is needed in order to positively influence the viability of both the yogurt cultures, as well as the probiotic bacteria present in them. Higher acidity negatively influences the growth of *S. thermophilus,* which ultimately affects the growth of *L. bulgaricus* (Shah, & Ravula, 2000). Processing techniques such as heat treatment, cooling, homogenization and pasteurization is observed to have influence on the microbiological aspects of yogurt. A heat treatment between 85-95$^0$C, kills the pathogenic bacteria, thereby reducing the chances of competition or inhibition of the culture bacteria (Tamime, & Robinson, 2007). Studies by Jankowska, Wiśniewska, & Reps (2005) has shown that high pressure treated yogurts are effective as good carried of probiotic bacteria throughout their storage period.

Increasing the inoculum and incubation temperature, increases the bacterial count and acid production, which positively influences the yogurt production. However, exceeding beyond an optimum level of 3% and an optimum temperature of 43$^0$ Celsius, is found to inhibit the bacterial growth and produce excess amounts of acid. Thus lower or higher incubation temperatures and inoculum quantity beyond the above mentioned optimum levels, would adversely affect the microbial qualities, and thus the textural and sensory properties of yogurt (Shu, Li, Chen, & Wang, 2014).

### 2.3.2 Microbial parameters

Microbial contamination during post-processing stage and storage has been found to influence yogurt characteristics, often causing it to lose its quality and become unacceptable for consumption. This can be attributed to reduction in the viability of starter bacteria (Mataragas, Dimitriou, Skandamis, & Drosinos, 2011). The main factors that adversely affect the shelf life of yogurt include the

contamination by a wide range of microorganisms, including the faecal coliforms like *Escherichia coli, Pseudomonas spp.,* and *Salmonella spp.* For instance, *Pseudomonas spp.,* is found to contaminate certain milk products and lower the food safety grade, making such products unacceptable for consumption (Nwamaka, & Chike, 2010). Microbial spoilage of milk products like yogurts, often lead to the production of certain metabolites in large amounts, such as the extracellular proteolytic and lipolytic enzymes which hydrolyses milk proteins and fat, respectively. Another adverse effect of microbial spoilage is the development of bitterness and off-flavours as a result of heat stable proteases and lipases by some bacteria (Sheehan, 2013).

Lactic acid bacteria in yogurt are found to have an antimicrobial effect on other pathogenic bacteria, especially the Gram negative pathogens, thereby reducing the risk of progression of contamination. The proposed mechanisms by which they have such an inhibitory action include the production of lactic acid, hydrogen peroxide, bacteriocins, and organic acids such as , ethanol, creation of low oxidation-reduction potential, and also via competition to colonize sites with the pathogens (Mirzaei, Lashani, & Davoodabadi, 2018). Adams, & Hall (1988) has explained that the antimicrobial action against pathogens can also be due to a combined effect of lactic acid and acetic acid produced by the LAB bacteria.

## 2.4 Gelation

Gelation in general is connectivity transition, wherein soft materials undergo the process of solidification leading to the formation of a viscous gel. The process of gelation is observed in many food products such as fermented milk products like yogurts, jellies, desserts etc. Yogurts start to become unacceptable when it shows significant syneresis, leading to visible formation of curd and whey, indicating that the shelf life of the yogurt has come to an end. Hence, to prolong the shelf life, scientists and manufacturers added stabilizers to maintain the gel viscosity over a longer period, thereby extending the shelf life of the product. Stabilizers are often used with certain types of yogurt more than the other types, as in the case of stirred yogurts over set type yogurts, to attain required texture, viscosity and for reducing the phenomena of syneresis (Priyashantha, Quintáns, Baixauli, & Vidanarachchi, 2019). Common stabilizers added to yogurt include gelatin, guar gum, carrageenans, pectins, starches and locust bean gum, and the amount added varies according to different standards followed in different nations. However, studies have pointed out that adding too much stabilizers, such as at or above the levels of 0.5% (w/w of yogurt) affects the flavour of yogurt and hence to maintain a higher quality of yogurts, it is often advised to keep the additions of such stabilizers to a minimum (Modler, Larmond, Lin, Froehlich, & Emmons, 1983).

### 2.4.1 Milk Proteins

The appearance and texture of yogurt is dependent on the total solids of the yogurt, protein content of the yogurt, and the amount of stabilizers added to the formula. Total solids and protein content play a significant role in determination of yogurt texture and stability.

Milk protein concentrates containing whey protein and caseins, is also found to extend the shelf life and produce a stable viscous gel. Such increase in milk proteins can supplement for a decrease in the stabilizer concentration, without adversely affecting the sensory qualities of yogurt, but rather significantly improving the flavour profiles of such yogurts (Tamime, & Robinson, 1999).

The total milk solids and protein content seems to have a significant impact on the yogurt stability and texture through the following mechanism. During pasteurization process of the milk, the high treatment employed completely denatured the whey proteins present in milk. An insoluble complex is formed when the alpha –casein in the milk react with the denatured beta-lactoglobulin of the whey protein in milk, thereby contributing to the yogurt texture. When culture is added to milk, it causes a decrease in pH, causing the casein micelles to form matrices in the milk, depending on the concentration of other proteins present in the yogurt. Earlier research in this field has shown that a thicker and more viscous gel is produced when yogurt is fortified with milk protein concentrates. The resultant gel formed is also discovered to exhibit less syneresis overtime, leading to extended shelf life (Modler, & Kalab, 1983).

### 2.4.2 Gelling agents

Gelling agents are those substances, added to foods like jellies, yogurts, desserts, etc, in order to thicken and stabilize them. These food additives, which includes certain stabilizers and thickening agents, are capable of providing the foods with a good texture through the formation of a gel. Only comparatively a few gums fall under the category of gels, with the frequently used gelling agents being natural gums, gelatin, agar-agar, pectins, carageenen, starches, along with certain proteins such as zein from corn, and milk protein concentrates such as whey proteins. These substances belong to a broad category of polysaccharides and are hydrocolloids in nature, having a regular or irregular unbranched structures, or irregular branched structure or structures that contain complex, well-defined repeating units (Banerjee, & Bhattacharya, 2012).

Gelling agents used in products like yogurt, often fall under the category of hydrocolloids that help in increasing the viscosity of gel and enhancing the physical stability. Interactions with milk proteins, however, can lead to negative impacts in the form of syneresis or strange mouth-feel, due to miscibility or complex coacervation (Hinrichs, Götz, Weisser, 2003). One gelling agent discovered by Li and Guo

(2006) to show reduced syneresis and improved gelating characteristics of yogurts made using goat's milk, is pre-polymerized whey protein, created from whey protein concentrate.

Gelatin is a common gelling agent added to yogurts to act both as a stabilizer and to create a perfect texture. This substance is capable of melting when heated and solidifying when cooled, and it forms a semi-solid colloidal gel when mixed together with water. Since gelatin is essentially a collagen, the mechanism by which gelatin acts as a gel base is determined by the partial reformation of triple helices found during the cooling of the collagen. Initially, a polypeptide chain orients itself to induce a reactive site, followed by which, condensation of two other chains near the reactive site occur, ultimately giving rise to a triple helix formation (Renard, van de Velde, & Visschers, 2006). In yogurts, gelatin not only functions to replace the emulsifier casein, but it also acts as a suspension stabilizer and protective colloid, binding the whey and providing texture. During the gelling process, the gelatine molecules added, tends to form a lattice in the casein gel, which is ultimately stabilized by hydrogen bonding. In turn, this change helps in preventing the proteins from clumping, thereby not allowing the whey to get separated out from the yogurt. Studies have shown that even when milk alone could not cause coagulation in the presence of culture, gelatin formed the basis of gel for yogurt formation (Fiszman, Lluch, & Salvador, 1999). Gelatin can also be used in combination with other hydrocolloids, such as starches and pectins, in order to prevent syneresis and produce a yogurt consistency of acceptable levels (Schrieber, & Gareis, 2007). However, since gelatin is obtained from animal origins as mentioned in chapter 1, and due to certain rare health impacts associated with it, there are on-going studies focusing on finding better alternatives to replace gelatin, without compromising the physical, antimicrobial and sensory attributes of yogurt.

## 2.5 Aims and objectives

The scope of this study encompasses the assessment of antimicrobial activity of a new gel used for yogurt manufacture. Activity of the gel, which is formed by the combination of Chemical X with lactic acid, is tested and compared against various controls to determine if it inhibits *Lactobacillus bulgaricus*, which is one of the lactic acid bacteria used as the starter culture during the production of yogurt. The antimicrobial activity against *Escherichia coli* is also assessed to ascertain the antimicrobial properties of new gel and gel forming compounds (Chemical X and lactic acid).

## 2.6 Hypothesis

- The gel (Chemical X+ lactic acid), has no effect on the activity of *L. bulgaricus* during the shelf life of yogurt.
- Chemical X alone does not affect the activity of yogurt culture over shelf life.
- The gel and its components inhibits bacterial contaminants such as *E. coli.*

# Chapter 3

# Materials and Methods

## 3.1 Experimental design

The experimental set-up consists of the following treatment samples and controls.

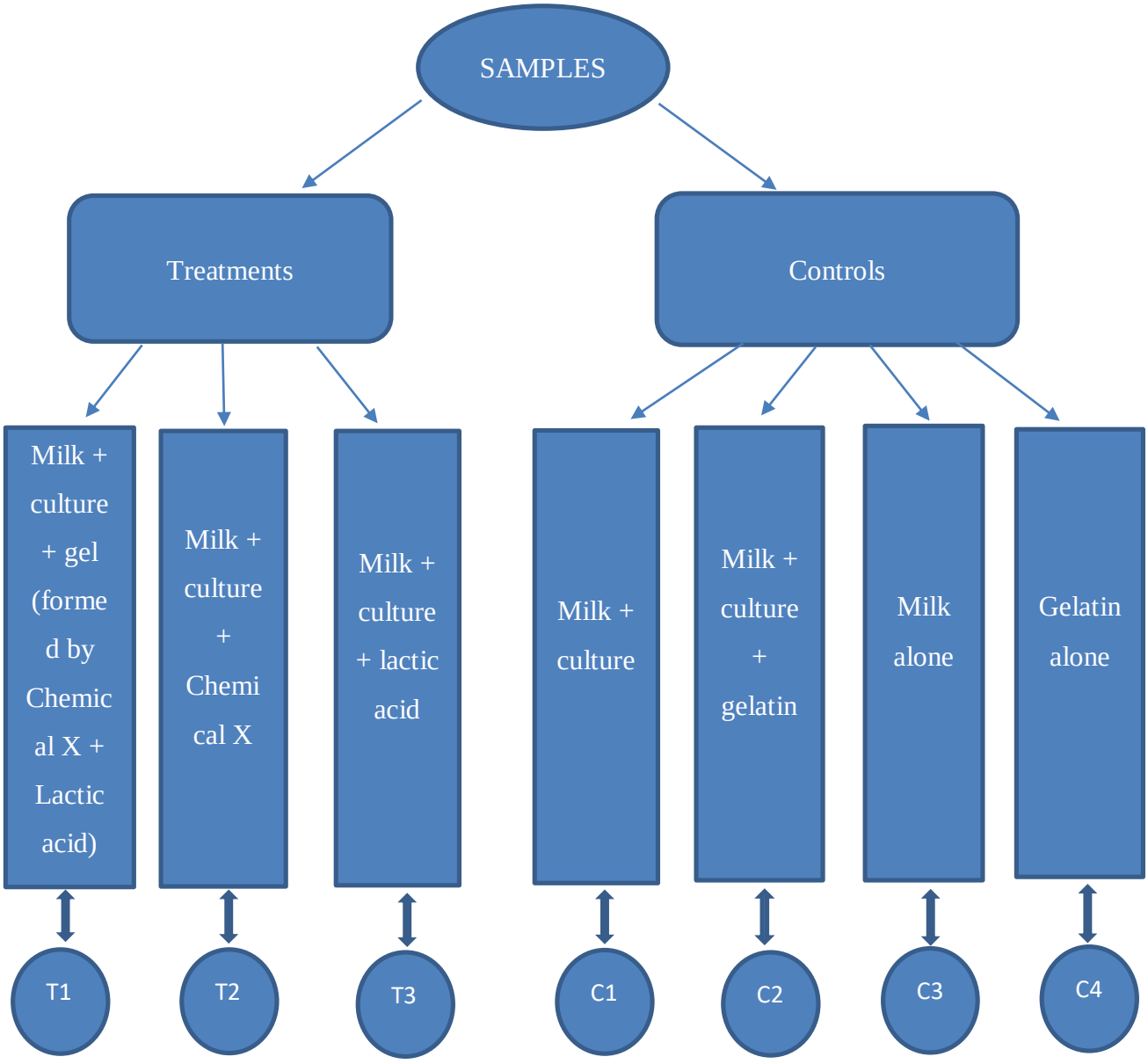



Figure 3.1.1. Flow chart explaining the experimental set-up

## 3.2 Preparation of samples

The procedure followed for yogurt preparation was adopted from the procedure used in previous studies of Chemical X in Lincoln University (unpublished). Chemical X stands for Disodium Guanosine 5'-Monophosphate. Meadow Fresh Original milk purchased from a local supermarket was subjected to pasteurization at $65^0$C for 30 minutes in a pre-heated water bath. Once the milk was cooled down to $43^0$C, which is the incubation temperature (Sodini & Béal, 2003, as cited in Karam, Gaiani, Hosri, Burgain, & Scher, 2013), 0.0072% of YoFlex® Mild 1.0 (50 U) thermophilic yogurt culture bacteria was added and mixed well. Various treatments and controls were prepared and added to the pasteurized milk later as per the composition in Table 3.2.1. The new gel was made by thoroughly mixing Chemical X, lactic acid and water, at $43^0$C and added at a concentration of 1% into the pasteurized milk at $43^0$C. The treatment containing Chemical X alone (T2) was prepared by adding Chemical X to pasteurized milk at a concentration of 0.83%. Similarly, Lactic acid alone as a treatment (T3) was made at a concentration of 0.21% (DL-Lactic acid, 85% w/w) by adding into the pasteurized milk at $43^0$C. Various controls were also made for comparisons. Milk and culture alone was taken as a control C1. A control C2 was prepared by adding commercially available gelatin (Nutra Organics TM Natural Gelatin) to pasteurized milk at 0.5% concentration, and a temperature of $43^0$C. The control C3 consisted of pasteurized milk alone, while C4 was made using 0.5% of gelatin in water as given in table 3.2.1.

## 3.3 Composition of samples

Table 3.3.1. Composition of the different treatments and controls

| Samples | Composition |
|---|---|
| T1 | 1% of Gel (5% of Chemical X, 1.08 % of lactic acid and 92% of water), culture, milk. |
| T2 | 0.83% of Chemical X, milk, and culture. |
| T3 | 0.21 % Lactic acid, milk, and culture. |
| C1 | Milk and culture |
| C2 | 0.5% gelatin, milk and culture. |
| C3 | Milk |
| C4 | 0.5% gelatin, water. |

## 3.4 Experiment protocol

The study was conducted in two parts. One part involved the microbiological shelf life testing of the new gel for *L. bulgaricus*, over four time points for all the treatments and controls. The next part consisted of the antimicrobial activity assessment against *E. coli* for one time-point for T1, T2, T3, C1, C2 and C3 (compositions as given in Table 3). All the experiments were conducted in triplicates.

### 3.4.1 Microbiological shelf life assessment of *Lactobacillus bulgaricus*

Each of the treatments and controls were divided into triplicates containing 30mL each. After the incubation at 43$^{o}$C for 5 hours, the samples were refrigerated at -40$^{o}$C. Since ideally, the culture bacteria counts in yogurt are viable and high in numbers upto 2 weeks of storage, the microbiological enumeration of *L. bulgaricus* was conducted on day 4, day 8, day 11 and day 15 of refrigerated storage. For the purpose of enumeration, De Man, Rogosa, and Sharpe (MRS) agar plates obtained from Fort Richards Laboratories Ltd, Auckland, New Zealand, was used. pH of the samples were also measured on the respective days to ascertain that the pH fell between 4.0- 5.0 range, with an ideal range between 4.0- 4.6 (Karam, Gaiani, Hosri, Burgain, & Scher, 2013).

Various serial dilutions were prepared for the samples. At each time point, dilutions of the sample were prepared by dissolving 1g of the sample in 9mL peptone water to obtain 10-1 dilution. This was then serially diluted upto 10-4, following which 100μL of the dilutions were poured onto respective properly labelled MRS agar plates (Dave, & Shah, 1996) and then spread plated. The plates were incubated under anaerobic conditions at 400C for 72 hours.

### 3.4.2 Assessment of antimicrobial activity against *Escherichia coli*

#### Inoculation of *E. coli*

*E. coli* ATCC 25922 strain obtained from ESR was used for inoculating the samples prior to incubation. An initial solution consisting of 1.2 x 10-5 CFU/mL was prepared. About 15μL of this *E. coli* solution was added into each of the 150mL samples prepared. All the treatments and controls were split into triplicates consisting of 30mL each, and incubated at 43$^{0}$C for 5 hours. The samples were then refrigerated at -4$^{0}$C. For the purpose of enumeration of *L. bulgaricus* and *E. coli*, MRS and Tryptone-Bile X-glucuronide (TBX) agar plates purchased from Fort Richards Laboratories ltd, Auckland, New Zealand was used.

Serial dilutions were prepared after 5 days of refrigeration in a similar way as done for the shelf life study. The samples were serially diluted upto 10-3 for T1 to C2 and upto 10-11 for C3. 100μL of 10-1 and 10-3 of the dilutions were spread plated onto MRS and TBX agar plates. The MRS plates were incubated under anaerobic condition for 72 hours at 40$^{0}$C, whereas the TBX plates were incubated

under aerobic conditions for 24 hours at $37^0$C, following which the colony counts were enumerated as Colony Forming Units (CFU) and expressed as log CFU/ mL. pH was also analysed after 5 days of refrigeration to check if the pH remained in the ideal range for yogurt.

## Data analysis

The average of log CFU/ mL and the mean standard error of each of the samples at different time points were calculated and plotted as a graph in Microsoft Excel. For statistical analysis, One-way ANOVA was performed using Minitab 2018 version. Tukey comparison was used to evaluate significant differences between the groups.

# Chapter 4

# Results & Discussion

## 4.1 Assessment of *L. bulgaricus* counts over shelf life

Enumeration of *L. bulgaricus* was done to ascertain that the new gel had no effect on the counts of LAB over a shelf life of 15 days. Enumeration done on MRS agar was converted as log CFU/ mL and plotted as a graph to show the change in trend in comparison to the other samples. Significant difference among the samples during each of the time points and over the shelf life are also portrayed in the graph and table below.

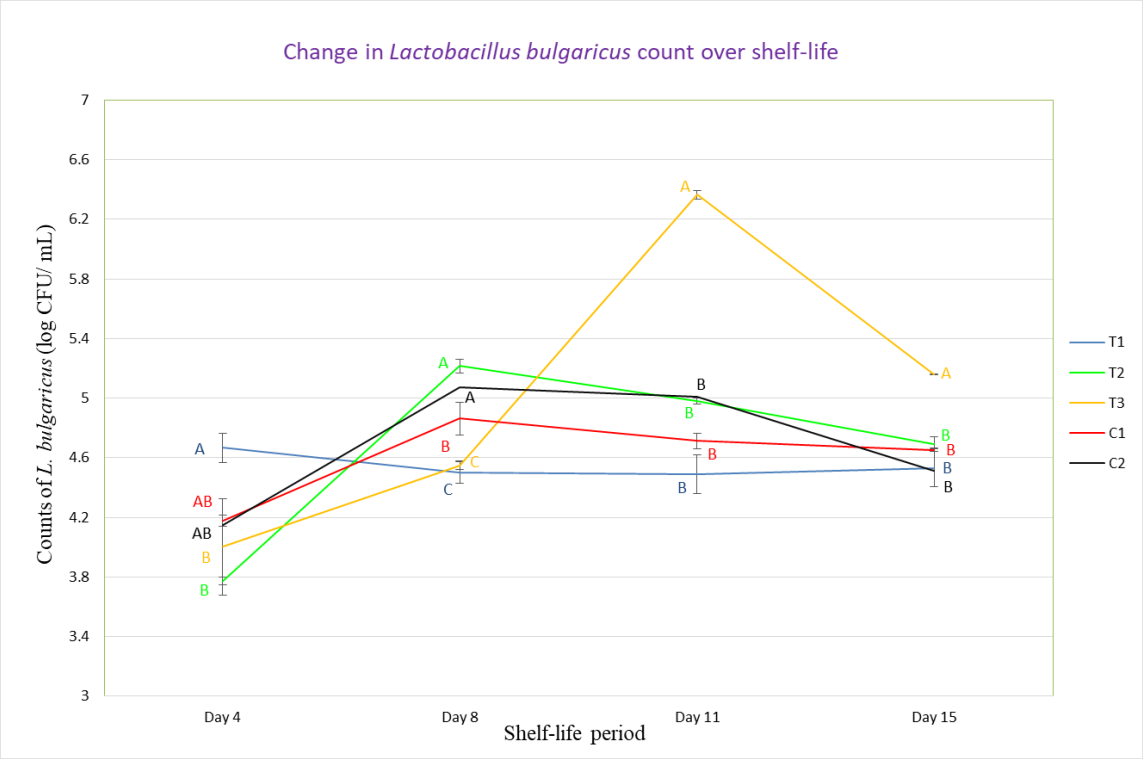



Figure 4.1.1 Graph showing the change in *Lactobacillus bulgaricus* count over shelf life. The average counts for the days 4, 8, 11 and 15 are expressed as log CFU/ mL for samples for treatments with new gel (T1), Chemical X (T2), lactic acid (T3), and controls with milk & culture (C1) and yogurt with gelatin (C2). The controls having milk alone (C3) & gelatin alone (C4) count taken for the first time point did not have any colonies. In a particular time point, the samples that are not significantly different are noted using the same letter,

while those that are significantly different from each other are noted using different letters. The mean of standard error is also depicted in the diagram.

| Sample | Day 4 | Day 8 | Day 11 | Day 15 |
|---|---|---|---|---|
| **T1** | 4.67± 0.097$^{a}$ | 4.5± 0.03$^{a}$ | 4.49± 0.32$^{a}$ | 4.53± 0.04$^{a}$ |
| **T2** | 3.78± 0.073$^{c}$ | 5.22± 0.04$^{a}$ | 4.98± 0.03$^{ab}$ | 4.69± 0.11$^{b}$ |
| **T3** | 4± 0.13$^{d}$ | 4.55± 0.02$^{c}$ | 6.37± 0.029$^{a}$ | 5.16± 0.05$^{b}$ |
| **C1** | 4.18± 0.13$^{b}$ | 4.86± 0.05$^{a}$ | 4.72± 0.004$^{a}$ | 4.65± 0.009$^{ab}$ |
| **C2** | 4.15± 0$^{c}$ | 5.08± 0.04$^{a}$ | 5.01± 0.03$^{a}$ | 4.51± 0.02$^{b}$ |

Table 4.1.2. Mean of counts with standard error for *L. bulgaricus* over shelf life. $^{a,b,c,d}$ Subscripts show the significant difference of the treatments and controls over the shelf life. Samples that share the same letter have no significant difference, while samples which do not share a letter are significantly different from one another.

From the graph it is evident that the treatment containing the new gel (T1) has the highest growth of *L. bulgaricus* on day 4 as compared to the other treatments and controls. It is also found to be statistically different ($p< 0.05$) from the treatments containing its individual components of Chemical X and lactic acid (T2 and T3), which possess the lowest counts of the microbe on that day. This could be indicative that the combined interaction of lactic acid with the Chemical X creates a conducive environment that promotes the growth of *L. bulgaricus*. The presence of lactic acid also helps the *L. bulgaricus* in having the highest numbers during the initial stages. This positive influence gradually decreases as time progresses and could possibly be explained due to the influence of lactic acid which slowly exerts a higher influence on the growth of the culture. Although the lactic acid produced by *S. thermophilus* positively influences *L. bulgaricus*, studies have shown that the external addition of lactic acid will inhibit the growth of *S. thermophilus* in the early stages of growth due to its low acid tolerance. As a consequence, the growth of *L. bulgaricus* is also delayed overtime, due to absence of the metabolites needed for its growth by *S. thermophilus* (Schmidt, 2009; Settachaimongkon et al., 2014). But the current findings show that the presence of Chemical X somehow balances inhibition by lactic acid and prevents the growth from getting very adversely affected. This could be why the growth of *L. bulgaricus* in T1 is maintaining a stable level of numbers till the end of the shelf life as indicated by no significant difference among the counts of different time points ($p> 0.05$). The maintenance of stable counts of *L. bulgaricus* through the storage period contradicts earlier findings which states the

number of viable counts of LAB keeps decreasing throughout the refrigerated storage period of yogurts (Hamann, & Marth, 1984).

Considering the case of the treatment containing Chemical X alone (T2), it is very clear from the graph that on Day 4, the lowest counts of *L. bulgaricus* is observed here. The absence of initial levels of lactic acid needed to boost the growth of *L. bulgaricus* may be the reason for it. Overtime, however, the *S. thermophilus* produces lactic acid which is needed for *L. bulgaricus* to reach its exponential phase. This can be correlated with previous researches, where they have explained that the growth of mixed starter cultures occur in 3 phases. In the first phase *S. thermophilus* grows faster and exponentially than *L. bulgaricus*, leading to a better acidification of milk. In the 2nd phase, the better levels of acidity and stabilized pH increases the *L. bulgaricus* counts, whereas the *S. thermophilus* remains stable. In the last phase, *L. bulgaricus* again undergoes a pause in their growth while the *S. thermophilus* undergoes another growth phase, with a reduced growth rate (Letort, Nardi, Garault, Monnet, & Juillard, 2002; Courtin, & Rul, 2004). Hence, after day 8, we can observe that there is a reduction in the growth of L. bulgaricus, due to it entering the stable phase while *S. thermophilus* undergoes its diauxic growth. Even though T2 has only Chemical X at first, due to the lactic acid produced by the culture bacteria, the Chemical X can interact with it, leading to the formation of the new gel at later stages. This can also explain why T2 changes over shelf life ($p < 0.05$) and exhibits a similar trend as T1 from Day 8.

For the treatment with lactic acid (T3), there is an initial lower numbers of *L. bulgaricus* on Day 4 due to the above explained effect of externally added lactic acid on lowering the numbers of *S. theromphilus*, which results in a consequent lower counts of *L. bulgaricus*. Although the growth is inhibited, the presence of lactic acid favours a slight increase in the *L. bulgaricus* populations on day 8. The growth behaviour of *L. bulgaricus* shoots upto a very high level due to unknown reasons on a day 11. Since we have only the data from day 4, conclusions cannot be made regarding this trend due to absence of data at the time of fermentation and on the initial days and the *S. thermophilus* counts. From day 11, there is a rapid decrease in *L. bulgaricus* counts. This is could be explained due to lack of proto-cooperation. Higher acidity would limit the counts of *S. thermophilus*. In the absence of essential metabolites such as pyruvic acid, formic acid, folic acid, ornithine and some long chain fatty acids, produced by *S. thermophilus*, the numbers of Lactobacilli decreases rapidly after day 11 as *L. bulgaricus* is always in need of these nutrients (Smid, & Lacroix, 2013). This is the only treatment that was found to completely change over shelf life ($p < 0.05$) and exhibit unpredicted changes at different time points.

Considering the control consisting of milk and culture alone (C1), a similar observation as the treatment with Chemical X alone (T2) is made, as they follow a similar trend. However, it is found that on day 4, C1 seems to have a slightly higher growth than T2, and T3, even if the difference among them is not

statistically significant ($p < 0.05$). This could be due to the apparent higher concentration of substrate (milk), in C1 as compared to T2 and T3. The positive influence on the growth of LAB by higher concentration of substrate availability, is backed up in a study done by Özen, & Özilgen (1992). Through their study they have proved that better proto-cooperation and favourable growth of culture bacteria occurs when there is a lower number of inhibitory substances and a large amount of substrate present. Their studies also provided proof of increase in numbers of *L. bulgaricus* in relation with higher substrate concentrations that provides higher amount of nutrients from milk for supporting its growth. The control with gelatin (C2) also follows a similar trend of T2 without being significantly different from T2 through out the storage period ($p > 0.05$). As expected, the *L. bulgaricus* counts is comparatively higher in C2 than in C1, with a difference that is statistically significant ($p < 0.05$). This could be due to the ability of gelatin, in association with the LAB, to decrease the pH, which helps in supporting the growth of *L. bulgaricus*. Such an increase in LAB counts were reported previously by García-Argueta et al. (2013) and was attributed to better interactions between the milk components and culture bacteria, made possible by the higher acidic medium with gelatin.

Overall, the results clearly indicate that the new gel and gel components (Chemical X and lactic acid), positively influences the growth of *L. bulgaricus*. The yogurt with new gel (T1) helps in maintaining stable numbers of *L. bulgaricus*population throughout the storage period without any significant difference in numbers till the end of the shelf life when compared to all the other samples. The yogurt with Chemical X alone, gradually forms the new and displays similar growth supporting properties as T1 (yogurt with new gel) for L. bulgaricus. Compared to yogurt with gelatin, at the end of shelf life, the yogurts containing the new gel and its components (T1 and T2) exhibited slightly higher counts, despite not being significantly different.

## *4.2* Assessment of antimicrobial effect against *E. coli*

Enumeration of *E. coli* and *L. bulgaricus* counts following *E. coli* inoculation into the samples, was done after 5 days of refrigerated storage. This gives an idea about the effectiveness of the new gel to inhibit yogurt contaminants like *E. coli*. The counts taken in TBX and MRS agar, are represented as log CFU/ mL, along with their significant difference in the following bar graph.

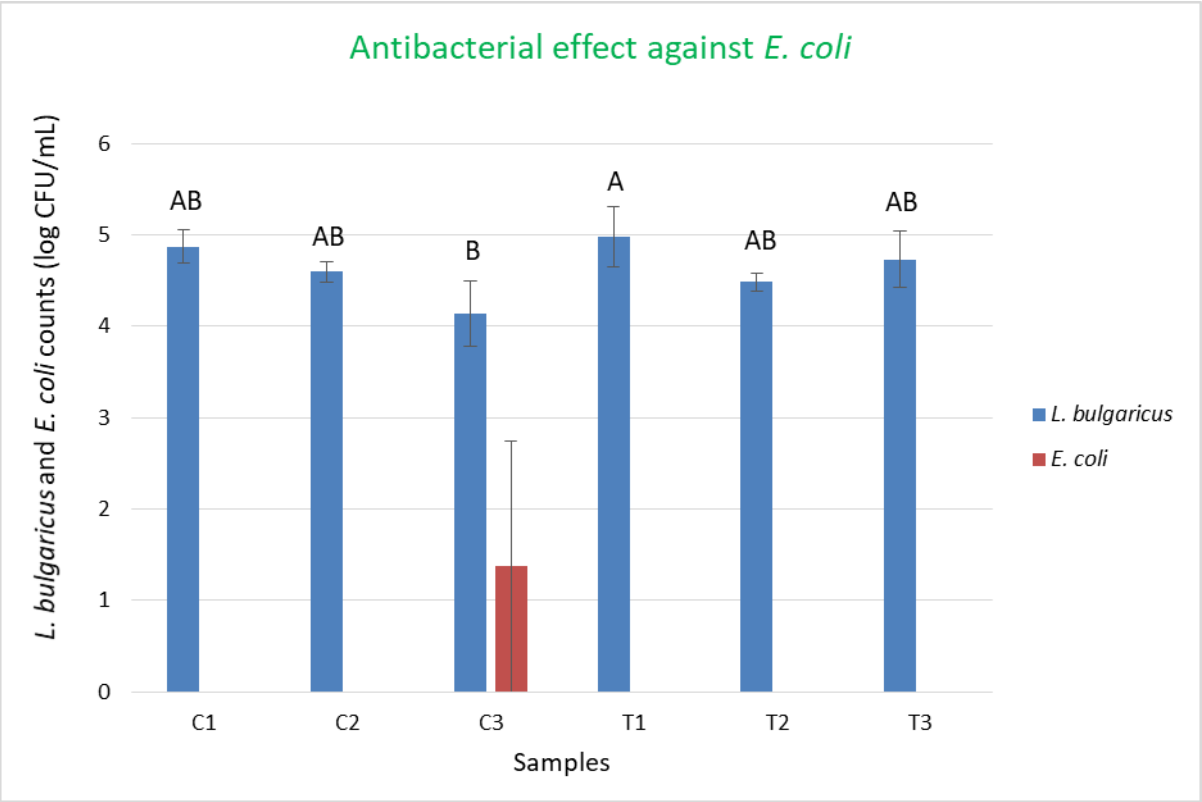



Figure 4.2.1. Graph depicting the assessment of antimicrobial effect against *E. coli*. The mean count of both *E. coli* and *L. bulgaricus* is noted as log CFU/ mL along with the mean of standard error. The significant difference of different samples after 5 days of refrigeration for *L. bulgaricus* counts is also represented, where the samples sharing the same letter (A, B) are statistically similar and the samples with different letters are significantly different. The samples under consideration are the samples with new gel (T1), Chemical X (T2), lactic acid (T3), milk & culture (C1), yogurt with gelatin (C2) and milk alone (C3). There was no significant difference observed for *E. coli* counts during statistical analysis and hence is not represented in the diagram.

*E. coli* growth was visible only in the control having pasteurized milk alone (C3), which did not have any starter culture added to it. The count in C3 was similar to the initial concentration of *E. coli* inoculated into the samples before incubation. But the difference among the samples with regard to *E. coli* count was not statistically significant from each other ($p > 0.05$; not depicted in the graph). This points out to the possible effectiveness of the new gel (T1) as being equally effective as any other yogurt samples in controlling the post-contamination of *E. coli* pathogens. *L. bulgaricus* counts under the influence of *E. coli* after 5 days of storage clearly shows that the treatment containing the new gel (T1) is significantly different from the milk only control (C3). T1 has the highest counts of *L. bulgaricus*, while C3 has the lowest count of the microbe on day 5.

In vitro antimicrobial action of yogurts against some *E. coli* strains were studied and reported in earlier research (Kotz, Peterson, Moody, Savaiano, & Levitt, 1990). The study explains that presence of live

cultures along with high concentrations of lactic acid are bactericidal to *E. coli*. Most of the action of LAB against pathogenic contaminants like *E. coli* was found to be due to the production of lactic acid, or due to synergistic action of lactic and acetic acid, or via competition to colonize sites with pathogens (Adams, & Hall, 1988; Mirzaei, Lashani, & Davoodabadi, 2018). Another study by Batdorj et al. (2007) using lactic acid bacteria, has reported similar antimicrobial activity against pathogens owing to the production of hydrogen peroxide, which is inhibitory against various pathogens, while not affecting the culture bacteria. Similar studies done against *Salmonella typhimurium, Staphyococcus aureus*, and *E. coli*, explains the bactericidal effects to be a cause of hydrogen peroxide and bacteriocins produced by *Lactobacilli* species in homemade yogurts (Yesillik, Yildirim, Dikici, Yildiz, & Yesillik, 2011).

# Chapter 5

# Conclusion

The present study to explore the antimicrobial properties of new gel and its gel components in yogurt system, has shown that the new gel and its components does not adversely affect the growth of *L. bulgaricus* species through shelf life. The yogurt with the new gel is in fact, found to be favourable for the growth during the initial stages of storage. Compared to other controls which showed a decrease in the *L. bulgaricus* counts, the new gel maintained stability in the number of *L. bulgaricus* till the end of shelf life. The gel component, Chemical X, was also found to exhibit a smilar trend as the new gel towards the later stages of storage, favouring the growth of *L. bulgaricus*. Current results also prove that the yogurts with the new gel and its components can maintain viable counts of *L. bulgaricus* needed to inhibit the growth of any pathogenic contaminants such as *E. coli*. The yogurt with the new gel was also found to be statistically different from the milk sample in which *E. coli* thrived. This suggests that the new gel can be used as a gelling agent in yogurts for maintaining the viable counts as well as for helping prevent contamination by pathogens.

To study the exact mechanism by which the new gel has better antimicrobial properties, future studies would require to analyse the changes that happen both during the fermentation and during the initial stages of storage. Enumeration of *S. thermophilus* along with *L. bulgaricus* and the quantification of biochemicals produced by the microbes during fermentation and storage would help to prove the above explained reasons. Since changes in culture population and interactions influence the sensory properties of the product, sensory analysis should also be done to assess its success as a food gel. Probiotics is an important on-going trend in the food industry. Therefore, the behaviour of new gel under the influence of probiotics in yogurt system would also have to be evaluated. Similarly, a study regarding different formulations and concentrations on new gel alone and in combination with other gelling agents, would help to produce a superior quality food gel, that could further be extended to other food products than yogurt alone.

# References


Adams, M. R., & Hall, C. J. (1988). Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. *International Journal of Food Science & Technology*, *23*(3), 287-292.

Banerjee, S., & Bhattacharya, S. (2012). Food gels: gelling process and new applications. *Critical reviews in food science and nutrition*, *52*(4), 334-346.

Batdorj, B., Trinetta, V., Dalgalarrondo, M., Prévost, H., Dousset, X., Ivanova, I., ... & Chobert, J. M. (2007). Isolation, taxonomic identification and hydrogen peroxide production by *Lactobacillus delbrueckii subsp. lactis* T31, isolated from Mongolian yoghurt: inhibitory activity on food-borne pathogens. *Journal of Applied Microbiology*, *103*(3), 584-593.

Baziwane, D., & He, Q. (2003). Gelatin: the paramount food additive. *Food Reviews International*, *19*(4), 423-435.

Behare, P., Singh, R., Kumar, M., Prajapati, J. B., & Singh, R. P. (2009). Exopolysaccharides of lactic acid bacteria: A review. *Journal of Food Science and Technology*, 46, 1–11.

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS Microbiology, 4*(4), 665-684.

Chandan, R. C., & Kilara, A. (2008). Role of milk and dairy foods in nutrition and health. Dairy Processing and Quality Assurance, 411-428.

Chandan, R. C., & O'Rell, K. R. (2006). Manufacture of various types of yogurt. *Manufacturing yogurt and fermented milks*, 211-236.

Chandan, R.C., & Nauth, K.R. (2012). Yogurt. In: Hui , Y.H. and Chandan , R.C. , editors. *Handbook of animal-based fermented food and beverage technology* (Vol. 1). Chapter 12. CRC press, 213-233.

Chen, C., Zhao, S., Hao, G., Yu, H., Tian, H., & Zhao, G. (2017). Role of lactic acid bacteria on the yogurt flavour: A review. *International journal of food properties*, *20*(sup1), S316-S330.

Chen, X. X., Lu, L. X., Lin, L. J., Yao, L. L., & Xiong, X. H. (2016). Effect of resistant dextrin on the quality of solidified yogurt. *Food and Fermentation Industry, 42*(9), 91-97.

Cheng, H. (2010). Volatile flavor compounds in yogurt: a review. *Critical reviews in food science and nutrition*, *50*(10), 938-950.

Courtin, P., & Rul, F. (2004). Interactions between microorganisms in a simple ecosystem: yogurt bacteria as a study model. *Le Lait*, *84*(1-2), 125-134.

Dave, R. I., & Shah, N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus*, and *Bifidobacteria*. *Journal of dairy science*, *79*(9), 1529-1536.

De Brabandere, A. G., & De Baerdemaeker, J. G. (1999). Effects of process conditions on the pH development during yogurt fermentation. *Journal of food engineering*, *41*(3-4), 221-227.

Donkor, O. N., Nilmini, S. L. I., Stolic, P., Vasiljevic, T., & Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. *International Dairy Journal*, *17*(6), 657-665.

Fiszman, S.M., Lluch, M.A., & Salvador, A. (1999). Effect of addition of gelatin on microstructure of acidic milk gels and yoghurt and on their rheological properties. *International Dairy Journal, 9*(12). 895-901.

Food Standards Australia New Zealand. (2016). *Australia New Zealand Food Standards Code – Standard 2.5.3 – Fermented milk products.* Retrieved from https://www.legislation.gov.au/Details/F2015L00413.

García-Argueta, I., Dublán-García, O., Quintero-Salazar, B., Dominguez-Lopez, A., Gómez-Oliván, L. M., & Salem, A. F. Z. (2013). Effect of lactic acid bacteria on the textural properties of an edible film based on whey, inulin and gelatin. *African Journal of Biotechnology*, *12*(19).

Garvie, E. I. (1984). 5 Separation of Species of the Genus *Leuconostoc* and Differentiation of the *Leuconostocs* from Other Lactic Acid Bacteria. In *Methods in microbiology* (Vol. 16, pp. 147-178). Academic Press.

Girard, M., & Schaffer-Lequart, C. (2007). Gelation of skim milk containing anionic exopolysaccharides and recovery of texture after shearing. *Food hydrocolloids*, *21*(7), 1031-1040.

Gómez-Gallego, C., Gueimonde, M., & Salminen, S. (2018). The role of yogurt in food-based dietary guidelines. Nutrition reviews, 76(Supplement_1), 29-39.

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. D. A. F., & Shah, N. P. (2010). Probiotic dairy products as functional foods. *Comprehensive reviews in food science and food safety*, *9*(5), 455-470.

Hamann, W. T., & Marth, E. H. (1984). Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yogurts. *Journal of Food protection*, *47*(10), 781-786.

Hinrichs, R., Götz, J., & Weisser, H. (2003). Water-holding capacity and structure of hydrocolloid-gels, WPC-gels and yogurts characterised by means of NMR. *Food chemistry*, *82*(1), 155-160.

Jankowska, A., Wiśniewska, K., & Reps, A. (2005). Application of probiotic bacteria in production of yoghurt preserved under high pressure. *High Pressure Research*, *25*(1), 57-62.

Karam, M. C., Gaiani, C., Hosri, C., Burgain, J., & Scher, J. (2013). Effect of dairy powders fortification on yogurt textural and sensorial properties: a review. *Journal of Dairy Research*, *80*(4), 400-409.

Kilara, A. (2017). Regulatory Aspects of Yogurt. In *Yogurt in Health and Disease Prevention* (pp. 107-132). Academic Press.

Kilara, A., & Shahani, K. M. (1976). Lactase activity of cultured and acidified dairy products. *Journal of Dairy Science*, *59*(12), 2031-2035.

Kotz, C. M., Peterson, L. R., Moody, J. A., Savaiano, D. A., & Levitt, M. D. (1990). In vitro antibacterial effect of yogurt on *Escherichia coli*. *Digestive diseases and sciences*, *35*(5), 630-637.

Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology*, *15*(2), 67-78.

Letort, C., Nardi, M., Garault, P., Monnet, V., & Juillard, V. (2002). Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Appl. Environ. Microbiol.*, *68*(6), 3162-3165

Lorenzen, P. C., Neve, H., Mautner, A., & Schlimme, E. (2002). Effect of enzymatic cross-linking of milk proteins on functional properties of set-style yoghurt. *International Journal of Dairy Technology*, *55*(3), 152-157.

Mataragas, M., Dimitriou, V., Skandamis, P. N., & Drosinos, E. H. (2011). Quantifying the spoilage and shelf-life of yoghurt with fruits. *Food Microbiology*, *28*(3), 611-616.

Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P., & Bardowski, J. (2010). Updates in the metabolism of lactic acid bacteria. *Biotechnology of lactic acid bacteria: Novel applications*, 3-33.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., ... & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging infectious diseases*, *5*(5), 607.

Mirzaei, E. Z., Lashani, E., & Davoodabadi, A. (2018). Antimicrobial properties of lactic acid bacteria isolated from traditional yogurt and milk against *Shigella* strains. *GMS hygiene and infection control*, *13*.

Modler, H. W., & Kalab, M. (1983). Microstructure of yogurt stabilized with milk proteins. *Journal of Dairy Science*, *66*(3), 430-437.

Modler, H. W., Larmond, M. E., Lin, C. S., Froehlich, D., & Emmons, D. B. (1983). Physical and sensory properties of yogurt stabilized with milk proteins. *Journal of Dairy Science*, *66*(3), 422-429.

Morgan, D., Newman, C. P., Hutchinson, D. N., Walker, A. M., Rowe, B., & Majid, F. (1993). Verotoxin producing Escherichia coli O 157 infections associated with the consumption of yoghurt. *Epidemiology & Infection*, *111*(2), 181-188.

Mullins, R. J., James, H., Platts-Mills, T. A., & Commins, S. (2012). Relationship between red meat allergy and sensitization to gelatin and galactose-α-1, 3-galactose. *Journal of allergy and clinical immunology*, *129*(5), 1334-1342.

Nishiyama, K., Kobayashi, T., Sato, Y., Watanabe, Y., Kikuchi, R., Kanno, R., ... & Suzutani, T. (2018). A Double-Blind Controlled Study to Evaluate the Effects of Yogurt Enriched with *Lactococcus lactis* 11/19-B1 and *Bifidobacterium lactis* on Serum Low-Density Lipoprotein Level and Antigen-Specific Interferon-γ Releasing Ability. *Nutrients*, *10*(11), 1778.

Ozcan, T., Horne, D. S., & Lucey, J. A. (2015). Yogurt made from milk heated at different pH values. *Journal of dairy science*, *98*(10), 6749-6758.

Özen, S., & Özilgen, M. (1992). Effects of substrate concentration on growth and lactic acid production by mixed cultures of Lactobacillus bulgaricus and Streptococcus thermophilus. *Journal of Chemical Technology & Biotechnology*, *54*(1), 57-61.

Peirera, M. J. F., & Luchese, R. H. (1988). The assessment of growth compatibility between strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *Rev. Inst. Lactic Cândido Tostes (Brasil)*, *43*, 11-13.

Priyashantha, H., Quintáns, A. P., Baixauli, R., & Vidanarachchi, J. K. (2019). Type of starter culture influences on structural and sensorial properties of low protein fermented gels. *Journal of texture studies*, *50*(6), 482-492.

Renard, D., van de Velde, F., & Visschers, R. W. (2006). The gap between food gel structure, texture and perception. *Food Hydrocolloids*, *20*(4), 423-431.

Rybka, S., & Kailasapathy, K. (1995). The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *Australian Journal of Dairy Technology*, *50*(2), 51.

Salvador, A., & Fiszman, S. M. (2004). Textural and sensory characteristics of whole and skimmed flavored set-type yogurt during long storage. *Journal of dairy Science*, *87*(12), 4033-4041.

Schmidt, K. A. (2009). Effect of acidulant addition on yogurt fermentation.

Schrieber, R., & Gareis, H. (2007). *Gelatine handbook: theory and industrial practice*. John Wiley & Sons.

Settachaimongkon, S., Nout, M. R., Fernandes, E. C. A., Hettinga, K. A., Vervoort, J. M., van Hooijdonk, T. C., ... & van Valenberg, H. J. (2014). Influence of different proteolytic strains of *Streptococcus thermophilus* in co-culture with *Lactobacillus delbrueckii subsp. bulgaricus* on the metabolite profile of set-yoghurt. *International journal of food microbiology*, *177*, 29-36.

Sfakianakis, P., & Tzia, C. (2014). Conventional and innovative processing of milk for yogurt manufacture; development of texture and flavor: A review. *Foods*, *3*(1), 176-193.

Shah, N. P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of dairy science*, *83*(4), 894-907.

Shah, N. P., & Ravula, R. R. (2000). Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. *Australian Journal of Dairy Technology*, *55*(3), 127.

Sheehan, J. J. (2013). Milk quality and cheese diversification. *Irish Journal of Agricultural and Food Research*, 243-253.

Shihata, A., & Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal*, *10*(5-6), 401-408.

Shu, G., Li, C., Chen, H., & Wang, C. (2014). Effect of inoculum and temperature on the fermentation of goat yogurt. *Advance Journal of Food Science and Technology*, *6*(1), 68-71.

Sieuwerts, S., De Bok, F. A., Hugenholtz, J., & van Hylckama Vlieg, J. E. (2008). Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl. Environ. Microbiol.*, *74*(16), 4997-5007.

Smid, E. J., & Lacroix, C. (2013). Microbe–microbe interactions in mixed culture food fermentations. *Current opinion in biotechnology*, *24*(2), 148-154.

Sodini, I., Remeuf, F., Haddad, S., & Corrieu, G. (2004). The relative effect of milk base, starter, and process on yogurt texture: a review. *Critical reviews in food science and nutrition*, *44*(2), 113-137.

Tamime, A. Y., & Robinson, R. K. (1999). *Yoghurt: science and technology*. Woodhead Publishing.

Tamime, A. Y., & Robinson, R. K. (2007). Yoghurt science and technology. Abington.

Teusink, B., Wiersma, A., Molenaar, D., Francke, C., de Vos, W. M., Siezen, R. J., & Smid, E. J. (2006). Analysis of growth of Lactobacillus plantarum WCFS1 on a complex medium using a genome-scale metabolic model. *Journal of Biological Chemistry*, *281*(52), 40041-40048.

Vinderola, G., Binetti, A., Burns, P., & Reinheimer, J. (2011). Cell viability and functionality of probiotic bacteria in dairy products. *Frontiers in microbiology*, *2*, 70.

Yesillik, S., Yildirim, N., Dikici, A., Yildiz, A., & Yesillik, S. (2011). Antibacterial effects of some fermented commercial and homemade dairy products and 0.9% lactic acid against selected foodborne pathogens. *Asian J Anim Vet Adv*, *6*(2), 189-195.

# Appendix A

## Composition of the different treatments and controls

| Samples | Composition |
|---|---|
| T1 | Gel- 196.4g (Chemical X- 9.84g, lactic acid- 2.56mL, water- 184mL). (Milk+ culture) is added to this gel to make upto 1200 mL. |
| T2 | Chemical X- 9.84g + (Milk+ culture) added to make upto 1200 mL. |
| T3 | Lactic acid- 2.56mL. (Milk+ culture) added to make upto 1200 mL. |
| C1 | 500mL of (Milk+ culture) solution |
| C2 | Gelatin- 4.5g (0.5%). (Milk+ culture) added to make upto 900mL. |
| C3 | 250mL of pasteurized milk |
| C4 | Gelatin -0.75g dissolved in 150 mL of water |
| | |

# Appendix B

# *L. bulgaricus* shelf life study

## B.1 First time point- Day 4

| Replicates | Count | Log CFU/mL |
|---|---|---|
| T1 | 58000 | 4.763428 |
| T1 | 37000 | 4.568202 |
| T2 | 7000 | 3.845098 |
| T2 | 5000 | 3.69897 |
| T3 | 17000 | 4.230449 |
| T3 | 10000 | 4 |
| T3 | 6000 | 3.778151 |
| C1 | 9000 | 3.954243 |
| C1 | 25000 | 4.39794 |
| C1 | 15000 | 4.176091 |
| C2 | 14000 | 4.146128 |
| C2 | 14000 | 4.146128 |
| C3 | | 0 |
| C3 | | 0 |
| C4 | | 0 |
| C4 | | 0 |

## Statistics

| Variable | replicates | N | N* | Mean | SE Mean | StDev | Minimum | Q1 | Median | Q3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Log CFU/mL | C1 | 3 | 0 | 4.176 | 0.128 | 0.222 | 3.954 | 3.954 | 4.176 | 4.398 |
| | C2 | 2 | 0 | 4.1461 | 0.000000 | 0.000000 | 4.1461 | * | 4.1461 | * |
| | C3 | 2 | 0 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | * | 0.000000 | * |
| | C4 | 2 | 0 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | * | 0.000000 | * |
| | T1 | 2 | 0 | 4.6658 | 0.0976 | 0.1380 | 4.5682 | * | 4.6658 | * |
| | T2 | 2 | 0 | 3.7720 | 0.0731 | 0.1033 | 3.6990 | * | 3.7720 | * |
| | T3 | 3 | 0 | 4.003 | 0.131 | 0.226 | 3.778 | 3.778 | 4.000 | 4.230 |

| Variable | replicates | Maximum |
|---|---|---|
| Log CFU/mL | C1 | 4.398 |
| | C2 | 4.1461 |
| | C3 | 0.000000 |
| | C4 | 0.000000 |
| | T1 | 4.7634 |
| | T2 | 3.8451 |
| | T3 | 4.230 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| replicates | 6 | 52.3543 | 8.72572 | 340.75 | 0.000 |
| Error | 9 | 0.2305 | 0.02561 | | |
| Total | 15 | 52.5848 | | | |

## Grouping Information Using the Tukey Method and 95% Confidence

| replicates | N | Mean | Grouping | | |
|---|---|---|---|---|---|
| T1 | 2 | 4.6658 | A | | |
| C1 | 3 | 4.176 | A | B | |
| C2 | 2 | 4.146 | A | B | |
| T3 | 3 | 4.003 | | B | |
| T2 | 2 | 3.7720 | | B | |
| C4 | 2 | 0.000000 | | | C |
| C3 | 2 | 0.000000 | | | C |

*Means that do not share a letter are significantly different.*

## B.2 Second time point- Day 8

| replicates | count | log cfu/ml |
|---|---|---|
| T1 | 28000 | 4.447158 |
| T1 | 33000 | 4.518514 |
| T1 | 34000 | 4.531479 |
| T2 | 148000 | 5.170262 |
| T2 | 182000 | 5.260071 |
| T3 | 34000 | 4.531479 |
| T3 | 37000 | 4.568202 |
| C1 | 82000 | 4.913814 |
| C1 | 65000 | 4.812913 |
| C2 | 129000 | 5.11059 |
| C2 | 109000 | 5.037426 |

### Statistics

| Variable | replicates | N | N* | Mean | SE Mean | StDev | Minimum | Q1 | Median | Q3 |
|---|---|---|---|---|---|---|---|---|---|---|
| log cfu/ml | C1 | 2 | 0 | 4.8634 | 0.0505 | 0.0713 | 4.8129 | * | 4.8634 | * |
| | C2 | 2 | 0 | 5.0740 | 0.0366 | 0.0517 | 5.0374 | * | 5.0740 | * |
| | T1 | 3 | 0 | 4.4991 | 0.0262 | 0.0454 | 4.4472 | 4.4472 | 4.5185 | 4.5315 |
| | T2 | 2 | 0 | 5.2152 | 0.0449 | 0.0635 | 5.1703 | * | 5.2152 | * |
| | T3 | 2 | 0 | 4.5498 | 0.0184 | 0.0260 | 4.5315 | * | 4.5498 | * |

| Variable | replicates | Maximum |
|---|---|---|
| log cfu/ml | C1 | 4.9138 |
| | C2 | 5.1106 |
| | T1 | 4.5315 |
| | T2 | 5.2601 |
| | T3 | 4.5682 |

### Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| replicates | 4 | 0.89885 | 0.224711 | 81.23 | 0.000 |
| Error | 6 | 0.01660 | 0.002766 | | |
| Total | 10 | 0.91544 | | | |

## Tukey Pairwise Comparisons

### Grouping Information Using the Tukey Method and 95% Confidence

| replicates | N | Mean | Grouping | | |
|---|---|---|---|---|---|
| T2 | 2 | 5.2152 | A | | |
| C2 | 2 | 5.0740 | A | | |
| C1 | 2 | 4.8634 | | B | |
| T3 | 2 | 4.5498 | | | C |
| T1 | 3 | 4.4991 | | | C |

*Means that do not share a letter are significantly different.*

## B.3 Third time point – Day 11

| samples | count | log cfu/ml |
|---|---|---|
| T1 | 7000 | 3.845098 |
| T1 | 66000 | 4.819544 |
| T1 | 64000 | 4.80618 |
| T2 | 102000 | 5.0086 |
| T2 | 90000 | 4.954243 |
| T3 | 2170000 | 6.33646 |
| T3 | 2480000 | 6.394452 |
| C1 | 51000 | 4.70757 |

| | | |
|---|---|---|
| C1 | 52000 | 4.716003 |
| C2 | 110000 | 5.041393 |
| C2 | 94000 | 4.973128 |

### Statistics

| Variable | samples | N | N* | Mean | SE Mean | StDev | Minimum | Q1 | Median | Q3 | Maximum |
|---|---|---|---|---|---|---|---|---|---|---|---|
| log cfu/ml | C1 | 2 | 0 | 4.7118 | 0.00422 | 0.00596 | 4.7076 | * | 4.7118 | * | 4.7160 |
| | C2 | 2 | 0 | 5.0073 | 0.0341 | 0.0483 | 4.9731 | * | 5.0073 | * | 5.0414 |
| | T1 | 3 | 0 | 4.490 | 0.323 | 0.559 | 3.845 | 3.845 | 4.806 | 4.820 | 4.820 |
| | T2 | 2 | 0 | 4.9814 | 0.0272 | 0.0384 | 4.9542 | * | 4.9814 | * | 5.0086 |
| | T3 | 2 | 0 | 6.3655 | 0.0290 | 0.0410 | 6.3365 | * | 6.3655 | * | 6.3945 |

### Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| samples | 4 | 4.6423 | 1.1606 | 11.05 | 0.006 |
| Error | 6 | 0.6300 | 0.1050 | | |
| Total | 10 | 5.2723 | | | |

### Grouping Information Using the Tukey Method and 95% Confidence

| samples | N | Mean | Grouping |
|---|---|---|---|
| T3 | 2 | 6.3655 | A |
| C2 | 2 | 5.0073 | B |
| T2 | 2 | 4.9814 | B |
| C1 | 2 | 4.71179 | B |
| T1 | 3 | 4.490 | B |

*Means that do not share a letter are significantly different.*

## B.4 Fourth time point- Day 15

| samples | count | log cfu/ml |
|---|---|---|
| T1 | 33000 | 4.518514 |
| T1 | 30000 | 4.477121 |

| | | |
|---|---|---|
| T1 | 40000 | 4.60206 |
| T2 | 31000 | 4.491362 |
| T2 | 73000 | 4.863323 |
| T2 | 53000 | 4.724276 |
| T3 | 140000 | 5.146128 |
| T3 | 120000 | 5.079181 |
| T3 | 180000 | 5.255273 |
| C1 | 46000 | 4.662758 |
| C1 | 44000 | 4.643453 |
| C2 | 34000 | 4.531479 |
| C2 | 31000 | 4.491362 |

## Statistics

| Variable | samples | N | N* | Mean | SE Mean | StDev | Minimum | Q1 | Median | Q3 | Maximum |
|---|---|---|---|---|---|---|---|---|---|---|---|
| log cfu/ml | C1 | 2 | 0 | 4.6531 | 0.00965 | 0.0137 | 4.6435 | * | 4.6531 | * | 4.6628 |
| | C2 | 2 | 0 | 4.5114 | 0.0201 | 0.0284 | 4.4914 | * | 4.5114 | * | 4.5315 |
| | T1 | 3 | 0 | 4.5326 | 0.0367 | 0.0636 | 4.4771 | 4.4771 | 4.5185 | 4.6021 | 4.6021 |
| | T2 | 3 | 0 | 4.693 | 0.109 | 0.188 | 4.491 | 4.491 | 4.724 | 4.863 | 4.863 |
| | T3 | 3 | 0 | 5.1602 | 0.0513 | 0.0889 | 5.0792 | 5.0792 | 5.1461 | 5.2553 | 5.2553 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| samples | 4 | 0.78363 | 0.19591 | 16.40 | 0.001 |
| Error | 8 | 0.09554 | 0.01194 | | |
| Total | 12 | 0.87917 | | | |

# Tukey Pairwise Comparisons

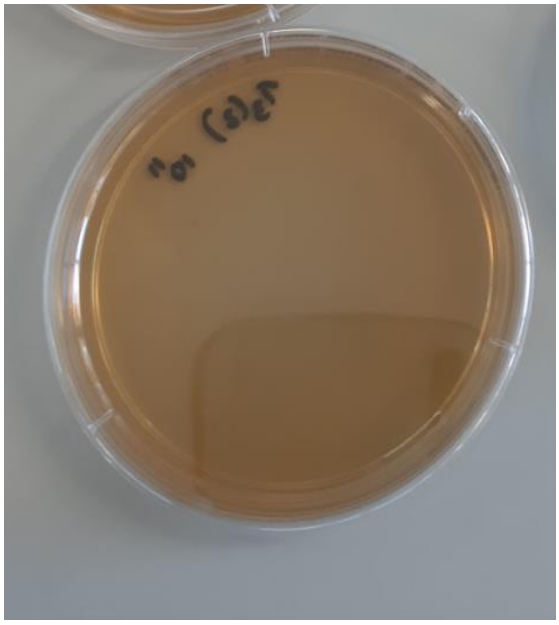
## Grouping Information Using the Tukey Method and 95% Confidence

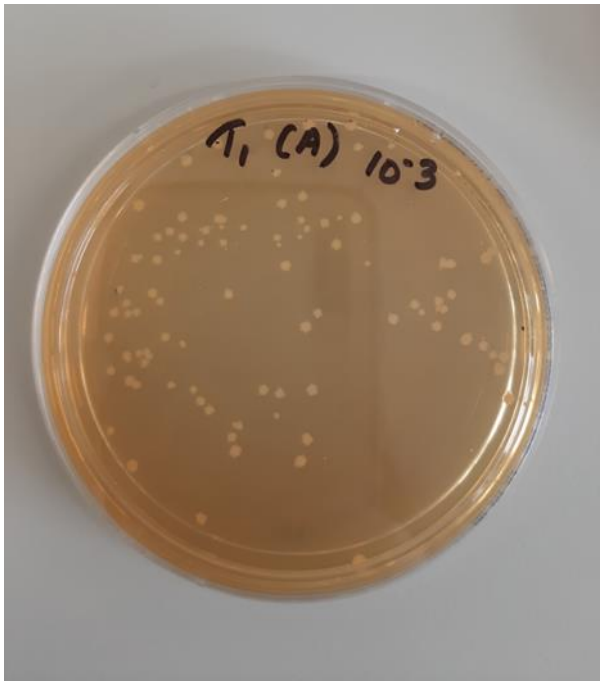
| samples | N | Mean | Grouping |
|---|---|---|---|
| T3 | 3 | 5.1602 | A |
| T2 | 3 | 4.693 | B |

| | | | |
|---|---|---|---|
| C1 | 2 | 4.65311 | B |
| T1 | 3 | 4.5326 | B |
| C2 | 2 | 4.5114 | B |

*Means that do not share a letter are significantly different.*

## B.5 Colony visualization on MRS agar plates





# Over shelf life

| days | sample | log cfu/ |
|---|---|---|
| 4 | T1 | 4.76 |
| 4 | T1 | 4.57 |
| 4 | T2 | 3.85 |
| 4 | T2 | 3.7 |
| 4 | T3 | 4.23 |
| 4 | T3 | 4 |
| 4 | T3 | 3.78 |
| 4 | C1 | 3.95 |
| 4 | C1 | 4.4 |
| 4 | C1 | 4.18 |
| 4 | C2 | 4.15 |
| 4 | C2 | 4.15 |
| 8 | T1 | 4.45 |
| 8 | T1 | 4.52 |
| 8 | T1 | 4.53 |
| 8 | T2 | 5.17 |
| 8 | T2 | 5.26 |
| 8 | T3 | 4.53 |
| 8 | T3 | 4.57 |
| 8 | C1 | 4.91 |
| 8 | C1 | 4.81 |
| 8 | C2 | 5.11 |
| 8 | C2 | 5.04 |
| 11 | T1 | 3.85 |
| 11 | T1 | 4.82 |
| 11 | T1 | 4.81 |
| 11 | T2 | 5.01 |
| 11 | T2 | 4.95 |
| 11 | T3 | 6.34 |
| 11 | T3 | 6.39 |
| 11 | C1 | 4.71 |
| 11 | C1 | 4.72 |
| 11 | C2 | 5.04 |
| 11 | C2 | 4.97 |
| 15 | T1 | 4.52 |
| 15 | T1 | 4.48 |
| 15 | T1 | 4.60 |
| 15 | T2 | 4.49 |
| 15 | T2 | 4.86 |
| 15 | T2 | 4.72 |
| 15 | T3 | 5.15 |
| 15 | T3 | 5.08 |
| 15 | T3 | 5.26 |
| 15 | C1 | 4.66 |
| 15 | C1 | 4.64 |
| 15 | C2 | 4.53 |
| 15 | C2 | 4.49 |

# General Linear Model: log cfu/ml for T1 versus T1 days

## Method

Factor coding (-1, 0, +1)

## Factor Information

| Factor | Type | Levels | Values |
|---|---|---|---|
| T1 days | Fixed | 4 | 4, 8, 11, 15 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| T1 days | 3 | 0.04265 | 0.01422 | 0.15 | 0.925 |
| Error | 7 | 0.65082 | 0.09297 | | |
| Total | 10 | 0.69347 | | | |

## Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.304916 | 6.15% | 0.00% | 0.00% |

## Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | 4.5477 | 0.0934 | 48.71 | 0.000 | |
| T1 days | | | | | |
| 4 | 0.117 | 0.179 | 0.66 | 0.533 | 1.69 |
| 8 | -0.048 | 0.156 | -0.31 | 0.768 | 1.56 |
| 11 | -0.054 | 0.156 | -0.35 | 0.737 | 1.56 |

## Regression Equation

log cfu/ml for T1 = 4.5477 + 0.117 T1 days_4 - 0.048 T1 days_8 - 0.054 T1 days_11 - 0.015 T1 days_15

## Fits and Diagnostics for Unusual Observations

| Obs | log cfu/ml for T1 | Fit | Resid | Std Resid | |
|---|---|---|---|---|---|
| 6 | 3.850 | 4.493 | -0.643 | -2.58 | R |

*R Large residual*

# Grouping Information Using the Tukey Method and 95% Confidence

| T1 days | N | Mean | Grouping |
|---|---|---|---|
| 4 | 2 | 4.66500 | A |
| 15 | 3 | 4.53257 | A |
| 8 | 3 | 4.50000 | A |
| 11 | 3 | 4.49333 | A |

*Means that do not share a letter are significantly different.*

# General Linear Model: log cfu/ml for T2 versus T2 days

## Method

Factor coding (-1, 0, +1)

## Factor Information

| Factor | Type | Levels | Values |
|---|---|---|---|
| T2 days | Fixed | 4 | 4, 8, 11, 15 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| T2 days | 3 | 2.38987 | 0.79662 | 45.39 | 0.000 |
| Error | 5 | 0.08775 | 0.01755 | | |
| Total | 8 | 2.47762 | | | |

## Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.132473 | 96.46% | 94.33% | 90.82% |

## Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | 4.6657 | 0.0448 | 104.05 | 0.000 | |
| T2 days | | | | | |
| 4 | -0.8907 | 0.0800 | -11.14 | 0.000 | 1.78 |
| 8 | 0.5493 | 0.0800 | 6.87 | 0.001 | 1.78 |
| 11 | 0.3143 | 0.0800 | 3.93 | 0.011 | 1.78 |

## Regression Equation

log cfu/ml for T2 = 4.6657 - 0.8907 T2 days_4 + 0.5493 T2 days_8 + 0.3143 T2 days_11 + 0.0272 T2 days_15

## Grouping Information Using the Tukey Method and 95% Confidence

| T2 days | N | Mean | Grouping | | |
|---|---|---|---|---|---|
| 8 | 2 | 5.21500 | A | | |
| 11 | 2 | 4.98000 | A | B | |
| 15 | 3 | 4.69299 | | B | |
| 4 | 2 | 3.77500 | | | C |

*Means that do not share a letter are significantly different.*

## Tukey Pairwise Comparisons: C1 days

### Grouping Information Using the Tukey Method and 95% Confidence

| C1 days | N | Mean | Grouping | |
|---|---|---|---|---|
| 8 | 2 | 4.86000 | A | |
| 11 | 2 | 4.71500 | A | |
| 15 | 2 | 4.65311 | A | B |
| 4 | 3 | 4.17667 | | B |

*Means that do not share a letter are significantly different.*

# General Linear Model: log cfu/ml for C1 versus C1 days

### Method

Factor coding (-1, 0, +1)

### Factor Information

| Factor | Type | Levels | Values |
|---|---|---|---|
| C1 days | Fixed | 4 | 4, 8, 11, 15 |

### Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| C1 days | 3 | 0.6859 | 0.22863 | 10.73 | 0.013 |
| Error | 5 | 0.1065 | 0.02130 | | |
| Total | 8 | 0.7924 | | | |

### Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.145947 | 86.56% | 78.50% | 68.60% |

### Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | 4.6012 | 0.0494 | 93.14 | 0.000 | |
| C1 days | | | | | |
| 4 | -0.4245 | 0.0774 | -5.48 | 0.003 | 1.38 |
| 8 | 0.2588 | 0.0881 | 2.94 | 0.032 | 1.46 |
| 11 | 0.1138 | 0.0881 | 1.29 | 0.253 | 1.46 |

### Regression Equation

log cfu/ml for C1 = 4.6012 - 0.4245 C1 days_4 + 0.2588 C1 days_8 + 0.1138 C1 days_11 + 0.0519 C1 days_15

## Grouping Information Using the Tukey Method and 95% Confidence

| T3 days | N | Mean | Grouping | | | |
|---|---|---|---|---|---|---|
| 11 | 2 | 6.36500 | A | | | |
| 15 | 3 | 5.16019 | | B | | |
| 8 | 2 | 4.55000 | | | C | |
| 4 | 3 | 4.00333 | | | | D |

*Means that do not share a letter are significantly different.*

## Grouping Information Using the Tukey Method and 95% Confidence

| C2 days | N | Mean | Grouping | | |
|---|---|---|---|---|---|
| 8 | 2 | 5.07500 | A | | |
| 11 | 2 | 5.00500 | A | | |
| 15 | 2 | 4.51142 | | B | |
| 4 | 2 | 4.15000 | | | C |

*Means that do not share a letter are significantly different.*

# General Linear Model: log cfu/ ml of C2 versus C2 days

## Method

Factor coding (-1, 0, +1)

## Factor Information

| Factor | Type | Levels | Values |
|---|---|---|---|
| C2 days | Fixed | 4 | 4, 8, 11, 15 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| C2 days | 3 | 1.14171 | 0.380570 | 266.85 | 0.000 |
| Error | 4 | 0.00570 | 0.001426 | | |
| Total | 7 | 1.14741 | | | |

## Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.0377647 | 99.50% | 99.13% | 98.01% |

## Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | 4.6854 | 0.0134 | 350.91 | 0.000 | |
| C2 days | | | | | |
| 4 | -0.5354 | 0.0231 | -23.15 | 0.000 | 1.50 |
| 8 | 0.3896 | 0.0231 | 16.85 | 0.000 | 1.50 |
| 11 | 0.3196 | 0.0231 | 13.82 | 0.000 | 1.50 |

## Regression Equation

log cfu/ ml of C2 = 4.6854 - 0.5354 C2 days_4 + 0.3896 C2 days_8 + 0.3196 C2 days_11 - 0.1739 C2 days_15

# General Linear Model: log cfu/ml for T3 versus T3 days

## Method

Factor coding (-1, 0, +1)

## Factor Information

| Factor | Type | Levels | Values |
|---|---|---|---|
| T3 days | Fixed | 4 | 4, 8, 11, 15 |

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| T3 days | 3 | 7.1423 | 2.38077 | 119.92 | 0.000 |
| Error | 6 | 0.1191 | 0.01985 | | |
| Total | 9 | 7.2614 | | | |

## Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.140900 | 98.36% | 97.54% | 96.26% |

## Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | 5.0196 | 0.0455 | 110.38 | 0.000 | |
| T3 days | | | | | |
| 4 | -1.0163 | 0.0733 | -13.86 | 0.000 | 1.63 |
| 8 | -0.4696 | 0.0839 | -5.60 | 0.001 | 1.74 |
| 11 | 1.3454 | 0.0839 | 16.04 | 0.000 | 1.74 |

## Regression Equation

log cfu/ml for T3 = 5.0196 - 1.0163 T3 days_4 - 0.4696 T3 days_8 + 1.3454 T3 days_11 + 0.1406 T3 days_15

# *Appendix C*

## Antimicrobial activity against *E. coli*

### C.1 *E. coli* study

| sample | L. bulgaricus mean | E. coli mean | L error | E error |
|---|---|---|---|---|
| C1 | 4.871 | 0 | 0.187 | 0 |
| C2 | 4.595 | 0 | 0.109 | 0 |
| C3 | 4.138 | 1.37 | 0.359 | 1.37 |
| T1 | 4.976 | 0 | 0.33 | 0 |
| T2 | 4.4841 | 0 | 0.0983 | 0 |
| T3 | 4.73 | 0 | 0.309 | 0 |

**Analysis of Variance**

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Sample | 5 | 1.8068 | 0.3614 | 3.09 | 0.037 |
| Dil | 1 | 2.6908 | 2.6908 | 23.00 | 0.000 |
| Error | 17 | 1.9886 | 0.1170 | | |
| Lack-of-Fit | 5 | 0.7653 | 0.1531 | 1.50 | 0.261 |
| Pure Error | 12 | 1.2233 | 0.1019 | | |
| Total | 23 | 6.4862 | | | |

*L. bulgaricus* counts

**Grouping Information Using the Tukey Method and 95% Confidence**

| Sample | N | Mean | Grouping | |
|---|---|---|---|---|
| T1 | 4 | 4.97550 | A | |
| C1 | 4 | 4.87054 | A | B |
| T3 | 4 | 4.72953 | A | B |
| C2 | 4 | 4.59488 | A | B |
| T2 | 4 | 4.48413 | A | B |
| C3 | 4 | 4.13798 | | B |

*Means that do not share a letter are significantly different.*

## Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Sample | 5 | 6.250 | 1.250 | 1.00 | 0.447 |
| Dil | 1 | 1.250 | 1.250 | 1.00 | 0.331 |
| Error | 17 | 21.249 | 1.250 | | |
| Lack-of-Fit | 5 | 6.250 | 1.250 | 1.00 | 0.458 |
| Pure Error | 12 | 14.999 | 1.250 | | |
| Total | 23 | 28.749 | | | |

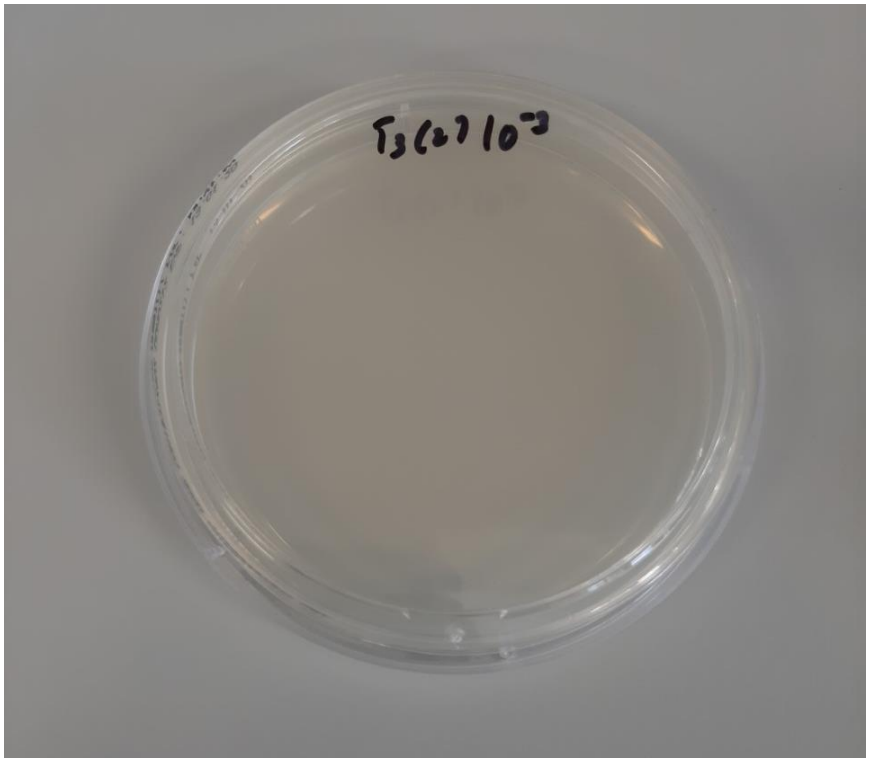
*E. coli* values

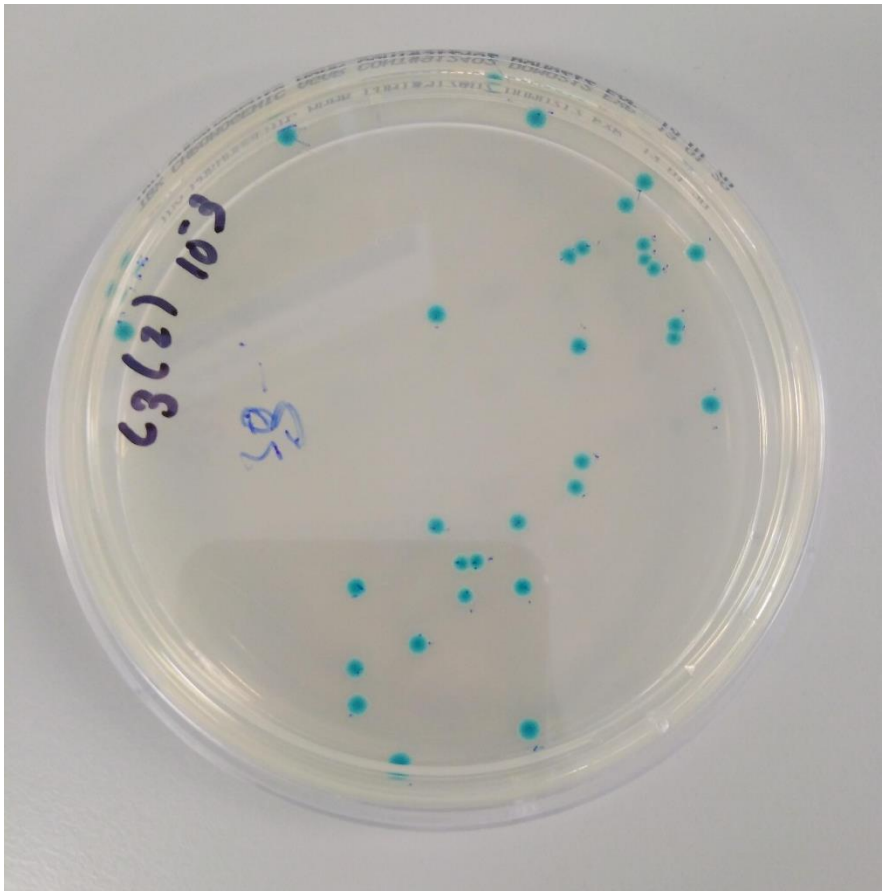
## Grouping Information Using the Tukey Method and 95% Confidence

| Sample | N | Mean | Grouping |
|---|---|---|---|
| C3 | 4 | 1.36928 | A |
| T2 | 4 | 0.00000 | A |
| T3 | 4 | 0.00000 | A |
| C1 | 4 | -0.00000 | A |
| C2 | 4 | -0.00000 | A |
| T1 | 4 | -0.00000 | A |

*Means that do not share a letter are significantly different.*

## C.2 Colony visualization in TBX plates




(3(2) 10-3