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# **Characterisation of starter culture for yoghurt design**

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## **Characterisation of starter culture for yoghurt design**

### **Abstract:**

Yoghurt is produced by the fermentation of milk by two primary thermophilic lactic acid bacteria - *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. These two bacteria are the most essential components of the starter culture that makes up this dairy product. The thesis work represents part of a larger project, where a number of yoghurt strains are characterized as an input to a modeling approach for genotype-to-phenotype predictions for the selection of the best consortia. In the current study, batch experiments in anaerobic bottles were carried out for one strain of each microorganism and these two strains in a consortium. Growth curves were collected by measuring the optical density of samples over time and extracellular metabolites were analysed using High-Performance Liquid Chromatography. As results, the growth of the strains is compared as monoculture and co-culture and a qualitative analysis of metabolites is performed. The specific growth rate of the co-culture is higher than that of the mono cultures. Compounds contributing to the flavour of the yoghurt are detected amongst others.

### **Keywords:**

*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, starter culture, yoghurt.

**CERCS:** T490 Biotechnology

## **Jogurtikujunduse stardikultuuri iseloomustus.**

### **Lühikokkuvõte:**

Jogurtit toodetakse kääritamise meetodil, kus põhikomponendiks on kaks primaarset termofiilset piimhappebakterit - *Streptococcus thermophilus* ja *Lactobacillus delbrueckii* subsp. *bulgaricus*. Need kaks bakterit on antud piimatoote koostises oleva algkultuuri kõige olulisemad komponendid. Lõputöö on osa suuremast projektist, kus kirjeldatakse mitmeid jogurtitüvesid, kui sisendit genotüübi ja fenotüübi ennustamise modelleerimisele, parima konsortsiumi valimisel. Selles uuringus tehti anaeroobsetes pudelites partiikatseid, iga

mikroorganismi ühe tüve ja nende kahe tüve kohta konsortsiumis. Kasvukõverad koguti, mõõtes proovide optilist tihedust aja jooksul ja rakuväliseid. Metaboliite analüüsiti kõrgtehnoloogilise vedelikkromatograafia abil. Tüvede kasvu võrreldakse monokultuuri ja ühiskultuurina ning viiakse läbi metaboliitide kvalitatiivne analüüs. Kopokultuuri erikasvumäär on kõrgem kui monokultuuridel. Muu hulgas tuvastatakse jogurti maitset soodustavad ühendid.

**Võtmesõnad:**

*Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. Bulgaricus*, algkultuur, jogurt

**CERCS:** T490 Biotehnoloogia

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## **TERMS, ABBREVIATIONS AND NOTATIONS**

***B.infantis:*** *Bifidobacterium Infantis*

***B.lactis:*** *Bifidobacterium Lactis*

**B-cells:** also known as B lymphocytes

**CAGR:** Compound Annual Growth Rate

**CDM:** Chemically defined medium

**EPSs:** Exopolysaccharides

**FDA:** Food and Drug Administration

**GRAS:** Generally recognized as safe

**HPLC:** High-Performance Liquid Chromatography

**IgA:** Immunoglobulin A

***L. acidophilus:*** *Lactobacillus acidophilus*

***L. bulgaricus:*** *Lactobacillus delbrueckii subsp. bulgaricus*

***L. paracasei:*** *Lactobacillus paracasei*

***L.casei:*** *Lactobacillus Casei*

***L.monocytogenes:*** *Listeria monocytogenes*

**LAB:** Lactic acid bacteria

**MQ water:** Milli-Q water

**OD:** Optical density

***S. thermophilus:*** *Streptococcus thermophilus*

## INTRODUCTION

A starter culture is a mixture of micro-organisms that is required to carry out the fermentation process. The starter culture needed to design yoghurt mainly comprises *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Several strains of these 2 species exist or can be synthesized. Hence, there are many variations about the way they can interact with each other to produce yoghurt. The properties of this product depend heavily on the growth of the culture and the types and concentrations of metabolites produced during the fermentation. For example, the concentration of lactic acid in yoghurt determines the sourness of the product in terms of flavour. It is important to characterize the strains being used as starter culture to monitor to kind of yoghurt being manufactured as product.

The growth of the microorganisms is observed in batch fermentation in bottles and bioreactors experiments. The batch cultivation experiment is first carried out in a rich medium and then in a chemically defined medium. The latter is in general a concoction of all the compounds needed to mimic the milk medium. We refrain to use milk in the primary stage to maintain control over the concentration of every compounds we supply to the culture to grow. We are therefore able to understand the change in a certain compound level better during analysis.

The organisms are first grown separately as monocultures and as a co-culture as well. *S. thermophilus* and *L. bulgaricus* are known, in the food industry, to exhibit proto cooperation. The latter is the foundation to create a symbiotic relation between the 2 species. The combined metabolism has many positive effects on the fermented product. These effects are explained in the “literature review” section. Additionally, the concomitant action of peptides cleavage by the culture is important to reduce allergic reactivity to  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. This allergy affects approximately 2% of adults and more that 5% of infants (Bu G et al., 2010).

All the goals were not achieved fully during the research. However, this thesis work is the base on which more conclusive work can be carried out such as a bioreactor experiment using the chemically defined medium and eventually milk to investigate the growth and characteristics of the *S. thermophilus* and *L. bulgaricus* strains.

# 1 LITERATURE REVIEW

## 1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of bacteria that convert carbohydrates such as lactose into lactic acid as the main metabolic end-product. Lactose is transported into the cells by a permease. It is broken down into glucose and galactose by  $\beta$ -galactosidase. Through the glycolytic pathway, glucose is quickly phosphorylated into pyruvic acid. The intermediate compounds of this reaction are 2 triose phosphates. Finally, lactate dehydrogenase converts pyruvic acid into lactic acid. Galactose is removed from the cell as a by-product and undergoes no fermentation (Ettore Baglio, 2014). The overall chemical equation of lactic acid formation is as follows:



These bacteria grow best in a slightly acidic environment. Lactic acid bacteria are prone to develop in anaerobic conditions. Another characteristic is that lactic acid bacteria are generally non-sporulating. The optimal temperature of lactic acid bacteria is within the range of 25 °C to 45 °C. The value differs for each bacterium. For example, *L. acidophilus* grows best at around 41 °C while *L. paracasei* at 30°C (Adamberg et al., 2002).

In a broader physiological meaning, lactic acid bacteria comprise 20 genera. However, the food-technology industry considers the following as the key LAB: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*.

Lactic acid bacteria are known to be ‘generally recognized as safe’ (GRAS). The latter is a legal term for any food additive that is approved by FDA to be safe under the conditions of its use. However, some species, most commonly members of the *Streptococcus* genus are pathogenic. For instance, in humans, *Streptococcus pyogenes* causes pneumonia and *Streptococcus mutans* lead to tooth decay (Mandell et al., 2015).

Lactic acid bacteria have contributed largely to the food industry. They are involved in the fermentation to manufacture food products such as yoghurt, cheese, pickles, and so forth. They contribute to the texture and aroma of the food items. Moreover, being one of the traditional ways of food preservation, they extend the shelf life of the products. Some lactic acid bacteria can produce antimicrobial compounds such as hydrogen peroxide, organic

acids and bacteriocins. They are heat-resistant and operate over a wide pH range. For example, the class IIa bacteriocins inhibit the growth of food-borne pathogen *L.monocytogenes* which is reported to spoil ready-to-eat food products. Lactic acid bacteria show an encouraging potential in the pharmaceutical and food preservation industries (Perez et al., 2014).

A starter culture is a microbial mixture which carries out fermentation. Some varieties of cultures consist of lactic acid bacteria. It is added so that desired characteristics of the medium (e.g. milk) may be observed in a controllable manner to obtain the end-products (e.g. cheese). Starter cultures can also be used in alcohol production. In yoghurt, the main task of the starter culture is to produce the optimum level of lactic acid within a certain period. Rapid acidification shortens the manufacturing time and reduces syneresis: whey separation during the milk fermentation. Hence, a high moisture content is achievable (Angelis et al., 2016). The level of acid that should be present in the yoghurt is around 0.85% to 0.95%. (Tamime and Robinsons, 2007). This amount determines relatively low pH values (4.0 to 4.5) since the fermentative pathway does not entail other processes and related by-products (Ettore Baglio, 2014).

Many lactic acid bacteria are auxotrophic for several amino acids. This means they do not have metabolic pathways to produce these compounds themselves. They must obtain them from the outer environment. Milk provides a favourable environment for the growth of LAB because it can provide proteins, lactose, minerals, and other elements. The amount of proteins present determines the viscosity of the yoghurt. For instance, yoghurt manufactured from unfortified mare's milk would be less viscous than that made from sheep's milk. The concentration of protein in sheep's milk (5.6 g 100 g<sup>-1</sup>) is higher than that of the mare's milk (2.6 g 100 g<sup>-1</sup>) (Tamime and Robinsons, 2007). In milk, lactose is the main energy source for the starter culture. Furthermore, it helps to control the type of growth which is needed for a product as we can assess the chemical compositions of milk from different sources. Generally, the cow's milk is used for yoghurt production as it is widely available (Tamime and Robinsons, 2007).

## **1.2 Yoghurt**

Yoghurt is produced by the fermentation of milk by two primary thermophilic lactic acid bacteria - *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chen et al., 2016). In many countries, the term *yoghurt* is allocated only to the products that contain a starter culture with both lactic acid bacteria (West et al., 2006). The

microstructure of yoghurt is made up of a protein matrix. It is composed of casein micelle chains and clusters. Fat globules are embedded in this protein matrix. (Tamime and Robinsons, 2007).

Yoghurt is recognised as a healthy product mainly due to the presence of probiotics. The addition of the microorganism to fermented milk products have gained popularity since the health benefits of bacteria from the *Lactobacillus* and *Bifidobacterium* species were recognised. Probiotics are living bacteria and yeasts that are often referred to as the “good” microorganisms for the human body. They are designed and added to food, especially in dairy products, to deliver beneficial bacterial cells to the human microbiota. Their main function is to strengthen the immune system against potentially dangerous microorganisms. Some other health benefits are the prevention of oxidative stress and improving the regular function of the colon and stomach (Shori et al., 2019). Some examples of probiotics, belonging to the lactic acid bacteria group, could be from the *Lactobacillus* species such as *L. acidophilus*, *L. casei* and *L. bulgaricus* and the *Bifidobacterium* species *B. infantis*, *B. lactis*, *L. casei* displays the highest level of effectiveness. An increase in the number of cells producing IgA (Immunoglobulin A) in the small intestines of mice was observed for each of the probiotics (Perdigon et al., 1995). IgA is an antibody produced by B-cells. It helps fight infections in the human body as part of the adaptive humoral immune defence. Other possible health benefits of probiotics include anticancer effects. It also relieves the symptoms of Crohn’s disease. (Prescott Harley Klein, 2008).

The practice of yoghurt fortification with iron is gaining acceptance among the manufacturers and consumers (Blanc 1981). Dairy food rich in iron is known to have a relatively high iron bioavailability. Iron bioavailability refers to the percentage of the mineral which is absorbed in the duodenum and used during metabolic pathways or stored over the amount of iron ingested. Yoghurt fortification can be used to complement a diet with low iron consumption. Besides, people who consume less iron-rich diets are more likely to consume more dairy products (Routray et al., 2011).

*Mordor Intelligence* carried out a yoghurt market overview during the forecast period of 2019-2024. Their results show that its growth scope, at a CAGR (Compound Annual Growth Rate) of 3.2 % is expected to reach EUR 35.89 billion by 2024. The report by *Mordor Intelligence* also depicts that Germany and France hold the major shares of the yoghurt market in terms of revenue while Scandinavian countries - despite low revenues- have the highest CAGR. The demand of this dairy product has increased mainly due to the associated

health benefits and recent trends. Yoghurt has also been modified to enter the beverage market. Drinking yoghurt has gained popularity because of its convenience for consumption. On a microbiology level, the trend may be turning towards milder-tasting yoghurts. The consumers are more likely to opt for less acidity. This may lead to a new-engineered starter culture. The combinations of the lactic acid bacteria would result in a high count of *S. thermophilus* and a lower count of *L. bulgaricus* (Tamime et al., 2002).

### 1.3 *Streptococcus thermophilus*

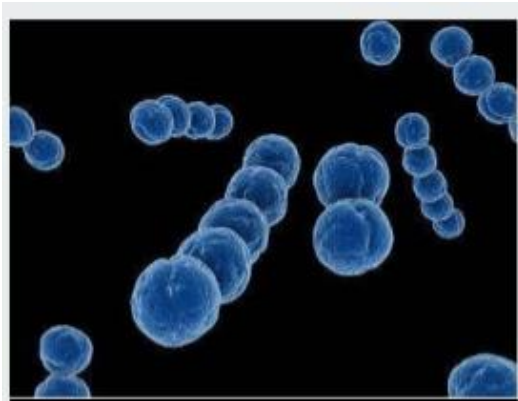


Figure 1. *Streptococcus thermophilus*

*Streptococcus thermophilus* is a spherical cell and they can be usually observed in pairs or chains (Figure 1). *S. thermophilus* is a Gram-positive bacteria which means it has a thick peptidoglycan layer in the bacterial cell wall. This feature may come in handy during the interaction with the environment, cell-to-cell communication and resistance to stress conditions (such as osmotic pressure) (Hols et al., 2005). This species is ranked as the second most important industrial dairy starter after *Lactococcus lactis* (Chaudron et al., 2002). The reason may be due to its extensive use for the past 2 decades following the increase in popularity of yoghurt and mozzarella cheese.

It is a facultative anaerobe. This means that it can produce ATP by aerobic respiration in the presence of oxygen but can switch to fermentation or anaerobic respiration if there is a lack of oxygen.

*S. thermophilus* prefers the temperature of 40°C for optimal growth. No growth can be observed at temperatures lower than 15°C. It can survive a high one (60°C) for approximately 30 minutes. This may be different depending on the strains (Tamime and Robinsons, 2007). According to Adamberg et al., *S. thermophilus* is more sensitive to pH change. A strain St20, stopped growing at pH 5.1. The experiment was carried out in a pH-auxostat with a smooth decrease in pH from 6.5 to 4.2 for different lactic acid bacteria. This research suggests that the higher the maximum growth rate of the strain, the more sensitive

its growth is to a decrease of external pH (Adamberg et al., 2002). The optimum pH is found to be 5.5 (Zisu and Shah, 2003).

Most strains of *S. thermophilus* produce exopolysaccharides (EPSs) (Cerning et al., 1995). EPSs are polymers made up of mostly sugar polysaccharides. They have a high molecular weight. Their role is to bring the cells in the medium closer to one another (Siewverts, 2009). In the yoghurt production, EPSs are likely to provide a likeable “mouth-feel” texture to the product (De Vuyst et al., 2001). Moreover, it may prevent syneresis. (Duboc et al., 2001).

The principal usage of *S. thermophilus* in dairy fermentation is to produce lactic acid. It is also associated with the production of formic acid, diacetyl, and acetate (Tinson et al., 1982). Formic acid acts as a cofactor in the purine biosynthesis. Diacetyl is a flavour compound that contributes to the buttery and creaminess flavour of yoghurt (Chen et al., 2016). Compounds such as ethyl acetate and butyl acetate are esters that provide a fruity flavour to yoghurt. Diacetyl (also known as 2,3-butanedione), together with acetaldehyde, is an important aromatic compound in yoghurt. At the start, due to the presence of small amounts of oxygen, *S. thermophilus* may undergo aerobic sugar metabolism which results in formation of carbon dioxide, alpha-acetolactate and acetoin. Alpha-acetolactate eventually undergoes chemical oxidative decarboxylation to produce diacetyl (Hols et al., 2005).

#### **1.4 *Lactobacillus bulgaricus***



*Lactobacillus bulgaricus* cells are in a rod shape with rounded ends (Figure 2). They are either observed as single cells or as chains. *L. bulgaricus* has similar characteristics to *S. thermophilus*. It is also facultative anaerobe, Gram-positive and non-motile. (Song et al., 2017).

In yoghurt, *L. bulgaricus* is the main producer of acetaldehyde which gives the yoghurt a typical yoghurt-like flavour. Acetaldehyde can be formed by breaking down threonine to acetaldehyde and glycine by the enzyme threonine aldolase (Zourari et al., 1992). The enzyme activity in *S. thermophilus* significantly decreases at high temperature during fermentation. However,

it remains constant in *L. bulgaricus*. Hence as yoghurt is produced at such temperature, we can assume that *L. bulgaricus* is forming this flavour compound.

This microorganism ferments fewer types of sugars and its optimum growth temperature is around 50°C. As compared to *S. thermophilus*, a slight growth can be detected at temperatures lower than 10°C. This indicates that *L. bulgaricus* may be more resistant to changes in temperature (Tamime and Robinsons, 2007). Acid tolerance is a property that is strain specific. It is important as the sugar present in milk is generally enough to decrease pH to a value of 4.5 or less. *L. bulgaricus* is more resistant to pH change than *S. thermophilus*. Adamberg et al. demonstrated that at pH 5 the growth rate of *L. bulgaricus* strain Lb12 was 51% while that of *S. thermophilus* St20 close to 0% of the maximum growth rate. Its optimum pH was found to be in the range of 5.8 to 6.2 where the specific growth rate was almost constant (Adamberg et al., 2002).

The main role of *L. bulgaricus* is to release amino acids such as valine, leucine, histidine, and methionine from the milk proteins. The expression of protease PrtB initiates this process. This enzyme catalyses the hydrolysis reaction of caseins into peptides (Juille et al., 2005). The peptides are broken down to amino acids in the cytoplasm by various endopeptidases (they break down peptide bonds within the protein molecules) and aminopeptidases (or exopeptidases; they catalyse the reaction from the N-terminal of the protein). Eventually, this leads to an additional source of amino acids (Rul et al., 1997).

## 1.5 Proto cooperation

Interactions between two different organisms can be distributed into 6 main groups depending on the influence caused on the effector and the target (Table 1). These are neutralism, amensalism, competition, commensalism, parasitism, and mutualism. Neutralism occurs when two species interact with each other without influencing fitness and growth on each other. Amensalism results in the destruction or inhibition of the target while the effector gets no detrimental effects. During a competitive growth both species in the consortium undergo limited growth and have adverse effects. Some of the aspects they may compete for are space or availability of nutrients. Parasitism is a type of symbiotic relationship where one species, the parasite or effector, benefits at the expense of the target.

The effector exhibits detrimental behaviour which obstructs the growth of the second species (Sieuwerts, 2016).

**Table 1: The six main classes of microbial interactions based on their beneficial and detrimental effects on fitness of the effector and target microorganisms.**

Effect on target	Effect on effector		
	Beneficial	Detrimental	Neutral
Beneficial	Mutualism		Commensalism
Detrimental	Parasitism	Competition	Amensalism
Neutral			Neutralism

*S. thermophilus* and *L. bulgaricus* have mutual communication. They may interact with each other by directly communicating via signalling molecules. Since, both are gram-positive, they probably use modified oligopeptides as autoinducers (Sturme et al., 2002). Another mode is by the division of labour by growth factor exchange or the aftermath of physico-chemical alterations of the environment.

During fermentation, *S. thermophilus* and *L. bulgaricus* perform various biochemical conversions of milk (Smit et al., 2005). The most important reactions are glycolysis, proteolysis, and lipolysis (G. Smit et al., 2005). Glycolysis refers to the conversion of lactose (more generally, carbohydrate) into lactic acid, ethanol, carbon dioxide and acetic acid. Proteolysis deals with the breakdown of caseins into peptides and free amino acids. Finally, lipolysis reduces milk fat into fatty acids (Endo et al., 2014).

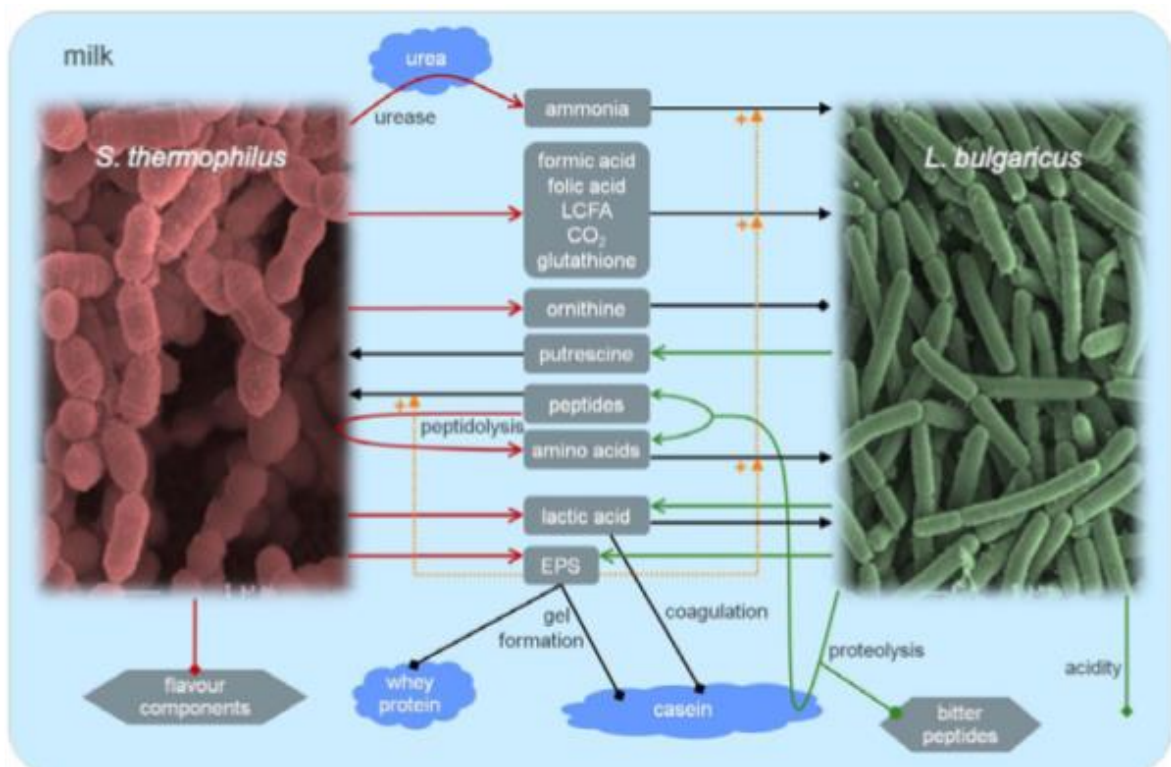


Figure 3. A representation of the interactions that occur between *S. thermophilus* and *L. bulgaricus* during fermentation in the milk medium (Sieuwerts, 2016)

Proto cooperation is the basis for creation of an associative relation between the two species and combined metabolism with positive effects on the fermented product (Figure 4). Although *S. thermophilus* and *L. bulgaricus* do grow separately and ferment milk in monocultures, the combined effect of these two lactic acid bacteria has more advantages. This means they are mutually beneficial but not interdependent. They both secrete and produce compounds along the fermentation that help the other bacteria to grow. The figure 3 from *Sieuwerts'* article provides a good illustration of the interactions. For example, ornithine, produced by *S. thermophilus*, affects the growth of *L. bulgaricus* while putrescine has a positive effect on *S. thermophilus*. The functions of the two compounds are not clarified yet. However, some possibilities are the formation of carbon dioxide and the usage of putrescine as a cofactor in cell division (Sieuwerts, 2009). The urease activity of *S. thermophilus* is hypothesised to increase *L. bulgaricus*'s growth by increasing the pH. (Arioli et al., 2016) From the urea present in milk, *S. thermophilus* produces carbon dioxide which is a precursor for the formation of amino acids (such as aspartate and arginine) and some nucleotides (Bringel et al., 2003). *S. thermophilus* being more aerotolerant and more tolerant to neutral pH than *L. bulgaricus* starts to grow first. By doing so, it consumes the oxygen found in the medium and produces carbon dioxide, making the external environment

more anaerobic. *L. bulgaricus* benefits from the deoxygenated environment and starts to grow (Sasaki et al., 2014). *S. thermophilus* produces folic acid and formic acid (Van de Guchte et al., 2006). During the transition phase, *S. thermophilus* stops growing due to a lack of amino acids. Moreover, *S. thermophilus* do not exhibit extracellular proteases (Hols et al., 2005). *L. bulgaricus* promotes its growth by enabling a source of amino acids which can be easily taken up by *S. thermophilus*. This promotes the second exponential growth of *S. thermophilus* (Sieuwerts et al., 2010). Glutathione (an antioxidant) production by *S. thermophilus* relieves the acid stress in *L. bulgaricus*, improving its growth. However, this deacidification process occurs only locally. This shows the importance of the bacteria being within a short distance in the consortium (Wang et al., 2016).

All these interactions lead to the final properties of yoghurt. It is therefore important to have the correct strains associated during the fermentation and in the correct ratio. This is to ensure that there is the most efficient mutual growth. For instance, high levels of *L. bulgaricus* is shown to be associated with the acidity and bitterness of yoghurt as it is mostly responsible for lactic acid production (Roudot Algaron F., 1996). *S. thermophilus* contributes to the flavour and aroma of yoghurt. It produces aroma compounds such as acetoin and diacetyl. These help to give the product its typical flavour, with the help of acetaldehyde (Pastink et al., 2008).

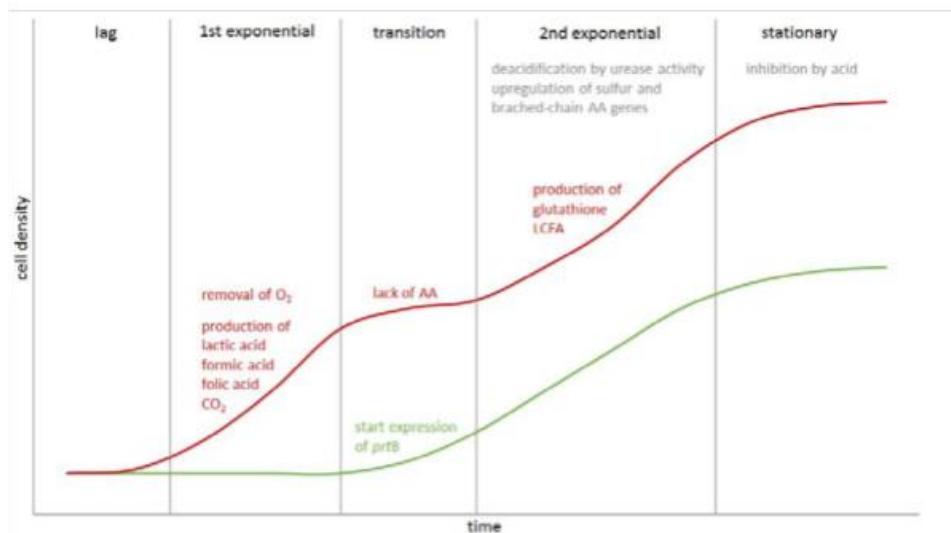


Figure 4. A picture of the graph illustrating proto cooperation between *S. thermophilus* and *L. bulgaricus* in milk. (Sieuwerts, 2016)

## 1.6 Batch cultivation

The manufacture of yoghurt is done in batch fermentation. A batch cultivation is a process where a microorganism or a consortium of microorganisms are inoculated to a fixed volume of medium (Xin et al., 2019) and it is a closed system. This means that the microorganisms and all the substrate are added to the system at 0 hours and nothing is removed from the system until the end of the fermentation. (Pumphrey et al., 1996). It gives us the possibility to observe the different growth phases of *S. thermophilus* and *L. bulgaricus* during the experiments. We can therefore have a better knowledge about the products formed at each level and carry out analyses to understand the activity and characteristics of the bacteria. We can carry out further optimisations to control the environmental parameters and the concentrations of raw materials (Xin et al., 2019).

The first growth state is known as the lag phase. In this phase the cells are active but there is almost no cell division. The cells are adapting to the new environmental conditions by synthesising ribosomes and enzymes that are needed to utilize the substrates from the culture medium. Using bacteria which were actively growing in a rich medium is of advantage at this stage. Such cells decrease the length of the latent phase as compared to one that was taken from a working stock in storage. As the reproduction process increases, the bacteria enter the exponential phase. At this stage, the bacteria achieve the maximum growth rate. The cells have fully adapted to the environment. They are now in the ideal parameters with a good amount of nutrients available. The log growth rate constant may be determined by the number of doublings per unit time. This varies with species. For example, *S. thermophilus*'s exponential rate constant is around 20 minutes for some strains. It is very useful to investigate the growth of the bacteria at this stage. They are actively growing and are excreting metabolites. However, the medium's composition is changing gradually, and this affects the growth. Eventually the cells enter the stationary phase. At this point, the nutrients are depleted, and the pH has decreased as more acids and carbon dioxide were built up in the exponential phase. The growth rate is static which means that the growth rate equals the death rate. The final stage is known as the death phase where more cells die than are produced. The overall growth declines. It is important to stop the fermentation process before this phase to prevent cell lysis (Jane Taylor, 2001).

There are many pros and cons of using a batch fermentation. Usually, an experiment in a batch cultivation has a shorter growth period as compared to a continuous or a fed-batch fermentation. Hence, the risk of contamination decreases because there is less handling of

the culture and the equipment. A batch fermentation offers a well-controlled growth period with a greater uptake of substrates. The substrates are added to the system and environmental parameters are fine-tuned. However, it may be time-consuming to maintain the fermenter (bottles or bioreactor) due to filling, sterilizing, cooling, and thorough cleaning of the vessel. Special attention must be given on the instrument used due to frequent sterilization. Industrially, there is a greater risk of exposure to pathogenic microorganisms and poisonous compounds because they are not gradually removed during the fermentation and will accumulate during the growth (Pumphrey et al., 1996).

## 2 THE AIMS OF THE THESIS

This thesis work is a part of a project whereby several strains of *S. thermophilus* and *L. bulgaricus* are investigated. The general aim of my research work is to carry out bottle experiments using one strain of each. The more specific aims of my research are as follows:

- Prepare the chemically defined medium (CDM) for experiments.
- Characterize the growth of *S. thermophilus* and *L. bulgaricus* in monocultures.
- Understand how *S. thermophilus* and *L. bulgaricus* interact with each other in a co culture: Proto cooperation

### 3 EXPERIMENTAL PART

#### 3.1 MATERIALS AND METHODS

##### 3.1.1 Bacterial strains

*S. thermophilus* strain and *L. bulgaricus* strain were obtained from a global bioscience company Chr. Hansen A/S. The *S. thermophilus* strain lacks protease activity and has a slower acidification rate. The *L. bulgaricus* strain has a faster acidification rate. The strains were stored as glycerol stocks at -80°C.

##### 3.1.2 Medium

Table 2. M-17 broth's composition

Compound	Concentration (g/l)
Tryptone	2.5
Meat peptone (peptic)	2.5
Soya peptone (papainic)	5
Yeast extract	2.5
Meat extract	5
Sodium glycerophosphate	19
Magnesium sulfate	0.25
Ascorbic acid	0.5
Lactose	5

Table 3. MRS broth' s composition

Compound	Concentration (g/l)
Enzymatic digest of casein	10
Meat extract	10
Yeast extract	5
Glucose	20
Dipotassium hydrogen phosphate	2
Sodium acetate	5
Triammonium citrate	2
Magnesium sulphate	0.2
Manganese sulphate	0.05
Molyoxyethylene sorbitan monooleate (Tween 80).	1.08

Table 4. Chemically defined medium' s composition

Stock solution name	Compound	Final concentration (mg/l) in CDM
Basal solution	$K_2HPO_4$	2500
	$KH_2PO_4$	3000
	Sodium Acetate	1000

	Tri-Ammonium citrate	600
	MnSO <sub>4</sub> x H <sub>2</sub> O	20
	FeSO <sub>4</sub> x 7 H <sub>2</sub> O	0.066
	Tween 80	1 ml
	Trace element solution (1000x)	1 ml
Trace elements	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> x 4H <sub>2</sub> O	0,0037
	CoCl <sub>2</sub> x 6H <sub>2</sub> O	0,007
	H <sub>3</sub> BO <sub>3</sub>	0,025
	CuSO <sub>4</sub> x 5 H <sub>2</sub> O	0,0025
	ZnSO <sub>4</sub> x 7 H <sub>2</sub> O	0,0029
Nucleobases	Adenine	10
	Guanine	10
	Uracil	10
	Xanthine	10
Amino acid stock	L-Tyrosine	120
	L-Isoleucine	360
	L-Tryptophan	102
	L-Leucine	600
	L-Valine	468
	L-Glutamate	330

	L-Aspartate	500
	L-Asparagine Monohydrate	342
	L-Phenylalanine	340
	L-Alanine	100
	L-Arginine	316
	Glycine	160
	L-Lysine	350
	L-Methionine	120
	L-Proline	920
	L-Serine	360
	L-Threonine	300
	L-Histidine.HCL.H2O	272
Vitamins	Folic acid (first dissolve in 1 ml NaOH 0,1M)	0.2
	Biotin	0.2
	Pyridoxal.HCl	1
	Riboflavin	0.5
	Thiamine-HCl	0.5
	Nicotinamide	0.5
	Cobalamin	0.5
	p-Aminobenzoic acid	0.5

	di-Ca-pantothenate	4
	Lipoic acid	0.5
	Lactose	20 000
	CaCl <sub>2</sub> ·H <sub>2</sub> O	135
	MgSO <sub>4</sub> ·7 H <sub>2</sub> O	200
	Urea	120
	Glutamine	290
	1M HCl	3 ml

### 3.1.3 Growth experiment workflow and conditions

The cultures were first grown in rich media. M-17 broth (56156-500G-F, Sigma-Aldrich) was used as a rich medium for *S. thermophilus*. Its initial pH was approximately 7.0. MRS broth (84613.0500, VWR chemicals) was used as the rich medium for *L. bulgaricus*. The initial pH of MRS broth was about 6.4. The medium was sterilised by autoclaving at 121 °C for 15 minutes. After autoclaving sterile lactose solution was added to the rich media to a final concentration of 20 g/l. The experimental cultures were grown at a temperature of 40 °C and an initial volume of 100 ml. For the rich media experiment, the cells, previously kept frozen at -80 °C, were inoculated into 100 ml of medium to an initial OD<sub>600nm</sub> of 0.05 for *S. thermophilus* and 0.1 for *L. bulgaricus* and the co-culture.

For the chemically defined medium experiments, the initial pH was approximately 6.35. The cells were taken from pre-inoculum bottles. The pre-inoculum bottles were prepared in 2 steps. First, a glycerol stock was injected to a bottle of 50 ml rich medium and allowed to grow to an exponential phase. An amount corresponding to the required optical density is then added to 50 ml of chemically defined medium as the second pre-inoculum column. The exponential cells are then transferred to 100 ml of chemically defined medium as the experimental bottles. The required optical density was 0.05 for *S. thermophilus* and 0.1 for *L. bulgaricus* and the co-culture. The strains were grown in triplicates. The bottle was gently shaken or stirred using the magnetic stirrer to obtain a homogenous mixture. A syringe was

used to remove the required volume of cell culture. The bottles were then placed back in the water bath at 40 °C.

During the rich media experiments, the cells grew till stationary phase and samples for optical density were taken every hour. The measurements were carried out in triplicates.

For the chemically defined medium, a certain amount of exponential phase cells from the first pre-inoculum (as shown in the equation below) is centrifuged at 14.8 rpm for 3 min, resuspended in sterile 0,9% NaCl and inoculated into the second pre-inoculum bottle.

$$\text{Volume}_{\text{STRAIN}} \text{ (ml)} = \frac{\text{Final optical density (OD}_{600\text{nm}}) \times \text{volume of new bottle (ml)}}{\text{Current optical density (OD}_{600\text{nm}})}$$

Exponential phase cells were then inoculated to the experimental bottle containing 100 ml of CDM.

### 3.1.4 Anaerobic cultivation

A series of steps were taken to ensure that the chemically defined medium in pressure bottles was sterile and anaerobic. First, 40 ml of MQ water and a magnetic stirrer were added to the pressure bottle and autoclaved at 121°C for 15 minutes. Sterile stocks of the CDM components were prepared and everything was added to the autoclaved MQ water under the laminar in sterile conditions. The bottles were closed air tightly and the headspace of the bottles containing the medium were flushed with a sterile gas mixture of 80% N<sub>2</sub> and 20% CO<sub>2</sub> at a pressure of 0,5 bars for 40 minutes. The content of the bottles was stirred continuously with a magnetic stirrer during the process. Finally, sterile, and anaerobic stock of cysteine-HCl was added into the medium. Cysteine has antioxidant properties and it can bind the free oxygen in the medium making the medium more anaerobic.

To inoculate the cells and to add cysteine into the anaerobic bottle, a syringe and needle were used. The latter is prepared under the flame to keep it sterile. A 1 ml syringe was used to take up all the glycerol stock under a flame. The stock was carefully injected into the bottle through the rubber cap. Furthermore, the rubber top part of the bottles such as pre-experimental bottles, solution bottles (for example, anaerobic lactose solution and cysteine-

HCl) and experimental bottles, were wiped with 96% of ethanol. The top section was heated lightly under the flame to disinfect the area.

### 3.1.5 Sample collection and storage

Samples were collected for measuring optical density (OD) at 600nm and High-Performance Liquid Chromatography (HPLC). For OD measurement, 1 ml per cuvette of sample was used. The sample for HPLC was obtained by centrifuging 1 ml of culture at maximum speed of 14.8 rpm for 3 minutes at 4°C. 900µl of the supernatant was added to a new Eppendorf tube and stored at -20°C until further analysis.

### 3.1.6 Data analysis

The OD data was used to plot growth curves over time. The specific growth rate was calculated from the curves obtained. An equation of the trendline was generated to which the gradient of the line corresponds to the specific growth rate. The y axis representing OD at 600nm was in exponential scale, base 10. This method can be further explained with the formula:

$$\mu = \frac{\ln \frac{x_2}{x_1}}{\Delta t}$$

whereby,

$\mu$  = specific growth rate,

$x_1$  = amount of cells (OD) at first data point,

$x_2$  = amount of cells (OD) at second data point,

$\Delta t$  = difference in time between first and second data points.

Analysis of organic acids, alcohols and sugars was performed by HPLC (Shimadzu Prominence- i LC-2030C 3D Plus) using a column (Rezex ROA-Organic Acids H+ 8% (00H-0138-K0)). 20µl of the stored sample were injected and eluted isocratically with 0.005 M sulfuric acid as mobile phase at 0.6 mL/min for 30 min at 45°C and detected by a refractive index detector (RID-20A, Shimadzu, Japan). The standards consisted of citric acid, pyruvic acid, lactic acid, formic acid, acetic acid, diacetyl, acetaldehyde, butyric acid, glucose, glycerol, ethanol lactose and galactose. An external standard calibration curve was

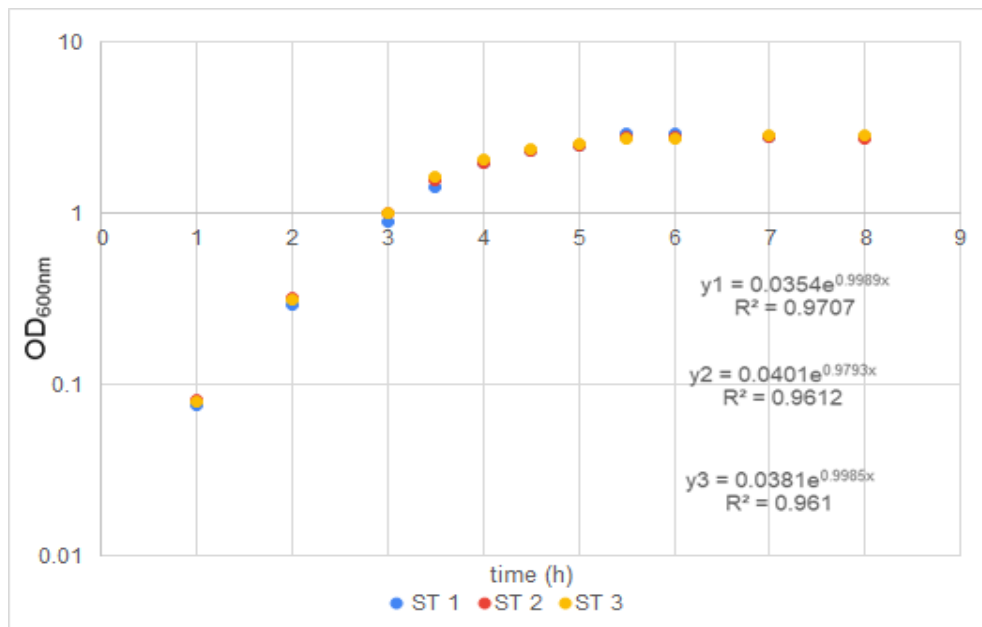
produced using these standards and the peaks of samples detected were compared against it. The analysis was carried out for all 3 replicates of each experiment. The data was analysed using *Microsoft Excel* software.

## 3.2 RESULTS

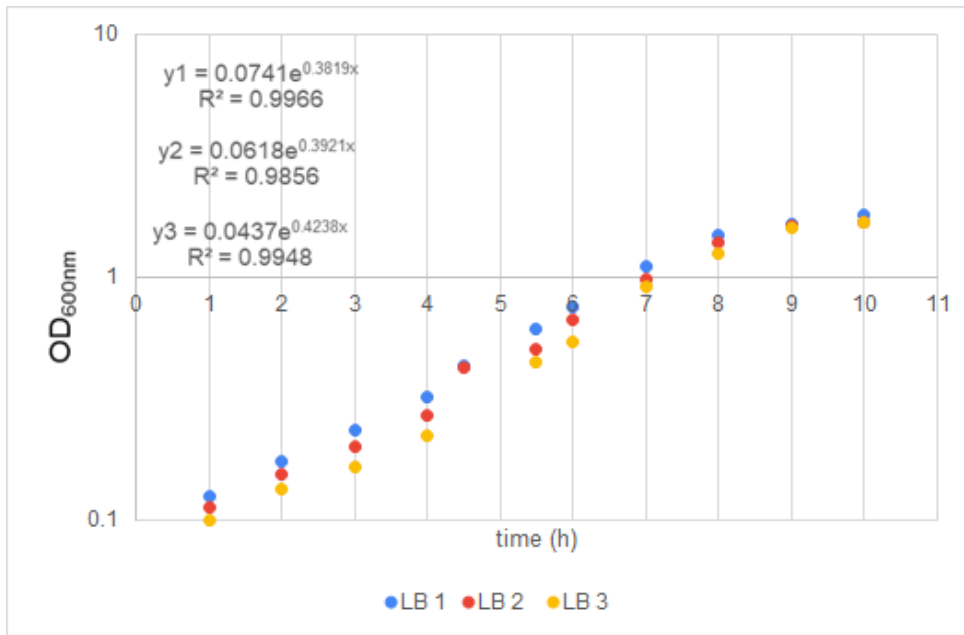
### 3.2.1 Growth experiments in the chemically defined medium

A graph of OD<sub>600nm</sub> in the exponential scale against time (h) was plotted to show the growth of the monocultures of *S. thermophilus* and *L. bulgaricus*, and the co-culture throughout the experiment.

a)



b)



c)

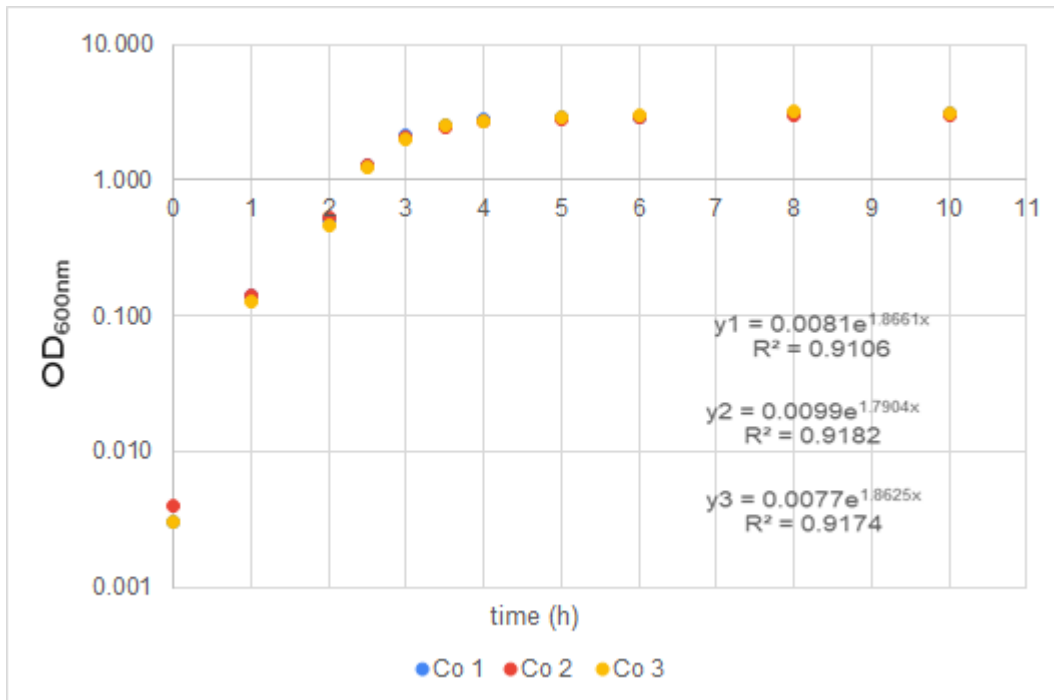


Figure 5 Growth curve of a) *S. thermophilus*, b) *L. bulgaricus* and c) co-culture in CDM. The set of equations for each replicate are added to the graph in a way that “y1” represents the first replicate, “y2” the second and “y3” the third one. “ST 1” represents *S. thermophilus* replicate 1. “LB 1” represents *L. bulgaricus* replicate 1. “Co 1” represents co-culture replicate 1.

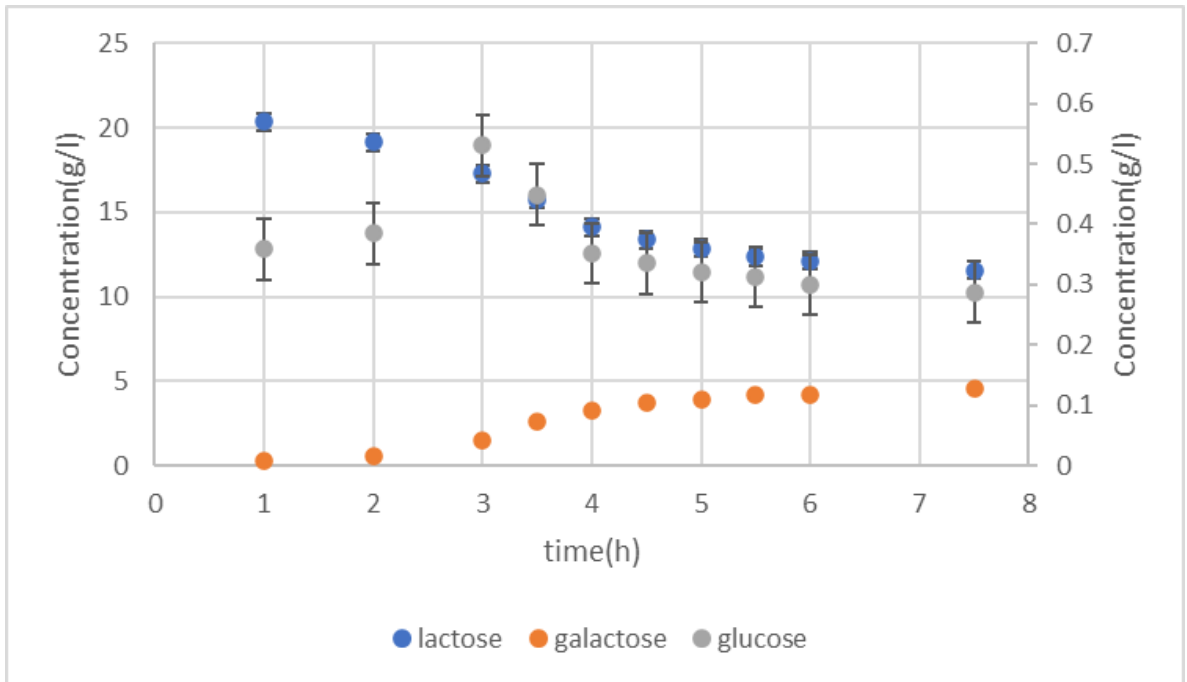
The *S. thermophilus* growth experiment lasted about 8 hours. *S. thermophilus* reached stationary phase in 5 hours. The maximum specific growth rate of *S. thermophilus* was  $0.992 \pm 0.011 \text{ h}^{-1}$  [average  $\pm$  standard deviation], *L. bulgaricus* grew to the exponential phase slower compared to *S. thermophilus*. The overall growth experiment lasted approximately 10 hours. *L. bulgaricus* was in lag phase during the first 3 hours. The cells were then in exponential phase for 5 hours approximately before reaching stationary phase. The maximum specific growth rate of this strain was  $0.399 \pm 0.022 \text{ h}^{-1}$ . The duration of the growth experiment for the co-culture was about 10 hours. The co-culture was in exponential phase for the first 4 hours before reaching stationary phase. The maximum specific growth rate was  $1.84 \pm 0.04 \text{ h}^{-1}$ . The rate of increase of biomass of the co-culture cells population per unit of biomass concentration was higher than that of the *S. thermophilus* and *L. bulgaricus* monocultures. The co-culture also reached stationary phase faster than the individual strains.

### **3.2.2 Extracellular metabolites analysis by HPLC**

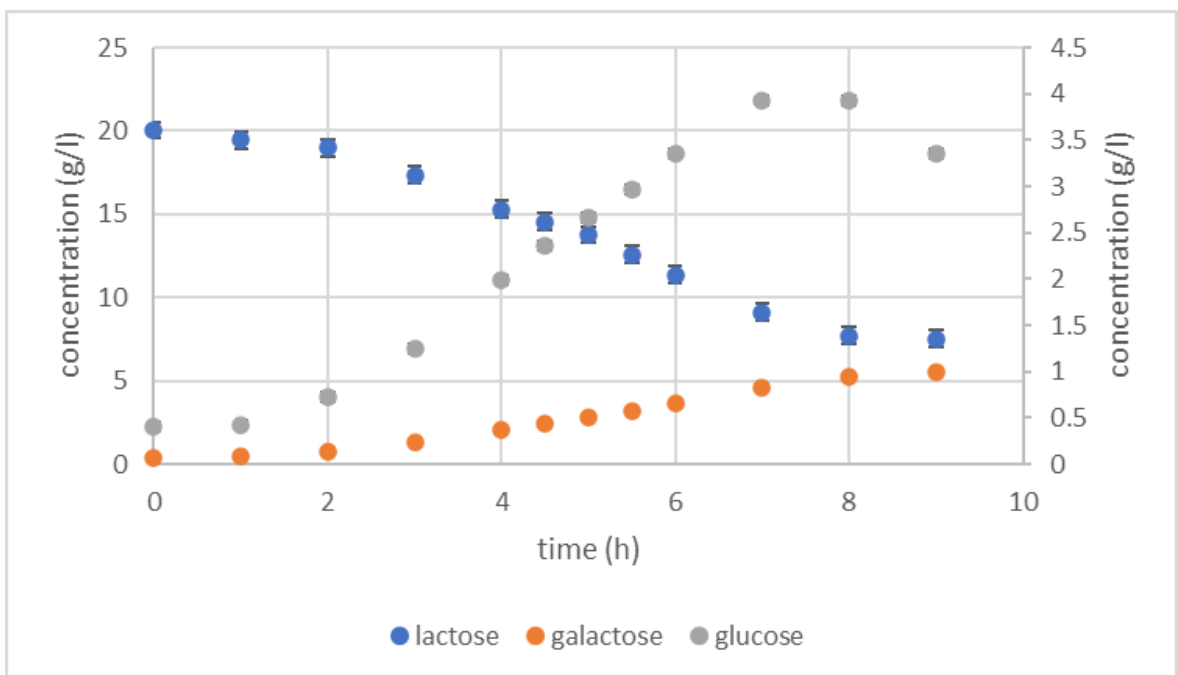
The graphs showed the metabolites that were either already present in the media, consumed or produced by the strains and those that changed in concentration levels over time.

Acetic acid, formic acid, acetaldehyde, glucose, galactose, and lactose were detected by the HPLC analysis. Certain irregularities could be observed in the trend of glucose uptake (Figure 6). The levels of glucose increased at certain time points in *S. thermophilus* and co-culture experiments while *L. bulgaricus* showed a gradual increase during the initial 7 hours. As a by-product of glycolytic breakdown of glucose, lactic acid was formed. Formic acid was also formed during the fermentation. However, the amount of lactic acid was 4 to 5 times that of formic acid. There was slight increase in the galactose production throughout the experiments. Acetaldehyde, an important flavour compound in yoghurt, had a slight change in concentration level over time. The concentration level of acetic acid slightly decreased over time.

a)



b)



c)

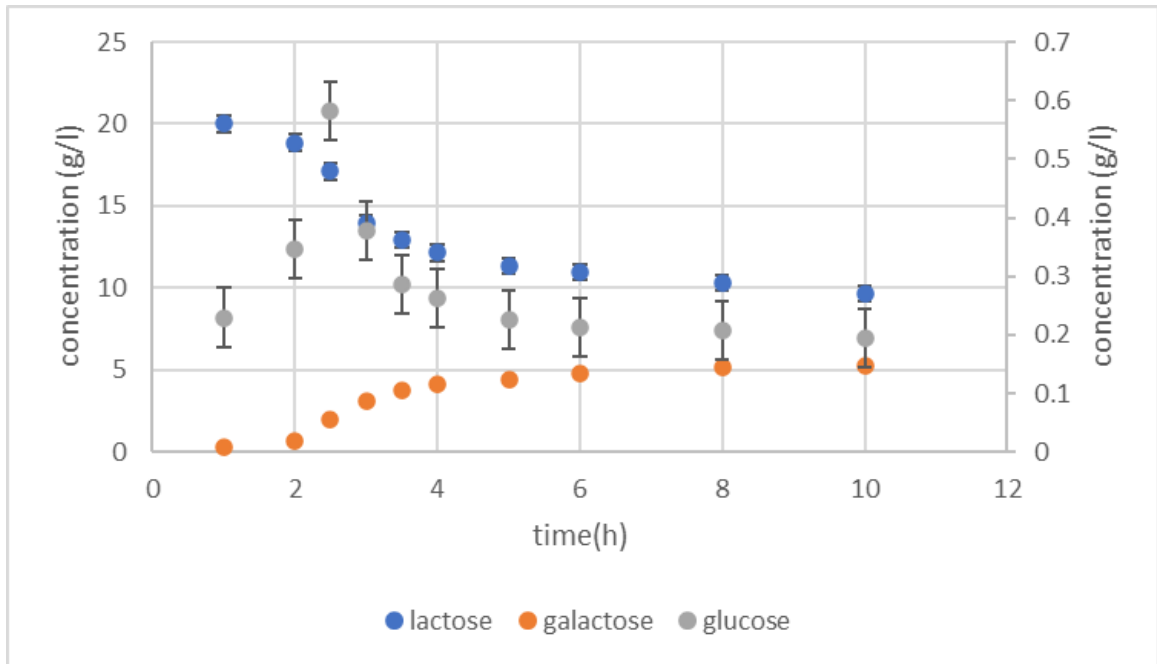
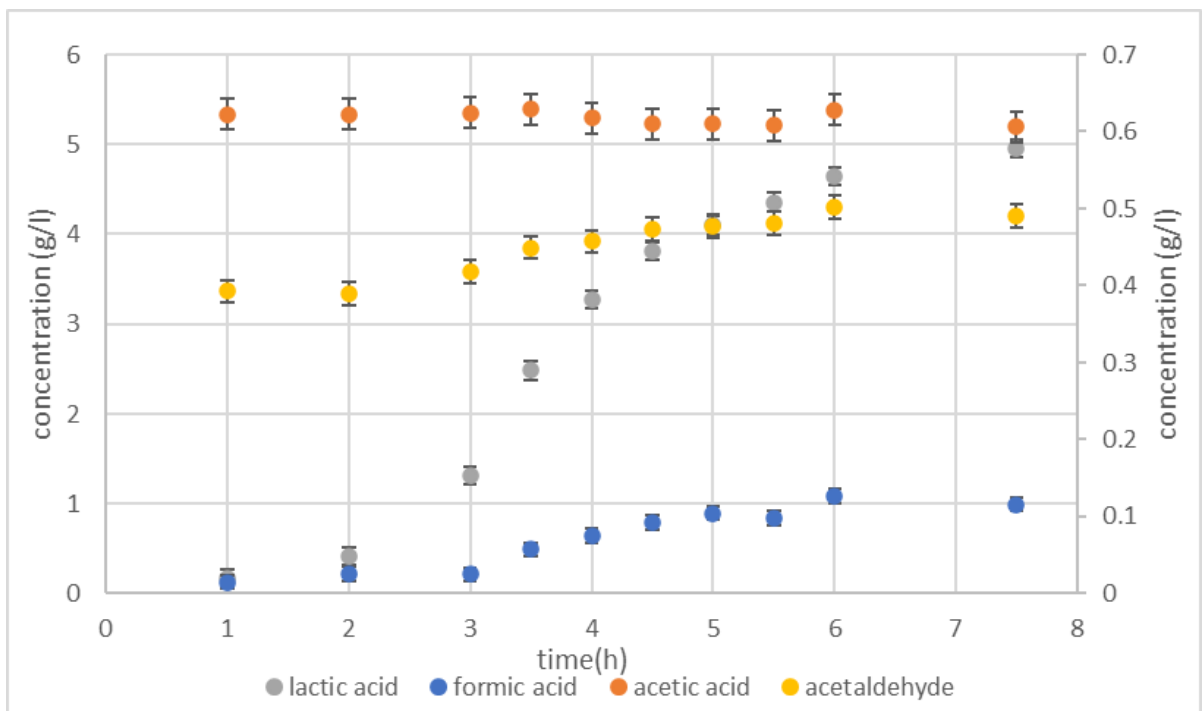
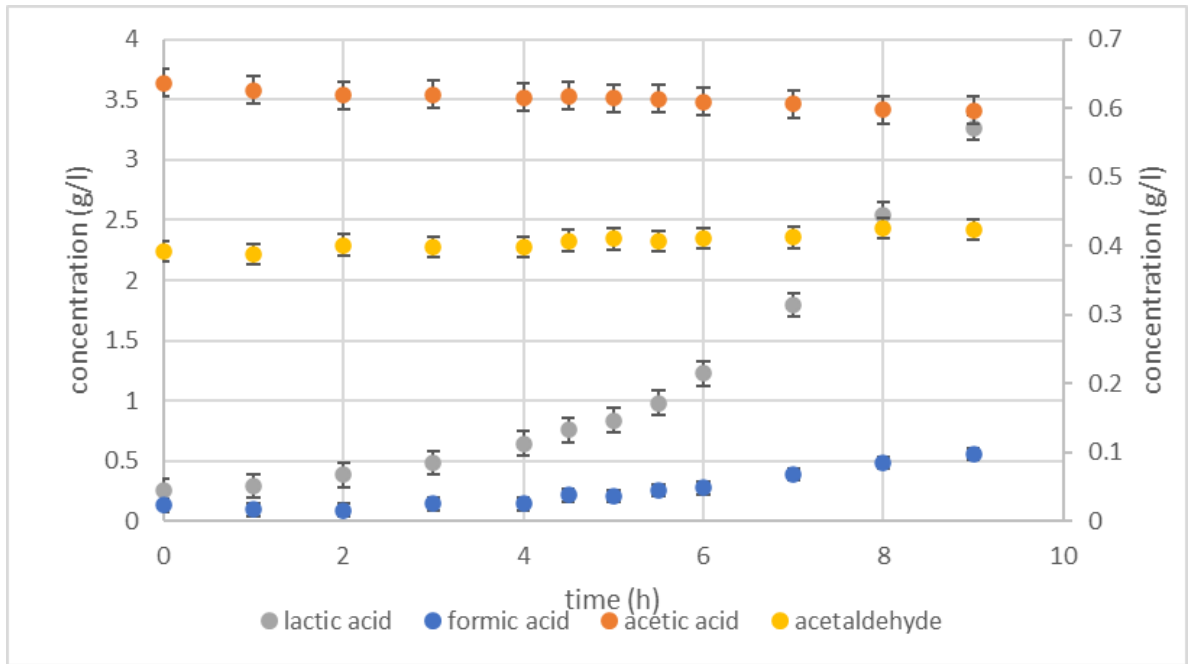


Figure 6. Sugars consumption and production by a) *S. thermophilus*, b) *L. bulgaricus* and c) the co-culture. Lactose and galactose are shown on the primary axis and glucose is shown on the secondary axis.

a)



b)



c)

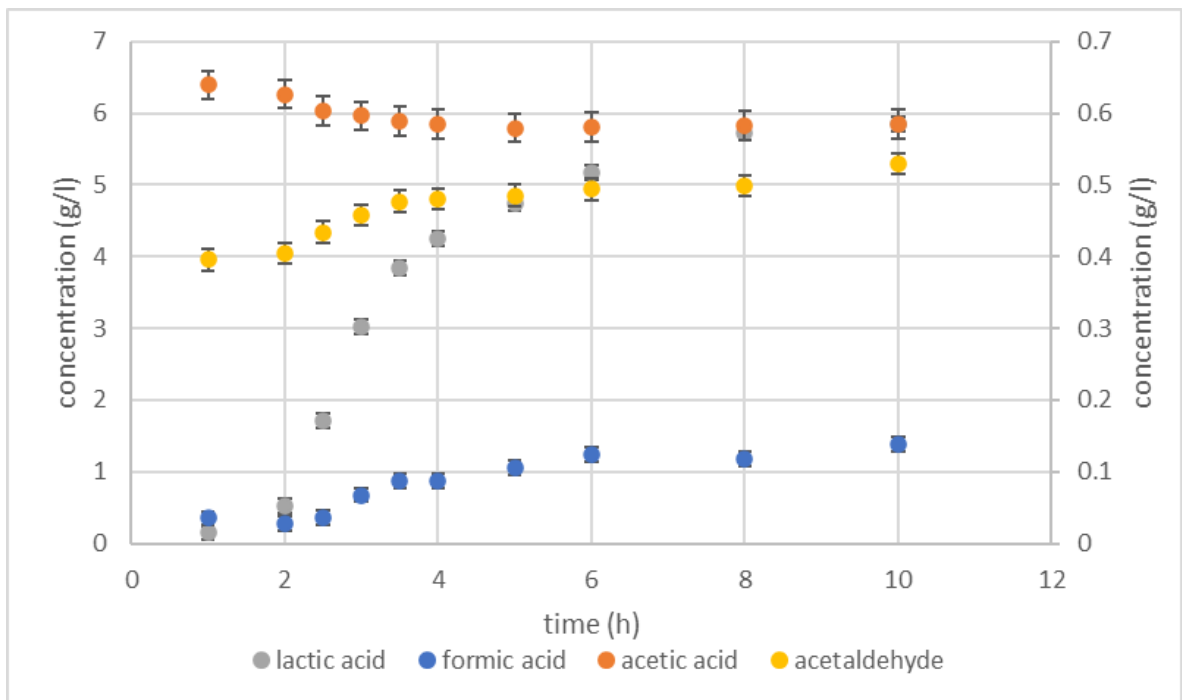


Figure 7. Concentration levels of acids and acetaldehyde over time in a) *S. thermophilus* monoculture, b) *L. bulgaricus* monoculture and c) co-culture. Lactic acid is shown on the primary axis. Acetic acid, formic acid and acetaldehyde are shown on the secondary axis.

### 3.3 DISCUSSION

From the growth graphs, we may conclude that the growth rate of the co-culture is higher than that of the monocultures. We did not observe a second exponential growth of *S. thermophilus* in the co-culture experiment as illustrated in Figure 4 in the “literature review” section. Nonetheless, we can deduce that it took less time for the strains to grow in a co-culture than a monoculture (Figure 5). This is because the cells to grow to exponential phase more rapidly in the co-culture than the monocultures. Hence, it may be hypothesized that these 2 strains had mutual beneficial effects or competitive behaviour. The latter means that both strains compete for the resources in the medium to promote their own growth. More research work is needed to provide a more specific conclusion.

A drop in pH might be a reason the strains stopped growing at a certain time even if lactose was still present in the medium. To overcome this issue, a bioreactor experiment could be carried out where pH level can be maintained.

Lactose was broken down to produce glucose and galactose (Figure 6). The increase of galactose along the experiment acted as an additional proof that showed the uptake of lactose by the bacteria. Glucose was used to synthesize ATP. However, the sudden peak and increase in glucose concentration could be because the intracellular and extracellular environment of the cells were saturated and therefore rejected the uptake of glucose. This occurrence might limit the growth of the cells. The presence of formic acid, acetic acid, lactic acid as acetaldehyde served as contributors to the typical flavour and aroma of yoghurt (Figure 7). Accounting for the loss of lactose, lactic acid and formic acid were produced as end-products of the fermentation. The slight decrease in acetic acid observed in the triplicates might be a measurement error.

## SUMMARY

In this thesis, a strain of *Streptococcus thermophilus* and one *Lactobacillus bulgaricus* strain obtained from Chr. Hansen A/S were used to carry out the experiments. They can mainly be separated in two parts according to the medium used: rich media experiments and chemically defined medium experiments.

The chemically defined medium was successfully prepared anaerobically, and precautions were taken to ensure the medium is sterile.

Growth curves of the strains could be plotted, giving an idea about the overall growth in monocultures and co-culture. However, more research can be done to improve these experiments. The volume of media used can be a limiting factor in bottle experiments because samples cannot be taken if the volume has dropped below 50%. It may also cause discrepancies in terms of pH and other compounds inhibiting the growth of the bacteria. Hence, a bioreactor may be used to upscale this experiment and observe the growth for a longer time. The pH could also be maintained at optimum level with a pH sensor.

Different compounds such as organic acids, sugars and flavour components could be detected by using High-Performance Liquid Chromatography (HPLC). As a future aim, quantitative work could be performed using the data obtained from the HPLC.

All the goals could not be fully achieved because of time constraints. However, this research work can serve as a good base for the upscaling of the project. The next step would be to carry out the growth experiments in bioreactors and use milk as a medium to gauge the growth of and interactions between these 2 strains.

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