Evaluating the behaviour of probiotic *Bacillus coagulans* XY1 in fruit juices and survival during heat treatment

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Abstract

It has become more important for people worldwide to eat and drink products that offer us health-promoting benefits, which traditional foods do not possess. These kinds of products are called functional foods and have been fortified using vitamins, minerals or probiotics among others. Today, there are a lot of probiotic strains on the market and the first part of the aim of this study was to evaluate the behaviour of a novel probiotic strain called *Bacillus coagulans* XY1 by incorporating it into two different fruit juices and follow its viability, stability and performance over a storage period of 10 weeks. In addition the physicochemical properties pH and lactic acid concentration was monitored in the samples throughout the storage period. The juices the probiotic strain was incorporated into were one orange and one pomegranate juice and they were both stored at 8-10°C in a refrigerator. The second part of the aim was to study the survival of *Bacillus coagulans* XY1 through different heat treatments.

It was found that in both juices, the probiotic strain was able to survive throughout the storage time. However, the viability of the strain in the juices did not keep the required concentration of 10^7 cfu/mL at every testing occasion. The pH value in the juices stayed stable indicating that the strain was metabolically inactive during storage and a sensory evaluation including aroma, appearance and colour, showed that the quality of the juices was not affected by the probiotic when refrigerated. The heat treatment did affect the viability of the strain greatly. The results showed that there was a great decrease in viability of the strain after the different heat treatments were performed. This needs to be further investigated in the future to find a suitable treatment for probiotic fruit juices and it needs to be performed more similar to industrial standards for more reliable results. In conclusion, fruit juices seemed to be an appropriate food matrix for inoculation of *Bacillus coagulans* XY1 to develop future probiotic beverages with a long shelf-life period.

Keywords: fruit juice; *Bacillus coagulans* XY1; probiotic; storage stability; heat treatment

Sammanfattning

Utvärdering av beteendet hos den probiotiska bakterien *Bacillus coagulans* XY1 i fruktjuicer och överlevnad under värmebehandling

Det blir allt viktigare för personer runt om i världen att äta och dricka livsmedelsprodukter som bidrar med hälsofrämjande fördelar som traditionella livsmedelsprodukter ej innehar. Den här typen av produkter kallas för mervärdesmat, vilket innebär att de har blivit berikade med bland annat vitaminer, mineraler eller probiotika. Idag finns det många probiotiska stammar på marknaden och det första syftet med det här projektet var att utvärdera beetendet hos en ny probiotisk stam kallad *Bacillus coagulans* XY1 genom att inkorporera den i två olika fruktjuicer och följa dess viabilitet, stabilitet och utförande under lagring i 10 veckor. Dessutom kontrollerades de fysiokemiska egenskaperna pH och mjölksyrakoncentration under lagringstiden. Juicerna som den probiotiska stammen inkorporerades i var en apelsinjuice och en granatäpplejuice, de båda lagrades i 8-10°C i kylskåp. Den andra delen av syftet innebar att undersöka hur den probiotiska stammen överlevde genom olika värmebehandlingar.

Det var tydligt i båda juicerna att den probiotiska stammen överlevde under lagringstiden. Dock var det några provtillfällen där den rekommenderade koncentrationen för probiotika i livsmedel var lägre än tillåtet. Juicernas pH värden var i stabila under lagringstiden, vilket indikerar att den probiotiska stammen var metaboliskt inaktiv vid lagring och en sensorisk analys (doft, utseende och färg) visade också att kvaliteten på juicerna ej påverkas avsevärt vid lagring i kylskåp. Värmebehandlingen påverkade den probiotiska stammens viabilitet avsevärt. Resultaten visade att det var en tydlig minskning i stammens viabilitet efter de olika värmebehandlingarna. Det här behöver undersökas ytterligare för att finna en passande behandling för probiotiska fruktjuicer och värmebehandlingen behöver utföras mer likt industriella standarder för mer trovärdiga indikationer och resultat. Slutligen, fruktjuicer fungerade bra för inkorporering av *Bacillus coagulans* XY1 för utveckling av nya probiotiska drycker med en lång hållbarhetstid.

Nyckelord: fruktjuice; *Bacillus coagulans* XY1; probiotika; hållbarhet; värmebehandling

Popular Scientific Summary

With an increase in health-related diseases, the importance of providing your body with healthy nutrients is more important than ever. People have therefore started to look into the area of foods that in one way goes beyond the traditional ones, in order to gain as many nutrients as possible from each meal. In order to further explore this exciting area of foods, a new probiotic strain has been investigated for the development of potential new health-promoting products, also called functional foods.

Traditionally, functional foods mostly consisted of dairy products but due to the ongoing trend of vegetarian and vegan diets, people have started to look at other options. Fruit juices have become a popular source for these kinds of foods, since they satisfy vegetarian and vegan diets and contain no allergens. They also come in a big range of flavours, which makes it easier for individuals to find something that suits them specifically. In this project a new probiotic is being investigated in fruit juices with the hope of developing it into new probiotic fruit juices. The probiotic microorganism used in this project had the ability to form spores, which means that it can go into a resting-state where it does not grow if it is being exposed to hostile growth environments. This ability comes in handy in many various ways and can make probiotic food products safer for the consumer. To minimize the risk of food poisoning, food products such as fruit juices are often heat treated to kill off possible pathogens and thanks to the spores of this new probiotic strain, the heat treatment will not be as harmful on this probiotic as it would have been on another probiotic.

The behaviour of the probiotic strain was tested during 10 weeks in storage to see if it could survive in fruit juices during a longer time and still be beneficial for the consumer. How the probiotic strain affected the fruit juices in terms of quality was also tested during the storage period, to ensure safe and high-quality products throughout the shelf-life period. The probiotic fruit juices were also heat treated in order to see how the probiotic strain survived during different treatments for future knowledge about the tolerance of high temperature of the probiotic strain.

It was possible to see that the probiotic was able to survive in fruit juices and remained at levels somehow satisfactory regarding the requirement for a food product to be considered a probiotic food product. By keeping the probiotic fruit juices in a refrigerator for storage, the probiotic did not affect the quality of the juices on a visual basis. The probiotic fruit juices were however never

tasted and therefore it is not possible to say if the probiotic did affect the taste in an unsatisfactory manner. The different heat treatment combinations showed that a lot of the probiotic strain did not survive in high enough amounts to be a satisfactory probiotic food product.

This project has been a first step into testing this new probiotic strain in fruit juices and more research will be needed to evaluate the probiotic even further. More heat treatment combinations need to be tested to find a more suitable treatment for this strain and the experiment needs to be performed in a larger scale, to mimic future industrial standards further.

Preface

This master thesis was a collaboration with Probi AB and the work took place at the Division of Applied Microbiology at the Department of Chemistry of the Faculty of Engineering, Lund University from March to August 2020. A big thank you to Probi for giving me the opportunity to perform this exciting master thesis project. I would like to say a special thank you to my two supervisors Divya Mohan and Jenny Schelin. Divya, thank you for all your help designing the experiments and discussing the project, and for the wonderful insights to the exciting area of product development. Jenny, thank you for always being there as a helping hand and encouraging me to do my best. Also a big thank you for your valuable inputs and feedback on the report throughout the project.

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Lund, August 2020
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List of Acronyms and Abbreviations

Word	Description
B. coagulans	*Bacillus coagulans*
cfu	Colony forming unit
EFSA	European Food Safety Authority
FDA	The US Food and Drug Administration
GRAS	Generally Recognized as Safe
GYEA	Glucose Yeast Extract Agar
HPLC	High-performance Liquid Chromatography
JO	Orange juice
JP	Pomegranate juice
L. plantarum 299v	*Lactiplantibacillus plantarum* 299v
MSDS	Material Safety Data Sheet
PCM	Plate Count Method
WHO	World Health Organization

1. Introduction

In recent years, people worldwide have started to become more and more invested in their health and lifestyle (Siró, Kápolna, Kápolna and Lugasi, 2008). This equals being more concerned about what they are eating and drinking, and how it is affecting their body. Therefore, an increased interest in healthy, safe and functional foods which offer health-promoting properties and overall make us feel better, has been possible to distinguish (Konuray and Erginkaya, 2018). Functional foods, such as foods fortified with minerals, vitamins or probiotics, can be regarded as functional if they affect one or more target functions in the body beneficially, in a way traditional foods cannot achieve. The evidence of the health benefits by intaking probiotics is increasing and some of the beneficial effects that can be observed are better gastrointestinal health, improved metabolism, enhanced immune response, reduced cholesterol levels and prevention of cancer (Kechagia et al., 2013). Functional foods not only give us basic nutrition by providing traditional nutrients to the consumer, but are also a source of well-being mentally and physically (Fernandes Pereira and Rodrigues, 2018; Syngai et al., 2015). It is possible to see that probiotics are commonly used in the functional foods market today (Granato et al., 2010).

Probiotics are live microorganisms that have been consumed through supplements and fermented foods for many years in order to improve our health. The first person known to mention the relationship between food and health was Hippocrates about 2500 years ago, who said "death sits in the bowels" (Gasbarrini, Bonvicini and Gramenzi, 2016). People have been eating fermented foods long before we could identify probiotic microorganisms and therefore they supplemented their diet with probiotics. Nobel Prize-winner Ilya Ilyich Metchnikoff saw how a consumption of fermented dairy products by Bulgarian rural people enhanced their lifespan, and suggested that lactobacilli might have gastrointestinal health benefits. He prescribed eating soured milk (e.g. yoghurt) as a treatment for old age and there the first intentional probiotic was born. In the 1990s research of the gut microbiome was initiated by a new generation of scientists. The understanding of how the microbes interacted with our bodies became deeper. With more understanding of the potential of probiotics, a formal definition was created by the World Health Organization (WHO) in 2001 later to be updated in 2014 (Gasbarrini, Bonvicini and Gramenzi, 2016; Lamb, 2019).

The present definition of probiotics is defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). There are currently a lot of products on the functional food market containing various sorts of probiotic strains (Mantzourani et al., 2018). Traditionally, probiotic microorganisms have been added mostly to fermented milks and other dairy products. With the ongoing trend of veganism and vegetarianism however, probiotic juices have now also become a suitable and popular source of these beneficial microorganisms (Granato et al., 2010). One of the benefits of using juice as the delivery medium is that juice already contains a lot of healthy nutrients, such as minerals, vitamins and antioxidants (Fernandes Pereira and Rodrigues, 2018). They also do not contain possible allergens as dairy products do (lactose intolerance or milk protein allergy). However, considering some of the

drawbacks with fruit juices include that they often contain a high level of sugar and lack fibres that can have several negative effects, such as a higher risk for obesity and type-2 diabetes (Torrens, 2019). Overall, together with the vegan and vegetarian trend, the consumer market is broader for juices. The possibility to choose from a variety of flavors of juices makes it easier to find something with a flavour profile that suits all different age and consumer groups (Colombo Pimentel et al., 2019).

Most food products on the market today that are fortified with probiotics do not normally undergo heat treatment after inoculation of the probiotic microorganisms, because it affects the probiotic microorganisms' viability and stability negatively. Two lactic acid genera of bacteria that are commonly used in probiotic food production are *Lactiplantibacillus* and *Bifidobacterium*. There is one disadvantage though with using these in food production, which is that they are not very heat-resistant and can therefore not undergo heat treatment such as pasteurisation after being inoculated into the desired product. Due to this, heat treatment is usually not a possibility for some probiotic food products, once the probiotic microorganism is added, that contains a commercial probiotic strain. Therefore, it may pose a risk for the consumers if the inoculation step of the process introduces some contamination that may be present in the final product. This has initiated the search for probiotic microorganisms that can withstand pasteurization and has led to the realisation that spore-forming probiotic microorganisms might be able to overcome that challenge, such as *B. coagulans* (*Bacillus coagulans*) (Konuray and Erginkaya, 2018).

1.1. Master Thesis Overview

This master thesis will look into the behaviour of one probiotic strain of *B. coagulans* for its potential use in fruit juices. The probiotic strain was tested in two different fruit juices that were chosen as good candidates, both with a 100% fruit concentration. One orange juice (Kiviks musteri) with pH 3.94 and one pomegranate juice (Dimes) with pH 3.30 (Figure 1). Evaluation of how the bacterium behaved in the desired products (fruit juices) over a shelf life period of 10 weeks in storage (storage temperature shifting between 8-10°C) was performed.

Figure 1. The two fruit juices used throughout the project, pomegranate to the left and orange to the right, were purchased at Willys in Lund.

The parameters that were evaluated were the viability of the probiotic strain inoculated in the two different fruit juices. The changes in pH, lactic acid concentration and a sensory evaluation (colour, aroma and texture) were also performed. All of these different parameters were studied and tested every other week during the complete storage time of ten weeks. In addition, various heat treatments of the juices, consisting of three different combinations of time and temperature, with the purpose to find the most beneficial one for the viability of the inoculated strain were performed. There have previously not been any studies performed for this specific strain regarding its behaviour in fruit juice, such as viability, as far as we know. Neither on its viability during different heat treatment steps.

1.2. Aim of the Master Thesis

The aim with this master thesis project was twofold. The overall aim was to evaluate the potential of using *B. coagulans* XY1 as a probiotic strain including the following specific objectives:

- Evaluate the behaviour of *B. coagulans* XY1 in two fruit juices by study its viability over a storage period of 10 weeks
- Investigate the viability of *B. coagulans* XY1 during heat treatment processes

1.3. Limitations and Focus

One limitation of this project is the heat treatment step and the equipment used for it. The commercial product will go through a pasteurisation step, which is not equivalent to the heat treatment method that was used in the lab setting. Therefore, the results obtained in this study may

vary when pasteurisation in the commercial scale is used. This implies that there may be differences in viability and other parameters of the inoculated strain in the juices, when produced commercially. The samples were stored in a temperature range between 8-10°C, therefore other temperatures/conditions that might influence the product during storage were not tested. The experimental part of this project focused on parameters such as the viability of the inoculated probiotic strain, as well as changes in lactic acid concentration and pH in the product during the storage time of 10 weeks. Parameters that were not tested, but would have been interesting to study were the survival and growth of other microorganisms in the fruit juices during storage.

2. Literature Review

A literature review was performed to gain a better understanding of the background to the topic. Here different areas such as probiotics, the species *B. coagulans*, probiotic fruit juices, heat treatment of food products and commercial probiotic fruit juices will be covered.

2.1. Probiotics

Probiotics are live microorganisms that can be beneficial to you in many different ways when they are consumed, most often improving the gastrointestinal environment of the body. Therefore, many food products have been fortified with probiotic microorganisms (Konuray and Erginkaya, 2018).

2.1.1. Probiotics in Food Products

In order for a probiotic food product to be beneficial for consumers it needs to contain a minimum of 10^7 - 10^9 colony forming units/mL (cfu/mL) depending of the efficacy of the probiotic culture and the probiotic microorganism needs to be viable and remain at a certain concentration until the end of shelf life (Konuray and Erginkaya, 2018). Naturally, there are microorganisms found in the human gastrointestinal microbiota that possess the abilities to be a protection against diseases in humans, enhance and modulate the immune system, prevent teeth deterioration, being anticarcinogenic, improve digestion of lactose and protect against coronary heart disease. These microorganisms are considered probiotics when they are administered. In Table 1 species of bacteria that are used as probiotics are listed (Saad et al., 2013). For a probiotic microorganism to be used in food products, it needs to be live when administered. It is also beneficial if it is able to pass through the gut and reach the digestive tract and colonise there for a certain time to support the growth of beneficial microbes. However a probiotic microorganism will still provide a health benefit even if this does not take place (Saad et al., 2013).

Table 1. Genus of bacteria and their species with probiotic health effects.

Genus of bacteria	**Species**
Lacticaseibacillus	*L. rhamnosus* *L. casei* *L. paracasei*
Lactiplantibacillus plantarum	*L. plantarum*
Lactobacillus	*L. gasseri* *L. johnsonii* *L. crispatus* *L. delbrueckii* *L.acidophilus*

	L.acidophilus *L. curvatus* *L. farciminis*
Limosilactobacillus	*L. reuteri* *L. fermentum*
Bifidobacterium	*B. infantis* *B.animalis* *B.bifidum* *B. longum* *B. breve* *B. adolescentis* *B. infantis* *B. lactis* *B. thermophilum*
Bacillus	*B. coagulans*
Saccharomyces	*S. boulardii* *S. cerevisae*
Lactococcus	*L. lactis*
Pediococcus	*P. acidilactici*
Leuconostoc	*L. mesenteroides*
Escherichia	*E. coli* Nissle

To ensure that the desired effect of the probiotic food will be accomplished, there have been guidelines established for evaluation of probiotics in food. A scheme of the main steps that outlines these guidelines is illustrated in Figure 2 (FAO/WHO, 2002).

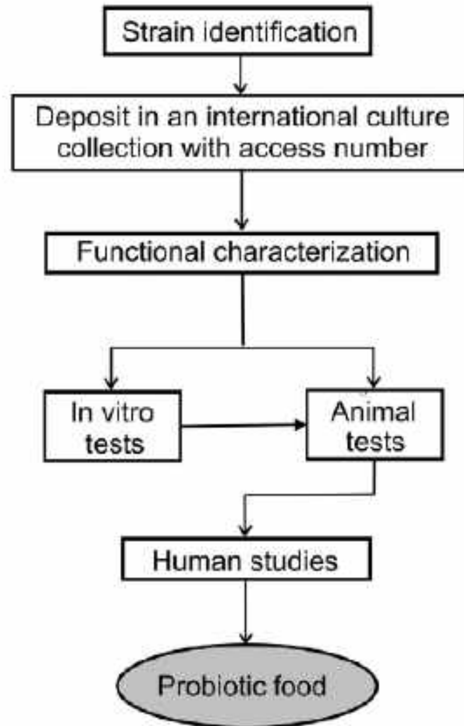

Figure 2. The main steps of probiotic food evaluation (Fernandes Pereira and Rodrigues, 2018).

2.1.2. Probiotic Microorganisms

It is possible to distinguish two different basic forms of probiotic microorganisms, the vegetative and the spore form, that are used in food products. A vegetative cell is the typical Gram positive or negative bacterial cell that consists of a cell membrane, cell wall, chromosomal DNA and ribosomes. All bacteria exist in the vegetative form, which is the active form of bacterial cells where they can grow and reproduce. Some genera that only exist in the vegetative form are *Lactiplantibacillus* and *Bifidobacterium* (Bruslind, 2020). The vegetative form is more sensitive to moisture, high temperatures and longer shelf life periods. There are various factors that influence the possibility for a probiotic microorganism to survive during production of probiotic food products. Different fermentation conditions, freezing and thawing are some factors that can have a negative impact on survival rates. Other factors e.g. food additives, pH, storage temperature, oxygen content and water activity affect the chance of survival during storage of probiotic microorganisms. Conditions in the gastrointestinal system and stress factors can also have a big impact on viability (Konuray and Erginkaya, 2018; Colombo Pimentel et al., 2019).

The spore form (endospore) can only be formed by a few genera of bacteria, such as *Bacillus* and *Clostridium*. Endospores are formed if the vegetative cells are exposed to hostile or stressful environments, such as shortage of nutrients, lowered water activity or high/low temperatures since

endospores are more resistant to these kinds of factors, by e.g. surviving at higher temperatures and being able to handle the tough conditions in the human gastrointestinal system (Majeed et al., 2016). The endospore is formed within the vegetative cell and when the vegetative cell lyses, the endospore is released. They consist of many different layers to withstand and be resistant to tough conditions. There is a core in the center, which is where the ribosomes, nucleoid and the cytoplasm of the cell are located in a very dehydrated form. Around the core there is an inner membrane (permeability barrier) and the inner membrane is surrounded by a cortex and then an outer membrane. Finally, there are some spore coats that protect against environmental stress (enzymes and chemicals) (Bruslind, 2020). Among probiotic spore-formers, such as the *Bacillus* species, the probiotic activity, where the bacteria can grow and reproduce, is most likely occurring when the microorganism is in its vegetative state after germination in the gastrointestinal tract. The microorganism can then return to its spore form when the conditions are insufficient in terms of nutrition or the ability to survive is challenged by harsh conditions (Sanders, Morelli and Tompkins, 2003). The sporulation of vegetative cells (Figure 3) is a complex process that takes several hours to complete, starting by the replication of DNA similar to what happens during cell division. There is a formation of a septum, where one copy of the chromosome is isolated at one of the poles of the cell (forespore). Substances that are endospore-specific are synthesized which results in more layers for the endospore plus dehydration. Finally, the "mother cell" lyses and the endospore is released (Bruslind, 2020).

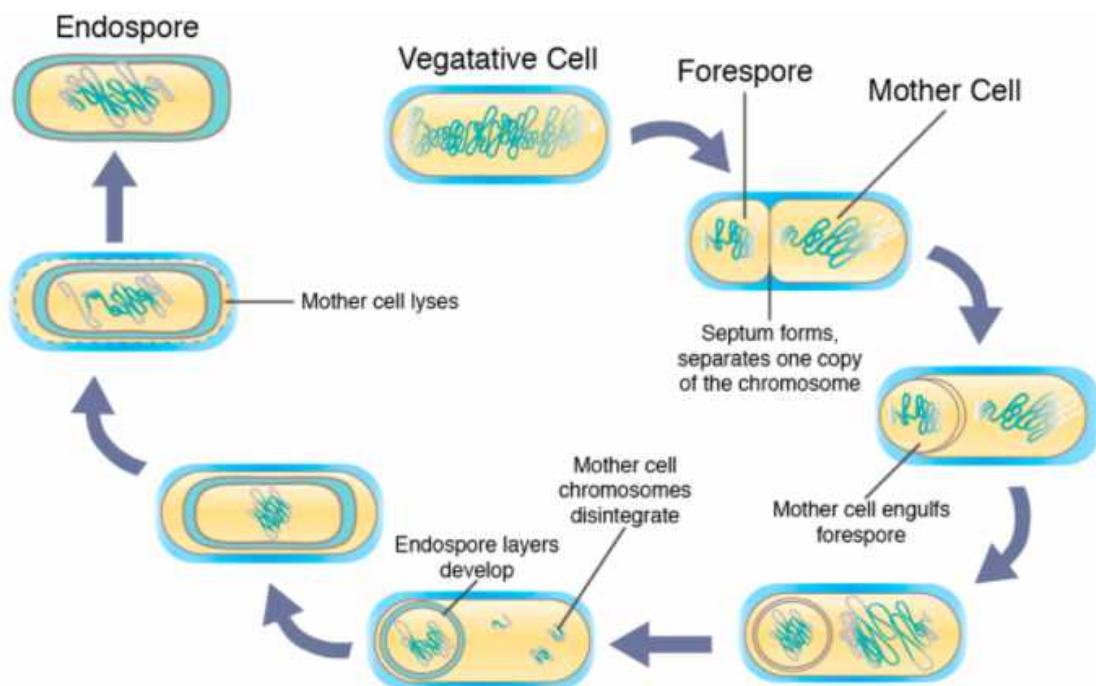

Figure 3. Sporulation of a vegetative cell into an endospore (Bruslind, 2020).

The endospore stays dormant for as long as it needs, until the harsh conditions improve. This initiates gene expression by causing a chemical change. There are three stages covering activation,

germination and outgrowth in order to go from an endospore to an active vegetative cell. The activation stage can be initiated by applying heat, during the germination the spore becomes more active and starts to gather water and then during the outgrowth the vegetative cell comes out from the shell of the endospore (Bruslind, 2020).

2.2. *Bacillus coagulans*

B. coagulans species belong to the genus *Bacillus*. *Bacillus* is a large group with diverse species of rod-shaped, gram-positive, aerobic or facultative anaerobic bacteria. All *Bacillus* species can form dormant endospores when exposed to unfavorable conditions. *B. coagulans* is a spore-forming, lactic acid-producing, nonpathogenic, facultative anaerobic bacterium (Konuray and Erginkaya, 2018). In Figure 4 it is possible to see a microscopic image of *B. coagulans* terminal spores and vegetative cells (A), colonies from *B. coagulans* grown on glucose yeast extract agar (GYEA) (B) and rod-shaped vegetative cells from *B. coagulans* (C). The colonies of *B. coagulans* are white to cream coloured and smooth (Majeed et al., 2016). The optimal growth temperature of the vegetative cells is between 35-50°C while the optimal pH for growth is between 5.5-6.5. Since the bacteria produce lactic acid it has been shown that it causes some decline in quality in products containing fruit, dairy and vegetables. It does not however produce any gas from fermentation of maltose, mannitol and sucrose. The spores that *B. coagulans* form are terminal meaning that the spores are seen at the poles of the cells, which differs from other species where they are more commonly central or subterminal meaning that the spores are closer to or in the middle of the cells (Konuray and Erginkaya, 2018). The species is similar to another well-known genus of bacteria used for its probiotic activity, *Lactiplantibacillus*, and has been referred to as a spore-forming version of *Lactiplantibacillus* (Keller, Farmer, McCartney and Gibson, 2010).

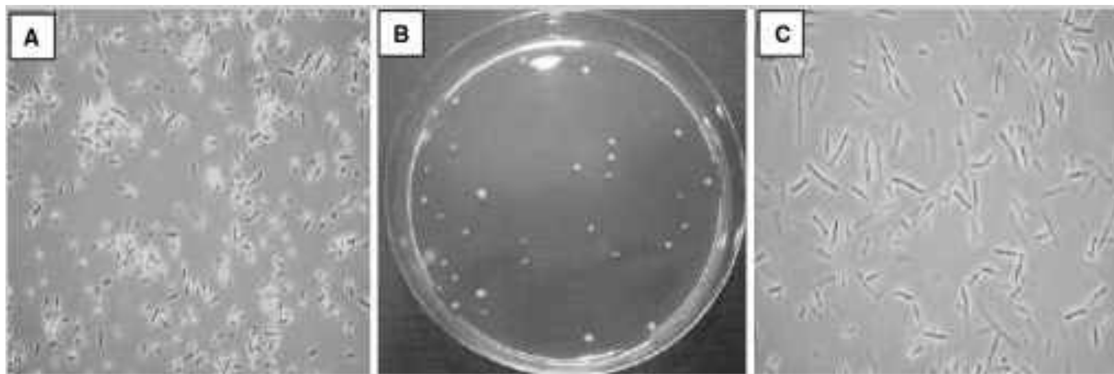

Figure 4. A) Microscopic image of a *B. coagulans* terminal spores and vegetative cells, B) colonies of *B. coagulans* grown on GYEA and C) vegetative cells of *B. coagulans* (Majeed et al., 2016).

B. coagulans being a spore-forming bacteria, has been given the advantage to grow spores if it experiences unfavorable growth conditions and can remain dormant for many years. Then, when the bacteria experience favourable growth conditions (pH, temperature and moisture) the spores become vegetative. In its vegetative form the bacteria returns to life by germination. Studies have

shown that the spores could germinate when they reach the gastrointestinal tract and the spores work as an effective way of delivering large amounts of bacteria to the small intestine for germination, due to survival through the acidic stomach environment (Casula and Cutting, 2002). However, some spores might be triggered to germinate by the stomach acid. The cell growth is likely to be limited by the high levels of bile salt in the small intestine and vegetative cells of *Bacillus* species have a high sensitivity to bile salts (Leser, Knarreborg and Worm, 2007). *B. coagulans* have been proven to have probiotic activity at pH as low as 2.0 (Abada, 2008). These spore-forming properties have made *B. coagulans* interesting when it comes to the functional food business since it overcomes some of the challenges other lactic acid bacteria face (Shinde et al., 2019). *B. coagulans* spores have been used and evaluated in several commercial preparations worldwide and have shown to be stable at conditions of ambient temperature which for the vegetative form is unlikely (Maajed et al., 2016).

Possible health benefits gained from intaking strains of *B. coagulans* have been studied. One study shows that subjects with irritable bowel syndrome (IBS) that received *B. coagulans* in order to relieve symptoms of pain, diarrhea and constipation, experienced significantly milder symptoms than the placebo group (Hun, 2009; Dolin, 2009). A small study examined if *B. coagulans* would ease symptoms of stomach distension and abdominal pain that were intestinal gas-related after meals compared to placebo. The subjects who received the probiotic showed improvement in pain, significantly in abdominal distension (Kalman et al., 2009). There has also been a study to analyse *B. coagulans* anti-inflammatory abilities on a smaller subject group. The probiotic was given in addition to standard medication to subjects with rheumatoid arthritis. By comparing with the placebo group, it was possible to see that the subjects who were given the probiotic addition reported less disability. The ability to participate in activities, such as longer walks, was improved and a marker for inflammation, C-reactive protein (CRP), was reduced (Mandel, Eichas and Holmes, 2010).

The US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) has stated that *B. coagulans* is safe and it is also on the Generally Recognized as Safe (GRAS) list as well as on the Qualified Presumption of Safety list (Konuray and Erginkaya, 2018). According to the Material Safety Data Sheet (MSDS) for the strain used during this master thesis provided by Probi, *B. coagulans* XY1 shows virtually no potential signs of negative health effects through inhalation, ingestion or skin contact.

2.3. Probiotic Fruit Juices

The consumption of fermented dairy products that have been the main source for probiotic delivery has been limited due to the large number of people who have allergies towards products containing lactose and milk proteins. The increase in individuals who chose to consume a vegan or vegetarian diet has also led to a limitation of these kinds of products. Non-dairy probiotic products and fruit-

based food matrices have therefore been increasingly studied (Šárka et al., 2018; Granato et al., 2010).

Utilising fruit juices as the probiotic delivery medium contributes with numerous advantages such as a rich naturally occurring source of nutrients. The digestion of fruit juices is faster in the stomach than for dairy products and therefore the time the probiotic microorganism spends in the acidic environment of the stomach is shorter than if it was inoculated in a dairy product (Ding and Shah, 2020). Even though fruit juices are a good matrix for growing probiotics, the complexity of the survival of these microorganisms is a bigger challenge in fruit juices than in dairy products (Fernandes Pereira and Rodrigues, 2018). The bacteria need to be able to survive in the more acidic environment of these food products (Granato et al., 2010). The fruit juices may contain substances that inhibit the survival of probiotic microorganisms, and have some additives that can lead to the loss of viability of probiotics. There is also a possibility that the sensory aspects of the fruit juices will be changed by the probiotics (Colombo Pimentel et al., 2019).

There are two different ways to turn a fruit juice into functional probiotic food: addition of the microorganisms to the fruit juice or fermentation with probiotics. Addition of the microorganisms has a higher chance of success if the strain is acid tolerant because then the chances for it to survive through the acidic gut environment and reach the intestines are higher. The advantages of fermentation is that the growth of the microbial strain in juices leads to a lower sugar content and more adapted for the strain, which can increase the survival rates. Fermentation can also help increase the quality of the product and decrease the risk of microbial contamination during storage (Fernandes Pereira and Rodrigues, 2018; Nguyen et al., 2019).

When choosing the juice flavor to inoculate probiotic strains in, it has been shown that it is favorable if the fruit juices have a high fiber and protein content (Fernandes Pereira and Rodrigues, 2018). Orange juice has a high acid concentration and fiber content and has been suitable for addition of probiotics. The fibers can physically protect the probiotic microorganisms from different types of damages. Since orange already contains vitamin C, it may reduce dissolved oxygen levels in the medium and enhance the possibility of the survival of the probiotics. Some fruits such as strawberry, kiwi and cranberry may promote loss of viability during storage. Cranberry has a very low pH (around 2.50) which contributes to a viability loss compared to fruits with a higher value of pH (Colombo Pimentel et al., 2019).

2.4. Heat Treatment of Probiotic Food Products

Heat treatment of food products is performed in order to ensure that the products are safe for people to consume and to enhance the shelf-life of food. Heat treatment reduces the presence of already existing microorganisms, either naturally present or through contamination, that has been introduced prior to the heat treatment step, e.g. harmful bacteria in the product (Wells-Bennik et al., 2016). There have been documented cases of foodborne illness, such as food poisoning, due to

the lack of heat treatment of fruit and vegetable juices. Between 1990-2010 there were 1700 people who got sick from foodborne illnesses and 2 deaths, due to unpasteurised juices and ciders in the US and Canada. In unpasteurised juices the most common pathogens are *E. coli* O157 and O111, *Salmonella, Cryptosporidium* and norovirus (Unpasteurized Fruit/Vegetable Juices and Ciders: A Potential Health Risk, 2019). Most people's immune systems are efficient enough to handle the effects of foodborne illness, but there are some risks for people with a weakened immune system to develop serious illnesses due to untreated juices (FDA, 2017). Therefore, heat treatment of fruit drinks is very important and should not be disregarded in the production of probiotic beverages either.

Unlike most other lactic acid bacteria, *B. coagulans* have a spore-forming mechanism (Konuray and Erginkaya, 2018). This species has therefore been considered for food products that need to be processed through heat treatment. The spore-forming abilities have shown an advantage regarding survival of these bacteria and they also have a higher stability. Therefore, they are ideal for production of functional foods that need to be heat treated since their viability is protected even at higher temperatures. By heat treating juices the storage time can also be increased (Hyronimus, Le Marrec, Hadj Sassi and Deschamps, 2000).

2.5. Available Commercial Probiotic Fruit Beverages

The market of probiotic foods has been increasing with new developments and applications. Food and beverage make up for the main area of application of the probiotics market, covering 73% of the total market share. The regions that consume these products to the greatest extent are Asia, Pacific, North America and Europe. There are some key participants that contribute to this probiotic market, which includes Biogaia, Danone, DuPont, Probi, Probiotics International and Christian Hansen Holding. Probiotic fruit juices are being produced by various companies on the probiotic market, such as Danone/ProViva, Tropicana and Naked Juice (Colombo Pimentel et al., 2019; Fernandes Pereira and Rodrigues, 2018).

ProViva is a brand of probiotic fruit juices and a part of Danone Nordic. The strain used in ProViva is *Lactiplantibacillus plantarum* 299v (*L. plantarum* 299v), patented by Probi and launched in 1994. *L. plantarum* 299v is used in the products of GoodBelly as well. ProViva is a cooperation that has been developed between Skånemejerier and Probi (Proviva, 2020; Colombo Pimentel et al., 2019). It consists of a whole line of probiotic fruit drinks. There is a regular product, which is possible to find in multiple flavors and fruit combinations e.g. "Passion Orange", which contains orange juice and pulp, grape juice, banana puree and passion fruit juice and "Raspberry Pomegranate", which contains a fruit mix of raspberry, pomegranate, sugar, banana, grape and chokeberry. ProViva also has a product called ProViva 50 which is a fruit juice without any added sugar and steviol glycosides is used as a sweetener that comes in four flavors (Fernandes Pereira and Rodrigues, 2018). Tropicana has a probiotic fruit juice that contains one billion live probiotic cultures per serving and comes in three flavors (Tropicana, 2020). Pepsi Company has the brand

Naked Juice which consists of one probiotic product called “Probiotic Machine Tropical Mango” and contains the probiotic genus *Bifidobacterium* (Fernandes Pereira and Rodrigues, 2018).

The most common genera of bacteria found in commercial probiotic fruit juices are as previously mentioned *Lactiplantibacillus* and *Bifidobacterium*, which do not contain any spore-formers. Since they are not very heat-resistant, heat treatment after addition of the probiotic is not common since it would decrease the probiotic effect of strains from these genera. Therefore, it is interesting to look into probiotic strains that can form spores, in order to provide consumers with safer and longer lasting probiotic fruit beverages. The previously documented health-benefits of *B. coagulans* and its spore-forming ability therefore makes it a very interesting candidate to enter the probiotic fruit juice market (Konuray and Erginkaya, 2018).

3. Materials and Methods

3.1. Materials

Chemicals, microorganisms and other media used during the experiment are presented in Table 2.

Table 2. The table shows all the chemicals and media, their CAS/product numbers, brands and suppliers used throughout the experiment.

Name	**CAS/product number**	**Brand**	**Supplier**
Bacillus coagulans XY1	-	-	-
Sodium chloride	7674-14-5	EMSURE®	Merck
Milli-Q water	-	Super-Q™ Plus Water System	Merck
Glucose yeast extract agar	M963-500G	HIMEDIA	HIMEDIA
100% orange juice	101197649_ST	Kiviks musteri	Willys
100% pomegranate juice	101293889_ST	Dimes	Willys

3.2. Pre-experimental Tests

3.2.1. Initial Viability Test of *B. coagulans* XY1 for Storage Samples

A sample strain of the probiotic *B. coagulans* XY1 (XY1 was a code number and not the name of strain) was received in the form of a spray-dried powder from Probi AB. The powder was stored at room temperature and kept away from direct light. 0.25 g of the spray-dried powder was dissolved in 50 mL sterile saline solution (0.9% NaCl, w/v) and homogenised by stirring it intensively by using a magnetic stirrer (MR 2000, Heidolph) for 10 minutes. From this sample, a 10x serial dilution in 0.9% NaCl was performed. The dilutions (10^{-5}, 10^{-6} and 10^{-7}) went through a heat-shock treatment in order to activate spore germination. At first it was incubated in a water bath (HMT200, Jouan Nordic) at 75°C for 30 minutes, followed by incubation on ice in order to cool down to around 45°C. When the heat-shock treatment was completed, one mL was pipetted carefully to three Petri plates each. Then, GYEA, which had been melted, sterilised and cooled to 45-50°C prior to experiment, was added by using the pour-plate method to each of the five plates.

Two additional plates were used as negative controls, one with growth medium and one with sterile saline solution. The plates were incubated at 37°C for 72 hours to allow colonies to grow. After the incubation time, the colonies were counted using the plate count method (PCM) (Figure A.1 in Appendix).

3.2.2. Initial Viability Test of *B. coagulans* XY1 for Heat Treatment Samples

For the heat treatment part of this project, an additional batch of sample strain *B. coagulans* XY1 spray-dried powder was provided in a different packaging material by Probi. This probiotic powder was prepared in the exact same manner as the one for the storage samples under section "3.2.1. Initial Viability Test of *B. coagulans* XY1 for Storage Samples".

3.2.3. Choice of Fruit Juices

The predetermined criteria for the juices were that the pH range should be between 3-4.2 in order to achieve a good analysis and that they preferably should consist of 100% fruit concentration (no additives). Prior to the sample preparation, 10 juices with different flavours, most of them containing 100% fruit concentration, were tested with the purpose to find two juices within the correct pH range. The 10 juices that were chosen for pH testing were based on previous literature of pH values of different fruit juices in the correct range (Master List of Typical pH and Acid Content of Fruits and Vegetables for Home Canning and Preserving, 2020). The two juices chosen were orange juice with 100% fruit concentrate and a pH of 3.94, and pomegranate juice with 100% fruit concentrate and water with a pH of 3.30. The table of contents of the juices are seen in Table 3. The orange juice had a shelf life of 6 months, if stored unopened in room temperature and the pomegranate juice had a shelf life of 7 months, if stored unopened in a cool and dry place. The recommendation for both of the juices after opening them, was to consume within 5 days and store them in the refrigerator.

Table 3. Table of contents of the two fruit juices.

Pomegranate juice	Orange juice
Pomegranate juice concentrate 100%	Orange juice concentrate 100%
Water	-

3.3. Storage Samples

This part of the project refers to the specific objective addressing the behaviour of the probiotic strain in the two fruit juices and analysis of the juices over the storage time of 10 weeks.

3.3.1. Sample preparation

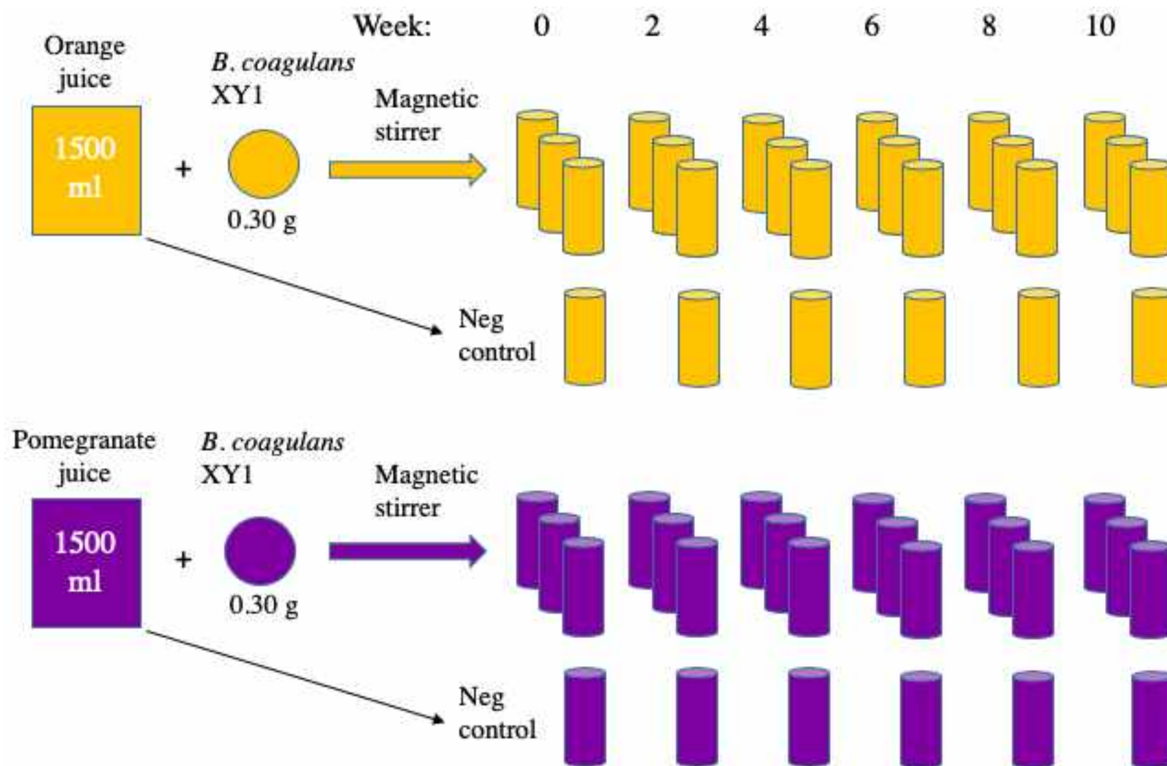

Figure 5. The experimental setup of the storage samples for 10 weeks stored in Falcon tubes. Negative control samples of each juice were first taken out from pure juice and then 1500 mL of each juice was inoculated with 0.30 g *B. coagulans* XY1. The juices were mixed using a magnetic stirrer and then divided into 24 samples each (n=3 for each testing week).

The overall process of preparing the storage samples for viability testing over 10 weeks can be seen in Figure 5. The two juices as previously mentioned were one orange and one pomegranate. Prior to inoculation of the probiotic strain, negative controls of the two juices were prepared. Six negative control samples (50 mL each) of each juice were transferred into Falcon tubes and stored in 8-10°C for future testing (Figure 3.1). The results from the initial viability test of the probiotic confirmed that 0.2 g of probiotic powder was needed for 1 L of each juice to obtain the desired initial concentration (cfu/mL) in the juices. 0.30 g of probiotic powder was added in 1.50 L of each juice to obtain the desired volume to cover the storage sample volumes. After the probiotic was inoculated in the juices, the two solutions were both mixed thoroughly with a magnetic stirrer (MR 2000, Heidolph) for 10 minutes. The volume of the inoculated juices were each divided into 24 Falcon tubes (50 mL each) with the purpose to minimize the head space and create an overall anaerobic environment, for storage over the shelf-life period of 10 weeks (0 + 10 weeks). The samples were made for testing every other week along with some extra samples (three of each juice) that were saved as well. The samples were stored in the same way as the negative juice control samples, at 8-10°C. The process of preparing the inoculation and the samples took place in the sterile bench.

3.3.2. Viability Test of *B. coagulans* XY1

The viability test of *B. coagulans* XY1 was performed by using PCM. The preparation of the experimental samples used for each testing week where described above. Samples of the two inoculated juices were stored in 50 mL Falcon tubes and both of the inoculated juices were analysed every other week along with negative control samples of the two juices, the sterile saline solution and the growth medium.

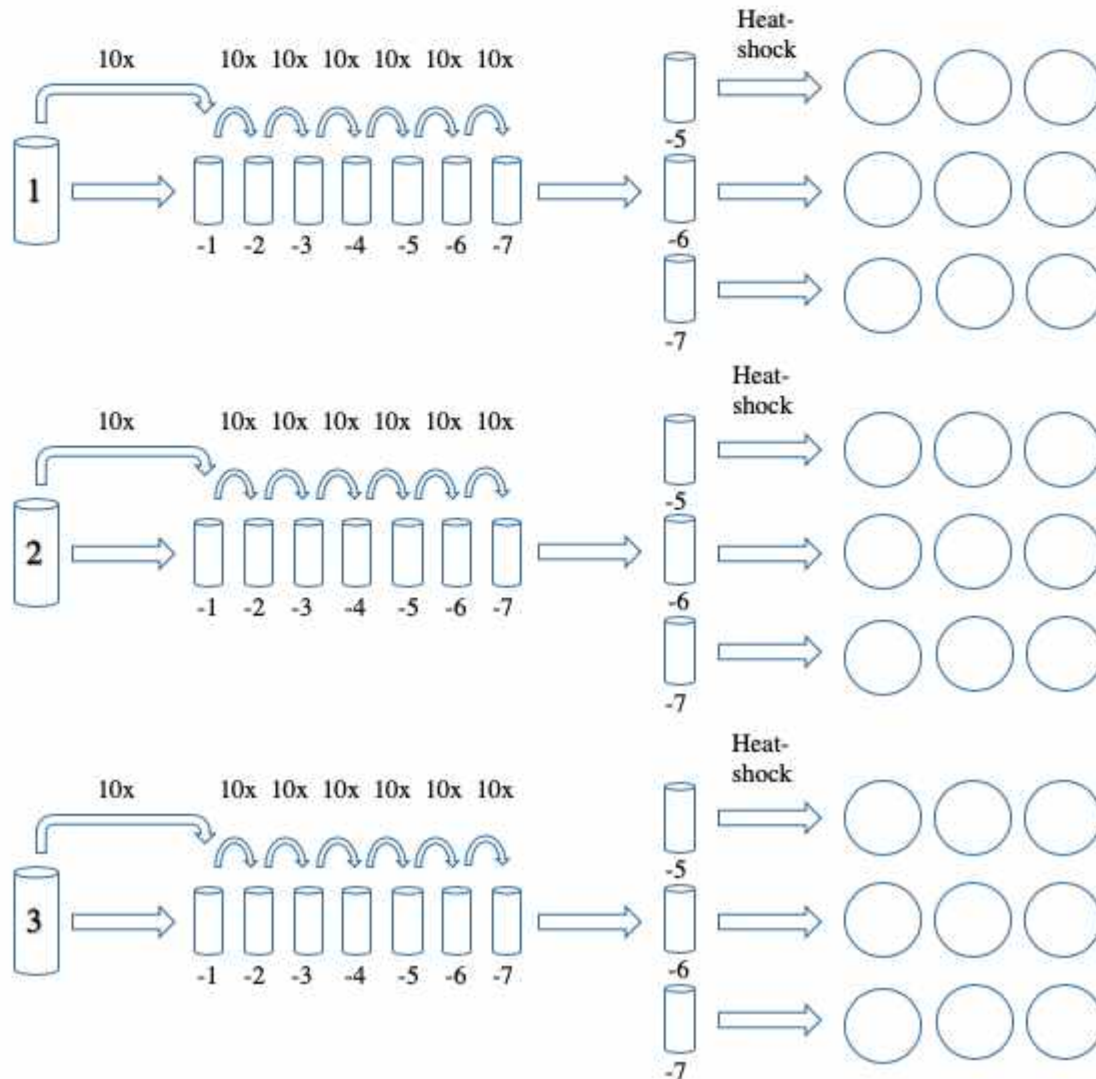

Figure 6. The process of preparing and performing the viability analysis for each juice in triplicates (1, 2 and 3, n=3). Each juice sample was diluted by a 10x serial dilution and three dilutions were put through heat-shock treatment and then plated in triplicates.

The process of preparing and performing the viability analysis is illustrated in Figure 6. One mL of each inoculated juice sample (in triplicates) from the Falcon tubes were pipetted into 9 mL of sterile saline solution. Then a 10x serial dilution in 0.9% NaCl solution was performed and three dilutions (10^{-5}, 10^{-6} and 10^{-7}) were chosen from each juice and put through a heat-shock treatment.

The dilutions were incubated in a water bath (HMT200, Jouan Nordic) at 75°C for 30 minutes, followed by incubation on ice in order to cool down to about 45°C.

After the heat-shock treatment, the samples were directly plated in triplicates using pour plate method, where one mL of the samples first were added to the plates and then about 15 mL GYEA were poured onto them. one mL of each of the control samples (juice controls, medium and 0.9% NaCl solution) were plated in the same way as the inoculated juice samples. The plates were incubated at 37°C for 72 hours and then the total cfu were counted per mL of sample. The viability test took place on a sterile bench.

3.3.3. pH

The pH of the inoculated juice samples and the juice control samples was tested every other week for 10 weeks at the same time as the viability test. After the desired volumes for the viability tests were collected, a sufficient volume for pH measuring was poured out of the Falcon tubes in storage for the correct testing week. The pH of the two juices used in the experiment was tested prior to the experiment initiated, as described above, and the pH meter (Five Easy™ FE20, Mettler Toledo) was calibrated prior to each measurement according to the initial pH of the juices, between pH 2-7. The pH was measured for both inoculated juice samples in triplicates as well as in the control juice samples.

3.3.4. Lactic Acid Concentration

The lactic acid concentration was tested three times in total throughout the storage time (week 0, 6 and 10) using QuantiQuik™ L-Lactic Acid Quick Test Strips (Universal Biologicals, Life Science Research Products) and High-performance Liquid Chromatography (HPLC), (LC-118, Beckman Instruments AB) with a Refractive Index detector (RID-6A, Shimadzu).

3.3.4.1. QuantiQuik™ L-Lactic Acid Quick Test Strips

Prior to the measurement the 100µL from each sample was transferred by pipetting to the provided Sample Development Tube which contained 400 µL solution to obtain a 5x dilution, which was recommended for fruit juice samples. The caps were then closed on all of the tubes as well as the vials, and the diluted samples were mixed by inverting the vials a few times. Thereafter, the caps were unscrewed and the test strips were put in the vials, while carefully making sure that the yellow reaction pads were fully submerged at the end of the strip. The strips were left for 5 seconds before being taken out and shaken to remove any excess drops on the strips. The colour of the reaction pads was left to stabilise for 5 minutes and then compared with the provided L-Lactic Acid Chart. The concentrations visible in the chart were multiplied with the 5x dilution to obtain the lactic acid concentration in the samples (QuantiQuik™ L-Lactic Acid Quick Test Strips, 2017). A positive control was also performed with a yoghurt sample, which is known to contain lactic acid (Niamsiri and Batt, 2009).

3.3.4.2. HPLC

As a complement to the test strips, HPLC (LC-118, Beckman Instruments AB) was run to detect the concentration of lactic acid and how it changed over time. One mL of the probiotic orange and pomegranate juice samples were transferred to transparent glass vials and placed in the HPLC instrument. The columns used were two RHM-Monosaccharide H+ [300 x 7.80 mm] (Rezex, Phenomenex) columns coupled after each other. The solvent was 100% 5mM H_2SO_4 and a flow rate of 0.6 mL/min. The injection volume was 20 µL and the analytes were eluted using a 60 minutes long gradient.

3.3.5. Sensory Evaluation

The sensory evaluation was performed three times in total during week 0, 6 and 10, in order to check whether the products underwent any considerate visual or aromatic changes after being inoculated with the probiotic for a longer time. The evaluation did not require any instruments or measurements, instead it was completely based on visual inspection. Results from the visual inspection therefore worked as a rough estimation of the appearance of the products. The analysis was performed using a sensory evaluation graded 1-9 on a hedonic scale (Table 4), which ranged from extreme dislike to extremely like for the different parameters tested (Hooda and Jood, 2005). The parameters tested were colour, texture and aroma and summarised in overall acceptability.

Table 4. Hedonic scale for sensory evaluation of food products.

1	Dislike extremely
2	Dislike very much
3	Dislike moderately
4	Dislike slightly
5	Neither like nor dislike
6	Like slightly
7	Like moderately
8	Like very much
9	Like extremely

3.4. Heat Treatments

To reach the second objective, three different heat treatments were performed in order to see how the viability of the probiotic strain would be affected by heat through a combination of different

times and temperatures. These heat treatments were performed two times in total (n=2), therefore twice for each juice during the experimental period of the project and the viability of the probiotic strain was evaluated.

3.4.1. Setup

The setup of the heat treatment consisted of a water bath (Lauda RM 6 B, Lauda) filled with a glycerol solution and connected to a jacketed glass double layer flask reactor bottle with a volume of 1000 mL placed on a magnetic stirrer (MR 2002, Heidolph). Glycerol solution from the water bath (Lauda RM 6 B, Lauda) filled up the space between the double layer of the glass flask and heated the content of the bottle to a desired temperature over a set time (Figure 7). Three different combinations of time and temperature were tested, one for each juice sample, based on values obtained from Probi, representing temperature and holding time combinations commonly used in the food industry during pasteurisation.

1. 80°C for 30 minutes
2. 88-92°C for 15-20 seconds
3. 95°C for 10 minutes

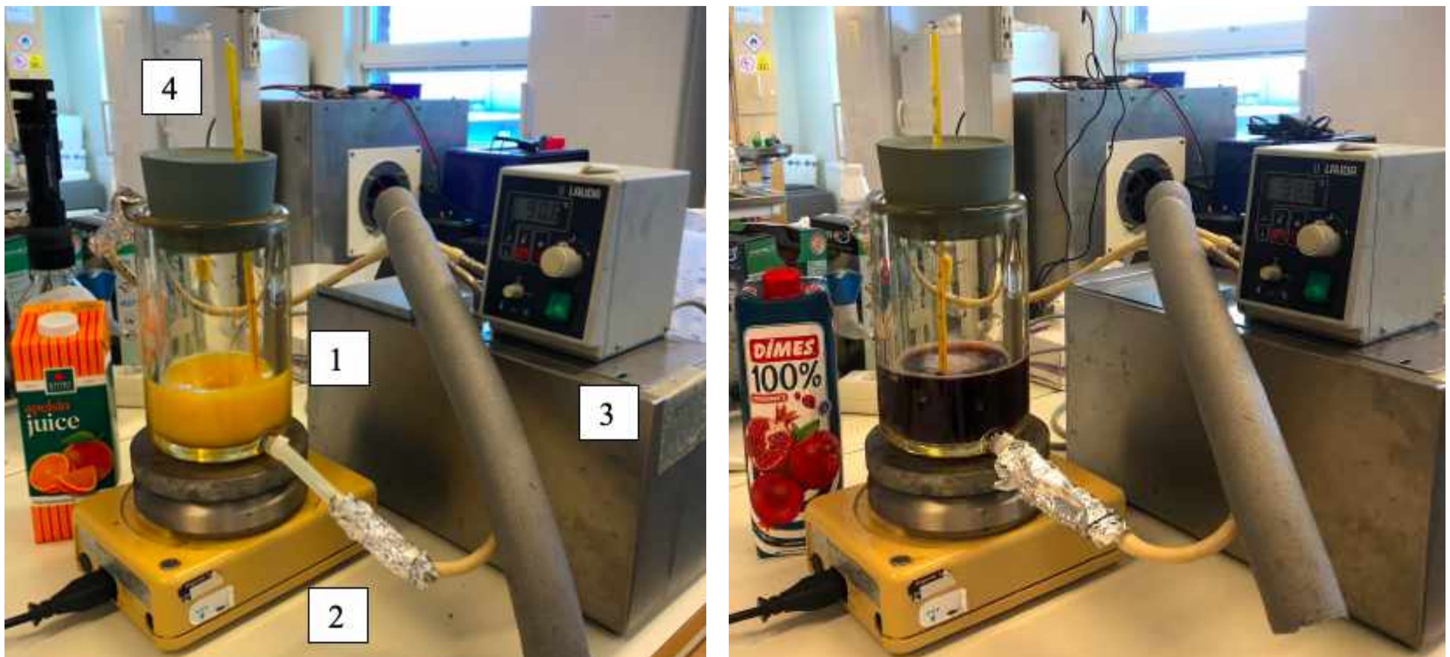

Figure 7. The setup of the heat treatment step with orange juice to the left and pomegranate juice to the right (1=jacketed glass double layer flask reactor bottle, 2=magnetic stirrer, 3=water bath and 4=thermometer).

3.4.2. Sample Preparation

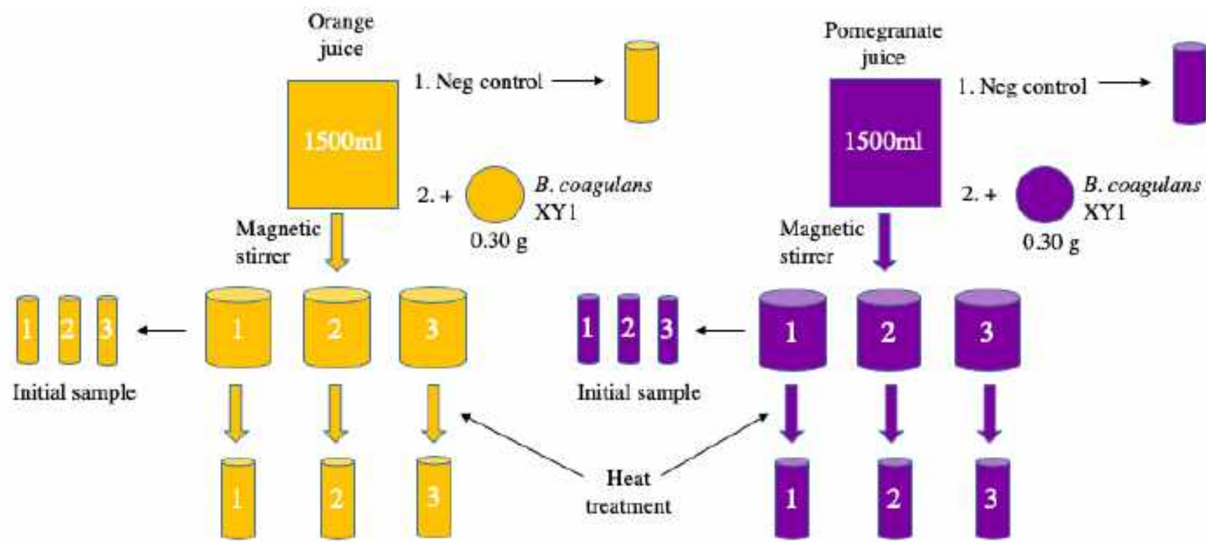

Figure 8. Preparation of heat treatment samples. First negative controls were taken out from the pure juices, then inoculated with *B. coagulans* XY1 and mixed with a magnetic stirrer. Then each juice were divided into three glass bottles for each time and temperature combination (1=80°C/30min, 2=88-92°C/15-20s and 3=95°C/10min) and samples were taken out before heat treatment "initial sample" for obtaining a start concentration (cfu/mL). Then the samples went through heat treatment and were each collected in new glass bottles.

The preparation process is illustrated in Figure 8. The two juices as previously mentioned were one orange and one pomegranate. Prior to inoculation of the probiotic strain, negative controls of the two juices were prepared. One negative control sample (50 mL each) of each juice was transferred into a Falcon tube. The results from the initial viability test of the probiotic confirmed that 0.2 g of probiotic powder was needed for 1 L of each juice to obtain the desired initial concentration (cfu/mL) in the juices. 0.30 g of probiotic powder was added in 1.5 L of each juice to obtain the desired volume for the heat treatment. After the probiotic was inoculated in the juices, the two solutions were both mixed thoroughly with a magnetic stirrer (MR 2000, Heidolph) for 10 minutes. The volume of the inoculated juices were each divided into three smaller flasks (400mL), each for one combination of time and temperature. Initial samples were taken out (50mL) to obtain a start concentration (cfu/mL). Then, 300mL of each juice sample went through heat treatment and was collected into Falcon tubes (50 mL) after. The process of preparing the inoculation and the samples took place in the sterile bench.

3.4.3. Viability Test of *B. coagulans* XY1

The viability test of *B. coagulans* XY1 was performed by using PCM. After all the samples of the two inoculated juices had been heat treated, the viability of *B. coagulans* XY1 was studied. In total there were three samples of each juice, each treated with a specific time and temperature.

Prior to the heat treatment step, samples were taken from all pre-divided samples of the juices (three for each juice) in order to determine the start concentration of *B. coagulans* XY1 (initial samples). One mL of each juice sample was pipetted into 9 mL of 0.9% NaCl solution. Then a 10x serial dilution was performed. Three dilutions (10^{-5}, 10^{-6} and 10^{-7}) were chosen from each juice sample. The dilutions were incubated in a water bath (HMT200, Jouan Nordic) at 75°C for 30 minutes, followed by incubation on ice in order to cool down to about 45°C. Thereafter, plated in triplicates using pour plate method, where one mL of the samples first were added to the plates and then about 15 mL GYEA were poured onto them. One mL of each of the control samples (juice controls, medium and 0.9% NaCl solution) were plated in the same way as the inoculated juice samples. The plates were incubated at 37°C for 72 hours and then the total cfu were counted per mL of sample.

After all the samples had gone through their specific temperature and time combination of the heat treatment, the viability test was performed in the same manner as described above but with plating of dilutions 10^{-4}, 10^{-5} and 10^{-6} for the first experiment and plating of dilutions 10^{-3}, 10^{-4} and 10^{-5} for the second experiment. Another difference was that the heat-shock treatment did not take place since the spores most likely had been activated during the heating of the juice samples which took about the same time as the heat-shock treatment. All of the viability tests took place in the sterile bench.

3.5. Statistical Analysis of Data

For the statistical analysis of data a 95% confidence interval was performed using Excel (Microsoft Office 365). The difference between the samples was the type of juice the probiotic was inoculated in (orange or pomegranate).

4. Results

The results presented in this section were obtained and documented from the different experimental tests of the two fruit juices inoculated with *B. coagulans* XY1 during the shelf-life period of 10 weeks (0 + 10 weeks), as well as during a number of heat treatments.

4.1. Pre-experimental Tests

4.1.1. Initial Viability Test of *B. coagulans* XY1 for Storage Samples

The results from the initial viability test of *B. coagulans* XY1 are seen in Table 5. The estimation according to the specification sheet for the spray-dried powder was that the 10^{-7} dilution was expected to produce 30 to 300 colonies per plate when the incubation time was over. In order to reach this number of colonies in the juices, 0.20 g of the spray-dried *B. coagulans* XY1 powder was needed for 1 L of each juice. The table was calculated according to section A.2 in Appendix.

Table 5. Viability results using PCM for *B. coagulans* XY1 spray-dried powder for storage samples.

Dilution	Plate 1 (cfu)	Plate 2 (cfu)	Plate 3 (cfu)	Average (cfu)	Mean cfu/g
10^{-6}	285	269	311	288	$5.7*10^{10}$
10^{-7}	39	65	41	48	$9.6*10^{10}$
Mean	-	-	-	-	$7.7*10^{10}$

4.1.2. Initial Viability Test of *B. coagulans* XY1 for Heat Treatment Samples

The results from the initial viability test of *B. coagulans* XY1 are seen in Table 6. The estimation according to the specification sheet for the spray-dried powder was that the 10^{-7} dilution was expected to produce 30 to 300 colonies per plate when the incubation time was over. In order to reach this number of colonies in the juices, 0.20 g of the spray-dried *B. coagulans* XY1 powder was needed for 1 L of each juice. The table was calculated according to section A.2 in Appendix.

Table 6. Viability results using PCM for *B. coagulans* XY1 spray-dried powder for the heat treatment experiments.

Dilution	Plate 1 (cfu)	Plate 2 (cfu)	Plate 3 (cfu)	Average (cfu)	Mean cfu/g
10^{-6}	332	331	316	326	$6.5*10^{10}$
10^{-7}	44	35	38	39	$7.8*10^{10}$
Mean	-	-	-	-	$7.2*10^{10}$

4.2. Viability and Stability of *B. coagulans* XY1 during Storage

This part of the results refers to the samples that were prepared for 10 weeks of storage in order to study their viability and stability during storage.

4.2.1. Viability of *B. coagulans* XY1

In order to investigate the viability of *B. coagulans* XY1 in orange and pomegranate juice during a storage period of 10 weeks, an experiment was designed where a large volume of each juice was inoculated with a defined amount of cells and aliquoted into a set of identical samples that were incubated at a storage time of 8-10°C. At regular intervals, every other week, samples of each juice were removed from the incubator and the viability of the inoculated *B. coagulans* XY1 was measured by PCM. The storage samples were plated using pour plate method and incubated at 37°C for 72 hours to let colonies grow. Different dilutions from the GYEA plates with 30-300 colonies on each plate were counted every other week using PCM during a total of 10 weeks and documented as cfu per mL juice sample. The viability of *B. coagulans* XY1 did go below the recommended minimum concentration of 10^7 cfu/mL at some testing times both for the orange and the pomegranate juice and there was not a clear pattern following how the viability changed in both juices (Figure 9). The average starting concentration at week 0 was $1.14 \pm 0.27*10^7$ cfu/mL for probiotic orange juice and $0.96 \pm 0.50*10^7$ cfu/mL for probiotic pomegranate juice. After the storage time of 10 weeks the average concentration was $1.12 \pm 0.12*10^7$ cfu/mL for probiotic orange juice and $0.87 \pm 0.087*10^7$ cfu/mL for probiotic pomegranate juice (Table A.1 in Appendix).

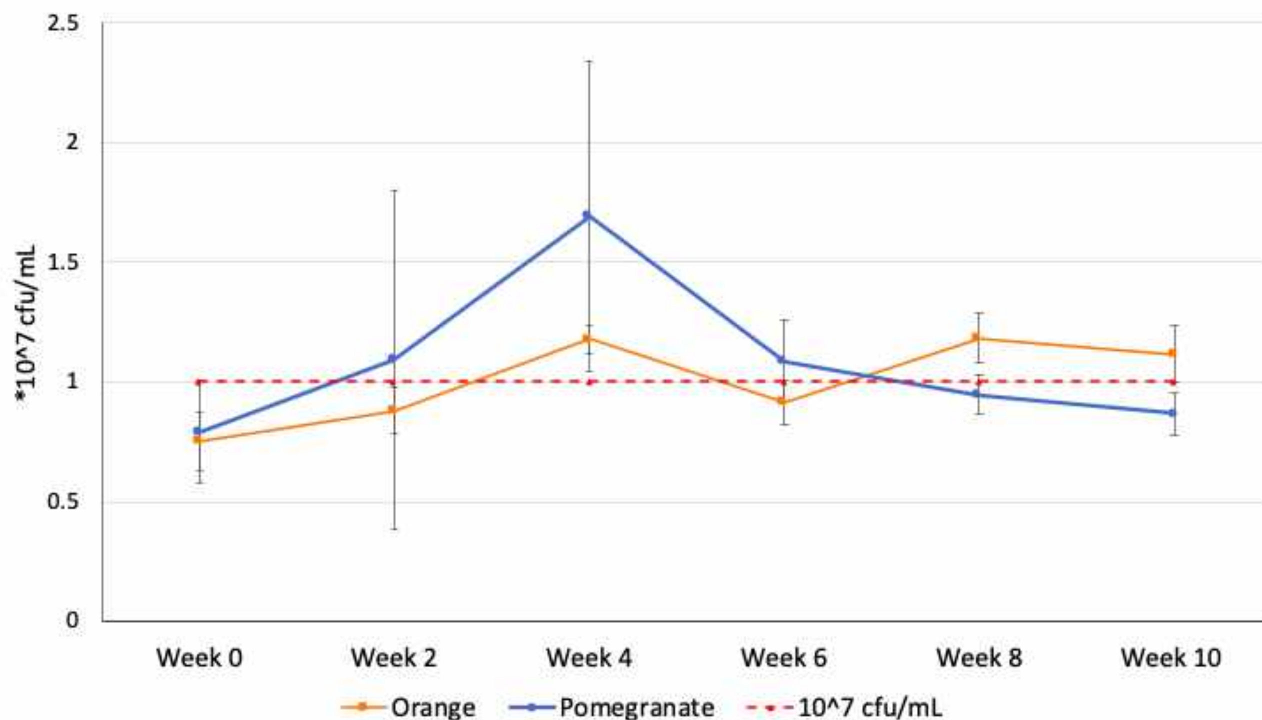

Figure 9. Viability of *B. coagulans* XY1 in orange and pomegranate juice over 10 weeks of storage shown in average probiotic cfu/mL. Average values and standard deviations of two independent biological replicates are shown and three technical replicates for each juice.

4.2.2. pH of Fruit Juices Inoculated with *B. coagulans* XY1 during Storage

The pH values of the orange, pomegranate and their corresponding negative control samples (no inoculation with *B. coagulans* XY1) were measured six times in total, during the same week as the viability tests were performed over 10 weeks in storage (Figure 10 and 11). The average starting pH at week 0 was 3.93±0.014 for probiotic orange juice and 3.32±0.014 for probiotic pomegranate juice. After the storage time, the average pH was 3.92±0.014 for probiotic orange juice and 3.34±0.014 for probiotic pomegranate juice (Table A.2 in Appendix). There was no clear difference in pH values between the two probiotic juices and their control samples throughout the storage time and it is possible to see that the pH values remained more or less stable during storage.

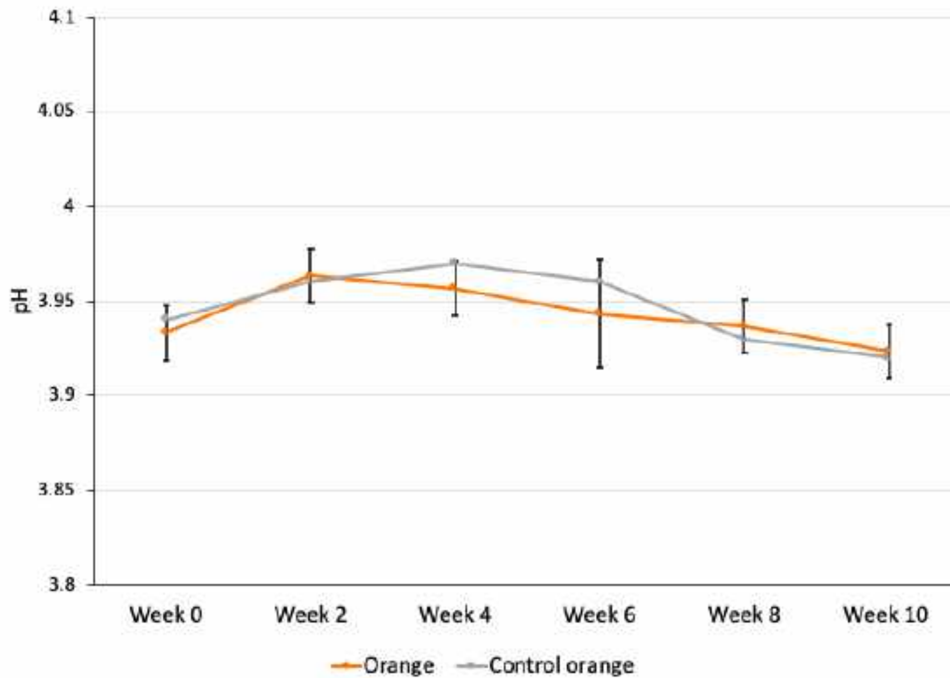

Figure 10. Average pH of orange juice inoculated with *B. coagulans* XY1 and control juice sample over 10 weeks of storage. Control samples (orange) were plotted against probiotic juice samples to analyse any change in pH over time. Average values and standard deviations from three technical replicates are shown.

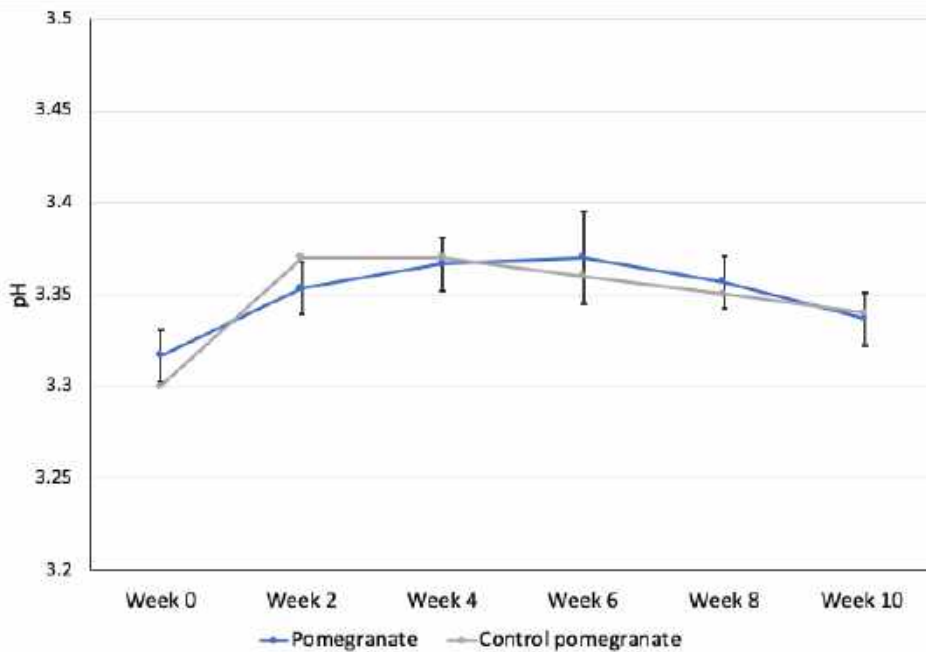

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Figure 11. Average pH of pomegranate juice inoculated with *B. coagulans* XY1 and control juice sample over 10 weeks of storage. Control samples (orange) were plotted against probiotic juice samples to analyse any change in pH over time. Average values and standard deviations from three technical replicates are shown.

4.2.3. Lactic Acid Concentration of Fruit Juices Inoculated with *B. coagulans* XY1 during Storage

The lactic acid concentration in the juices was measured using two methods, one with test strips and the other by HPLC to obtain data from two independent methods that could be compared. The lactic acid concentration was tested three times in total in the inoculated juice samples and their controls, during testing week 0, 6 and 10, to see whether or not it changed over the shelf-life period of 10 weeks.

4.2.3.1. QuantiQuik™ L-Lactic Acid Quick Test Strips

The strips did not give any reliable results at any of the testing occasions (week 0, 6 and 10) regarding the lactic acid concentration in neither the inoculated fruit juice samples or their negative control samples. The strips did not indicate whether there were any lactic acid in the juices, negative or positive controls at any testing occasion. Therefore, no values can be obtained from using these strips and no results can be presented.

4.2.3.2. HPLC

The HPLC results showed that there was a small peak (at the grey line for orange juice) for lactic acid in the probiotic orange juice samples (Figure 12) indicating that there was a low concentration of lactic acid that remained stable without any change throughout the storage period. When compared to a peak of a standard sample of L-lactic acid (175 mg/L), the concentration of lactic acid in probiotic orange juice could be determined to <100 mg/L. In the chromatogram for probiotic pomegranate juice samples, no peak of lactic acid could be detected (Figure 13). Therefore, this concentration was negligible throughout the storage period.

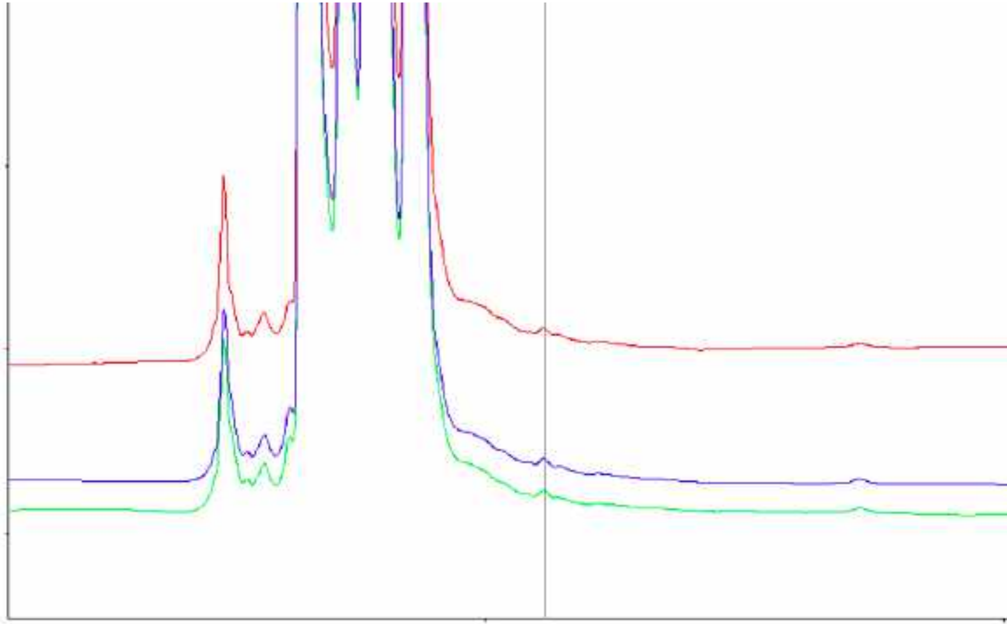

Figure 12. Chromatogram obtained from HPLC for detection of lactic acid in probiotic orange juice. A small peak is visual during all three testing occasions (week 0, 6 and 10) indicating that the lactic acid concentration remained stable throughout storage with a concentration of <100 mg/L when compared to a standard. The grey line indicates where the peaks for lactic acid are in the chromatogram. (Red = week 0, green = week 6 and blue = week 10)

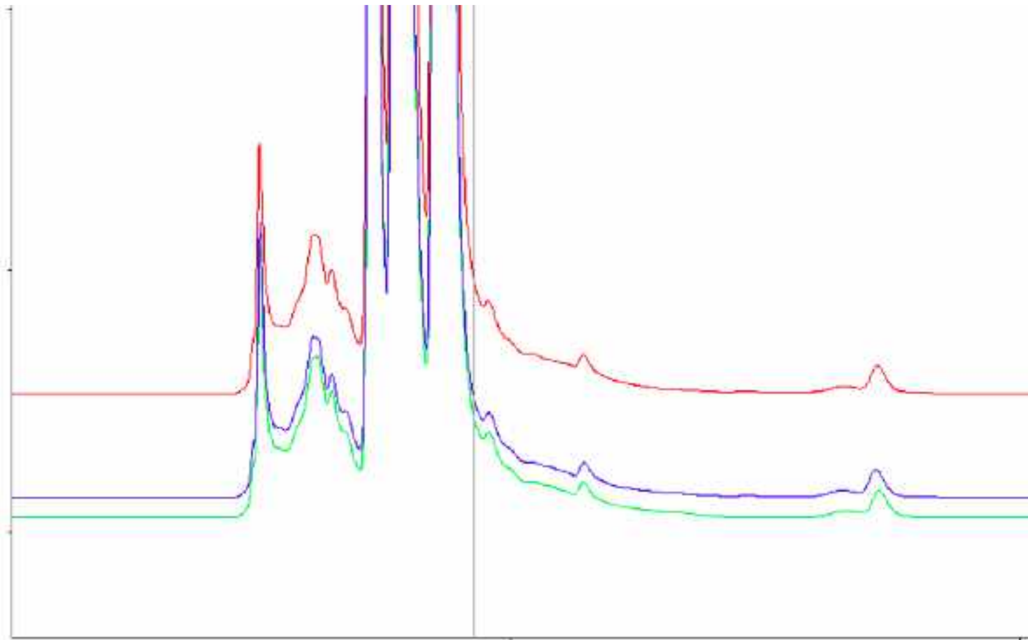

Figure 13. Chromatogram obtained from HPLC for detection of lactic acid in probiotic pomegranate juice. No peak was seen during all three testing occasions (week 0, 6 and 10) indicating that the lactic acid concentration was negligible. (Red = week 0, green = week 6 and blue = week 10).

4.2.4. Sensory Evaluation of Fruit Juices Inoculated with *B. coagulans* XY1 during Storage

The sensory evaluation was performed three times in total over the shelf-life period of 10 weeks, during week 0, 6 and 10 in order to see if the two juices underwent any changes (Table 7). The appearance, colour and aroma did not change in any alarming way during the storage period and the quality of the juices were stable throughout the storage period. Neither the appearance, aroma or colour changed to any alarming levels and the overall acceptability of the juices was still satisfying at the end of shelf life with clear colours, satisfying textures and sweet/sour aromas as you would expect a juice to obtain. Two of the changes that were possible to detect were that the pomegranate juice had a milder arome at the end of shelf life and the orange juice samples were a bit darker. This was however the same in the control samples. There was a very small difference between the probiotic juice samples and the control samples indicating that the addition of *B. coagulans* XY1 did not affect the quality of the juices to any unsatisfactory or unlikeable conditions.

Table 7. The average results from the sensory evaluation of the two juices and their control samples during week 0, 6 and 10 of storage. The scale ranging from extremely dislike (1) to extremely like (9) according to the hedonic scale in Table 4.

	Week 0				**Week 6**			
Juice type	Appear ance	Colour	Aroma	**Overall accepta bility**	Appear ance	Colour	Aroma	**Overall accepta bility**
Orange	9	9	8	**9**	9	8	8	**8**
Orange control	9	9	8	**9**	9	9	8	**9**
Pomegra nate	8	9	8	**8**	8	9	7	**8**
Pomegra nate control	8	9	8	**8**	8	9	8	**8**

	Week 10			
Juice type	Appearance	Colour	Aroma	**Overall acceptability**
Orange	8	8	8	**8**
Orange control	8	8	8	**8**
Pomegranate	8	9	7	**8**
Pomegranate control	8	9	7	**8**

4.3. Survival of *B. coagulans* XY1 during Heat Treatments

This part of the results refers to the samples that were put through heat treatments in order to study their survival at different time and temperature combinations. This was performed twice in total for each juice (experiment 1 and 2), using the same three different time and temperature combinations at each occasion.

4.3.1. Survival of *B. coagulans* XY1 during Heat Treatment Experiment 1

In order to investigate the survival of *B. coagulans* XY1 in fruit juices, an experiment was designed where three different time and temperature combinations (80°C/30min, 88-92°C/15-20s and 95°C/10min) were used for treatment of orange and pomegranate juice inoculated with *B. coagulans* XY1. After performing each respective heat treatment, the probiotic juice samples were plated using pour plate method and incubated at 37°C for 72 hours to let colonies grow. Different dilutions from the GYEA plates containing 30-300 colonies, were counted using PCM and documented as cfu per mL juice sample for samples from before the heat treatment "initial samples" and after the heat treatment. The viability of *B. coagulans* XY1 did decrease in different proportions depending on which combination for temperature and time was used (Figure 14, 15, 17 and 18).

Heat treatment at 80°C for 30 minutes

The average starting concentrations for JO:1 (JO symbolizing probiotic orange juice) and JP:1 (JP symbolizing probiotic pomegranate juice), the samples that later underwent heat treatment by 80°C for 30 minutes, were $1.34\pm0.90*10^7$ cfu/mL for JO:1 and $1.03\pm0.30*10^7$ cfu/mL for JP:1. After the heat treatment, the average concentrations were $0.57\pm0.21*10^6$ cfu/mL for JO:1 and $0.56\pm0.10*10^6$ cfu/mL for JP:1 (Table A.3 in Appendix). This heat treatment led to a decrease of

probiotic concentration in both juices and the probiotic concentrations in both juices went below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:1 was 1.37 and for JP:1 it was 1.26. This indicates that less than 95% of the probiotic was reduced in both JO:1 and JP:1 after heat treatment at 80°C for 30 minutes (Figure 16 and Figure 19).

Heat treatment at 88-92°C for 15-20 seconds

The average starting concentrations for JO:2 and JP:2, the samples that later underwent heat treatment by 88-92°C for 15-20 seconds, were $1.06\pm0.15*10^7$ cfu/mL for JO:2 and $1.77\pm0.52*10^7$ cfu/mL for JP:2. After the heat treatment, the average concentrations were $0.13\pm0.063*10^6$ cfu/mL for JO:2 and $0.52\pm0.13*10^6$ cfu/mL for JP:2 (Table A.3 in Appendix). This heat treatment led to a big decrease of probiotic concentration in both juices and the probiotic concentrations in both juices went below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:2 was 1.90 and for JP:2 it was 1.54. This indicates that around 99% of the probiotic was reduced in JO:2 and around 95% in JP:2, after heat treatment at 88-92°C for 15-20 seconds (Figure 16 and Figure 19).

Heat treatment at 95°C for 10 minutes

The average starting concentrations for JO:3 and JP:3, the samples that later underwent heat treatment by 95°C, for 10 minutes were $1.00\pm0.31*10^7$ cfu/mL for JO:3 and $1.09\pm0.97*10^7$ cfu/mL for JP:3. After the heat treatment, the average concentrations were $0.023\pm0.038*10^5$ cfu/mL for JO:3 and $0.013\pm0.014*10^5$ cfu/mL for JP:3 (Table A.3 in Appendix). This heat treatment led to a huge decrease in probiotic concentration of both juices and the probiotic concentrations in both juices went far below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:3 was 2.63 and for JP:3 it was 2.91. This indicates that around 99.9% of the probiotic was reduced in both JO:3 and JP:3 after heat treatment at 95°C for 10 minutes (Figure 16 and Figure 19).

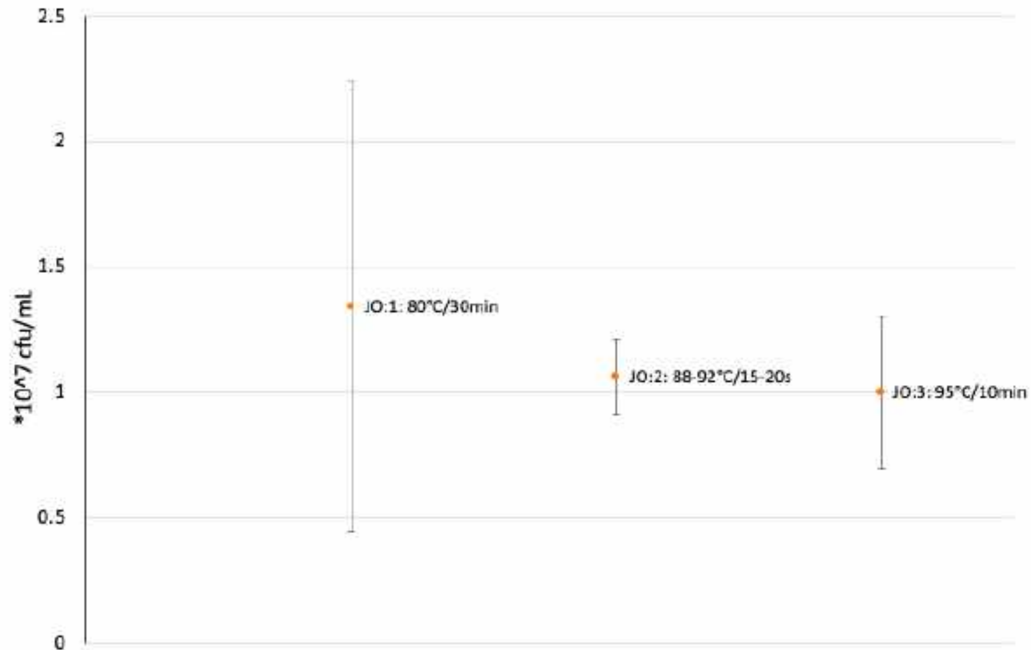

Figure 14. Starting concentration of *B. coagulans* XY1 in the "initial samples" of orange juice before heat treatment was performed. JO:1 represents the juice sample which later underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which later underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which later underwent heat treatment by 95°C/10min.

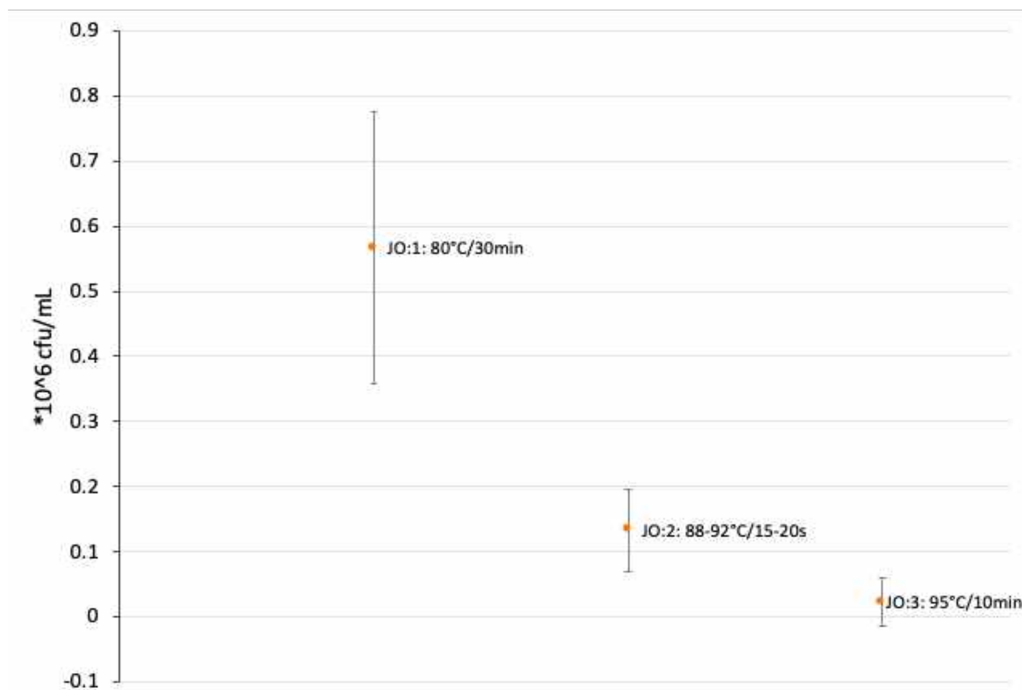

Figure 15. Viability of *B. coagulans* XY1 in orange juice after heat treatment was performed. JO:1 represents the juice sample which underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which underwent heat treatment by 95°C/10min.

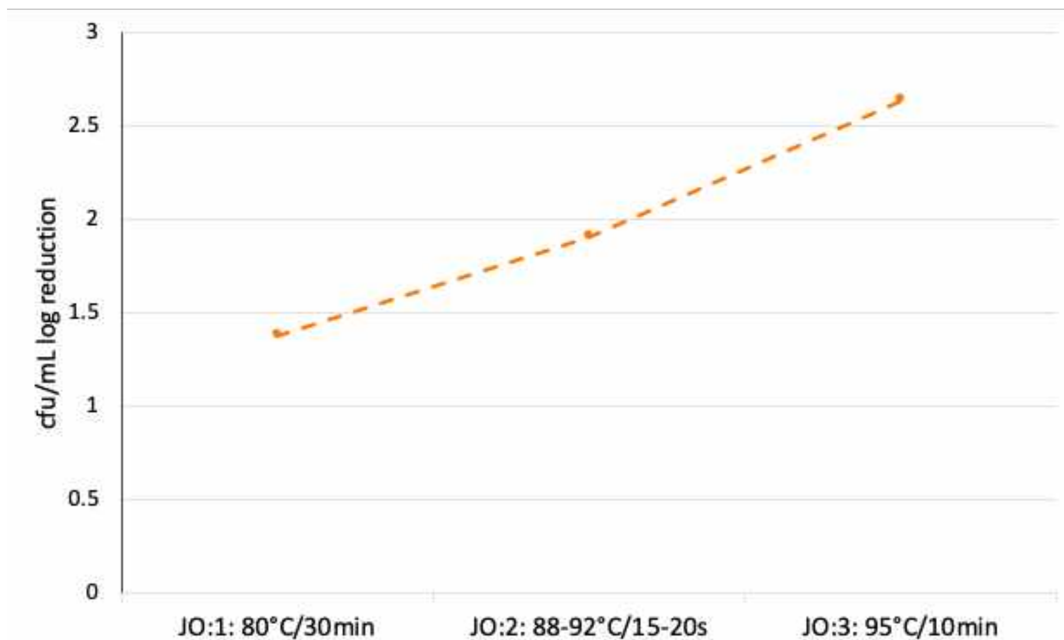

Figure 16. Log reduction of the viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JO:1 represents the juice sample which underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which underwent heat treatment by 95°C/10min.

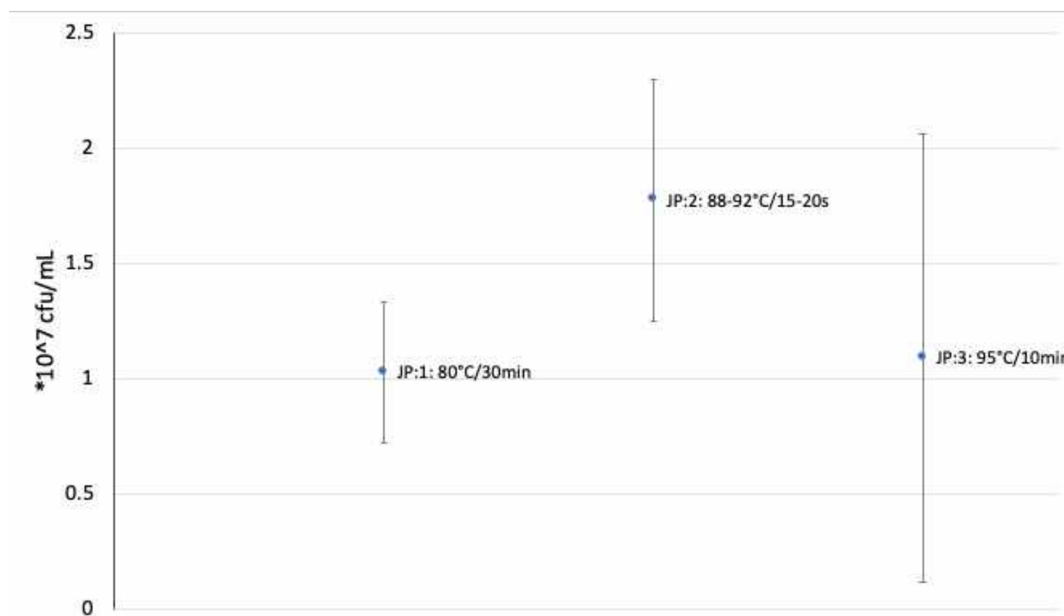

Figure 17. Starting concentration of *B. coagulans* XY1 in the "initial samples" of pomegranate juice before heat treatment was performed. JP:1 represents the juice sample which later underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which later underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which later underwent heat treatment by 95°C/10min.

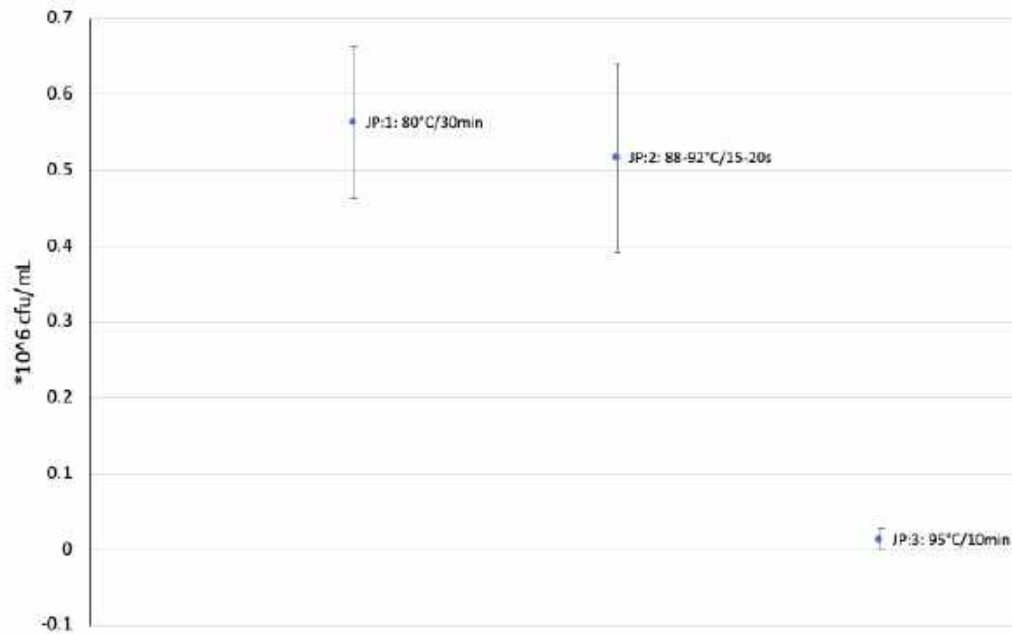

Figure 18. Viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JP:1 represents the juice sample which underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which underwent heat treatment by 95°C/10min.

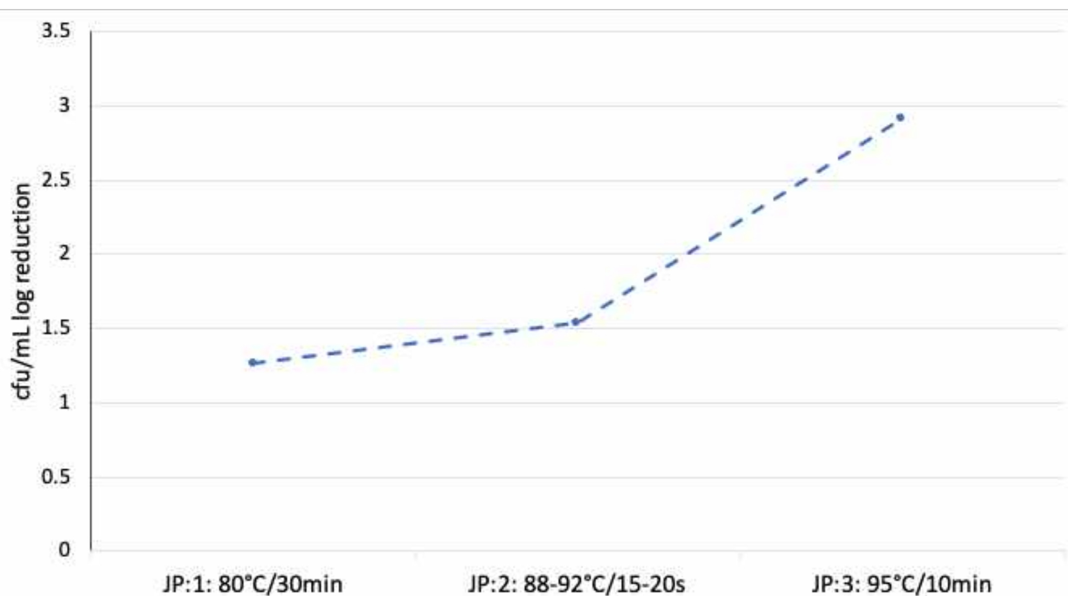

Figure 19. Log reduction of the viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JP:1 represents the juice sample which underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which underwent heat treatment by 95°C/10min.

4.3.2. Survival of *B. coagulans* XY1 during Heat Treatment Experiment 2

In order to obtain results from two independent biological replicates, the heat treatments were performed two times in total with the exact same time and temperature combinations used during heat treatment experiment 2. After the heat treatments were performed, plates containing 30-300 colonies were counted using PCM and documented as cfu per mL juice sample for samples from before the heat treatment "initial samples" and after the heat treatment. The viability of *B. coagulans* XY1 did decrease in different proportions depending on which combination for temperature and time was used (Figure 20, 21, 23 and 24).

Heat treatment at 80°C for 30 minutes

The average starting concentrations for JO:1 and JP:1, the samples that later underwent heat treatment by 80°C for 30 minutes, were $1.47 \pm 0.29 * 10^7$ cfu/mL for JO:1 and $1.21 \pm 0.069 * 10^7$ cfu/mL for JP:1. After the heat treatment, the average concentrations were $0.57 \pm 0.34 * 10^6$ cfu/mL for JO:1 and $0.21 \pm 0.069 * 10^6$ cfu/mL for JP:1 (Table A.3 in Appendix). This heat treatment led to a decrease of probiotic concentration in both juices and the probiotic concentrations in both juices went below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:1 was 1.41 and for JP:1 it was 1.76. This indicates that around 95% of the probiotic was reduced in JO:1 and more than 95% was reduced JP:1 after heat treatment at 80°C for 30 minutes (Figure 22 and Figure 25).

Heat treatment at 88-92°C for 15-20 seconds

The average starting concentrations for JO:2 and JP:2, the samples that later underwent heat treatment by 88-92°C for 15-20 seconds, were $1.81 \pm 0.17 * 10^7$ cfu/mL for JO:2 and $0.93 \pm 0.38 * 10^7$ cfu/mL for JP:2. After the heat treatment, the average concentrations were $3.16 \pm 0.93 * 10^6$ cfu/mL for JO:2 and $2.12 \pm 0.38 * 10^6$ cfu/mL for JP:2 (Table A.3 in Appendix). This heat treatment led to a big decrease of probiotic concentration in both juices and the probiotic concentrations in both juices went below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:2 was 0.76 and for JP:2 it was 0.64. This indicates that less than 90% of the probiotic was reduced in JO:2 and JP:2, after heat treatment at 88-92°C for 15-20 seconds (Figure 22 and Figure 25).

Heat treatment at 95°C for 10 minutes

The average starting concentrations for JO:3 and JP:3, the samples that later underwent heat treatment by 95°C, for 10 minutes were $1.55 \pm 0.22 * 10^7$ cfu/mL for JO:3 and $1.54 \pm 0.0075 * 10^7$ cfu/mL for JP:3. After the heat treatment, the average concentrations were $0.021 \pm 0.0087 * 10^5$ cfu/mL for JO:3 and $0.018 \pm 0.0075 * 10^5$ cfu/mL for JP:3 (Table A.3 in Appendix). This heat treatment led to a huge decrease in probiotic concentration of both juices and the probiotic concentrations in both juices went far below the required minimum of 10^7 cfu/mL. The log reduction from before to after heat treatment of JO:3 was 2.86 and for JP:3 it was 2.93. This

indicates that around 99.9% of the probiotic was reduced in both JO:3 and JP:3 after heat treatment at 95°C for 10 minutes (Figure 22 and Figure 25).

Figure 20. Starting concentration of *B. coagulans* XY1 in the "initial samples" of orange juice before heat treatment was performed. JO:1 represents the juice sample which later underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which later underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which later underwent heat treatment by 95°C/10min.

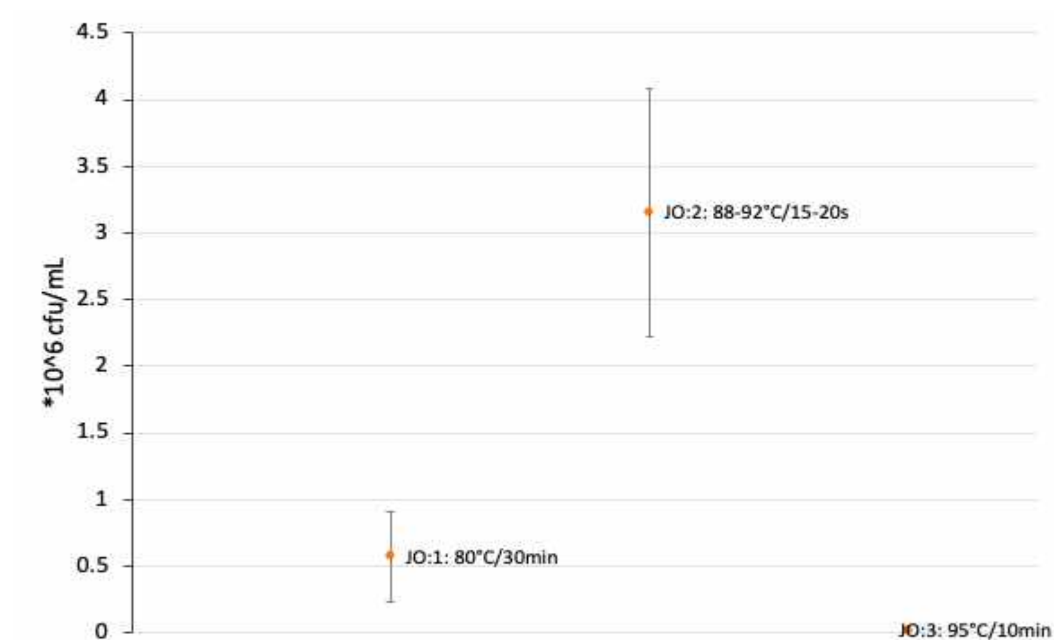

Figure 21. Viability of *B. coagulans* XY1 in orange juice after heat treatment was performed. JO:1 represents the juice sample which underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which underwent heat treatment by 95°C/10min.

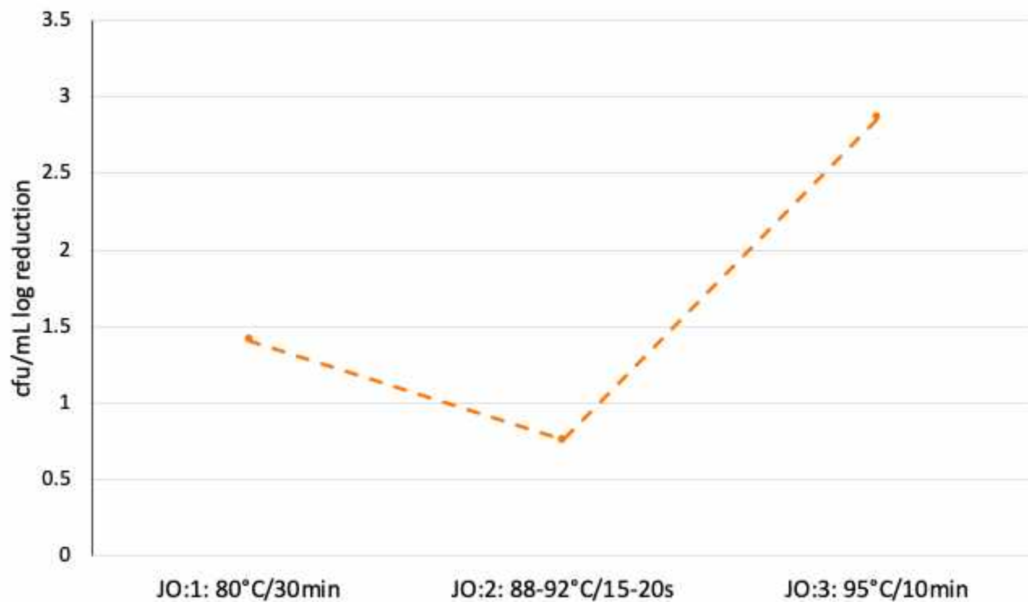

Figure 22. Log reduction of the viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JO:1 represents the juice sample which underwent heat treatment by 80°C/30min, JO:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JO:3 represents the juice sample which underwent heat treatment by 95°C/10min.

Figure 23. Starting concentration of *B. coagulans* XY1 in the "initial samples" of pomegranate juice before heat treatment was performed. JP:1 represents the juice sample which later underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which later underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which later underwent heat treatment by 95°C/10min.

Figure 24. Viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JP:1 represents the juice sample which underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which underwent heat treatment by 95°C/10min.

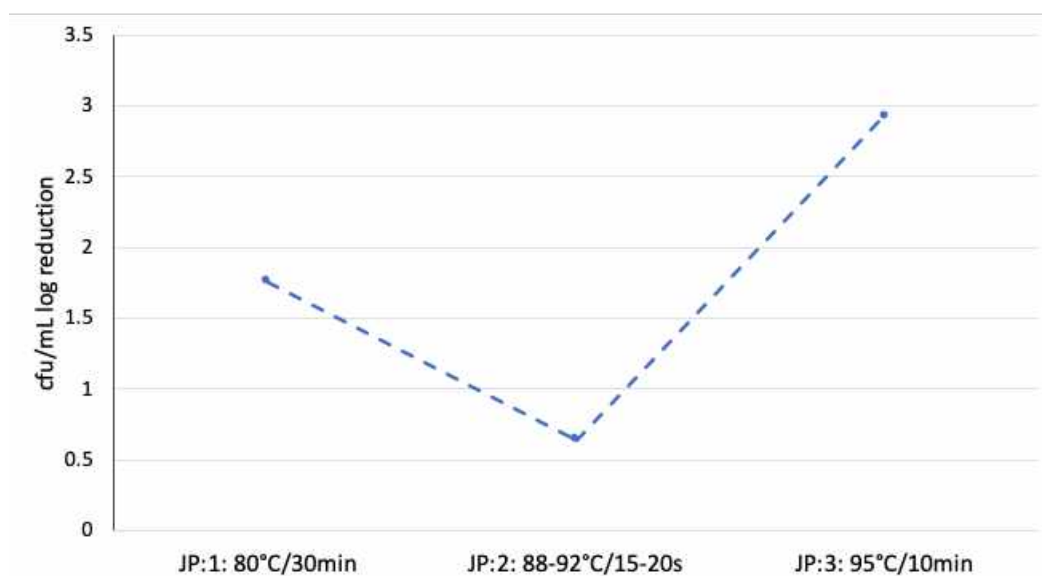

Figure 25. Log reduction of the viability of *B. coagulans* XY1 in pomegranate juice after heat treatment was performed. JP:1 represents the juice sample which underwent heat treatment by 80°C/30min, JP:2 represents the juice sample which underwent heat treatment by 88-92°C/15-20s and JP:3 represents the juice sample which underwent heat treatment by 95°C/10min.

5. Discussion

Most probiotic fruit beverages today consist of probiotic strains that cannot undergo heat treatment after inoculation of probiotics due to heat sensitivity. Therefore, there is a need for new probiotic strains that can withstand these kinds of treatments and provide consumers with safer products with a longer shelf-life. The investigation of *B. coagulans* XY1 during this project deepens the understanding of how this spore-forming probiotic behaves both in fruit juices during storage and after heat treatments. Fruit juices seemed to be suitable as a food matrix for incorporation of *B. coagulans* XY1 spores, which a previous study with apple juice has shown (Maajed et al., 2016). Fruit juices usually have a lower pH than other food matrices and even though there are differences between different species and strains of bacteria, when the pH gets closer to 3 there is an overall increased sensitivity towards the environment for the bacteria (Corcoran, Stanton, Fitzgerald and Ross, 2005). The strain viability results of the storage samples remained overall stable during storage but the minimal recommended dosage of 10^7 cfu/mL was not fulfilled at some occasions. One source of error is the analysis technique used, PCM which could have affected the outcome of the results to a certain extent. This is a very manual technique including dilutions and counting plates which leaves many opportunities for errors to occur that are not reflecting the actual result but more the weakness of the analysis method. However, there are no alternative methods to replace this one since this is the only one that takes into account if the cells are dead or alive and therefore errors need to be considered when using this technique.

By looking at another study regarding inoculating a *B. coagulans* strain into apple juice, the amount of probiotic strain used in that experiment was much higher (1g in 1 L) (Maajed et al., 2016). In this project 0.2g in 1 L was used and therefore an increase in the amount of probiotic added would increase the concentration of cells throughout storage. That would most likely keep the probiotic concentration above the recommended dosage. By studying the two juices inoculated by the probiotic strain, there were no obvious differences between the viability of the strain in both juices throughout the storage time of 10 weeks. There was a pH difference of around 0.6 between the juices and apart from the pH difference, the contents of the juices were very similar containing only their respective fruit juice from concentrates (100%). The sugar content differed with 5.5 g/100mL, with pomegranate having the highest sugar content of 14 g (Kiviks musteri; Dimes). Even though there were a few differences between the two juices, the survival of the strain in both juices was similar and did not seem to be affected by the pH and sugar content differences.

B. coagulans XY1 was provided in the form of spores, which are highly acid tolerant (Konuray and Erginkaya, 2018), and therefore the low pH of the juices did not become an issue for the survival of the probiotic strain. This was expected due to previous results where a strain of *B. coagulans* was able to keep its probiotic activity at pH 2.0 (Abada, 2008). The pH during storage stayed similar to the start pH in both juices as well as the pH control samples. Therefore, it can be assumed that the refrigerated conditions resulted in metabolic inactivation of the strain. This

metabolic inactivation kept the spores dormant during storage. Since fruit juices are quite complex matrices with a lot of components that can induce germination, refrigerating the juice samples containing the probiotic strain will minimise the risk of germination (Maajed et al., 2016).

The purpose of measuring changes in lactic acid concentration in the two juices, was to investigate if any production of lactic acid by the probiotic could affect the quality of the products. It was also checked to see whether or not the strain was metabolically active during the storage period. The HPLC results indicated that there was no change in lactic acid concentration in either of the juices throughout the storage period. It was only possible to detect a peak of lactic acid concentration in the orange juice samples, which was about <100 mg/L when compared to a standard of L-lactic acid of 175 mg/L. In the pomegranate sample, no peka could be detected in all of the samples indicating very low levels of lactic acid that did not change throughout storage. Since there was no change in lactic acid concentration in the probiotic juice samples throughout storage, the strain remained metabolically inactive during storage in the refrigerator. Therefore, the lactic acid did not affect the quality of the probiotic juice samples either which can be seen through the results of the sensory evaluation.

The sensory evaluation was performed visually and therefore there might have been changes not possible to see without any use of instruments, such as colour changes. Overall, the quality of the juices in terms of aroma, colour and appearance did not change to any greater extent during the time in storage. The juices chosen for the experiments did have a long shelf-life period and even though the packages were opened for the experiments, they visually stayed fresh in the refrigerator at 8-10°C throughout the project. The juices incorporated with the probiotic strain were however never tasted, because of the slight risk of cross-contamination from other microorganisms in the lab. Therefore it was not possible to evaluate the possible change in taste which also is an important factor to consider over the storage time. If the probiotic strain affects the taste in a negative way, the probiotic juices will not be successful products.

The heat treatment showed that the probiotic strain was affected differently depending on which temperature and time combination that was used. Since the setup in the lab did not include a real pasteuriser, the results from lab scale to industrial scale can differ quite a lot. One of these factors may be the time of the heating. When the juices were poured into the glass bottle, it took some time for them to reach the desired temperature for the heat treatment and due to that the juices have been heated for a longer time than stated before, by taking the heating time into account. This may also have affected the viability of the strain. For the heat treatment combination 80°C/30min it took about 20 minutes to reach 80°C for experiment 1 and 10 minutes for experiment 2 and then it was heated at 80°C for 30 minutes. For the combination 88-92°C/15-20s it took about 15 minutes to reach 88°C for experiment 1 and 5-7 minutes for experiment 2 and then during the 15-20 seconds it got heated up to 92°C before the heating was finished. For the combination 95°C/10min it took about 25 minutes to reach 95°C for experiment 1 and 12-15 minutes for experiment 2 and then it

was heated at 95°C for 10 minutes. Since the actual heating time was longer than the suggested ones, there might have been some unnecessary loss of viability. It is possible to see however that the heating times were a lot shorter during experiment 2 than 1 which is visible from the results. During experiment 1, the highest survival rates were achieved after combination 80°C/30min while during experiment 2, the combination 88-92°C/15-20s was clearly most efficient with the highest survival rates of all combinations/experiments. The reason for this might depend on that during experiment 2 the strain was heated during a much shorter time until it reached the goal temperature and therefore the cells did not get exposed to as much unnecessary heating and managed to survive better. When doing this on an industrial scale, the heaters will most likely be more efficient than the setup used during this experiment and the time to reach the desired temperatures will therefore be much shorter. Therefore, this might result in a higher viability, since the heating times will be shorter and not be as demanding for the strain. The way this experiment was performed made it difficult to pre-heat the juices before inoculation and therefore the probiotic strain was exposed to more heating than necessary for the treatments.

According to the log reduction of the number of viable cells in both juices, it was possible to see a pattern among both juices during both experiments. For experiment 1, there was around 1 log reduction in the first heat treatment combination, around 1.5-2 log reduction for the second combination and around 3 log reduction for the third combination. For experiment 2, there was around 1.5 log reduction in the first heat treatment combination, around 0.5 log reduction for the second combination and around 3 log reduction for the third combination. Since the heating time to reach the heat treatment temperature differed between experiment 1 and 2, there was a difference in log reduction values between the two experiments. For combination 80°C/30min, the results were similar in experiment 1 and 2 indicating that the extended heating time did affect the survival, when compared to the heat-shock treatment, which otherwise is similar to this combination. For combination 88-92°C/15-20s, the results differed between experiment 1 and 2 due to very different heating times to heat treatment temperature. During experiment 2, where the heating time was much shorter, *B. coagulans* XY1 had a higher chance of survival since the actual holding time for the heat treatment was very short. For combination 95°C for 10 minutes, the results were again similar between both experiment 1 and 2 indicating that this combination did affect the survival greatly and since it also had a longer heating time it was difficult for *B. coagulans* XY1 to survive in those conditions.
It was expected that the survival of *B. coagulans* XY1 would be higher after heat treatment, but due to long heating times that did not represent the desired heat treatment it did not perform as well as expected. In order to increase the viability after heat treatments, a greater amount of the probiotic strain needs to be added to the juices before the heat treatments are performed to make up for the loss of viable cells and the heat treatments also need to be more efficient to reduce the heating times.

6. Conclusions and Future Perspectives

In conclusion, this study shows that it is possible to incorporate probiotic strain *B. coagulans* XY1 into the food matrix fruit juices. The strain was able to survive in the fruit juices throughout storage of 10 weeks in refrigerated conditions. However, there were some occasions where the probiotic strain did not fulfill the minimum recommendation of 10^7 cfu/mL in both of the juices and overall the viability in both juices were stable with some changes throughout the storage period. The pH remained stable during storage, indicating that the refrigerating conditions kept the strain inactive and showed that the strain has a possibly long shelf-life in fruit juices which the sensory evaluation also confirmed. The heat treatment experiments all indicated a reduction of viable cells after the samples had been exposed to heat treatment. Each heat treatment combination led to a probiotic concentration less than the required concentration (10^7 cfu/mL). By adding a greater amount of the probiotic strain prior to the heat treatment, this loss of viable cells can be decreased and result in the required concentration being fulfilled.

In order to further develop the investigation of this probiotic strain, more fruit juices could be tested with other pH values. It would be interesting to see how the strain behaves in a juice with a higher pH value, to test if the viability and growth activity will increase. It would also be worth increasing the amount of probiotic strain in the fruit juices in order to increase the viability. The sensory evaluation can be developed further, and the taste of the inoculated juices should be considered. Regarding the heat treatment part, more combinations of time and temperature should be tested in order to find combinations that are more mild on the probiotic and still efficient enough to eliminate possible pathogens. The heat treatment experiment also needs to be performed more similar to an industrial approach to obtain more accurate and reliable results. There is also a need for testing whether other microorganisms are present during the storage of fruit juices, which could possibly be harmful for consumers or have a negative impact on the quality and shelf life of the product. The heat treatment part could also be performed for a *Lactiplantibacillus* strain to show that *B. coagulans* is more stable during heat treatment due to its spore-forming ability. By studying *B. coagulans* in a microscope before and after heat treatment, it would give an estimation of how many spores that have germinated. This can also be done after the heat-shock treatment for an indication how well it actually works in triggering the spores to germinate.

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Appendix

A.1. Initial Viability Test of *B. coagulans* XY1

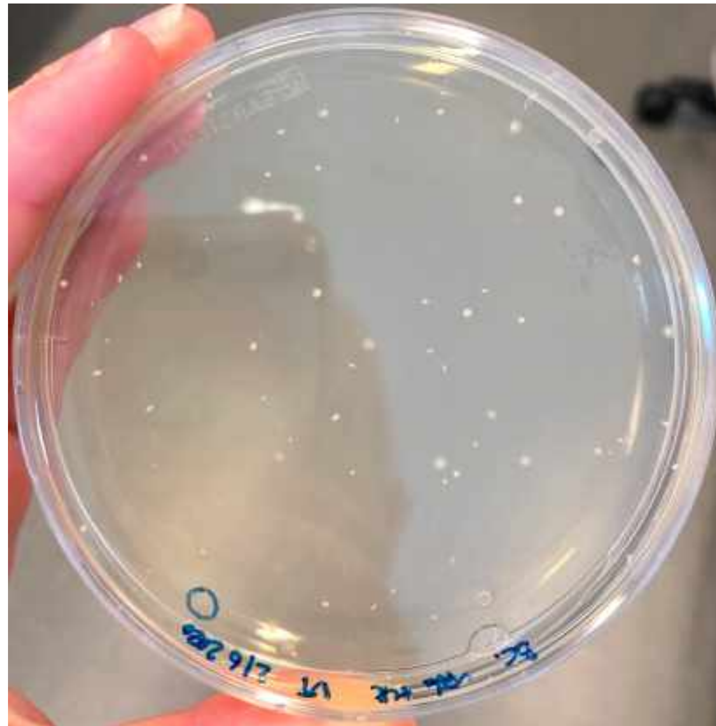

Figure A.1. Colonies of spray-dried *B. coagulans* XY1 from a 10^7 dilution using pour plate method on GYEA. The colonies are documented to be white and a bit translucent which is possible to see on the plate (Ansari, 2017).

A.2. Calculations of Initial Viability Test of *B. coagulans* XY1

Calculations for cfu/g (1) in Table 5 and Table 6 in the Results section.

$$(Average\ cfu\ *\ 50\ mL\ *\ dilution\ factor)\ /\ 0.25g = cfu/g \qquad (1)$$

A.3. Supplementary Data for Storage Samples

A.3.1. Viability of *B. coagulans* XY1

The number values of the average cfu/mL of the two juices can be seen in Table A.1.

Table A.1. The average viability of the orange and pomegranate juice samples in cfu/mL over the shelf-life period of 10 weeks.

Juice Type	**Week 0** (*10^7 cfu/mL)	**Week 2** (*10^7 cfu/mL)	**Week 4** (*10^7 cfu/mL)	**Week 6** (*10^7 cfu/mL)	**Week 8** (*10^7 cfu/mL)	**Week 10** (*10^7 cfu/mL)
Orange	0.75±0.12	0.88± 0.096	1.18±0.063	0.92± 0.093	1.18±0.11	1.12±0.12
Pomegranate	0.79±0.22	1.09±0.71	1.70±0.65	1.09±0.17	0.95± 0.082	0.87±0.087

A.3.2. pH

The number values of the average pH of the two juices can be seen in Table A.2.

Table A.2. The average pH of the orange and pomegranate juice samples over the shelf-life period of 10 weeks.

Juice Type	**Week 0**	**Week 2**	**Week 4**	**Week 6**	**Week 8**	**Week 10**
Orange	3.93± 0.014	3.96± 0.014	3.96± 0.014	3.94± 0.029	3.94± 0.014	3.92±0.014
Orange control	3.94	3.96	3.97	3.96	3.93	3.92
Pomegranate	3.32± 0.014	3.35± 0.014	3.37± 0.014	3.37± 0.025	3.36± 0.014	3.34±0.014
Pomegranate control	3.30	3.37	3.37	3.36	3.35	3.34

A.4. Supplementary Data for Heat Treatment Samples

A.4.1. Viability of *B. coagulans* XY1

The number values of the average cfu/mL of the two juices before and after heat treatment can be seen in Table A.3.

Table A.3. The average viability of the orange and pomegranate juice samples before and after heat treatment in cfu/mL for the different combinations of temperature and time. Number 1 and 2 represents the two different occasions performing the heat treatment experiment.

Juice Type	**1. Before heat treatment** ($*10^7$ cfu/mL)	**1. After heat treatment** ($*10^6$ cfu/mL)	**2. Before heat treatment** ($*10^7$ cfu/mL)	**2. After heat treatment** ($*10^6$ cfu/mL)
Orange 80°C/30 min	1.34±0.90	0.57±0.21	1.47±0.29	0.57±0.34
Orange 88-92°C/15-20s	1.06±0.15	0.13±0.063	1.81±0.17	3.16±0.93
Orange 95°C/10 min	1.00±0.31	0.023±0.038	1.55±0.22	0.021±0.0087
Pomegranate 80°C/30 min	1.03±0.30	0.56±0.10	1.21±0.069	0.21±0.069
Pomegranate 88-92°C/15-20s	1.77±0.52	0.52±0.13	0.93±0.38	2.12±0.38
Pomegranate 95°C/10 min	1.09±0.97	0.013±0.014	1.54±0.0075	0.018±0.0075