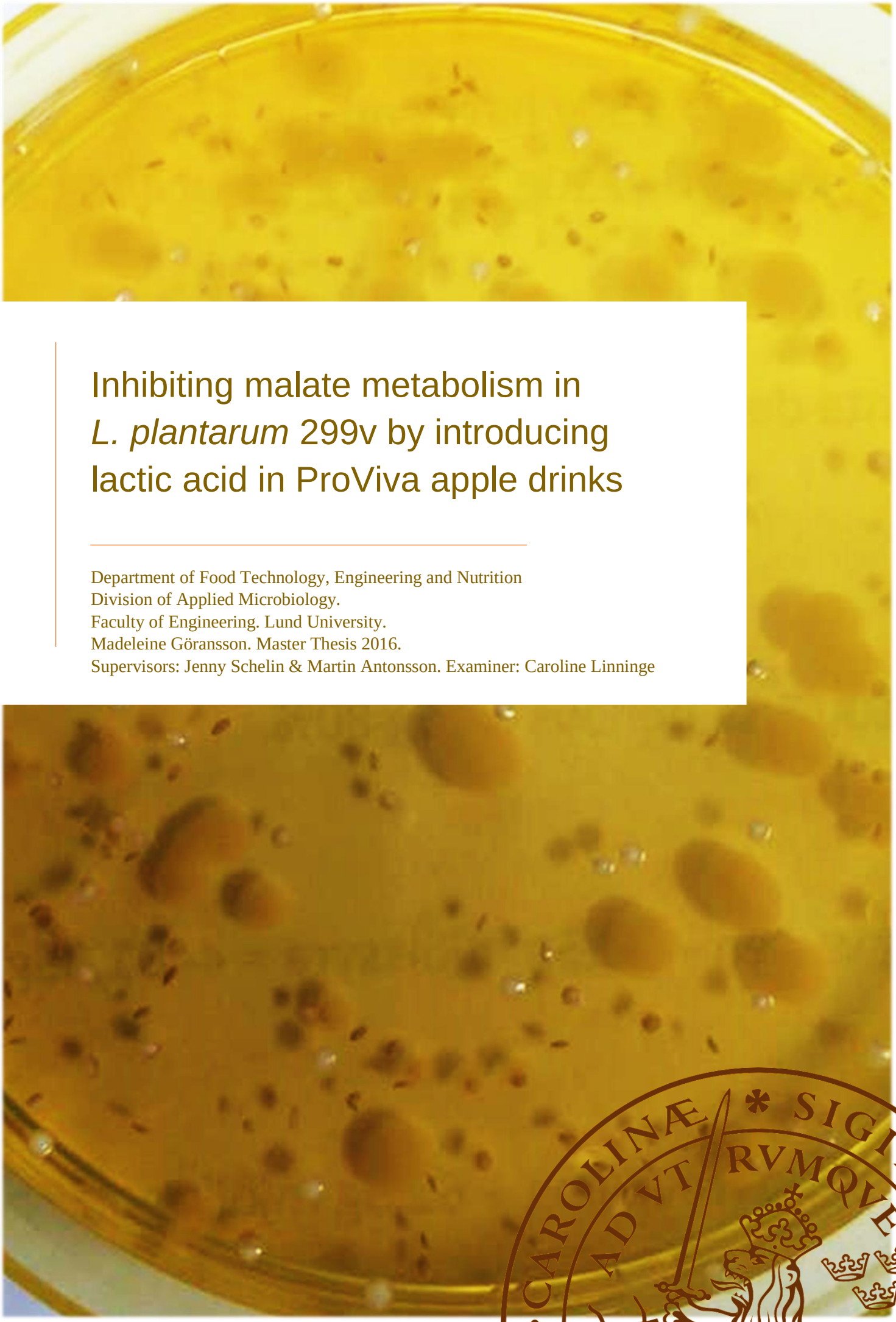


Inhibiting malate metabolism in *L. plantarum* 299v by introducing lactic acid in ProViva apple drinks

Department of Food Technology, Engineering and Nutrition
Division of Applied Microbiology.
Faculty of Engineering. Lund University.
Madeleine Göransson. Master Thesis 2016.
Supervisors: Jenny Schelin & Martin Antonsson. Examiner: Caroline Linninge

Abstract

This master thesis aimed to evaluate the possibilities of inhibition of *Lactobacillus plantarum* 299v metabolism of malate by introduction of lactic acid in ProViva apple drinks. The malate metabolism of *L. plantarum* have been found to promote an overproduction of carbon dioxide in ProViva apple drinks and thereby the metabolism was tried to be inhibited. The experiment included three different concentration of apple juice in combination with three different concentrations of lactic acid. This lead to a total of nine different apple drinks to examine and the drinks were evaluated with respect to carbon dioxide production, pH changes and organic acids alternations. Besides this, the amount of live probiotics and the absence/presence of molds and yeast were documented. The analyses were performed specific days from the drinks had been produced until the drinks had been stored in 8°C for 36 days. Sensorial analysis were performed to highlight possible flavor differences during storage and also to grade the different drinks with respect to how tasty they were.

It was found that the concentration of lactic acid does affect the *L. plantarum* and its metabolism. The bacterium was inhibited in growth due to the addition of lactic acid and the gas formation was decreased. It was also found that with a lactic acid concentration equal to or above 7g/l the *L. plantarum* started to die. At the same time a lactic acid concentration of 4g/l seemed to be a promising concentration for a future ProViva apple drink. The sensory analysis showed that no significant difference in organoleptic properties could be found during the storage of the drinks. The amount of added lactic acid to the apple juices did not significantly matter for the shelf life of the drinks since the yeast and mold present in the analysis appeared to occur randomly.

Sammanfattning

Svensk titel på examensarbetet: Inhibering av malatmetabolismen hos *L. plantarum* 299v genom tillsats av mjölksyra i ProVivas äppeldricka.

I det här examensarbetet har mjölksyrans förmåga att inhibera malatmetabolismen i *L. plantarum* 299v i ProViva äppeldricka undersökts Det har tidigare påvisats att malatmetabolismen i *L. plantarum* leder till en överproduktion av koldioxid och därför försökte metabolismen inhiberas. Nio olika ProViva äppeldrycker med olika koncentrationer av äppeljuice och mjölksyra tillverkades och sedan har dessa drycker analyserats. Dryckerna har analyserats med avseende på koldioxidproduktion, pH och uppsättning av organiska syror och detta för att se hur metabolismen har påverkats. Dryckerna har också analyserats gällande mängden *L. plantarum* och förekomst/frånvaro av jäst och mögel. Analyserna har gjorts med jämna mellanrum från det att dryckerna producerades till det att de förvarats i 8^{o}C i 36 dagar. Sensoriska analyser har genomförts för att notera eventuella smakförändringar allteftersom dryckerna förvarats och också för att gradera hur goda de olika dryckerna var.

I den här studien kunde det tydligt påvisas att tillsatsen av mjölksyra till ProViva äppeldricka har en inhiberande effekt på *L. plantarum* och dess malat metabolism. Detta genom att antalet *L. plantarum* minskade i de drycker där en högre koncentration av mjölksyra tillsattes och också genom att koldioxidproduktionen blev lägre. En mjölksyrakoncentration på 4g/l såg ut att vara en lovande koncentration för en framtida ProViva äppeldryck. Under de genomförda sensoriska analyserna så kunde det fastställas att ingen signifikant smakförändring uppstod i dryckerna under förvaringstiden. Det kunde i studien inte ses någon koppling mellan mängd tillsatt mjölksyra och dryckernas hållbarhet, det vill säga tillväxten av jäst och mögel. Detta då jäst och mögel tycktes förekomma slumpmässigt i dryckerna.

Preface

In this project the inhibition effect of malate metabolism in *L. plantarum* 299v by introduction of lactic acid in ProViva apple drinks was examined. The idea of the project came from my supervisor at ProViva, Martin Antonsson, and since I thought the idea was very interesting I was happy to do this project as my master thesis. All work done have been performed at ProViva AB, except for some analysis of organic acid that were analyzed externally. During the project I have really tried to do my best to combine facts known from literature with the obtained result from the study and by that formulated a discussion and explanation for the obtained results. The project has been performed during the autumn year 2016 and I am very happy to have had the possibility to perform this project as a last part of a five year long education.

I would like to thank Martin Antonsson for giving me the opportunity to do my master thesis project at ProViva AB. The work at ProViva has provided me not only with knowledge regarding working with product development but also how the system and different tasks and employees work together in a food company. A big thank you for that, and also for all the support and for all input during my project work.

I also want to thank the two other persons working in the product development department at ProViva, Anna Tyrberg and Marie Berger. Thank you for all the support and advice during my work. A thank you also to the employees in the lab who have supported me and helped me with the analysis performed in the lab.

I would like to thank Jenny Schelin for being my supervisor and for all the advice throughout my project. Thank you for all your detailed comments and for being very much dedicated in my work. I would also like to thank Caroline Linninge for accepting my project and for giving me advice.

Finally, I would like to thank friends and family who have supported me all the time and in all situations during these 5 months of intense project work and report writing.

Popular Abstract

ProViva Apple drink – Might soon be the drink to consume for promoting a healthy stomach!

An increasing awareness of health leads to an increasing awareness of probiotics. Probiotics are live microorganisms which promote health benefits to its host when ingested. Probiotics have in the recent years become popular as ingredients in food products, such as in fruit drinks – which ProViva AB produce. Apple juice is a popular drink to consume in Sweden, however, it has so far in the production of ProViva drinks been impossible to produce a ProViva drink with apple. This is because the probiotics love apples – just like we do – which leads to an overproduction of carbon dioxide in the drinks. Though, after research, it has now been found that the over production of carbon dioxide can be inhibited which open up doors for a production of a ProViva apple drink!

A lot of people are having stomach problems and diseases such as irritated bowel disease gets more and more familiar for us. In the same time the awareness for healthiness and healthy food increases in society. More and more people tend to care about what they are eating and thereby the market of healthy food alternatives increases. The ProViva fruit drinks provide the body with two positive factors. First of all they contain fruits which are an important part of the diet and should, together with vegetables as a recommendation from the Swedish National Food Agency, be consumed in at least five portions every day (for an adult equal to approximately 500 grams). Secondly, they contain the probiotic bacterium *Lactobacillus plantarum* 299v (*L. plantarum* 299v) which has been seen to promote a healthy gut microbiota in different ways. For example *L. plantarum* have been found to modulate the immune system to produce more antibodies. The bacterium has also been seen to compete pathogenic bacteria and also to strengthen the epithelial layer and thereby inhibit the invasion of pathogenic bacteria to the blood stream. The fruit content of ProViva, and the presence of *L. plantarum*, make ProViva drinks a good choice when preferring a healthy lifestyle.

L. plantarum is a non-spore forming, gram positive bacterium that grows anaerobically. *L. plantarum* has a high tolerance to low pH and can grow in pH below four. Due to its high tolerance to low pH it can survive throughout the acidic environment in the stomach and start to colonize in the gastrointestinal tract of humans. *L. plantarum* is commonly present in a lot of different fermented food products thereby the bacterium is usually a resident in the human body, from the mouth to the column.

Apple juice is a widespread popular drink to consume all over the world. China is the country that produces the most apple juice concentrate in the world, this with a production of 1 million tons year 2007/2008. In Sweden apples also have been seen to be a popular choice and apples are the second most popular fruit to consume after bananas. An apple consists of a lot of different nutrient, as for example vitamin C and different minerals. It also consists of dietary fibers and is rich in different phenolic compounds. Apples also contains organic acids, and the most common organic acid in apples is the malic acid.

So far, in the ProViva drink catalog, there are a lot of different fruit and berry drinks. However, never has a ProViva drink with

apple been able to be produced. This due to that an overproduction of carbon dioxide occurs when apple is combined with *L. plantarum*. The presence of malic acid in apple makes the *L. plantarum* ferment this organic acid in what is called the malolactic fermentation. *L. plantarum* uses the malic acid as a substrate and forms lactic acid and carbon dioxide as products. The carbon dioxide production due to the malolactic fermentation explains the overproduction of carbon dioxide that has been seen. This overproduction of carbon dioxide does affect the flavor of the drinks, as well as the shelf-life and also limits the possibility of packaging the drinks. The packages get all filled with gas- to that point that they even blow up and burst!

An additional problem that also has been noticed when combining apple and *L. plantarum* is that formation of off-flavors occurs. The upcoming off-flavors are known to taste as medical, glove or smoked.

Research has now found that it soon might be possible to buy a ProViva Apple drink nearby in your store. It has been found that the addition of lactic acid to a ProViva apple drink inhibit the over production of carbon dioxide. The *L. plantarum* and the metabolism are affected/modified in different ways when lactic acid is introduced. It has also been noticed that the appearance of off-flavors are avoided after introduction of lactic acid. In figure 1 it can be seen how a package of pure apple drink looks after 6 storage days in 25°C compared to a package with a ProViva apple drink (without lactic acid). The package of the drink with ProViva apple drink has lost its structure due to carbon dioxide formation, and can not stand straight up anymore. This picture gives an understanding of the problems with the malolactic fermentation. However, after addition of lactic acid to the ProViva apple drink it has been found that the package will keep its original structure due to the inhibition of malolactic fermentation.

Figure 1. A plastic bottle with 50% apple juice to the left and a bottle with ProViva apple drink to the right. The bottle to the right has a changed structure and can't stand up straight anymore due to gas formation.

The addition of lactic acid to the ProViva apple drinks is believed to affect the cells of *L. plantarum* in different ways. As for example it is hypothesized that the lactic acid interacts with the cell membrane which disrupt its function. The entrance of lactic acid into the cells does also promote a decrease in the intracellular pH. This pH decrease is believed to cause inactivation of enzymes that are important for a proper function of the bacterium. However, at the same time as an inhibition of the malolactic fermentation has been noticed it should be said that *L. plantarum* still survive and live in the ProViva apple drinks. The probiotic properties of the drink are thereby preserved.

What more influences does the addition of lactic acid have to the ProViva apple drink? Since it is an acid that is added the taste of the drink off course gets a bit sour. However, the sweetness from the apple is still noticeable and who does not like the thought of a fresh drink with taste of the late summer's first new Swedish sour apples!

Table of Contents

List of abbreviations

4-EG: 4-ethyl guaiacol

4-EP: 4-ethyl phenol

4-VG: 4-vinyl guaiacol

4-VP: 4-vinyl phenol

CFU: Colony Forming Units

FA: Ferulic acid

L. plantarum: Lactobacillus plantarum

LAB: Lactic acid bacteria

MLF: Malolactic fermentation

MRS-agar: de Man, Rogosa and Sharpe – agar

PCA: p-coumaric acid

PDA: Potato Dextrose Agar

Wt. %: Weight percentage

1.0 Background

In the food industry, there is always an existing need to please consumers' demands and follow food trends that arise in the society (Zink, 1997). Product development is therefore a vital part of a food company and it is an important working procedure for obtaining growth, success and to remain competitive (Costa & Jongen, 2006). One of the food trends present in society is the consumers' demands for new tastes and requirement of a variety of the food (Zink, 1997). When developing a new taste of an already existing product this product development is a so called line extension (Brody & Lord, 2007). Another trend that has been observed recently is the increased human awareness of healthiness and thereby also for a healthy diet. This has brought attention to probiotics, and probiotics in different foods (Zink, 1997). Probiotics are live microorganisms which, when added in an adequate amount, are believed to give health benefits to the host (Molin, 2013). The initiation and implementation of new products is a result from the combination of the consumers' demand, the available technologies at a company and food science research (Winger & Wall, 2006).

ProViva AB is a company that produces fruit and berry drinks including the probiotic bacterium *Lactobacillus plantarum* 299v (*L. plantarum* 299v) (ProViva, 2016). To stay competitive strong on the market, and to continue to catch consumers' interest, a company like ProViva AB needs to extend and innovate the product catalog, by for example developing a new flavor of the fruit drinks. Apple is a popular fruit to consume in Sweden, and so also apple juice, therefore the production of an apple ProViva fruit drink is believed to be a promising product. However, it has been found that apple in combination with *L. plantarum* leads to an overproduction of carbon dioxide which limits the possibility of packaging the product. The overproduction of carbon dioxide is due to the bacterial decarboxylation of malic acid to lactic acid and carbon dioxide. It has also been found that the bacterial fermentation of apple leads to off-flavor due to the decarboxylation of phenolic compounds. If inhibiting the malate metabolism in *L. plantarum* by introduction of lactic acid, and so also the decarboxylation of phenolic acids, the two problems connected to bacterial decarboxylation might be avoided, which would enable a production of an apple ProViva drink.

1.1 Danone and ProViva

ProViva AB is a company located in the south of Sweden, in a small village called Lunnarp. ProViva is a part of the companies Danone (51%) and Skånemejerier Economic Association (49%). Danone is an international company with three main categories of products: dairy, waters and early life nutrition. ProViva is an example of a big trademark of Danone, and so are also trademarks such as Activia and Danonino (Danone Sverige, 2016). The products of ProViva consist of fruit and berry drinks which contain the probiotic bacterium *L. plantarum* 299v (ProViva, 2016). These fruit drinks, including the probiotic bacterium, promote two different positive effects in the body when consumed (Francois, Portier, Crepel & Faurie, 2010). First they contain fruits which are an important part of the diet and should, together with vegetables as a recommendation from the Swedish National Food Agency, be consumed in at least five portions every day (for an adult equal to approximately 500 grams) (Livsmedelsverket, 2015). Secondly, *L. plantarum* provides the body

with positive effects connected to its probiotic properties (Francois et al., 2010).These two positive health aspects make ProViva drinks a popular choice to consume both as a part of the breakfast and for promoting a healthy stomach.

1.1.1 Ingredients and Production

The fruit drinks of ProViva have some main ingredients present in all different drinks. First of all, all ProViva drinks have a certain percentage of fruit juice. These percentages differs in the different drinks but should preferable be higher than 35% or at least 35%. Secondly, all ProViva drinks consists of 5 weight % oatmeal base, which includes the probiotic bacterium *L. plantarum* 299v. All drinks also contain enzymes, which degrade the starch in the oat and makes it possible for *L. plantarum* 299v to metabolize it (ProViva AB, 2016).

Besides this, the drinks commonly also contain sugars, or sweeteners, some kind of thickening agent, vitamins, and add backs for increasing the flavor of the drink. Add backs are aromas which are naturally occurring and obtained from fruit and berries.

ProViva fruit drinks are produced in the following way: The fruit mixture is produced separately and heated to 93°C. After the heat treatment the mixture is cooled down again. The oatmeal base mixture is also created separately. The enzymes are added to the oatmeal base and after a while the enzymes are inactivated by heat treatment. After heat treatment the oat swell is cooled to 37°C and thereafter the *L. plantarum* 299v is added and the fermentation starts. After fermentation, the fruit mixture and the oatmeal base is combined and the ProViva drink is finished, see flow chart in figure 1. The storage of the drink should be in maximum 8°C and the shelf life of the drink is set to 35 days. The amount of *L. plantarum* 299v in the fruit drinks should be targeting $5x10^7$ CFU/ml.

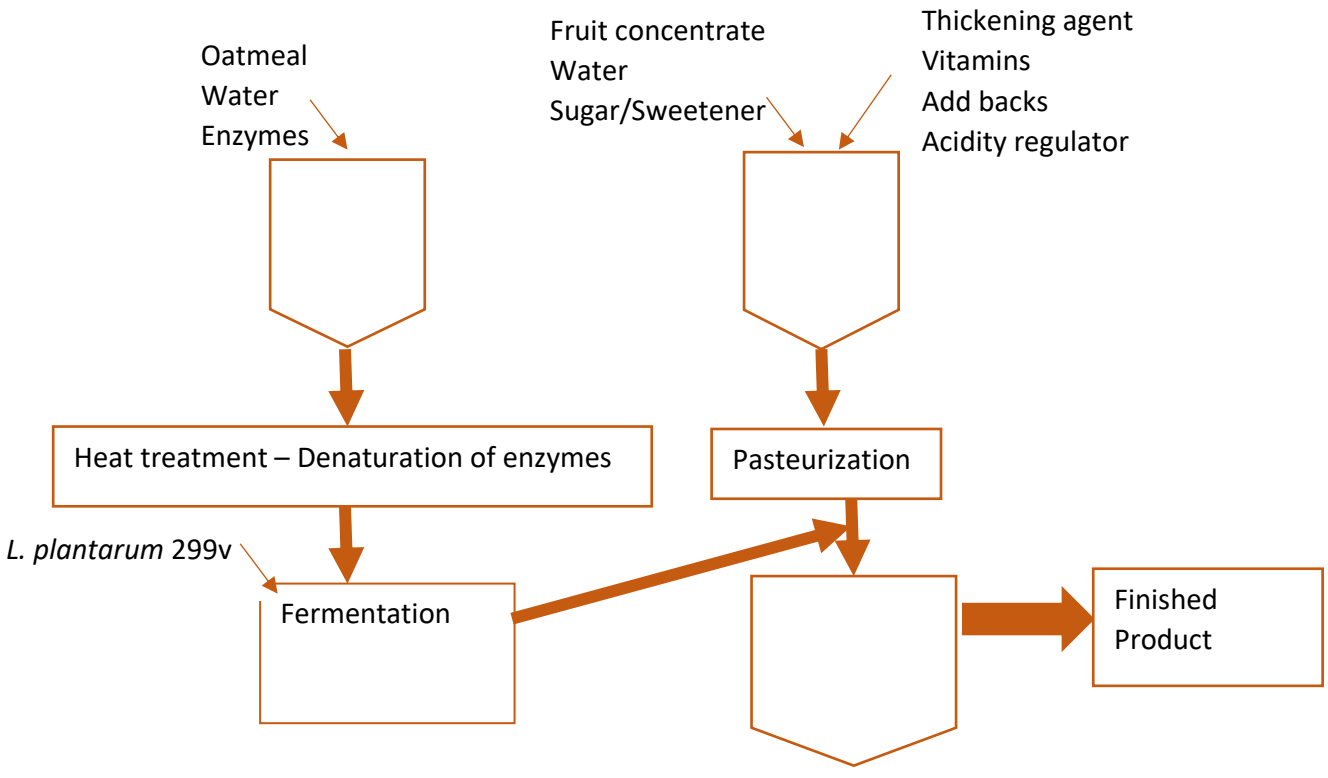


Figure 1. A flow chart of the production of ProViva fruit drinks.

1.2 Apple juice

Apple juice is a widespread popular drink to consume all over the world. China is the country that produces the most apple juice concentrate in the world, with a production of 1 million tons year 2007/2008 (World Market and Trade, 2008; Hyson, 2011). In the U.S, year 2014, apple was the second most consumed fruit, after bananas, and also the second most consumed fruit juice, after orange (Produce For Better Health Foundation, 2015). The same result has been seen in Sweden, where apples are the second most popular fruit to consume after bananas (Wilhelmsson, 2013).

Recently, research has shown that apple, apple juice and its ingoing components possess important beneficial effects on the risk and markers of diseases such as cancer, Alzheimer's disease and cardiovascular disease. Apple may also contribute to bone health, normal aging, weight management and gastrointestinal protection (Hyson, 2011).

An apple consists of a lot of different nutrient, as for example vitamin C and different minerals. It also consists of dietary fibers and is rich in different phenolic compounds (Gerhauser, 2008). The health benefits seen with apple have frequently been coupled to the presence of phytochemicals, as for example polyphenols, and their strong antioxidant effects. In laboratory trials apples have been seen to have a lowering effect of cholesterol, to promote a decrease in lipid oxidation and also to inhibit cancer cells to proliferate (Boyer & Liu, 2004).

The presence of organic acid in apples contributes to the freshness of apples. It also contributes to the overall taste and is important for the overall flavor experience. The most common organic acid in apples is the malic acid. Malic acid is not only important for the flavor of the apple, it is also important for the digestion process of humans and helps to maintain a healthy liver. The average amount of malic acid in 15 different types of apples have been determined to 919mg/100g (Nour, Trandafir & Ionica, 2010).

1.3 Probiotic bacterium

1.3.1 Definition

The definition of probiotics is, according to the World Health Organization "probiotics are live microorganisms that, when administrated in an adequate amount, confer health benefits to the host" (WHO, 2002). These health benefits are commonly connected to either the immune system or changes of the gut flora (Sanders, 2008). *Lactobacillus* and *Bifidobacterium* are the two genera that are mostly used as probiotics and only a few strains of the different species have been used successfully as ingredients in food or as food supplements. To confer health benefits, the total ingested dose of probiotics should be 10^9, preferable 10^{10} CFU daily (Molin, 2013).

1.3.2 Gut microbiota

In the human gastrointestinal tract, including stomach, small intestine and large intestine, there are a lot of different bacteria which together create an environment that is called the gut microbiota. The gut microbiota consists of around 10^{14} bacteria grouped in approximately 1000 different species (Musso, Gambino & Cassader, 2010). Some of the resident bacteria in the gastrointestinal tract have positive effects in the body, as for example promoting a healing effect

on injuries, while other can worsen injuries (Molin, 2013). It is the resident bacteria together with the structural and functional characteristics of the gastrointestinal tract in combination with the diet that creates a gut ecosystem. Probiotics have the possibilities to reach the intestine alive and thereby affect the present conditions in positive directions towards health benefits in different ways (Charalampopoulos & Rastall, 2009). As for example probiotics have the ability to stimulate the production of antibodies, work as competition against the more unbeneficial bacteria present, and inhibit the invasion of unwanted bacteria (Goktepe, Juneja & Ahmedna, 2006; Molin, 2013).

1.3.3 Prebiotics

Prebiotics are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora, which confer benefits" (Slavin, 2013). More exactly, prebiotics are for example fibers and other food products that the human body itself can nog digest. However, some microorganisms have the ability to degrade these food components which make it possible for the human body to absorb nutrients from food that it otherwise would not. The products of the microbial digestion of prebiotics, mostly so called short chain fatty acids, make up 10% of the total energy and nutrients required by humans (Charalampopoulos & Rastall, 2009). The most abundant short chain fatty acids are acetate, butyrate and propionate and these acids have been found to have positive influences on treatment and prevention of diseases such as bowel disorders, metabolic syndromes and some types of cancers (den Besten et al. 2013). The addition of prebiotics to the body support the growth and proliferation of the beneficial resident bacteria in the gastrointestinal tract (Charalampopoulos & Rastall, 2009).

1.4 Lactic acid bacteria

To apply probiotics in food, the probiotics need to survive in both the food product and in the gastrointestinal tract. Since centuries back, some bacteria have been used as important ingredients in different kind of foods. These bacteria, commonly grouped as lactic acid bacteria (LAB), and the food products including these, are believe to be good targets when searching for new probiotics.

LAB are bacteria that ferment carbohydrates to form carboxylic acids, frequently lactic acid. These kinds of bacteria are commonly used in different food applications, as for example in dairy products, wine production, and sourdough fermentation. The definition of LAB is by Molin (2013) stated to be "LAB is a functional group and applies to bacteria that are harmless to both food quality and human health, and that occurs spontaneously in high number in traditional lactic acid fermented foods". In these food products the lactic acid has the properties of being a preservative, however it also affects the flavor and the texture of the food product and promotes an acidic environment (Liu, 2003). Lactic acid bacteria are non-spore forming, gram positive bacteria that grow anaerobically (de Vries, 2006).

The LAB commonly involved in food include the genera *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Weissella, Streptococcus* and *Pediococcus* (Stiles & Holzapfel, 1997).

1.4.1 Lactobacillus and *Lactobacillus plantarum*

The genus *Lactobacillus* is connected to a lot of different habitats and is present in human in the oral cavity, intestinal tract and vagina. *Lactobacillus* is also related to spoilage of a lot of different foods, such as fruits, fermented beverages, sugars, beers and milk. The attributes of *Lactobacillus* are different from the different species. However, a characteristics present in all species is the acidophilic properties. When *Lactobacillus* is present in food which include a fermentable carbohydrate the *Lactobacillus* will ferment the carbohydrate and lower the pH towards four. This acidic influence of the food leads to a competitive environment and likely a suppressed growth for other bacteria (Stiles & Holzapfel, 1997). Strains from *Lactobacillus*, and also the genera *Bifidobacterium*, are the most known to be applied as probiotics in food (Molin, 2013).

L. plantarum is one of the species of the *Lactobacillus* genus. It is commonly present in lactic acid fermented food based on plant material but also in products based on meat and dairy (de Vires, 2006). *L. plantarum* have a high tolerance to low pH and can grow in pH below four. Due to its high tolerance to low pH it can survive throughout the acidic environment in the stomach. Since *L. plantarum* is commonly present in a lot of different fermented food products it is a bacterium usually present in the human body, from the mouth to the column (Molin, 2015). Complete genome sequencing has quite recently been done on *L. plantarum* WCFS1. From this it could be seen that *L. plantarum* can grow on a lot of different substrates and that a lot of genes related to regulatory functions were present which indicates that the bacterium also can stand and live in a lot of altered conditions and environments (Kleerebezem et al. 2003). There are some strain of *L. plantarum* that today are stated as probiotics and this includes *L. plantarum* 299v which is the one used in ProViva fruit drinks (de Vries, 2006).

L. plantarum 299v has been isolated from the intestinal mucosa of healthy humans and is one of the species that commonly dominates the *Lactobacillus* flora in a healthy person. This strain has a high tolerance to pH and can survive in pH from 2 up to 9 (Molin, 2015). In a study performed by Johansson et al. 1993 the effect of colonization by different strains of *Lactobacillus* on healthy human individuals was evaluated. This was examined by consumption of an oat soup including 19 different types of *Lactobacillus* strains for 10 days. It was during analyzes of mucosal samples performed day 11 shown that *L. plantarum* 299v and *L. plantarum* 299 were the two dominating bacterial strains which indicates that these strains have the best colonization properties of the different strains included in the study (Johansson et al. 1993). *L. plantarum* 299v has a high tolerance for bile salts and can grow in conditions with bile salts up to 2% (Molin, 2015; de Vries, 2006).

1.4.2 Carbohydrate metabolism of *Lactobacillus*

The metabolism used by different *Lactobacillus* in different foods are important to consider in food applications. In some food products, ethanol is for example a wanted product after fermentation and in other products an ethanol production would mean a spoiled product. Also, in some products a too high production of carbon dioxide may lead to problem of packaging the product. Conditions leading to a high production of acetic acid may affect the taste of the product since acetic acid can contribute to a sticky and vinegar-like taste. It is thereby always important to

consider what metabolic pathways that will be used when combining different kind of *Lactobacillus* with different foods (Molin, 2013; Francois et al. 2010).

Lactobacillus metabolize different compounds, such as amino acids, organic acids and carbohydrates, and forms lactate and pyruvate (see figure 2). The most common substrates are sugars, like pentoses and hexoses, and the pathways of fermentation of these sugars can both be heterofermentative or homofermentative (Liu, 2003). Three different categories are used when classifying the different *Lactobacillus* species based on type of metabolism. What differs in the different groups are which substrates that are fermented, what metabolic pathways that are used and what products that are formed. The three groups of *Lactobacillus* are:

(1) Obligate homofermenters: the bacterium ferments hexoses (not pentoses nor gluconate) to form lactic acid as the primary product.

(2) Obligate heterofermenters: the bacterium ferments hexoses to lactic acid, carbon dioxide and acetic acid/ethanol.

(3) Facultative heterofermenters: the bacterium, just like obligate homofermentative, ferments hexoses to lactic acid by the Embden-Meyerhoff-Parnas (EMP) pathway. The bacterium is also able to ferment pentoses throughout the phophoketolase pathway to lactic acid and acetic acid. It can also ferment gluconic acids such as citrate to diacetyl, acetoin and carbon dioxide, and malic acid to lactic acid and carbon dioxide (Kleerebezem et al. 2003; Stiles & Holzapfel, 1997; Molin, 2013).

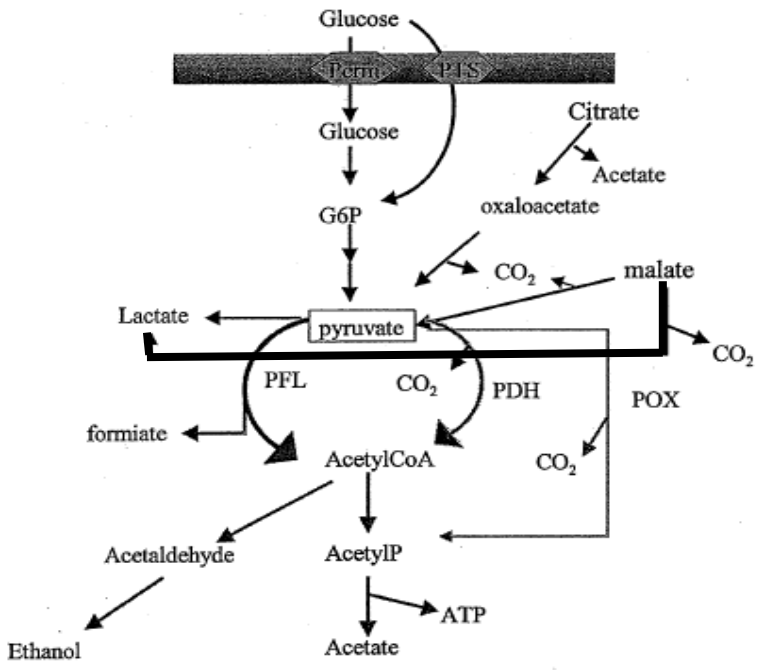


Figure 2. An overviewing scheme of the metabolism of the *Lactobacillus* genus (Beverini, Lacorre, Francois & Labbe, 2014).

1.4.3 Malolactic fermentation

The first problem that have been observed when producing a ProViva drink with apple is the overproduction of carbon dioxide. This overproduction have been found to be a cause due to the bacterial fermentation of malic acid.

The genus *Lactobacillus* ferment organic acids in different metabolic pathways. Malate is one organic acid which is either metabolized directly to lactate and carbon dioxide, or to pyruvate and carbon dioxide (see figure 2). *L. plantarum* belongs to those LAB that ferment malate directly to lactate and carbon dioxide, without pyruvate as a transitional compound (Liu, 2003). The fermentation of malic acid is called malolactic fermentation (MLF) and more exactly this means the enzymatic reaction when L-malic acid is metabolized to L-lactic acid and carbon dioxide, see figure 3 (Cabrita et al. 2007).

Malic acid is an organic acid with a pKa of 3.46 in 25°C (SigmaAldrich, 2016). The low pKa value of malic acid leads to that the acid decrease the pH in an external environment with neutral pH. A decrease in the external environment promotes a pH decrease intracellularly of *L. plantarum* as well. The presence of malate also affects and changes the permeability of the cell membrane and reduces the ion movement through the membrane of *L. plantarum*. Based on this fact, it is in a study performed by Filannino et al. (2014) stated that the carbohydrate metabolism is used by *L. plantarum* for growth while the malolactic fermentation is used for maintenance depending on if the present condition is favorable or unfavorable for the bacterium (Filannino et al. 2014). In a low external pH a switch from carbohydrate metabolism to malolactic fermentation has been seen in *L. plantarum* (Mozzi, Raya & Vignolo, 2015). The MLF and the intracellular decarboxylation of malate to lactate (see figure 3) leads to an increase in intracellular pH, this since lactic acid has a pKa of 3.86 which is higher than the pKa of malic acid. The increase of intracellular pH due to the decarboxylation of malate to lactate promotes energy advantages for the bacterium (Featherstone & Rodgers, 1981; Filannino et al. 2014).

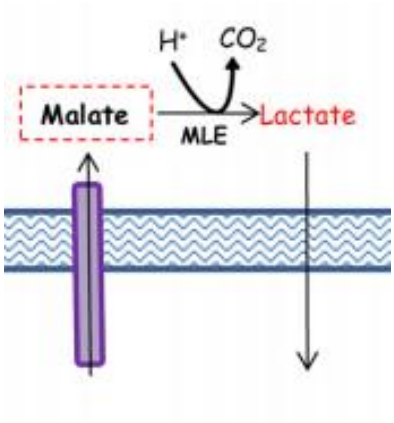


Figure 3. Transport of malate over the cell membrane and intracellular reduction to lactate (Filannino et al. 2014).

In the same study performed by Filannino et al. (2014) it was evaluated what different substrates that are consumed and products that are formed when strains of *L. plantarum* are added to different fruit drinks. What could be seen was that the consumption of sugar differed in all the juices, in some juices the sugar decreased a lot during fermentation and storage, while in another juices the sugar content only slightly decreased. This was explained with the fact that the juices differed in acidity. In the drinks with lowest pH the consumption of carbohydrates tended to decrease since the malolactic fermentation was favorable. In juices with higher pH the fermentation of carbohydrates was increased. Common for all the juices was a clear decrease of malic acid concentration after fermentation compared to before. It could also been seen that lactic acid was the major end product and also that the acetate concentration was increased in all juices after fermentation and storage (Filannino et al. 2014).

1.4.4 Off-flavors – Decarboxylation of Cinnamic acids

The second problem that have been observed in the connection between apple juice and *L. plantarum* is the production of off-flavors. The off-flavors are produced due to the decarboxylation of phenolic acids. Phenolic acids are compounds that are important as nutrients and antioxidants in food. The properties of being antioxidants are especially important due to its activity against carcinogenesis (Rodriquez et al. 2009). Phenolic acids are present throughout the whole plant kingdom. There are mainly two types of phenolic acids, the hydroxycinnamic acid and the hydroxybenzoic acids. Hydroxycinnamic acids mainly include the acids ferulic acid (FA), p-coumaric acid (PCA), shikimic acid and caffeic acid. These acids are commonly present in fermented products, and in food that have undergone sterilization and freezing (Rodriquez, Landete, de las Rivas & Munoz, 2008). When the hydroxycinnamic acids FA and PCA are combined with certain microorganisms they are decarboxylated by enzymes present in the microorganism and thereby they form volatile phenols named 4-vinyl derivatives. These volatile phenols are unwanted in a lot of different food applications because they are considered to be off flavors (McMurrough et al. 1996; Cabrita et al. 2007). Further, these volatile phenols can by some microorganism be reduced even more and this by a vinylphenol reductase, and then form 4-ethyl derivatives (Cabrita, Palma, Raquel & Freitas, 2010). FA is decarboxylated to 4-vinyl guaiacol (4-VG) and further to 4-ethyl guaiacol (4-EG) and PCA is decarboxylated to 4-vinyl phenol (4-VP) and further to 4-ethyl phenol (4-EP) (see figure 4 below) (van Beek & Priest, 2000). The 4-VP and 4-VG are known to give off-flavors recognized as medicinal, glove, smoked and phenolic while the 4-EP gives a false taste of earthy hay (Francois et al. 2010).

A lot of microorganisms are able to decarboxylate cinnamic acid derivatives by the use of decarboxylase enzymes to form 4-vinyl derivatives. However, fewer microorganisms are thereafter able to reduce the 4-vinyl derivatives to their corresponding 4-ethyl derivatives. It is stated by van Beek and Priest (2000) that it is likely that a combination between microorganism,

in this example yeast and bacteria, increase the reduction capability of 4-vinyl derivatives to 4-ethyl derivatives compared to when having either microorganism alone (van Beek & Priest, 2000).

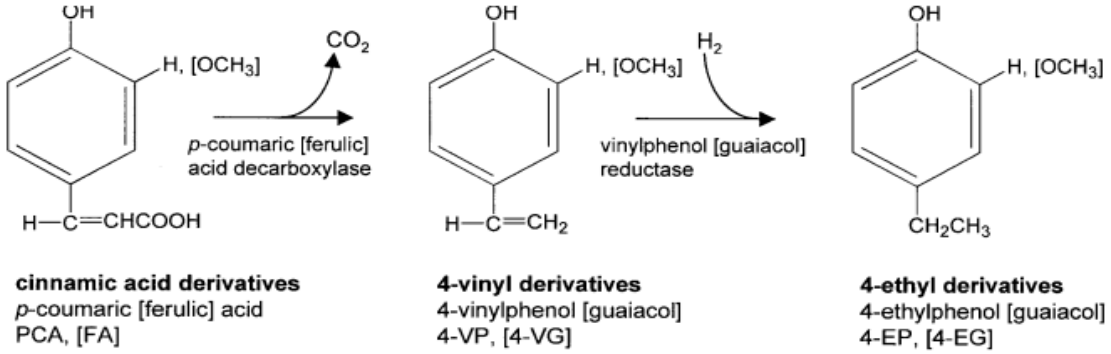


Figure 4. The decarboxylation of cinnamic acids to 4-vinyl derivates and 4- ethyl derivates (van Beek & Priest, 2000).

It is clear that *L. plantarum* includes enzymes that are responsible for the decarboxylation of the two cinnamic acids; PCA and caffeine acids. However, when it comes to *L. plantarum* and its decarboxylation of FA the results from different studies are controversial. In a study performed by Beek and Priest (2000) no decarboxylation of FA was seen. However, in another study performed by Rodriquez et al. (2008) a decarboxylation of FA was noticed and thereby also the production of 4-VG, but no further reduction to 4-EG. When it comes to decarboxylation of PCA the two studies have the same results; in the presence of *L. plantarum* PCA is decarboxylated to 4-VP and further to 4-EP (van Beek & Priest, 2000; Rodriquez et al. 2008).

1.5 The inhibition effect of lactic acid

The two problem observed connected to the production of an apple ProViva drinks, the malolactic fermentation and the decarboxylation of cinnamic acids, will in the study be tried to be inhibited with the introduction of lactic acid to the ProViva apple drinks.

During fermentation, lactic acid bacteria produce lactic acid as the main fermentation product. Lactic acid is a weak organic acid and the production of lactic acid leads to a decrease in pH which promotes a more acidic environment for the fermentation bacteria to live within (Leroy & de Vuyst, 2004; Beverini et al. 2014).

Weak organic acids have since centuries back in history been used as preservatives including acids such as lactic acid, acetic acid, propionic acid and sorbic acid. These weak organic acids have been found to promote antimicrobial effects and can thereby prolong the shelf life of food products. The mechanism for how a weak organic acid is bacteriostatic, bactericidal and antimicrobial is not yet really understood, however, it is clear that the pH has a central role in the effect (Hirshfield, Terzulli & Byrne, 2003; Russell & Diez-Gonzalez, 1997). A weak organic acid can be either protonated and deprotonated depending on the pH of a medium and the pKa value of the acid. This according to the formula (1) below:

(1). $pH = pKa + log_{10}\frac{[A-]}{[HA]}$

[A-] stands for the concentration of base and [HA] for the concentration of protonated conjugated acid (Francois et al. 2010). The pKa of lactic acid is in 25°C equal to 3.86 (Featherstone & Rodgers 1981).

A weak acid that is protonated is uncharged and liposoluble and can thereby diffuse into cells and their cytoplasm. Once inside the cell the acid can affect and reduce the intracellular pH. The decrease in pH is a result of the release of the acid's proton since the acid come from a more acidic environment from outside the cell to a more alkali environment inside the cell. A decrease in intracellular pH leads to for example inactivation of different enzymes which affect bacterial functions (Francois et al. 2010). The decrease in intracellular pH is one possible explanation for the antimicrobial effect of weak acids, however there are also other possible explanations for this effect. Another explanation may be the impact of weak organic acid in the cell membrane which may create a perturbation of its function. The liposoluble protonated organic acids can interference with the liposoluble proteins in the cell membrane and thereby contribute to growth inhibition of the microorganism (Hirshfield, Terzulli & Byrne, 2003; Ricke, 2003). Finally, another explanation for the toxicity effects of weak organic acids may be the anion accumulation in the cytoplasm of the cell which increases the osmotic concentration in the cytoplasm. The increase of osmotic concentration may increase the flow of external water into the cytoplasm which can promote a lethal effect due to a too high increase in turgor pressure (Hirshfield, Terzulli & Byrne, 2003).

In a patented study performed by Francois et al. (2010) the inhibition of *Lactobacillus* metabolism by introduction of lactic acid in fruit juices is discussed. It has been found that a combination of keeping a low pH of the fruit drink together with adding a weak organic acid, as for example lactic acid, can reduce the metabolic activity of *Lactobacillus* and thereby also prevent production of off-flavors and gases in fruit/berry drinks. In the study a recommended concentration of protonated organic acid (in this case lactic acid) to use in a fresh food product which will be kept cold (between 0 and 15°C) is set to at least 2.2g/l and up to 20g/l. It is also recommended to add a basic compound to the fruit drink to increase the pH after a weak organic acid has been introduced. The recommended final pH of a fruit drink after introduction of a basic compound is set to between 3.4 and 3.7 (Francois et al. 2010).

2.0 Aim

The aim of the project was to evaluate the inhibition effect on malate metabolism in *L. plantarum* 299v by introduction of lactic acid in ProViva apple drinks. This was done by creating nine different ProViva apple drinks in which the apple juice concentration and the concentration of added lactic acid was altered so that nine different combinations were produced and examined. One of these nine drinks was produced in duplicate to eliminate and discuss differences found between batches.

The inhibition effect was evaluated by examining the differences in gas formation (carbon dioxide), pH and metabolite production in the nine different apple drinks. The project also evaluated the effect of apple juice concentration/lactic acid concentration with respect to live probiotics, shelf life and by performing a sensory analysis.

3.0 Material and Methods

To learn the equipment and methods used at ProViva two screening experiments were performed. These screening experiments aimed to identify and determine which parameters and concentrations to use for the experiment performed later on. The purpose was also to get to know the different tastes and how they were affected when adding lactic acid and oat swell to the apple drinks.

The results obtained from the screening experiments have been used to determine the lactic acid concentration used in the experiment.

3.1 Calculations of ingoing ingredients in the apple drink

The amounts of the different ingredients used in the apple drinks in the experiments are based on literature and on preferences from ProViva. The weight percent of the different ingredients are also based on the fact that a 100% apple juice should have the BRIX value of 11.2. This is the starting point for all calculations. In appendix 1 an example is presented how the amounts of the different ingredients were calculated to obtain a certain drink. All drinks of ProViva should have an oatmeal base concentration of 5 % (w/w), and a fruit juice concentration of at least 35% (w/w).

3.2 Preparation of drinks

The apple concentrate used in the experiments was produced and bough from RAUCH POLSKA SP. Z.O.O. and named Apple Juice Concentrate item number 4004. The lactic acid used, concentration 80%, was called PURAC FCC 80 L(+) Lactic Acid FCC Special 80% (Corbion purac, Netherlands). The fermented oatmeal base was produced at ProViva and the oatmeal base included the probiotic bacterium *L. plantarum* 299v. The oatmeal base including the bacterium was stored in and retrieved from production tanks at ProViva. The apple juice concentrate was stored and used from tank trucks.

All drinks produced in the project were according to the following procedure:

* The apple concentrate was mixed with water from the tap, according to the calculated % (w/w) for every drink.

* The correct percentage (w/w) of lactic acid was added to the apple drinks and the drinks were mixed.

* All apple drinks, including the lactic acid, were heat treated to 93^{o}C and then cooled down in a cold water bath to approximately 10^{o}C.

* After cooling, the oatmeal base was added to the drinks.

* The drinks were mixed and thereafter filled into plastic bottles samples, 250 (±0.5) grams per bottle.

3.3 Analytical Measurements

3.3.1 pH-Measurements

The pH measurements were performed using a WTW pH 340i from Christian Berner AB, Sweden. A two-point calibration (pH 4 and pH 7) of the pH electrode was performed before every measuring day. Approximately 100 ml of each sample was poured into a plastic cup and the pH electrode was immersed into the sample. After approximately 5 minutes, when the measured pH was stable, the result was recorded. Two to four samples of each drink were analyzed and the average value was calculated and presented in the report.

In screening experiment 2 the impact of adding a basic compound in the ProViva apple drinks was evaluated using a pH measurement equipment Seven Compact pH (Mettler Toledo, Sweden). For evaluating the impact of a basic compound 0.1 mol/l sodium hydroxide ((0.1N, product number 31770, VWR International, Belgium) was added in small portions to a 250 g drink and the pH differences were measured until the pH reached 3.3-3.4.

3.3.2 Headspace Gas-Measurements

All carbon dioxide measurements were performed with a CheckPoint Handheld gas analyzer from 2008 from PBI densensor, Denmark. The used needles were 100 Sterican Ø0.80X50mm B.BRAUN (Sweden) and Henke Sass WOLF 1x100 Fine-ject 0.6x25mm (Germany). Septums were used and these were bought from Densensor A/S, Denmark, and called Septum, Ø15, grey. Before every analyzing day the Checkpoint Handheld gas analyzer was calibrated.

The head space gas measurements were performed as follows: A plastic bottle sample was gently carried from the storage place to the desk where the analysis was performed. A gentle hand was needed since shaking of the bottle might affect the results. The plastic bottles were not opened before the measurements took place. It was important to not have any leakage of the gas in the headspace.

A septum was placed on the upper part of the sample bottle, where the headspace of the gas was located. The needle was put through the septum and the plastic bottle. Thereafter the measurement of the headspace was started by pushing the analyzing bottom. Two to four samples of each drink were analyzed. An average value of the different sample of the same drink was calculated and presented.

The carbon dioxide amount was set as an initial value = 0 for all drinks produced in the project.

3.3.3 Metabolite analysis

Analyses of organic acids in samples were performed by Eurofins (Jönköping, Sweden). These plastic bottle samples were taken from the fridge a certain day of the experiments, see detailed day in section 3.5.3 below. The samples were transferred to a freezer for storage until shipment to Eurofins for analysis. Eurofins was responsible for analyzing different organic acids in the samples, and for this experiment the organic acids: lactic acid, malic acid and acetic acid, were the most relevant and presented in this report.

3.4 Microbiological Analysis

3.4.1 *Lactobacillus*

The amount of colony-forming units (CFU) of *L. plantarum* 299v per milliliter drink was determined by using the method viable count. The samples were grown on MRS-agar plates and after storage in 35°C for 48 hours plate/colony count was performed.

A dilution series was performed for all samples. Some samples were only diluted to 10^{-3} while others to 10^{-8}. The dilution was determined based on previously obtained results. One milliliter of the sample was added to the first cup of dilution (Dilutioncup Elegance-BPW 9.0ml, LabRobot, Sweden) which contained 9 milliliters prepared buffered peptone (20g/l) and de-ionized water. A shaker (Dilushaker III Digital, product number: 4ODS21/42, LabRobot, Sweden) was used to mix samples homogenously. Each cup was mixed for a while before pipetting one milliliter from the first cup to the second. The second cup also contained 9 milliliters buffered peptone solution leading to the dilution 10^{-2}. This procedure was continued until the required dilution was reached. Thereafter 1 milliliter of every dilution which were aimed to be analyzed, as for example dilution 10^{-5}, 10^{-6}, 10^{-7}, was spread on empty petri plates which had been marked with the sample name, the dilution, the agar and the date. When all samples had been put on the empty agar plates approximately 15ml of MRS-agar pH 5.7 (Biomerieux, France) was poured onto the samples in the plates. The MRS agar was prepared by employees in the lab. After five minutes the agar had solidified and the plates were incubated at 35°C for approximately 48 hours. The colonies obtained were counted and the amount of CFU *L. plantarum* 299v per milliliter apple drink could be determined. This by using the formula:

$$\frac{\sum CFU}{(n_1 + 0.1 * n_2) * d}$$

where $\sum CFU$ are the total number on colonies on both dilutions, n_1 the number of agar plates in the lowest dilution, n_2 the number of agar plates in the highest dilution, and d is the lowest dilution.

3.4.2 Mold and Yeast Analysis on PDA-agar

The shelf life of the ProViva apple drinks was analyzed by evaluation of the presence of yeast and mold in the samples by growing the samples on PDA-agar. The presence of yeast and mold were also an indication and an explanation for potential off flavors and could tell when the samples should be avoided to consume.

One milliliter of the sample to be analyzed was placed on an empty petri plate. Prepared PDA agar (approximately 15 milliliters) (Potato Dextrose Agar 400782ZA, VWR chemicals, Belgium) was then poured into in the plate. After 5 minutes, when the agar had congealed, the plate was incubated at 25°C for five days for colonization of yeast and molds to occur. After five days the amount of colonies were counted and the amount of yeast/molds per milliliter drink could be determined.

3.5 Sensorial Analysis

Two different types of sensorial analyses were performed in the experiment:

The first one consisted of one to four people who discussed the taste of the drink orally together.

The second sensorial analysis consisted of a consumer test panel which was represented by 17 persons. All members were placed individually to taste and judge the apple drinks in silence. The drinks were marked with random numbers and every person got a unique order to taste the drinks in. After one drink had been tasted, the test panel member should mark in a scale from 1-9, where 1 was “I don’t like it at all” and 9 was “I love it”, depending on what this person thought of the flavor of the drink. After all drinks had been rated, the members of the panel were free to go and the answers from all members were collected. Average values of all results for each type of drink were calculated and presented in the report. The survey that was filled in by the test panel is included in appendix 2.

3.6 Experimental Setup

3.6.1 Screening experiment 1

In the first screening experiment performed, four different ProViva apple drinks were produced. All of them containing an apple juice percentage of 50% (w/w). The four different drinks consisted of the following:

1. 50% apple juice
2. 50% apple juice with 16 g/l lactic acid
3. 50% apple juice with 5 % (w/w) oatmeal base
4. 50% apple juice with 5 % (w/w) oatmeal base and 16 g/l lactic acid

3 kg of each drink was produced and filled into nine plastic bottles.

The apple drink, number 1 above, was produced to work as a reference with respect to taste and appearance compared to the other drinks performed in this screening experiment.

The used percentage of apple juice in the drinks, 50% (w/w), was chosen due to two important reasons. Firstly, 50% was a higher percentage than the 35%, which was the percentage that ProViva sets to a minimal limit of fruit juice content and the drinks should preferably contain a higher fruit juice percent than that. Secondly, 50% juice was thought to be a high enough percentage to promote the differences in gas formation to investigate the impact of lactic acid. A higher percentage than 50% apple juice was excluded in this screening experiment since it was believed to be a risk that the gas formation would increase too rapidly.

The concentration of lactic acid used in the drink was based on the recommendations by Francois et al (2010) and to ensure a distinct impact the concentration 16g/l lactic acid was used. The different ingredients, expressed in wt. %, of the four drinks can be seen in table 1 below.

Table 1. In screening experiment 1 four different drinks were produced, all of them with 50% (w/w) apple juice. Drink 1 only consisted of apple juice, drink 2 consisted of apple juice and lactic acid, drink 3 consisted of apple juice and oatmeal base and drink 4 consisted of apple juice, lactic acid and oatmeal base. The amount of the different ingredients are given in % (w/w).

Type of drink	Apple Concentrate	Lactic acid 80%	Oatmeal base	Water
Drink 1	8.180	-	-	91.82
Drink 2	8.180	2.000	-	89.83
Drink 3	8.180	-	5.000	86.82
Drink 4	8.180	2.000	5.000	84.82

All the different drinks were produced and the pH as immediately measured in the drinks (direct analysis).

After production, one set of drinks were stored in a fridge, 8°C, and one set of drinks were stored at 25°C. The drinks were observed every day and after six days all drinks, both the ones in the fridge and the ones at 25°C, were analyzed with respect to carbon dioxide formation and pH. The apple drink number 4 (with juice, oatmeal base and lactic acid) stored at 25°C, were also evaluated with respect to amount of live probiotics to study whether or not *L. plantarum* 299v could support the high amount of lactic acid (16g/l) in combination with the low pH of the drink. The analyses were performed by serial dilution of the drink from 10^0 to 10^{-8} and growth on MRS agar.

The amount of protonated and deprotonated lactic acid were calculated with the formula $pH = pKa + log_{10}\frac{[A-]}{[HA]}$ for drink number 4, see appendix 3 for an example of this kind of calculation.

3.6.2 Screening experiment 2

In screening experiment 2 four different drinks were produced, all of them with 100% (w/w) apple juice and 5% (w/w) oatmeal base. The difference between the drinks was the amount of added lactic acid, see list below.

1. 100% apple juice with 5g/l lactic acid
2. 100% apple juice with 10g/l lactic acid
3. 100% apple juice with 15g/l lactic acid
4. 100% apple juice with 20g/l lactic acid

The purpose of this study was to see how these four different ProViva apple drinks were affected with respect to pH and taste after the addition of different concentrations of lactic acid. The aim was also to discuss and calculate the amount of lactic acid that was in its protonated state or deprotonated state. Finally, the purpose was to change the pH of all the four drinks, to a pH that reached approximately 3.4, by addition of a basic compound, in this case sodium hydroxide, and to see how this affected the taste of the drink. Sodium hydroxide was used as the basic compound since this chemical was the chemical usually used when performing titration analysis in the lab at

ProViva. The accessibility of this chemical was therefore the decisive parameter. Sodium hydroxide is a base that is commonly used as food additive, E524, in a lot of different food applications. It is used as an acidity regulator in products such as jam, marmalade and snacks. It is also commonly used in bakery products as an important ingredient to contribute to the appearance of the products.

1 kg of each drink, 1-4, were produced and filled into plastic bottles. The different ingredients, expressed in wt. %, of the four drinks can be seen in table 2 below.

Table 2. In screening experiment 2 four different drinks were produced, all of them containing 100% (w/w) apple juice and 5 % (w/w) oatmeal base. The difference between the drinks was the amount of added lactic acid. The amounts of the different ingredients are given in % (w/w).

	Apple Concentrate	**Water**	**Oatmeal base**	**Lactic Acid 80%**
Drink 1	16.35	78.03	5.000	0.6250
Drink 2	16.35	77.40	5.000	1.250
Drink 3	16.35	76.78	5.000	1.875
Drink 4	16.35	76.15	5.000	2.500

After the four drinks had been produced, during the same day, one plastic bottle sample of every drinks were titrated with 0.1mol/l sodium hydroxide to that point were the samples reached the pH 3.4. Thereafter a test panel of four people tasted all the different drinks and discussed the organoleptic properties of the drinks.

The amount of protonated and deprotonated lactic acid were calculated which the formula $pH = pKa + log_{10}\frac{[A-]}{[HA]}$ for all drinks before and after titration with sodium hydroxide.

As a part of the experiment, a pure apple juice (100%) (only consisting of apple concentrate and water) were produced in the total volume of 500 ml and the pH of the drink were measured. This was done to be able to compare the other obtained pH measurements with this as a control.

3.6.3 Experimental set-up to investigate inhibition of malate metabolism by introducing lactic acid

In this experiment 9 different drinks were produced and analyzed. The obtained results from the screening experiments were used to define concentrations and decide parameters of the experiment. The drinks were evaluated with respect to produced amount of carbon dioxide, the pH of the drinks were measured, amount of live probiotics was determined, shelf life with considerations for mold and yeast growth was considered, changes in organic acid composition were analyzed and finally the drinks were evaluated by a consumer test panel regarding the flavor of the drinks.

All drinks consisted of apple juice, water, oatmeal base and lactic acid. The differences in the drinks were the concentration of added lactic acid and the percentage of apple juice. The drinks were produced according to the matrix found below in table 3.

In total 9 different apple drinks were produced, however, one of the drinks, the apple 60% and lactic acid 7g/l were produced two times leading to a total of 10 different drinks. A duplicate of this drink was performed to be able to evaluate the variation between different batches.

Table 3. Nine different apple drinks were produced and analyzed. Three different concentrations of lactic acid were combined with three different percentages (w/w) of apple juice leading to a total of nine different drinks. The drink in the middle of this matrix was determined to be produced in duplicate, this to study differences between batches.

		Apple juice percentage (w/w)		
		35%	**60%**	**100%**
Lactic acid concentration	**4g/l**	35% Apple 4g/l Lactic acid	60% Apple 4g/l Lactic acid	100% Apple 4g/l Lactic acid
	7g/l	35% Apple 7g/l Lactic acid	60% Apple 7g/l Lactic acid In duplicate!	100% Apple 7g/l Lactic acid
	10g/l	35% Apple 10g/l Lactic acid	Apple 60% Lactic acid 10g/l	100% Apple 10g/l Lactic acid

Each drink was produced in a total weight of 5kg, enough to fill 18 plastic bottle of every drink. This amount of samples of every drink were estimated to be enough for all following analyzes. The wt. % of the different ingredients in the drinks is presented in table 4 below.

Table 4. The different ingredients of the nine different drinks expressed in % (w/w).

Type of drink	Apple concentrate	Lactic acid 80%	Oatmeal base	Water
35% Apple, 4g/l Lactic acid	5.723	0.5000	5.000	88.78
35% Apple, 7g/l Lactic acid	5.723	0.8750	5.000	88.40
35% Apple, 10g/l Lactic acid	5.723	1.250	5.000	88.03
60% Apple, 4g/l Lactic acid	9.811	0.5000	5.000	84.69
60% Apple, 7g/l Lactic acid	9.811	0.8750	5.000	84.31
60% Apple, 10g/l Lactic acid	9.811	1.250	5.000	83.94
100% Apple, 4g/l Lactic acid	16.35	0.5000	5.000	78.15
100% Apple, 7g/l Lactic acid	16.35	0.8750	5.000	77.77
100% Apple, 10g/l Lactic acid	16.35	1.250	5.000	77.40

After production of all 10 drinks, all plastic bottles of each drink were stored in 8°C. The analyses of the drinks were performed several times for each drink, this according to the scheme listed below:

Day 0: Carbon dioxide production was assumed to be 0 for all drinks as an initial value. The pH was measured in all drinks. Samples for LAB were grown on MRS agar in the dilution series 10^{-6} to 10^{-8}, one plate for every dilution and sample. Samples for yeast and mold were grown on PDA agar plates, one plate for every sample. One sample of each drink was stored in the freezer until analysis of organic acids.

Day 2: The results from the MRS-agar plates were calculated.

Day 6: The results from the PDA-agar plates were obtained.

Day 9: Carbon dioxide production and pH were measured in all drinks. The consumer test panel sensorial analysis was performed.

Day 17: The carbon dioxide production and the pH were measured in all drinks. Samples were grown on MRS agar in the dilution series 10^{-5} to 10^{-8}, based on the results obtained from the first analyzes of *Lactobacillus*, see table 5.

Table 5. The dilutions for the different drinks for MRS-agar analysis performed day 17.

Type of drink	Dilutions		
35% Apple, 4g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
35% Apple, 7g/l Lactic acid	10^{-5}	10^{-6}	10^{-7}
35% Apple, 10g/l Lactic acid	10^{-5}	10^{-6}	10^{-7}
60% Apple, 4g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
60% Apple, 7g/l Lactic acid No. 1	10^{-5}	10^{-6}	10^{-7}
60% Apple, 7g/l Lactic acid No. 2	10^{-5}	10^{-6}	10^{-7}
60% Apple, 10g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
100% Apple, 4g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
100% Apple, 7g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
100% Apple, 10g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}

One plate for each dilution was prepared. Samples were also grown on PDA agar plates, one plate for every sample. One sample of each drink was stored in the freezer until analysis of organic acids. A sensorial analysis was performed, one person tasted the drinks and noticed the flavor experience.

Day 20: The results from the MRS-agar plates were calculated. Since some dilution series of some of the drinks did not give any colonies new plates were planned for the following day.

Day 21: In four of the drinks, no growth was detected on the agar plates performed day 17. Therefore new agar plates were made, and this time according to the dilutions below:

35% Apple, 7g/l Lactic acid: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}
35% Apple, 10g/l Lactic acid: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}
60% Apple, 10g/l Lactic acid: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}
100% Apple, 100g/l Lactic acid: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}

One plate for every dilution and sample was performed.

Day 22: The results from the PDA agar plates were obtained and a suspected contamination was observed. New agar plates were grown for all drinks. This time the plastic bottle samples were taken immediately from the fridge and were unopened until that point when 1ml was placed on the PDA agar plate.

Day 23: The results from the MRS-agar plates were calculated.

Day 27: The results from the PDA-agar plates were obtained. Carbon dioxide production and pH was measured in all drinks. The flavor of the three drinks: 100% Apple 4g/l Lactic acid, 100% Apple 7g/l Lactic acid, 60% Apple 4g/l Lactic acid was discussed among three people.

Day 36: The carbon dioxide production and the pH was measured in all drinks. The drinks were grown on MRS agar in the dilution series 10^{-1} to 10^{-8}, this based on the results obtained from the first analyzes of *Lactobacillus*, see table 6.

Table 6. The dilutions for the different drinks for MRS-agar analysis performed day 36.

Type of drink	Dilutions		
35% Apple, 4g/l Lactic acid	10^{-5}	10^{-6}	10^{-7}
35% Apple, 7g/l Lactic acid	10^{-3}	10^{-4}	10^{-5}
35% Apple, 10g/l Lactic acid	10^{-1}	10^{-2}	10^{-3}
60% Apple, 4g/l Lactic acid	10^{-5}	10^{-6}	10^{-7}
60% Apple, 7g/l Lactic acid No. 1	10^{-4}	10^{-5}	10^{-6}
60% Apple, 7g/l Lactic acid No. 2	10^{-4}	10^{-5}	10^{-6}
60% Apple, 10g/l Lactic acid	10^{-1}	10^{-2}	10^{-3}
100% Apple, 4g/l Lactic acid	10^{-6}	10^{-7}	10^{-8}
100% Apple, 7g/l Lactic acid	10^{-4}	10^{-5}	10^{-6}
100% Apple, 10g/l Lactic acid	10^{-1}	10^{-2}	10^{-3}

One plate for each dilution was grown. The drinks were also grown on PDA agar plates, one plate for every sample. One sample of each drink were stored in the freezer until analysis of the organic acids.

Day 37: Samples that were stored in the freezer were sent to Eurofins for analysis of organic acids: lactic acid, acetic acid, malic acid. Depending on obtained results some of the stored samples were not sent to Eurofins for analysis. The samples that were sent were the following:

35% Apple, 4g/l Lactic acid: samples from day 0, 17 and36
35% Apple, 7g/l Lactic acid: samples from day 0, 17 and 36
60% Apple, 4g/l Lactic acid: samples from day 0, 17 and 36
60% Apple, 7g/l Lactic acid No. 1: samples from day 0, 17 and 36
60% Apple, 7g/l Lactic acid No. 2: samples from day 0, 17 and 36
100% Apple, 4g/l Lactic acid: samples from day 0, 17 and 36
100% Apple, 7g/l Lactic acid: samples from day 0, 17 and 36

In total 27 different samples were sent for analysis.

Day 38: The results from the MRS-agar plates were calculated. The flavor of the drink 100% Apple 4g/l Lactic acid was discussed among two people.

Day 41: The results from the PDA-agar plates were obtained.

4.0 Results

In this study, two screening experiment were performed. These screening experiments aimed to identify and determine what limits to use for the experiment in which it the inhibition effect of lactic acid in apple ProViva drinks was evaluated.

4.1 Screening experiment 1

In screening experiment 1 four different drinks were produced and examined. All four drinks were analyzed with respect to pH, carbon dioxide and organoleptic properties.

The drinks that were produced and analyzed were the following:

1. 50% apple juice
2. 50% apple juice with 16g/l lactic acid
3. 50% apple juice with 5 % (w/w)oatmeal base
4. 50% apple juice with 5 % (w/w) oatmeal base and 16g/l lactic acid

One set of the four drinks were stored in 25°C for 6 days. Another sets of drinks were stored in 8°C for 6 days. Drink number 4 was also evaluated with respect to amount of *L. plantarum.*

The parameters CO_2, pH and flavor were measured and the results are presented in table 7, 8 and 9. The organoleptic properties of the different drinks are also presented in these tables. In table 7 the initial measurements are presented, in table 8 the measurements after 6 days storage in 25°C are presented and in table 9 the measurements after 6 days storage in 8°C are presented.

Table 7. Measurement of pH and carbon dioxide and the appearance of the four different apple drinks immediately after production (direct analysis).

Type of drink	CO_2 Direct analysis	pH Direct analysis	Flavor Direct analysis
Drink 1	0	3.88	Tastes good, a mild taste of apple.
Drink 2	0	2.75	Very sour! No other taste can be noticed except the sourness
Drink 3	0	3.87	A tiny taste of apple, tastes good.
Drink 4	0	2.78	Very sour! No other taste can be noticed except the sourness

Table 8. Measurement of pH and carbon dioxide and the appearance of the four different apple drinks after storage in 25°C for 6 days.

Type of drink	CO_2 Day 6	pH Day 6	Flavor Day 6.
Drink 1	2.30	3.87	Tastes as an apple juice but has a slightly taste of stale.
Drink 2	2.60	2.75	Can just feel the taste of sour and nothing else.
Drink 3	15.9	3.55	Feels old in the taste, like the shelf life is over. Taste a bit stale and musty.
Drink 4	4.10	2.78	Can just feel the taste of sour, no other flavors.

Table 9. Measurement of pH and carbon dioxide and the appearance of the four different apple drinks after storage in 8°C for 6 days.

Type of drink	CO_2 Day 6	pH Day 6	Flavor Day 6
Drink 1	2.30	3.85	Good nice taste of an apple drink
Drink 2	3.40	2.75	Just tastes sour
Drink 3	5.15	3.85	Tastes good, pretty much taste of oat, but probably due to low apple concentration
Drink 4	5.35	2.78	Just tastes sour

Calculation of the amount of protonated/deprotonated lactic acid showed that an added lactic acid concentration of 16g/kg and a pH of 2.78 resulted in 14,78g/l protonated lactic acid when assuming the pKa of lactic acid to be 3.86.

The amount of *L. plantarum* was after storage for 6 days in 25°C analyzed in drink number 4. The result showed that there was no detection of *L. plantarum* on the MRS agar plates. This probably indicated that the amount of lactic acid (16g/l) together with the low initial pH value of 2.78 created an environment which was too tough for the bacterium to live and grow in.

Visually it could be seen that six days after production of the drinks the package with drink 3, stored in 25°C, was really swollen. The package, a plastic bottle, was changed in structure due to the carbon dioxide formation and the bottle could no longer stand straight up (see figure 5). All the other drinks, both in storage temperature 25°C and storage temperature 8°C, seemed to be unchanged. No noticeable changes could be seen with the eyes.

Figure 5. A plastic bottle sample with 50% apple juice to the left and bottle with 50% apple juice and oat swell to the right. The bottle to the right has a changed structure and can't stand up straight anymore due to gas formation.

4.2 Screening experiment 2

In screening experiment 2 four different drinks were produced, all of them with 100% apple juice. These drinks were evaluated with respect to organoleptic properties by four people. The buffering capacity of the drinks were also evaluated by the addition of sodium hydroxide to that point when the pH reached 3.3-3.4. The drinks that had been titrated with sodium hydroxide were also evaluated with respect to their organoleptic properties. The amount of deprotonated/protonated lactic acid in each separate drink were calculated. A 100% pure apple juice were produced and the pH were measured.

The drinks to analyze were the following:

1. 100% apple juice with 5g/l lactic acid
2. 100% apple juice with 10g/l lactic acid
3. 100% apple juice with 15g/l lactic acid
4. 100% apple juice with 20g/l lactic acid

The 100% pure apple juice had the pH 3.694.

In table 10 below the initial pH of the different drinks are presented, and so also the calculated values of protonated lactic acid before and after titration. The amount of added sodium hydroxide for the titration is listed to the right. All calculations were based on a pKa of lactic acid at 25°C = 3.86.

Table 10. Measurements of the pH before and after addition of sodium hydroxide. Calculated values of protonated lactic acid before and after the titration. The amount of added sodium hydroxide in each drink to obtain the pH of 3.3-3.4.

	pH before titration	Amount of protonated lactic acid before titration (g/l)	pH after titration (goal pH=3.4)	Amount of protonated lactic acid after titration (g/l)	Amount of added NaOH 0.1mol/l (g)
Drink 1	3.20	4.10	3.41	3.80	8.50
Drink 2	3.00	8.78	3.34	7.67	34.8
Drink 3	2.86	13.6	3.33	11.6	69.9
Drink 4	2.77	18.5	3.30	15.5	95.4

During the organoleptic analysis it was found that 10g/l will be the highest lactic acid concentration to use in the experiment performed later on. This was determined due to a clear acidity in all drinks with a higher concentration of lactic acid even though sodium hydroxide was added. The drink with the least amount of lactic acid, and without added base, was the drink that tasted the best. Therefore it was also determined to exclude sodium hydroxide in the following experiment.

4.3 Experiment- inhibiting malate metabolism by introducing lactic acid

In this experiment, different concentration of lactic acid were combined with difference percentages of apple juice which resulted in nine different drinks to produce and evaluate. One of these drinks, the 60% apple 7g/l lactic acid, was produced in duplicate. All drinks were stored in 8°C for 36 days. The drinks were evaluated with respect to pH, carbon dioxide production, organoleptic properties, growth of *L. plantarum*, yeast and molds growth and the organic acids lactic acid, malic acid and acetic acid were measured.

The 10 drinks that were examined were composed of the following:

1. 35% Apple juice, 4g/l Lactic acid
2. 35% Apple juice, 7g/l Lactic acid
3. 35% Apple juice, 10g/l Lactic acid

4. 60% Apple juice, 4g/l Lactic acid
5. 60% Apple juice, 7g/l Lactic acid, No. 1
6. 60% Apple juice, 7g/l Lactic acid, No. 2
7. 60% Apple juice, 10g/l Lactic acid

8. 100% Apple juice, 4g/l Lactic acid
9. 100% Apple juice, 7g/l Lactic acid
10. 100% Apple juice, 10g/l Lactic acid

All of the drinks also contained 5% (w/w) oat swell.

4.3.1 pH Measurements

The pH was measured in all 10 drinks five times during the experiment: day 0, 9, 17, 27 and 36. The temperature of the drinks were approximately 10°C since the samples were taken from the storage temperature of 8°C for the analysis to be performed. Figure 6 shows the pH measurements of the drinks with 35% apple juice, figure 7 shows the pH measurements of the drinks with 60% apple juice and figure 8 shows the pH measurements of the drinks with 100% apple juice.

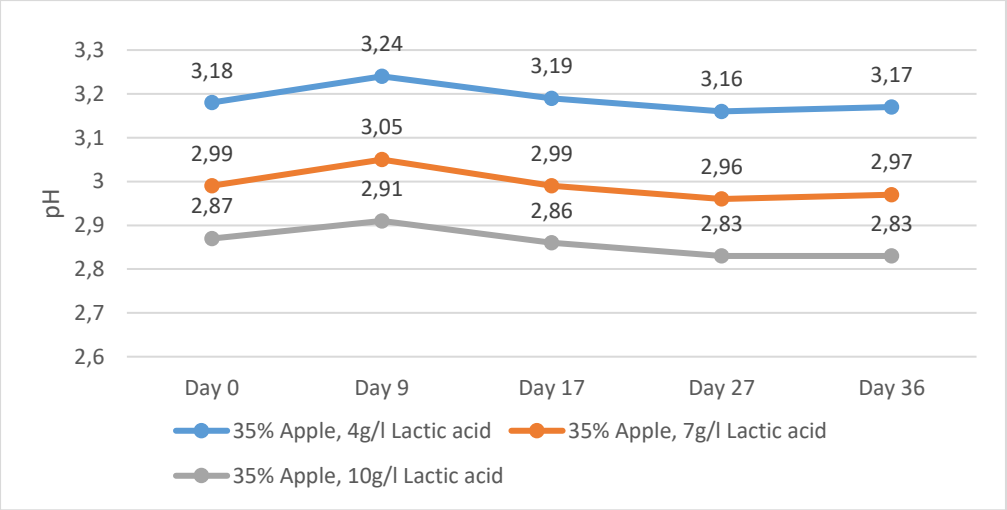


Figure 6. The pH measurements and the pH changes in the three drinks composed of 35% apple juice. The measurements were performed five times during the storage time of the drinks, day 0, 9, 17, 27 and 36.

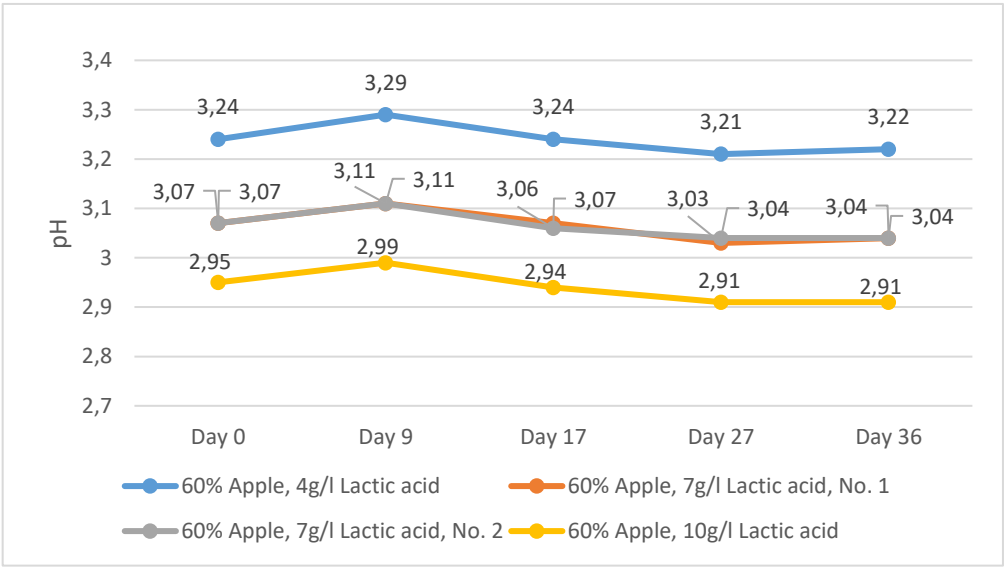


Figure 7. The pH measurements and the pH changes in the three drinks composed of 60% apple juice. The measurements were performed five times during the storage time of the drinks, day 0, 9, 17, 27 and 36.

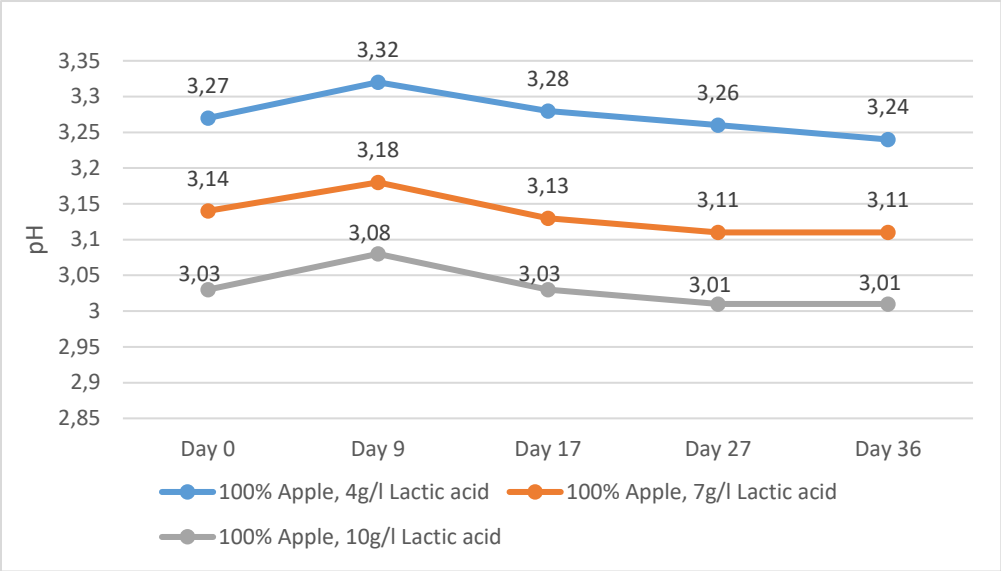


Figure 8. The pH measurements and the pH changes in the three drinks composed of 100% apple juice. The measurements were performed five times during the storage time of the drinks, day 0, 9, 17, 27 and 36.

4.3.2 Carbon Dioxide Measurements

The production of carbon dioxide in the drinks was measured by measuring the carbon dioxide percentage in the headspace of the samples. A low production of carbon dioxide indicated that the malolactic fermentation had been inhibited. The measurements were performed five times, storage day 0, 9, 17, 27 and 36, see figure 9.

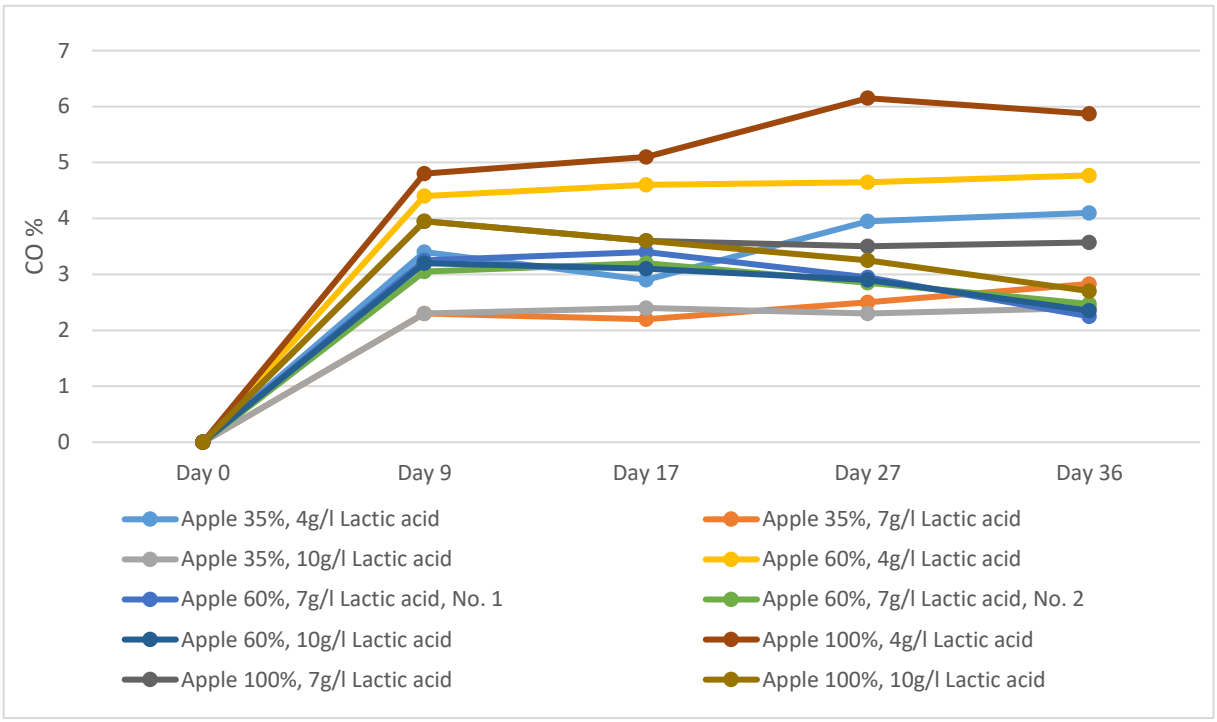


Figure 9. Measurement of the carbon dioxide production of the headspace for all 10 drinks. The head space measurements were performed five times during the storage time, day 0, 9, 17, 27 and 36.

4.3.3 Sensorial analysis

Two different sensorial analyses were performed in the experiment. One of the sensorial analyses consisted of a consumer test panel and this test panel was evaluating all 10 different drink during storage day 9. A survey seen in appendix 2 was completed by 17 participants and the average value was calculated for each drink. The result for each drink is presented in figure 10 below.

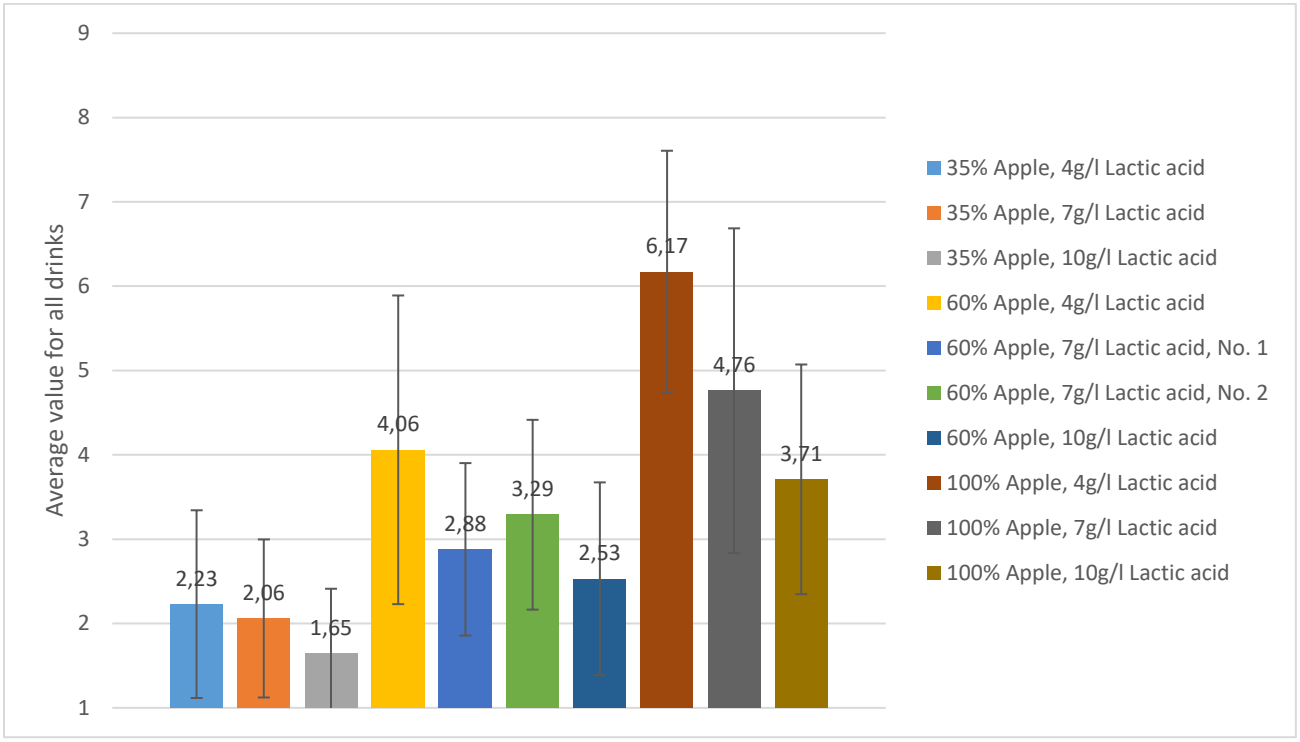


Figure 10. The different drinks according to the list on the right. The presented values are average values calculated from 17 participants in the test panel. 1= I do not like it at all, 9= I love it! The line in each staple represent the standard derivation.

The second sensorial analysis that were performed consisted of one to three persons who all tasted the drinks. The result achieved from these analysis were as follows:

Storage day 17: The drinks were tasted and the result was that no clear differences could be noticed compared to the sensorial analysis day 9 for all the 10 drinks.

Storage day 27: The drinks: 100% Apple 4g/l Lactic acid, 100% Apple 7g/l Lactic acid and 60% Apple 4g/l Lactic acid were tasted by three people. These drinks were selected to be analyzed due to the outcome of the consumer sensorial analyses were these drinks were the only ones that had an acceptable grade. It was stated that no significant differences in flavor could be noticed compared to the sensorial analysis performed day 9. The three drinks tasted pretty much the same since day 9 and only one possible difference could be detected by one of the three person who thought that there might existed a small stickiness in taste in the drink with 100% Apple and 4g/l lactic acid.

Storage day 38: The drink composed of 100% apple 4g/l lactic acid were tasted by two persons. It was found that the drink still appeared unchanged in flavor compared to day 9 when the sensorial test panel analyzed the drinks.

4.3.4 Growth of *L. plantarum* 299v

The amount of *L. plantarum* 299v in CFU/ml were determined three times during the experiment for all 10 drinks. First an initial value of *L. plantarum* for all drink was determined. Microbial analysis were then performed at day 17, day 21 and at day 36. In figure 11, 12 and 13 below the growth curves for all 10 ProViva apple drinks are presented. In figure 11 it is shown how the amount of *L. plantarum* was changed in the drinks containing 35% apple juice, in figure 12 in the drinks containing 60% apple juice and in figure 13 in the drinks containing 100% apple juice.

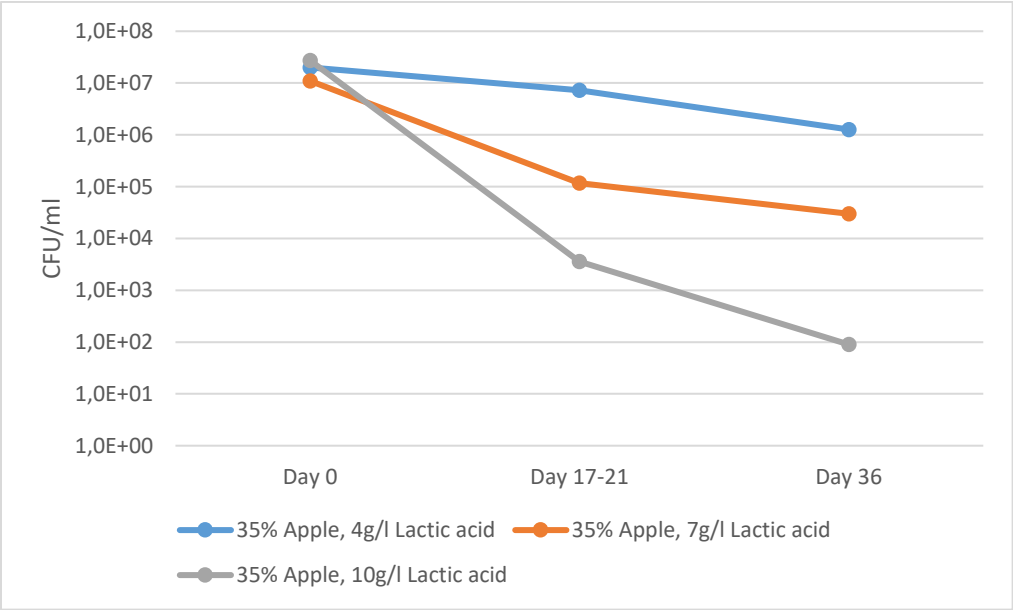


Figure 11. Growth of *L. plantarum* in drinks with 35% apple juice and varying concentrations of lactic acid at storage day 0, 17 and 36.

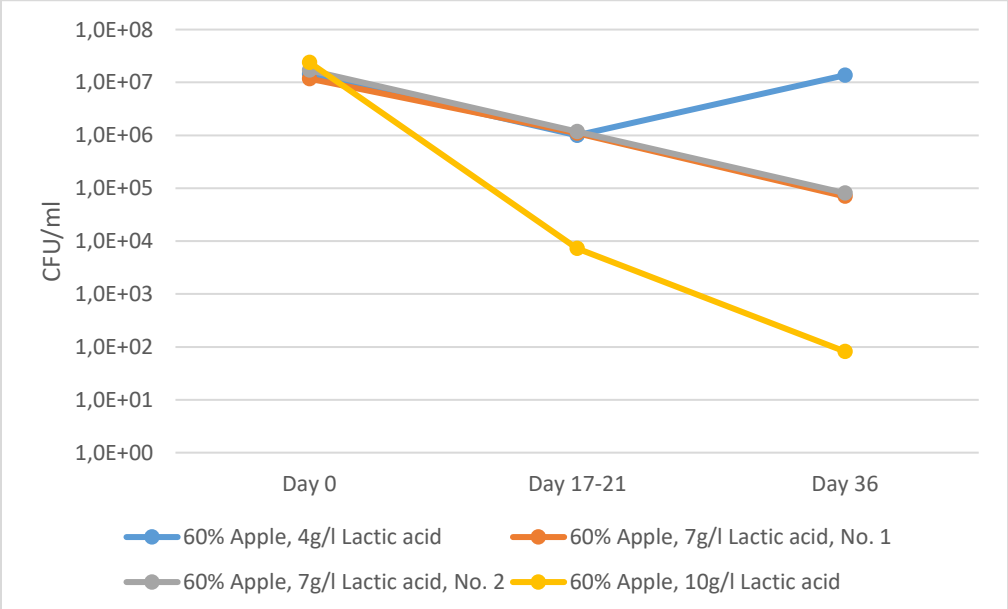


Figure 12. The growth of *L. plantarum* in the drinks with 60% apple juice and varying concentrations of lactic acid at storage day 0, 17 and 36.

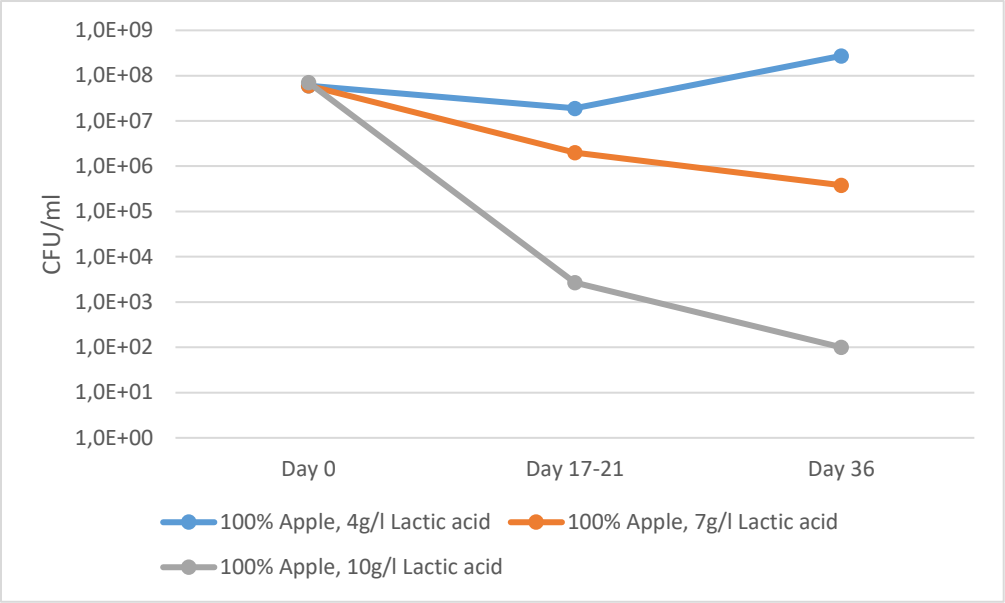


Figure 13. The growth of *L. plantarum* in the drinks with 100% apple juice and varying concentrations of lactic acid storage day 0, 17 and 36.

4.3.5 Yeast and Mold Analysis

The presence of yeast and molds in the 10 drinks were evaluated four times during the experiment; day 0, 17, 22 and 36. The analysis day 22 were performed as an extra control since the results from day 17 were believed to be contaminated due to an unexpected growth of a lot of yeast and mold in the majority of the ten drinks. After obtaining the results from day 22 it was assumed that a contamination had occurred in the samples from day 17 since the growth of yeast and molds were decreased in the samples. In table 11 below the results of growth on PDA-agar are shown in CFU/ml for all four samplings.

Table 11. In this table the results from all PDA-agar analysis during the experiments are presented. The numbers written in orange are believed to be samples that have been contaminated and therefore these results are not discussed further in the report.

	Day 0 (CFU/ml)	**Day 17 (CFU/ml)**	**Day 22 (CFU/ml)**	**Day 36 (CFU/ml)**
35% Apple, 4g/l Lactic acid	<1	<1	<1	3
35% Apple, 7g/l Lactic acid	<1	<1	<1	18
35% Apple, 10g/l Lactic acid	<1	5	<1	<1
60% Apple, 4g/l Lactic acid	<1	<1	<1	<1
60% Apple, 7g/l Lactic acid Nr 1	<1	1	<1	<1
60% Apple, 7g/l Lactic acid Nr 2	<1	<1	<1	<1
60% Apple, 10g/l Lactic acid	<1	1	<1	<1
100% Apple, 4g/l Lactic acid	<1	>100	3	<1
100% Apple, 7g/l Lactic acid	<1	4	4	<1
100% Apple, 10g/l Lactic acid	<1	6	<1	<1

4.3.6 Metabolite Analysis

During the storage period of 36 days samples of every drink were put in the freezer to be stored for metabolite analyzes to be performed externally at Eurofins during day 0, 17 and 36. After obtaining some of the other results in the experiment it was decided that the drinks with the most promising results were the ones to be sent for analysis. The drinks including 10g/l lactic acid were thereby excluded. The drinks were analyzed with respect to amount acetic acid, malic acid and lactic acid. The amount of acetic acid in all drinks, independently of the storage time, was measured to <0.5g/100g. The obtained result regarding the amount of lactic acid and malic acid can be seen in figure 14, 15 and 16 below.

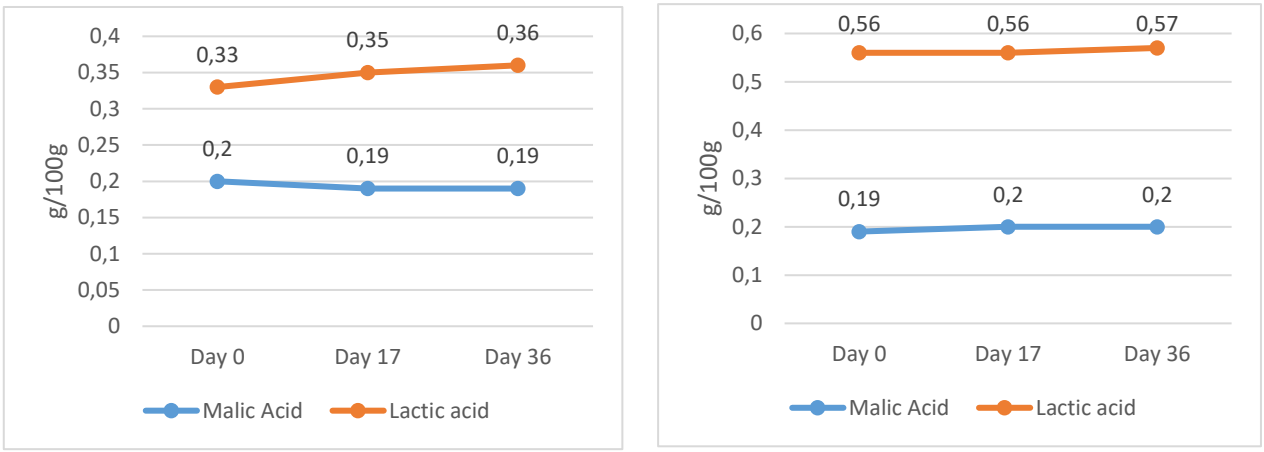


Figure 14. To the left: The measurements of malic acid and lactic acid in the drink with 35% apple juice and 4g/l lactic acid. To the right: The measurement of malic acid and lactic acid in the drink with 35% apple juice and 7g/l lactic acid.

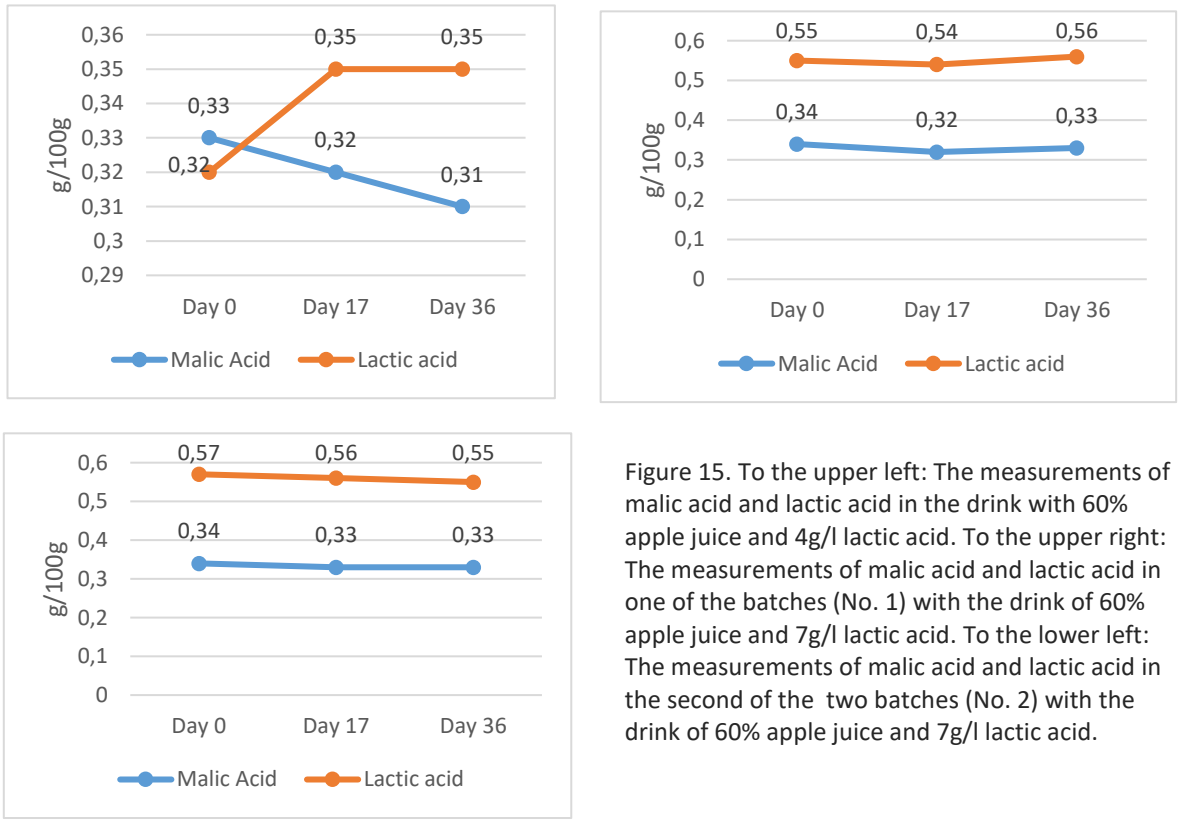
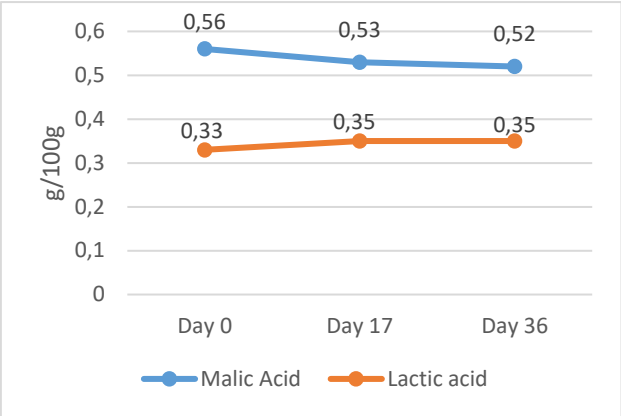


Figure 15. To the upper left: The measurements of malic acid and lactic acid in the drink with 60% apple juice and 4g/l lactic acid. To the upper right: The measurements of malic acid and lactic acid in one of the batches (No. 1) with the drink of 60% apple juice and 7g/l lactic acid. To the lower left: The measurements of malic acid and lactic acid in the second of the two batches (No. 2) with the drink of 60% apple juice and 7g/l lactic acid.



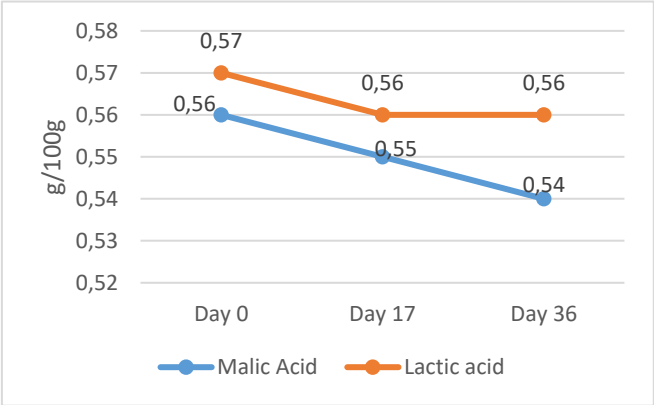


Figure 16. To the left: The measurements of lactic acid and malic acid in the drink with 100% apple juice and 4g/l lactic acid. To the right: The measurement of lactic acid and malic acid in the drink with 100% apple juice and 7g/l lactic acid.

5.0 Discussion

This study has provided several different interesting findings. The results showed that the amount of added lactic acid to the ProViva apple drinks do affect the metabolism of *L. plantarum* 299v. This was demonstrated in different ways, both due to the avoidance of an overproduction of carbon dioxide and also from an inhibited growth of *L. plantarum*.

During the performed screening experiments the maximum amount of added concentration of lactic acid could be determined for the experiment. In screening experiment 1 it was seen that a lactic acid concentration of 16g/l lead to a total loss of *L. plantarum* 299v after storage at 25°C for six days in a 50% apple juice drink. In the patent by Francois et al. (2010) it is recommended to add a concentration of 2.2g/l to 20g/l dietary protonated weak mono-acid to achieve an inhibition effect of the metabolism of organic acids. However, it is also recommended to increase the pH to approximately 3.4-4.0 by adding a basic compound. The increase in pH leads to a decrease of the amount of protonated lactic acid and thereby a decrease in possible inhibition effect. The result obtained in pre-trail 1 may be explained by the fact that a lactic acid concentration of 16g/l in combination with the low pH of the drink, 2.78, resulted in a protonated lactic acid concentration of 14.78 which might create a too harsh environment for the bacteria to live in and creates too much damaging effect on the bacteria. These obtained results promoted the decision that the used concentration of lactic acid in the experiment should be below 16g/l.

In screening experiment 2 a sensory analysis was performed which further lead to limitations regarding the acceptable concentration of lactic acid used in the apple drinks. It was during the analysis decided that a concentration of 10g/l was the maximum concentration to be evaluated in the experiment since concentrations above this lead to a too distinguished sourness of the drinks. It was also determined that a basic compound should not be used to increase the pH of the drink. The intrinsic buffering capacity of the drinks was high enough and also since the addition of a basic compound promotes a risk to have an impact of the flavor. It was also seen that the sourness still was overwhelming in the drinks with a lactic acid concentration above 10g/l even though a basic compound had been added.

It is believe that the result found in screening experiment 2, regarding the decision that no basic compound should be added, were a beneficial result for the product popularity. It is in society today commonly not appreciated when food contain E-numbers and therefore the addition of E-numbers to the ProViva apple drink might have created a discussion and also promoted a non-satisfactory response from the consumers.

The screening experiments were performed to be able to determine some of the limits for the different parameters for the experiments, including the maximal concentration of added lactic acid. However, the minimal concentration of lactic acid, 4g/l, was chosen since it was believed from experiments and knowledges achieved earlier at ProViva, and also based on literature, that this concentration should be the minimum needed to promote a distinctive inhibitory effect. Still the hypothesis of the project was that this concentration would be too low and that malolactic fermentation of *L. plantarum* would still occur with an overproduction of carbon dioxide. The results found in the study were therefore a bit surprising. The concentration 4g/l lactic acid was the only lactic acid concentration used in the experiment that actually did not kill off the bacteria.

A lower concentration than 4g/l would therefore be interesting to evaluate, since lactic acid is very intense in taste and the lowest concentration possible included in the future ProViva apple drink.

The concentrations of both 7g/l and 10g/l were found to be too high concentrations to use in the drinks because these concentration lead to a decrease of *L. plantarum*. It was found that during storage day 36 an increase of amount *L. plantarum* could be noticed for two of the drinks, 100% apple 4g/l lactic acid and 60% apple 4g/l lactic acid, while for the rest of the drinks *L. plantarum* continued to decrease. The drinks with a lactic acid concentration of 10g/l only had a *L. plantarum* amount of 100CFU/ml. At such high lactic acid concentration it could be seen that the drinks were independent of the apple juice concentration, the lactic acid still suppressed the growth in all drinks to around 100 CFU/ml. In the drinks with 7g/l lactic acid it could be noticed that the amount of apple juice affected the results. The drink with 35% apple juice had the least amount of *L. plantarum*, $3.0x10^4$CFU/ml, and the two drinks with 60% the second least, $7.0x10^4$ and $8.1x10^4$, and finally the drink with 100% apple juice the most, $3.8x10^5$CFU/ml. However, these drinks are unacceptable as ProViva products since the ProViva drinks should contain around $5x10^7$CFU/ml and are rejected at $1x10^7$CFU/ml.

When it comes to the three drinks with 4g/l added lactic acid it was also seen that the percentage off apple juice affected the results. The drink with 35% apple juice had the lowest amount of *L. plantarum*, $1.27x10^6$ CFU/ml, the drink with 60% apple juice was in between with $1.37x10^7$ CFU/ml and the drink with 100% apple juice had the highest amount of *L. plantarum*, $2.73x10^8$. Probably these results indicated that the nutrients found in apple juice are more available for the bacteria in drinks with higher apple juice percentage and thereby the bacteria have a bigger possibility to survive. However, in the drinks with 10g/l lactic acid the damaging effect are overwhelming and the amount of apple juice is not able to compensate for this effect.

In the same time as an increase in growth could be seen in the two drinks: 100% apple juice 4g/l lactic acid and 60% apple juice 4g/l lactic acid, from storage day 17 to storage day 36, the carbon dioxide production appeared unchanged. It was in the measurement of carbon dioxide found that the two drinks with the highest amount of *L. plantarum* had the highest production of carbon dioxide. Though, from the result obtained day 17 and 36 the gas production did not change that much in the same time as the amount of bacteria increased a lot. This was an unexpected result since a rapid increase of *L. plantarum* was believed to be connected to a rapid increase in gas formation due to malolactic fermentation. The malolactic fermentation was believed to be the main metabolism used by the bacterium since the pH in all ProViva apple drinks was low. The highest pH observed in the 100% apple 4g/l lactic acid drink was the initial pH of 3.27. According to Filannino et al. (2014) it is stated that *Lactobacillus* switch from its carbohydrate metabolism to a malic acid metabolism when located in an unfavorable environment. An unfavorable environment is for example a too high concentration of carbohydrates which promote an inefficient metabolism. Mozzi, Raya and Vignolo (2015) also refer to this kind of metabolism switch when the *Lactobacillu*s is in an environment with low pH. A switch to malolactic fermentation in low pH should leads to an increased production of carbon dioxide in the drinks. This due to the fact that for every lactic acid that is created, as a result of the malolactic fermentation, a carbon dioxide is produced as well (Filaninno et al. 2014).

However, since it also was found in this study that the lactic acid concentration in these two drinks remained constant from day 17 to day 36, it might suggest that the bacterium used an altered metabolism instead of the malolactic fermentation, in with the lactic acid concentration was expected to increase. An altered metabolism may have used the carbohydrates present in the drinks as the main substrate to form alternative end products besides than lactic acid. Though, at the same time it could be seen that the malic acid concentration continued to decrease in this two drinks, a decrease in malic acid is believed to be strictly related to the formation of lactic acid, and the absence of an increase of lactic acid concentration seemed therefore difficult to understand and explain.

In this experiment the malolactic fermentation was believed to be inhibited due to the lack of carbon dioxide production compared to the drink produced in screening experiment 1. The carbon dioxide production was found to be the highest in the drinks with the least amount of lactic acid, 4g/l, but still this production was less than in the drink in the screening experiment which consisted of 50% (w/w) apple juice.

When studying the results from the pH measurements of the drinks it could be seen that from the measurements performed day 0 to measurements performed day 9 there was an increase in pH for all drinks. This might be due to that an equilibrium was reached in the drinks after some time and that the pH obtained in the measurements at day 0 were yet not stabilized. Results from measurement performed later during storage showed that the pH started to decrease slightly in all drinks between day 9 and day 27. This post-acidification is a result due to the metabolic activity of the bacteria, in which substrates, such as carbohydrates or malic acid are fermented and form carboxylic acids. The pH decrease of the different drinks was in an interval of 0.06 till 0.09 pH units. Between day 27 and 36 the pH in some drinks increased, but in most of the drinks the pH remained unchanged and only in the drink with 100% apple juice 4g/l lactic acid the pH continued to decrease. The final post acidifications found in this study, between day 9 and 36, were thereby in the interval of 0.07 (35% apple 4g/l lactic acid, 60% apple 4g/l lactic acid, 60% apple 7g/l No. 1 and No. 2, 100% apple 7g/l lactic acid and 100% apple 10g/l lactic acid) to 0.08 (35% apple 7g/l lactic acid, 35% apple 10g/l lactic acid, 60% apple 10g/l lactic acid and 100% apple 4g/l lactic acid) pH units. This post acidification can be compared with post acidifications found in literature. As for example in a study performed by Donkor, Henriksson, Vasiljevic & Shah (2005) it was found that cold storage of yoghurt including probiotics for 28 days resulted in a post acidification of 0.153-0.230 pH units when the initial pH value were 4.45-4.60. In another study performed by Donkor, Henriksson, Singh, Vasiljevuc & Shah (2007) the post acidification of a probiotic yoghurt was found to be 0.1-0.34 pH units when having an initial value of 4.55 during cold storage for 28 days. In the same study it was also found that an increase in pH occurred in the yoghurt during the first 12 hour. The pH of the yoghurt increased from pH 4.50 to 4.55. When comparing the result found in this study with the result found in literature it can be stated that a post acidification of 0.07-0.08 pH unit in cold storage for 36 days seems to be a rather low acidification. This low post acidification may be explained by the fact that the initial pH was lower in the ProViva apple drinks compared to the yoghurt found in literature. It can also be stated that there was no significant difference in the post acidification between all different drinks in the study since all the drinks had a post acidification of between 0.07-0.08 pH units. The low post acidification found in the study may be

explained by the intracellular reduction of malic acid to lactic acid which occurs during the malolactic fermentation. The reduction of malic acid to lactic acid promotes an increase in pH.

In the patent written by Beverini et al. (2014) the term stable probiotics is stated to be probiotics that lack activity when stored in 10°C for 30 days which results in three properties of the drinks:

1) The drinks should have the same taste and flavor during the whole storage time. With other words, no off-flavors should be noticeable.
2) The post acidification of the drinks should be <0.5 pH units.
3) After packaging of the product no gas production should be able to be detected.

When comparing the results found in the study with the criteria for being stable probiotics it could be stated that the probiotics in the drinks are stable probiotics. Stable probiotics is wanted in different applications since it ensure the product quality. The drinks in the study had a post acidification below 0.5 pH units, the carbon dioxide production was not noticeably high in any of the drinks, and overall no flavor alternations could be detected during the whole storage time. However, these three properties do not mention anything about the level of *L. plantarum* in the drinks, which is essential for providing good probiotic qualifications. In the study it was found that only the two drinks; 100% apple 4g/l lactic acid and 60% apple 4g/l lactic acid had acceptable amounts of *L. plantarum*.

The content of malic acid, lactic acid and acetic acid were determined for all drinks with a lactic acid concentration of 4g/l and 7g/l. The results showed a pattern for a decrease of malic acid in the drinks and an increase of lactic acid in the drinks. However, this pattern was only significant in the drinks with 4g/l lactic acid. In the drinks with 7g/l the lactic acid concentration also decreased and in one drink, 35% apple and 4g/l lactic acid, the malic acid concentration increased. The acetic acid level was shown to be <0.5g/100g for all drinks that were analyzed during the whole storage time. The detection limit for the method used might be too low to notice changes or there were no changes in the drinks. The result obtained from the sensorial analysis also showed that there was no clear change in the organoleptic properties of the drinks during the storage time.

In order to eliminate differences between batches the drink with 60% apple juice 7g/l lactic acid were produced in duplicate. It was found that the results were similar regarding the formation of carbon dioxide, amount of *L. plantarum* and pH changes. However, minor differences could be found in the measurement of organic acids and in the sensorial test panel analysis. Regarding the measurements of organic acids the differences are too small to be significant. The difference observed in the sensorial analysis however was a bit confusing. The changes seen between the two batches may be important to consider in future analyzes.

All different ProViva drinks included in the product catalog today contains sugar or sweeteners. However, in this study performed neither sugar nor sweeteners were added to the drinks. The sugar and sweeteners are usually added to the ProViva drinks to enhance the flavor of the drinks, though, in society today all negative consequences connected to a high consumption of high sugar is constantly highlighted and thereby a ProViva drink without sugars or sweeteners would be preferable. The results obtained from the study showed that the drink with 100% apple juice and 4g/l lactic acid does have the best possibilities in succeeding as a ProViva apple drink when

combining all different aspects together. I think that a production of this drink would not only be an advantage due to the fact that it would be an apple drink. It would also promote advantages due to being the first and only ProViva drink with 100% fruit juice and also for being a drink without sugar or sweeteners.

6.0 Conclusion

In this study the malate metabolism of *L. plantarum* 299v was inhibited by the introduction of lactic acid in ProViva apple drinks. Nine different ProViva apple drinks were produced. The drinks differed with respect of two parameters. First of all the concentration of added lactic acid to the drinks were altered. The used lactic acid concentrations were 4g/l, 7g/l or 10g/l. Secondly the percentage (w/w) of apple juice used were either 35%, 60% or 100%. In the study it was shown that the addition of lactic acid both affected the carbon dioxide production and also the survival of *L. plantarum* 299v. Further this study found that:

- A lactic acid concentration of 10g/l resulted in ~100 CFU/ml *L. plantarum* after storage for 36 days. The concentration of 10g/l was found to be a too high concentration for the bacterium to survive within. At this concentration it was also found that the bacterium was independent of the percentage of apple juice in the drink, the survival rate of the bacterium was still the same.

- At a lactic acid concentration of 7g/l or 4g/l *L. plantarum* was dependent on the percentage of apple juice in the drinks. The drinks with higher percentage of juice had a higher survival rate of the bacterium.

- A lactic acid concentration of 4 g/l was the only concentration where the amount of *L. plantarum* (CFU/ml) increased during storage of 36 days. The drink with 100% apple juice and 4g/l lactic acid and the drink with 60% apple juice and 4g/l lactic acid were the only drinks that had amounts of *L. plantarum* to be considered acceptable as a ProViva drink after storage of 36 days.

- The pH of all drinks decreased in the same ratio (0.07-0.08 pH units) during the storage time. Thereby no significant difference could be seen between the differently composed drinks.

- In the drinks with the lowest amount of lactic acid, 4g/l, the carbon dioxide production was the highest indicating that malate metabolism was not inhibited to the same extent as the in the drinks with 7g/l and 10g/l lactic acid. Out of the three different drinks with 4g/l lactic acid the drink with the most percentage of apple juice (100 wt. %) had the highest carbon dioxide production. However, still the production of carbon dioxide was found to be low enough to not have any impact on the organoleptic properties and the packaging of the drinks.

- No significant impact of the amount of lactic acid on the shelf life could be found. The presence of yeast and mold seemed to occur randomly in the drinks.

- No off-flavors off the drinks could be noticed during the sensorial analysis. The amount of acetic acid in the drinks was below 0.5g/100g during the storage period.

- No clear differences could be noticed between the two batches including 60% apple juice and 7g/l lactic acid. The carbon dioxide production, pH changes and amount of *L. plantarum* were similar in the two batches. However, in the sensorial analysis batch No. 2 had a slightly higher grade than No 1.

- The lactic acid concentration showed a tendency to increase in the drinks during storage while the malic acid concentration tended to decrease. However, this pattern was only significant in the drinks with the lowest amount of added lactic acid, 4g/l.

- When combining all obtained results the drink with 100% (w/w) apple juice and 4g/l lactic acid was the most promising drink for production of a ProViva apple drink.

7.0 Future Outlook

In the study it was found that the apple drink with the composition of 100% apple juice and 4g/l lactic acid gave the most promising results. The results implied from all different points of views that this kind of ProViva apple drink would be possible to produce. The next step in the innovation process is therefore to perform an industrial trial with the drink. In this way it would be possible to see if a large scale production of the drink would give the same result as seen in this small scale study.

There would be some different suggestions for improvement for a recipe of the drink. As for example the sourness of the drink might be able to be decreased with the addition of aroma add backs. If the drink, after addition of add backs, still would be considered slightly sour it could be an alternative to add sweeteners or sugar. The recipe of the apple drink today gives a drink with a watery consistence. If a thicker drink is desired it could be interesting to add some kind of thickening agent to see how this would affect the drink.

However, it would also be interesting to evaluate what the lowest concentration of lactic acid would be before the carbon dioxide formation gets out of control. It would be valuable to produce for example a drink with 100% apple juice and 2g/l lactic acid, and also an 80% apple juice and 2g/l lactic acid. This to find the most optimal combination of lactic acid concentration and apple juice percentage for the avoidance of both carbon dioxide and a sour taste of the drink.

8.0 References

Beverini, M., Lacorre, M., Francois, A. & Labbe, M. (2014). Probiotic enriched and low organic acid food products. *United States Patent Application Publication, Pub. No: US 2014/0087027 A1*

Boyer, J. & Liu, R. H. (2004). Apple phytochemicals and their health benefits. *Nutritional journal,* 3:5

Brody, A. L. & Lord, J. B. (2007). *Developing New Food Products for a Changing Marketplace.* Boca Raton: CRC Press, 2. ed. p. 48-50

Cabrita, M. J., Palma, V., Raquel, P. & Freitas, A. M. C. (2010). Conversion of hydroxycinnamic acids into volatile phenols in a synthetic medium in red wine by *Dekkera bruxellensis, Ciência e Tecnologia de Alimentos*, 32, 106-112

Cabrita, M. J., Torres, M., Palma, V., Alves, E., Patão, R. & Costa Freitas, A. M. (2007). Impact of malolactic fermentation on low molecular weight phenolic compounds. *Talanta,* 74, 1281-1286

Charalampopoulos, D. & Rastall, R.A. (2009). *Prebiotics and Probiotics Science and Technology.* New York: Springer Science& Business Media. p. 1-2, 12, 14

Costa, A. I. A. & Jongen, W. M. F. (2006). New insights into consumer-led food product development. *Trends in Food Science and Technology,* 17, 457-465

Danone Sverige. (2016). *Danone i korthet.* [Online]. Available: http://danone.se/om/ [Accessed: 2016-08-13]

de Vries, M. C. (2006). *Analyzing global gene expression of Lactobacillus plantarum in the human gastrointestinal tract*. Thesis Wageningen University. p. 10-16

den Besten, G., Van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J. & Bakker, B.M. (2013). The role of the short chain fatty acids in the interplay with diet, gut, microbiota, and host energy metabolism. *Journal of lipid research,* 54, 2325-2340

Donkor, O. N., Henriksson, A., Singh, T., Vasiljevic, T., Shah, N.P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal,* 17, 1321-1331

Donkor, O. N., Henriksson, A., Vasiljevic, T., Shah, N. P. (2005). Effect of acidification on the activity of probiotics in yoghurt during cold storage. *International Dairy Journal,* 16, 1181-1189

Featherstone, J. D. B. & Rodgers, B. E. (1981). Effect of Acetic, Lactic and other Organic Acids on the Formation of Artificial Carious Lesions. *Caries Research,* 15, 377-385

Filannino, P., Cardinali, G., Rizzello, C. G., Buchin, S., De Angelis, M., Gobbetti, M. & Di Cagno, R. (2014). Metabolic Responses of Lactobacillus plantarum Strains during Fermentation and Storage of Vegetables and Fruit Juices. *Applied and Environmental Microbiology,* 80, 2206-2215

Francois, A., Portier, R., Crepel, P. & Faurie, J-M. (2010). Probiotic-containing food product and a protonated weak monoacid. *United States Patent Application Publication, Pub. No: US 2010/0086646 A1*

Gerhauser, C. (2008). Cancer chemopreventive potential of apples, apple juice, and apple components. *Planta medica,* 74, 1608-1624

Goktepe, I., Juneja, V. K. & Ahmedna, M. (2006). *Probiotics in Food Safety and Human Health.* New York: CRC Press, Taylor and Francis Group. p. 20-21

Hirshfield, I. N., Terzulli, S. & O´Byrne, C. (2003). Weak organic acids: a panoply of effects on bacteria. *Science Progress,* 86, 245

Hyson, D. A. (2011). A Comprehensive Review of Apples and Apple Components and Their Relationship to Human Health. *Advances in Nutrition*, 2, 408-420

Johansson M. L., Molin, G., Jeppsson, B., Noneak, S., Ahrné, S. & Bengmark, S. (1993). Administration of different Lactobacillus strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora. *Applied and Environmental Microbiology,* 59, 15-20

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., Tarchini, R., Peters, S. A., Sandbrink, H. M., Fiers, M. W., Stiekema, W., Lankhorst, R. M., Bron, P. A., Hoffer, S. M., Groot, M. N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W. M. & Siezen, R. J. (2003). Complete genome sequence of Lactobacillus plantatum WCFS1. *Proceedings of the National Academy of Science of the United States of America,* 100, 1990-1995

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

Liu, S. Q. (2003). Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *International Journal of Food Microbiology,* 83, 115-131

Livsmedelsverket. (2015). *Frukt, grönt och baljväxter.* [Online]. Available: http://www.livsmedelsverket.se/livsmedel-och-innehall/mat-och-dryck/frukt-gront-och-baljvaxter/ [Accessed: 2016-08-13]

McMurrough, I., Madigan, D., Donnelly, D., Hurley, J., Doyle, A-M., Hennigan, G. & McNulty, N. (1996). Control of ferulic acid and 4-vinyl guaiacol in brewing. *Journal Institution of Brewing,* 102, 327-332

Molin, G. (2013). Lectures in Probiotics-*Physiologic and physiopathologic effects of the human microbiota.* Lund University, p. 16, 49, 68, 76, 85-86, 95, 119

Molin, G. (2015). *Lactobacillus plantarum* 299v. Lund University. p. 5-7

Mozzi, F., Raya, R. R. & Vignolo, G. M. (2015). *Biotechnology of Lactic Acid Bacteria: Novel Applications*. 2nd Edition., USA: Wiley-Blackwill. p. 224

Musso, G., Gambino, R. & Cassader, M. (2010). Obesity, Diabetes, and Gut Microbiota. *Diabetes Care,* 33, 2277-2284

Nour, V., Trandafir, I. & Ionica, M. E. (2010). Compositional Characteristics of Fruits of several Apple (Malus domestica Borkh.) Cultivars. *Notulae Botanicae Horti Agrobotanici Chij-Napoca,* 38, 228-233

Produce For Better Health Foundation. (2015). *2015 Study on America's Consumption of Fruit and Vegetables.* State of the Plate

ProViva. (2016). *Om ProViva. Magens lilla vän. Så kallas ProViva.* [Online]. Available: https://proviva.se/om-proviva/ [Accessed 2016-08-13]

ProViva AB. (2016). *Havre- ProViva innehåller havre.* [Online]. Available: https://proviva.se/fragor-svar/havre/ [Accessed 2016-10-03]

Ricke, S. C. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry science,* 82, 632-639

Rodriquez, H., Curiel, J. A., Landete, J. M., de las Rivas, B., López de Felipe, F-. Gómez-Cordovés, C., Mancheño, J. M. & Munoz, R. (2009). Food Phenolics and lactic acid bacteria. *International Journal of Food Microbiology,* 132, 79-90

Rodriquez, H., Landete, J. M., de las Rivas, B. & Munoz, R. (2008). Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}. *Food Chemistry,* 107, 1393-1398

Russell, J. B. & Diez-Gonzalez, F. (1997). The Effects of Fermentation Acids on Bacterial Growth. *Advances in Microbial Physiology,* 39, 205-234

Sanders, M.E. (2008). Probiotics: Definition, Sources, Selection, and Uses. *Clinical Infectious Diseases,* 46, 58-61

SigmaAldrich. (2016). *L-(-)-Malic acid.* [Online]. Available: http://www.sigmaaldrich.com/catalog/product/sigma/m1000?lang=en&region=SE [Accessed 2016-11-15]

Slavin, J. (2013). Fiber and Prebiotics: Mechanism and Health Benefits. *Nutrients,* 5, 1417-1435

Stiles, M. E. & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology,* 36, 1-29

van Beek, S. & Priest, F. G. (2000). Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolation during malt whiskey fermentation. *Applied and environmental microbiology,* 66, 5322-5428

WHO. (2002). Guidelines for the Evaluation of Probiotics in Food. *Joint FAO/WHO*

Wilhelmsson, W. (2013). *Banan Populäraste Frukten i Sverige.* [Online]. Available: http://tjock.se/strupen/art/276322/banan_populraste_frukten_i_sve/ [Accessed 2016-10-21]

Winger, R. & Wall, G. (2006). Food Product innovation- a background paper. *Food and agriculture organization of the United Nations*

World Market and Trade. (2008). 2007/08 Global Concentrated Apple Juice. *United State Department of Agriculture*

Zink, D. L. (1997). The Impact of Consumer Demands and Trends on Food Processing. *Emerging of infectious Disease,* 3, 467-469

9.0 Appendix

Appendix 1. Calculation of a 100% apple ProViva with 4g/kg lactic acid

Apple juice concentrate: 68.5 BRIX
BRIX for being an apple juice: 11.2 Brix

Dilution factor=68.5 BRIX / 11.2 BRIX = 6.116.
Which gives: 1 part apple concentrate + 5.116 parts water = 100% apple juice.
In weight %: 16.35 weight % apple concentrate and 83.65 weight % water.

5 weight % of the drink should be oat swell, and this should be a part of the dilution → 83.65weight % water – 5 weight % = 78.65 weight % water.

Lactic acid concentration is in this drink set to 4g/l (Assuming the density in the drink to be 1kg/l → (4g/l) / (1kg/l) = 4g/kg

If having one kg in total this leads to (4g/1000g) = 0.4 weight% lactic acid.

However, the lactic acid used is 80%, so to obtain equally 100% the weight % added needs to be: 0.4/0.8= 0.5 weight %.

The lactic acid is also a part of the dilution → 78.65 weight% water – 0.5 weight % lactic acid = 78.15 weight % water.

In total:

16.35 % apple concentrate + 0.5 % lactic acid + 78.15 % water + 5% oat swell = 100% ProViva apple drink

Appendix 2. Consumer test panel survey

Datum: 2016-09-29
Bedömare: 1 Namn:_____

Markera på skalan med ett X.
Vad tycker du om drycken?
1= Tycker extremt illa om
9= Tycker extremt bra om

1 2 3 4 5 6 7 8 9

708
Tycker extremt illa om Tycker extremt bra om

417
Tycker extremt illa om Tycker extremt bra om

612
Tycker extremt illa om Tycker extremt bra om

409
Tycker extremt illa om Tycker extremt bra om

501
Tycker extremt illa om Tycker extremt bra om

395
Tycker extremt illa om Tycker extremt bra om

254
Tycker extremt illa om Tycker extremt bra om

986
Tycker extremt illa om Tycker extremt bra om

565
Tycker extremt illa om Tycker extremt bra om

211
Tycker extremt illa om Tycker extremt bra om

Appendix 3. Calculation of protonated/deprotonated lactic acid

Uträkningar på protonerad mjölksyra i dryck 4 i pre-trail 1.

pH=pKa+log[A-]/[HA]

uppmätt pH=2,78

pKa=3,86 för mjölksyra

Tillsätt mjölksyra totalt: [HA] + [A-] = 16g/l -> [A-] = 16 – [HA]

2,78 = 3,86 * log (16-[HA]) / [HA]

→ [HA] = 14,78 Vid tillsatts av 16 g/l mjölksyra vid ett pH på 2,78