Optimization of ingredients for *Lactobacillus* fermented beverages

Impact of different fermentation parameters on the final product's sensory and shelf life

Department of Food Technology
Lund University
Rebecka Olsson. Master Thesis 2017
Supervisors: Anna Andrys & Elisabeth Uhlig
Examiner: Åsa Håkansson

Abstract

The ingredients generally used in the fermentation of probiotics tend to give an off-flavour to the fermentation product which is not ideal when producing e.g. fruit drinks. The aim of this master thesis was to evaluate if the concentration of such ingredients could be lowered and also to investigate whether there are other ingredients that could act as substitutes to those ingredients giving off-flavours. The growth, stability and taste of the fermentation products containing the ingredients and probiotics, as well as the fermentation product put in a fruit model system, was evaluated. The experimental work included changing the concentration of the ingredient giving the off-flavour and exchanging it with substituting ingredients during the fermentation of three different probiotic bacteria. The bacterial content was measured using a plate count method and the stability of the fermentation product was evaluated after one week of storage in 4°C. After putting the fermentation product in a fruit juice, the stability was measured during four weeks of storage and a simplified sensory evaluation was done.

It was found that the ingredients that gave the significantly highest growth was generally the ingredient normally used during the fermentation and none of the substituting ingredients were as successful in supporting growth. It was also concluded that the type of bacteria will have an effect on the final bacterial content in the fermentation product and the sensory properties where some strains had a better taste profile than others.

Preface and Acknowledgement

The idea for this master thesis originated at Probi AB and all experimental work was performed there. The work was done during spring 2017 and is the final submission of my master's degree in Biotechnology with Food Technology specialization at LTH.

This thesis had not been possible without the help of the following people.

Anna Andrys, my supervisor at Probi AB, who granted me the opportunity to do my thesis at Probi AB. I would also like to thank her for all her valuable inputs and help with the experimental work, I have learned so much. I would also like to thank Jenny Rudolfsson who acted as a stand in for when Anna was not available, I apologize to you both for making you taste the sometimes-bad tasting drinks. Doing my thesis at Probi AB have provided me with much appreciated experience of how it works when developing new food products and I have enjoyed every minute of it.

I would also like to thank my supervisor at LTH, Elisabeth Uhlig, who always had good advice in store. Thank you for your help with the statistics, for your dedication and for all your valuable comments.

Finally, I would like to thank my friends and family who have stood by me during this entire project, I could not have done this without you.

Table of content

Abstract 2

Preface and Acknowledgement 3

1 Introduction 6

2 The *Lactobacillus* genus 7
2.1 Carbohydrate metabolism 7
2.1.1 Glucose 7
2.1.2 Maltose 8
2.1.3 Fructose 8
2.1.4 Pentose 8
2.1.5 Citrate 9
2.1.6 Pyruvate and end products 9
2.2 Malolactic fermentation (MLF) 9
2.3 Proteolysis 9
2.4 Lactobacillus rhamnosus 9
2.5 Lactobacillus paracasei 9
2.6 Lactobacillus plantarum 10

3 Substituting ingredients and fermentation conditions 12
3.1 Plant seed powder 12
3.2 Pea Protein 12
3.3 Rice Protein 12
3.4 Increasing the temperature for L. rhamnosus 12

4 Material and method 13
4.1 Organisms and inoculum 13
4.2 Cultivation conditions and ingredients 13
4.2.1 Varying ingredient 2 and 3 14
4.2.2 Ingredient 4 14
4.2.3 Ingredient 5 14
4.2.4 Combining ingredient 3 and 5 14
4.2.5 Ingredient 6 and 7 14
4.2.6 Ingredient 8 14
4.2.7 Ingredient 9 14
4.2.8 Increasing the fermentation temperature for *L. rhamnosus* 14
4.2.9 Increasing the fermentation time for *L. paracasei* 14
4.3 Plate count analysis 15
4.4 Storage stability 15
4.5 Sensory evaluation 15
4.6 Statistics 15

5 Result and discussion 16
5.1 pH 16
5.2 Growth and stability 19
5.3 Stability in juice 24
5.3.1 Varying the concentration of ingredient 2 and 3 24
5.4 Sensory evaluation 26
5.4.1 Varying the concentration of ingredient 2 and 3 26
5.4.2 Ingredient 4 26
5.4.3 Ingredient 5 26
5.4.4 Combining ingredient 3 with ingredient 5 27

5.4.5 Ingredient 6 and 7 27
5.4.6 Ingredient 8 27
5.4.7 Ingredient 9 27
5.4.8 Increasing the fermentation temperature for *L. rhamnosus* 28

6 Conclusion 29

7 Future suggestions 30

8 References 31

9 Appendix 32

9.1 Plate count analysis 32
9.2 Refrigerator temperature 32
9.3 Varying the concentration of ingredient 2 and 3 33
9.4 Ingredient 4 34
9.5 Ingredient 5 35
9.6 Ingredient 6 and 7 35
9.7 Ingredient 8 36

1 Introduction

The field of probiotics is growing constantly and the rise of consumer awareness for healthy and tasty products are increasing the demands on probiotic products. For the probiotic bacteria to grow and survive in the harsh environment of e.g. a fruit drink, additional ingredients must be added that supports growth. Those ingredients are components which might provide off-flavours.

The aim of this thesis is to investigate whether it is possible to lower the concentration of the ingredients that are creating off-flavours or exchange them for something that has a better taste profile. The taste, growth and stability of the fermentation product containing different *Lactobacillus* species will be evaluated as well as putting the fermentation product in a fruit model system.

Lactic acid bacteria (LAB) is defined as a functional group of lactic acid producing and harmless bacteria, both to human health and to the quality of food, that are spontaneously present in lactic acid fermented food. The consumption of lactic acid bacteria might have been invented 1.5 million years ago, as suggested by archaeologists. (Molin, 2013) The preservative function of LAB works when the food product containing carbohydrates are stored in an environment without oxygen. The bacteria existing on the food product will start to multiply resulting in an increased concentration of carbon dioxide, a decrease in oxygen tension and a decrease in pH. At the end of the lactic acid fermentation, the food product will be dominated by lactic acid bacteria and have a final pH of around 3.5-4.0. The low pH will inhibit the growth of most bacteria. In addition, LAB can produce inhibitory factors such as carboxylic acids, nitrogen oxide, hydrogen peroxide and bacteriocins. (Molin, 2013)

Probiotics are defined by a working group from FAO and WHO as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host". (FAO/WHO, 2002)
For the probiotics to have an effect, the daily dose should be at least 10^9 CFU but 10^{10} is preferred. No upper limit of the probiotic dose exists. (Molin, 2013)

Probiotics can often be found in different kinds of fermented milk, such as yoghurt. The possibility of using fruit juices are currently being studied. Pasteurized fruit juices are beneficial for the bacteria since it contains various nutrients and no other bacteria that may compete with the supplemented probiotics. Another benefit is that fruit juices often contains oxygen scavenging ingredients, e.g. ascorbic acid, that will promote an anaerobic environment. Fruit juices are also rich in sugar, which promotes the growth of the probiotics. (Garcia Maia Costa, et al., 2013)

How well bacteria adapt to fruit and vegetable systems will vary according to the strain and species of lactobacilli. The reason for this is that the natural environment in which they grow differs greatly and will therefore affect the ability to distribute the energy used for metabolism between maintenance and biosynthesis, i.e. between for example responses to stress and the utilization of alternative substrates. (Filannino, et al., 2014) Finding the balance between growth and survival is therefore of great importance.

2 The *Lactobacillus* genus

The bacteria in the genus *Lactobacillus* are non-motile, normally rod-shaped and do not form spores. The growth temperature is between 2 and 53°C where the optimal temperature is in the range of 30 to 40°C while the pH can range from 3 to 8. *Lactobacillus* species tolerates oxygen but will also grow anaerobically and the main product from the fermentation of sugar is lactic acid. (Holzapfel & Wood, 2014) Lactobacilli can be divided into three different functional groups; facultatively heterofermentative, obligately homofermentative and obligately heterofermentative, which will give different end products when fermenting hexoses:

- Obligately homofermentative: glucose → lactic acid
- Obligately heterofermentative: glucose → lactic acid, carbon dioxide, ethanol and/or acetic acid
- Facultative heterofermentative:
  - Glucose → lactic acid
  - Pentoses → lactic acid and acetic acid
  - Malic acid → lactic acid and carbon dioxide
  - Citrate → diacetyl, acetoin and carbon dioxide

Lactobacilli that are growing on glucose and are facultative heterofermentative will, in presence of oxygen, have lactic acid, diacetyl, acetic acid and acetoin as end products. (Molin, 2013)

2.1 Carbohydrate metabolism

2.1.1 Glucose

The metabolism of glucose is either done through the Embden-Meyerhof-Parnas (EMP) pathway, also called glycolysis, or the pentose phosphate pathway, also called the phosphogluconate pathway. The homofermentative lactobacilli uses the EMP pathway when lactic acid is produced from hexoses while the obligately heterofermentative bacteria uses the heterolactic phosphogluconate pathway. Facultatively heterofermentative LAB uses the EMP pathway. (Holzapfel & Wood, 2014) A simplification of the glucose fermentation can be seen in Figure 1 where the EMP pathway/glycolysis is located to the left and the pentose phosphate pathway to the right. All the bacteria used in the experiment are able to utilize Glucose as a carbon source. (Probi AB, 2017)

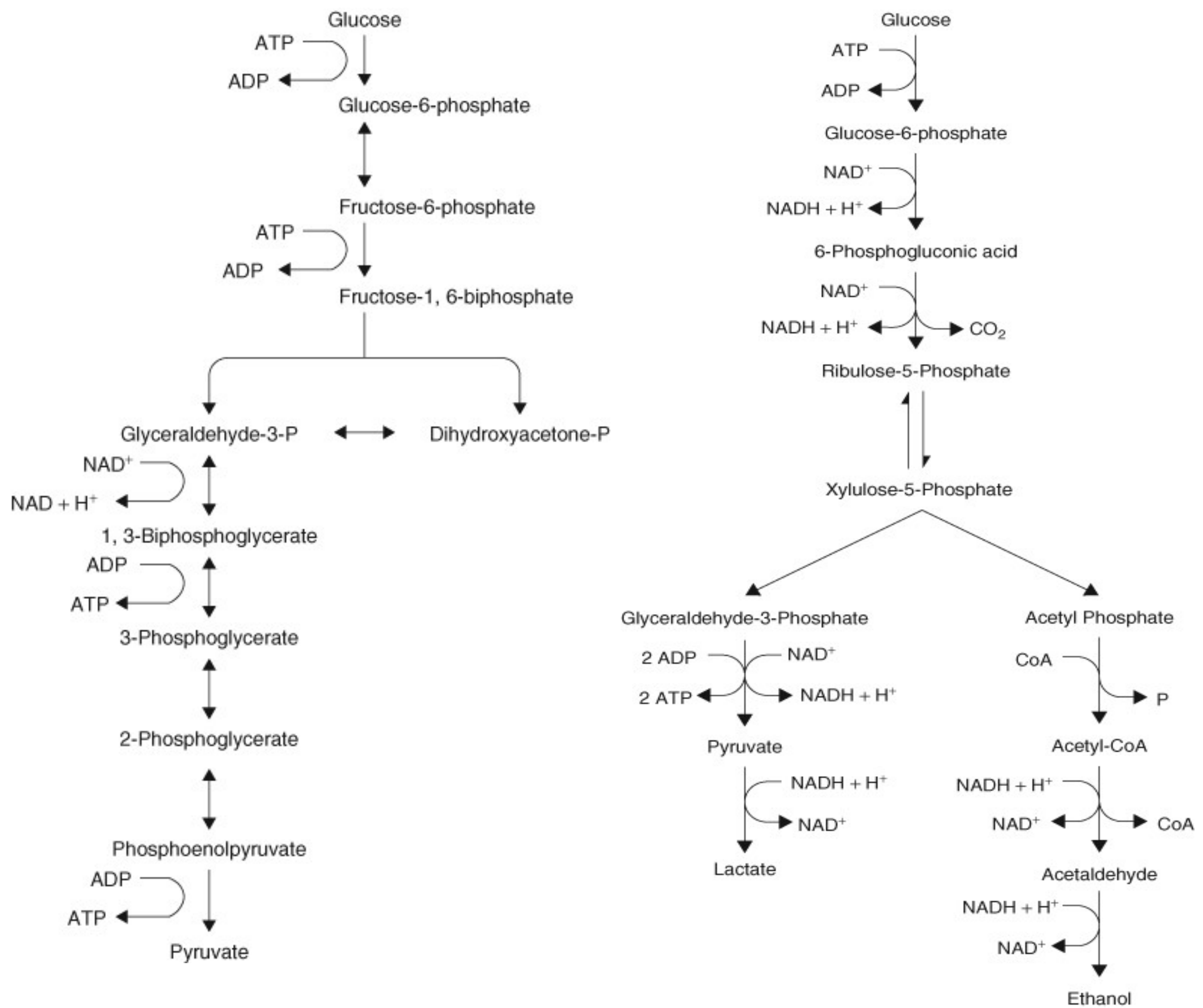

Figure 1. Glucose fermentation in lactic acid bacteria, the EMP pathway/glycolysis to the left and the pentose phosphate pathway to the right. (Butler, et al., 2010)

2.1.2 Maltose

Maltose is cleaved into glucose and β-glucose-1-phosphate which is converted to glucose-6-phosphate and can then enter the pentose phosphate pathway. However, the hexokinase responsible for this conversion is not present in exponentially growing cells in a media containing only maltose. This means that the glucose that is un-phosphorylated can not be utilized and is excreted into the medium. Although, the hexokinase activity is believed to be induced when fructose or glucose is in the medium. (Holzapfel & Wood, 2014) The three bacteria used in the experiments are all able to utilize Maltose as a carbon source. (Probi AB, 2017)

2.1.3 Fructose

L. sanfranciscensis and *L. pontis* are able to utilize fructose as a carbon source. However, when there is an oxygen depletion and available maltose, the fructose is mainly used as an electron acceptor and mannitol will be produced. Acetic acid is the main metabolic product when the molar ratio between fructose and maltose is 4:1. *L. sanfranciscensis* produce mannitol from fructose while *L. pontis* produces ethanol and lactic acid in small amounts. (Holzapfel & Wood, 2014) The bacteria used in the experiment are also able to utilize fructose as a carbon source. (Probi AB, 2017)

2.1.4 Pentose

The pentoses are phosphorylated to xylulose-5-phosphate or ribulose-5-phosphate which are then metabolized in the pentose phosphate pathway. (Holzapfel & Wood, 2014)

2.1.5 Citrate

The utilization of citrate depends on a transporter gene present in an endogenous plasmid. Citrate can be converted into oxaloacetate and acetate which are decarboxylated into pyruvate and it can also be transformed into lactate. Carbon dioxide is produced during the breakdown of citrate. (Holzapfel & Wood, 2014)

2.1.6 Pyruvate and end products

Since LAB can alter their metabolism depending on the environment in which they live, this will also result in different end products. Pyruvate is generally used for the reduction to lactic acid, however, pyruvate can be converted into many different end products, e.g. diacetyl, ethanol and acetate. Depending on the lactobacilli strain and the conditions in which it is under, different end products will be produced. (Axelsson, 2004)

2.2 Malolactic fermentation (MLF)

Many LAB can convert malate to lactate and carbon dioxide which is an important reaction when fermenting fruits and vegetables, e.g. in wine making, since they contain high concentrations of malate. LAB utilizing the MLF in combination with the fermentation of carbohydrates are generally showing e.g. higher growth rates. The reason for this is not entirely known but it has been suggested that the deacidification of the external environment in combination with providing small amounts of electron acceptors creates the energy benefits. (Axelsson, 2004)

2.3 Proteolysis

The capability of LAB to use inorganic nitrogen to synthesize amino acids is limited and it is therefore necessary to have pre-formed amino acids present as a nitrogen source in the medium used for growth. Which amino acids that needs to be supplemented differs among different species and strains. (Axelsson, 2004)

2.4 Lactobacillus rhamnosus

Lactobacillus rhamnosus belongs to the phylogenetic group casei. It is facultatively heterofermentative and grows at 15°C and 45°C. They are non-motile, rod shaped with a size of 0.8-1.0x2.0-4.0 μm with square ends and exist in chains or singly. They do not hydrolyse arginine and they are urease negative. (Holzapfel & Wood, 2014) *L. rhamnosus* can be found in the digestive tract of humans and will, as well as *L. paracasei*, grow spontaneously in milk products during lactic acid fermentation. (Molin, 2013)

A study on *L. rhamnosus* ATCC 10863 concluded that the amino acids, out of the 20 added, that had the highest utilization during growth was Cysteine, Serine, Asparagine and Glutamine. (Berry, et al., 1999) *L. rhamnosus* does not possess the enzyme to synthesise amino acids and vitamin B by itself which means that that must be added for sufficient growth. (Cui, et al., 2010)

For *L. rhamnosus* GG, grown in a water-based pudding containing rice flour, maize flour, fructose, water and NaCl and fermented for 12 hours at 37°C, the utilization and production of citric acid, acetion and ethanol was investigated after 21 days of storage at 4-6°C. The study showed a decrease in citric acid and an increase in acetoin and ethanol. The concentration of diacetyl during the fermentation was increased to around 7 mg/kg which is above the threshold of taste which is 0.03 mg/kg. Diacetyl will give a flavour of butter. Lactic acid was also produced while orotic acid was consumed. (Helland, et al., 2004)

2.5 Lactobacillus paracasei

Lactobacillus paracasei is facultatively heterofermentative and belongs to the phylogenetic group casei. It grows at 10°C and 40°C but some strains grow at 5°C and 45°C, is rod shaped (0.8-1.0x2.0-4.0 μm) with squared ends and exist in chains or singly. (Holzapfel & Wood, 2014) *L. paracasei* can be

found in the digestive tract of humans but it also occurs and grows spontaneously in the lactic acid fermentation of milk products, e.g. in cheese. (Molin, 2013)

L. paracasei strain 8700:2 can utilize and grow rapidly on long-chain inulin and oligofructose which are considered prebiotics. The metabolic end products during growth on these energy sources are mainly lactic acid but also acetic acid, ethanol and formic acid. (Makras, et al., 2005)

2.6 Lactobacillus plantarum

Lactobacillus plantarum belongs to the phylogenic group plantarum which is divided into six species and subspecies; *L. plantarum* subsp. *argentoratensis, L. plantarum* subsp. *plantarum, L. paraplantarum, L. xiangfangensis, L. fabifermentans,* and *L. pentosus.* They are facultatively heterofermentative, grow at 15°C but not at 45°C and has a G+C content within the group of 44 and 47 mol%. They are straight rods with a size of 0.9-1.2x3-8 μm with rounded ends, non-motile and exist singly or in pairs or chains. *L. plantarum* is recognised by its pseudocatalase activity, the reduction of nitrate and also its incapability to utilise α-methyl-D-mannoside. (Holzapfel & Wood, 2014) *L. plantarum* exist spontaneously in most of the foods that are lactic acid fermented, especially plant based fermented food, and will therefore also exist in the digestive tract of humans. (Molin, 2013)

Since *L. plantarum* can pass through the acidic stomach in humans and occur spontaneously in pH less than 4.0, it is highly tolerant to low pH. The large genome of *L. plantarum* and its ability to ferment a large number of carbohydrates indicate that it can adapt to different environments. Manganese is required for growth of *L. plantarum* since it will provide protection against oxygen radicals. The radicals will be reduced to hydrogen peroxide which in turn will be converted to oxygen and water by manganese cofactored pseudocatalase. The microorganism can break up tannins into flavonoids and phenolic acids which has health beneficial antioxidant properties. (Molin, 2013)

The carbon metabolism of *L. plantarum* is fairly simple, generally providing lactic acid as its main product. However, they are very flexible regarding the substrates. A study investigating growth in fruit and vegetable juices concluded that the growth of *L. plantarum* strains CIL6, C2, POM1, 1MR20 and CC3M8 had a negative correlation with the concentrations of carbohydrates (fructose and glucose) and malic acid. A high concentration of carbohydrates, in e.g. pineapple juice, leads to either an inefficient metabolism or catabolite repression (Filannino, et al., 2014), i.e. the prevention of expression of catabolic systems that makes the usage of secondary substrates possible when a preferred carbon source is present. (Görke & Stülke, 2008) This is not optimal since the bacteria needs to have an equilibrium between the concentrations both inside and outside of the cell. The study observed that the bacteria in vegetable juices had similar consumption of carbohydrates to the bacteria in favourable environments, such as MRS broth, compared to juices which were more acidic and had higher concentrations of fructose and glucose, e.g. pineapple. This means that the strains of *L. plantarum* will change its metabolism from one that favours growth, e.g. carbohydrate fermentation, to one that favours the maintenance of the cells, e.g. malolactic fermentation. The presence of malic acid will result in a decrease in pH, both external and internal, and a change in the permeability of the membrane. However, the increased pH in the cell and the synthesis of reducing power from the decarboxylation of malic acid will create energy advantages. (Filannino, et al., 2014)

The study also showed that during the fermentation, the concentration of branched-chained amino acids (Valine, Leucine and Isoleucine) decreased and might have been converted to branched alcohols (2-methyl-1-butanol, 2-methyl-1-propanol and 3-methyl-1-butanol) since the concentration of those increased. All the *L. plantarum* strains had, when fermented in tomato juice, an increased level of glutamic acid and GABA. The study determined that the free amino acid catabolism is more pronounced in a higher pH environment, as in the vegetable juices, while in an environment with

lower pH, the malolactic fermentation is more pronounced, as in the cherry juice. It was also shown that the amino acid Tyrosine act as a stimulatory agent on *L. plantarum* growth and that during stress, the bacteria will synthesize alcohols, ketoacids, terpenes and ketones. The fermented juices all contained acetic acid and diacetyl. (Filannino, et al., 2014)

Genome sequencing of *L. plantarum* WCFS1 showed a fermentation pattern almost completely homolactic during growth on glucose as the carbon source. Pyruvate is produced via the EMP pathway from glucose and the pyruvate is then converted into lactate. It was also shown that this strain of *L. plantarum* had genes that encodes enzymes related to the conversion of pyruvate into e.g. acetoin, ethanol, formate, 2,3-butanediol and acetate. Since *L. plantarum* can utilize a wide variety of carbon sources, it can be concluded that the bacteria are very flexible, versatile and can adapt to many different environments. (Kleerebezem, et al., 2003)

L. plantarum are vitamin and amino acid auxotrophs, which means that they cannot synthesize certain vitamins and amino acid that are essential for their growth. A study by Ma et al. (2016) showed that for *L. plantarum* ST-III, six amino acids (Leucine, Isoleucine, Valine, Methionine, Tyrosine and Phenylalanine) and one purine (guanine or adenine) are essential for its ability to ferment milk. In addition, mineral salts had a stimulating effect on growth but was not essential. Since *L. plantarum* is vitamin auxotrophs, they will also need vitamins for growth. However, milk is very rich in vitamins which means that no vitamins needed to be supplemented. (Ma, et al., 2016)

3 Substituting ingredients and fermentation conditions

3.1 Plant seed powder

There have been studies where the use of vegetal carbon and nitrogen sources have been investigated to substitute conventional MRS medium. Plant seed powder was used instead of peptone, beef extract and yeast. The study was done on *L. lactis* and it was observed that the growth was enhanced when using the vegetal sources compared to MRS. The sample seeds used were mung beans, chickpea, lentil, Bengal gram, wheat and peanut powder. The recipe contained 10 g of the respective seed powder, 20 g Glucose, 1 g Tween-80, 2 g K_2HPO_4, 5 g Na-acetate, 2 g $(NH_4)_2$ citrate, 0.2 g $MgSO_4$-7H2O and 0.05 g $MnSO_4$-H2O. All the ingredients were then dissolved in distilled water (850 ml) and the pH was then adjusted to 6.5 after which the medium was autoclaved. The medium containing lentil seed powder showed the best result for growing *L. lactis*, even better than the conventional MRS medium. (Pathak & Martirosyan, 2012)

3.2 Pea Protein

The use of pea seed as a protein source is getting more attention. One reason for this might be that it has less anti-nutritive components, e.g. phytic acid, and is less allergenic than e.g. soybean. To get a pea protein hydrolysate, the pea protein isolate is hydrolysed until a specific degree of hydrolysis (DH). The DH is important since an excess in hydrolysis will e.g. cause a decline in solubility while the right degree of hydrolysis will improve solubility. The properties of the hydrolysate will depend on the enzyme doing the hydrolysis. (Barać, et al., 2015)

One study grew *L. acidophilus* 10 in MRS broth with an addition of pea protein hydrolysate in a concentration of 1 mg/ml. Even though the pea protein hydrolysate stimulated the growth of *L. acidophilus* during the first part of the cultivation, it was directly followed by a drastic decline in total bacterial number at the end of the fermentation. (Świątecka, et al., 2010)

3.3 Rice Protein

Rice protein has a high nutritional value and is a hypoallergenic food component. However, there are only few studies on the nutritional value of rice bran proteins. One study concluded that the rice bran protein showed a nutritional quality that was considered superior to other vegetable proteins, such as soy. However, the biological availability is lower than proteins originating from animals. (Han, et al., 2015)

The lactic acid production of *L. rhamnosus* NBRC 3863 was investigated while growing on rice bran. The rice bran was hydrolysed with acid and showed a productivity similar to 8 g/L yeast extract. However, the lag phase was prolonged as much as to 40 hours for the rice bran hydrolysed at pH 0.5. At pH 1 and 2, the lag phase was shorter. An alternative, suggested by the authors of the study, would also be to combine the rice bran hydrolysate with yeast extract, e.g. 30 g/L rice bran in combination with 3 g/L yeast extract. (Gao, et al., 2008)

3.4 Increasing the temperature for *L. rhamnosus*

The lactic acid production by *L. rhamnosus* grown on whey permeate was investigated in one study. The growth temperature that obtained the highest cell count ($2.98*10^{10}$ CFU/mL) were 42°C compared to ~$1.7*10^{10}$ CFU/mL for 37°C. (Cui, et al., 2010)

4 Material and method

All trials with different cultivation characteristics were done in duplicates, in two fermenters (Probi AB, Lund, Sweden), with the same ingredients. One sample is taken from each fermenter on which one dilution series is done. From the dilution series, 0.1 ml of the diluted sample are put on plates in duplicates, see Figure 2.

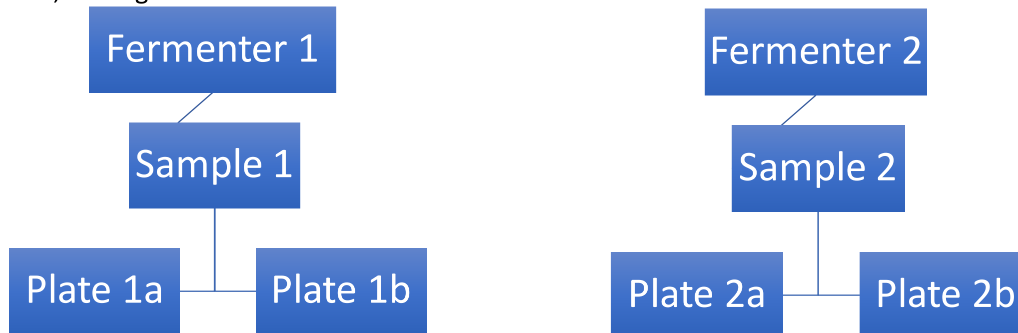

Figure 2. The experimental set-up of the fermentation done in duplicates, with the same ingredients in two fermenters.

The original recipe used for fermentation at Probi AB can be seen in Table 1. The recipe is yeast based.

Table 1. The ingredients used in the original recipe at Probi AB and the corresponding amounts.

Ingredients	Amount
Ingredient 1	39.5 g
Ingredient 2	1.5 g
Ingredient 3	1.5 g

Water was then added up to 600 ml. The cultivation medium was then heated to 90°C for 15 minutes after which the temperature was lowered to fermentation temperature.

The juices used in the trials are LOKA crush, with strawberry or raspberry flavour, and mango juice (Rubicon). The dose of probiotics in the juices are $5*10^7$/ml and $1.25*10^8$/ml for LOKA crush and mango juice respectively. The reason for this is that the estimated daily dose of the LOKA is 200 ml while the dose of the mango juice is 80 ml, which will give a cell count of 10 billion probiotic bacteria.

4.1 Organisms and inoculum

The bacteria used in the fermentation were *L. plantarum* 299v (Probi AB, Lund, Sweden), Probi Rhamnosus 6594 (Probi AB, Lund, Sweden) and Probi Paracasei 13434 (Probi AB, Lund, Sweden). The number of bacteria added depends on which strain that was used. A volume of 0.05 ml of *L. plantarum* was going to be added to the culture medium containing 600 ml. However, since adding such a small volume will increase errors, the bacteria are diluted 1:10 which means that 0.5 ml should be added instead. For *L. rhamnosus*, 8.57 µl should be added. For the same reason as previously mentioned, the sample is diluted 1:100 which means that the added volume is 0.857 ml. The added amount of *L. paracasei* was 1.333 ml after a 1:10 dilution.

4.2 Cultivation conditions and ingredients

The fermentation was done batch-wise and anaerobically in a fermenter with a working volume of 600 ml. The pH and temperature were measured with a pH meter (Mettler-Toledo, Stockholm, Sweden) online with LabVIEW 8.6 (National Instruments, Austin, USA) with temperature, time and stirring speed according to the standard fermentation settings used at Probi AB. A water bath (Julabo, Seelbach, Germany) was used to adjust the temperature.

4.2.1 Varying ingredient 2 and 3

Ingredient 2 and Ingredient 3, which are yeast based, were used. The recipe for the cultivation medium was done according to the original recipe, see Table 1, except for the varying amounts of ingredient 2 and 3. The trials were done in duplicates as explained in Table 2.

Table 2. Explanation of the trials with the ingredients used and the amount. The trials were done in duplicates.

Trial	Ingredient 2 (g/600 ml)	Ingredient 3 (g/600 ml)
1	0.5	1.5
2	1.5	0.5
3	1.0	1.5
4	1.5	1.0

4.2.2 Ingredient 4

Ingredient 4, a yeast based component, has been recommended as a substitute to both ingredient 2 and 3. It was therefore hypothesised that ingredient 4 could be used to stimulate sufficient growth on its own. The composition of ingredient 4 can be seen in Table 11 in the appendix, section 9.4. Ingredient 4 was used in a concentration of 3 g/600 ml and 1.5 g/600 ml for *L. plantarum* and the concentration that achieved the best results was further tested for *L. rhamnosus* and *L. paracasei*. Otherwise, the original recipe as described in Table 1 was used.

4.2.3 Ingredient 5

Ingredient 5, a component based on yeast, has also been recommended as a substitute to both ingredient 2 and 3. The composition of ingredient 5 can be seen in Table 14 in the appendix, section 9.5. Ingredient 5 was used in a concentration of 3.0 g/600 ml for all the bacteria in combination with the ingredients, except ingredient 2 and 3, presented in Table 1.

4.2.4 Combining ingredient 3 and 5

Exchanging ingredient 2 with ingredient 5 in combination with ingredient 3 has also been recommended. 1.5 g ingredient 5 in combination with 1.5 g ingredient 3 was used during the experiment. Due to lack of time, the fermentation product was not put in juice for storage stability measurements.

4.2.5 Ingredient 6 and 7

The study described in the background, section 3.1, used 10 g of plant seed powder in approximately 850 ml, which corresponds to 7.0 g/600 ml of ingredient 6 and 7 respectively and ingredient 1 was also added to the fermenter, see the original recipe in Table 1. The composition of the ingredients can be seen in Table 17 in the appendix.

4.2.6 Ingredient 8

Ingredient 8, a plant based component, was used in a concentration of 3.0 g/600 ml. Ingredient 8 was used in combination with ingredient 1 and water as presented in Table 1.

4.2.7 Ingredient 9

3.0 g/600 ml of ingredient 9, a plant based component, was added to the cultivation medium. Ingredient 2 and 3 in the original recipe in Table 1 was substituted with ingredient 9.

4.2.8 Increasing the fermentation temperature for *L. rhamnosus*

The temperature was increased to 42°C, which is the temperature that the study in the background, section 3.4, suggested. The fermentation was done with the original recipe, as presented in Table 1. Due to lack of time, the fermentation product was not put in juice for storage stability measurements.

4.2.9 Increasing the fermentation time for *L. paracasei*

During the experiments, it was observed that the lag-phase of *L. paracasei* was approximately 40% longer than for the other two bacteria. Hence, an experiment with a fermentation time that was 12.5% longer than the standard fermentation time used at Probi AB was added. The fermentation was done with the original recipe, Table 1. Due to lack of time, no stability measurements in juice was done for this experiment.

4.3 Plate count analysis

When analysing the bacterial count in the fermentation product and in the juice, the bacteria are diluted using Dilucups (labrobot, Stenungsund, Sweden), containing 9 ml peptone water, where 1 ml of sample is transferred between the cups with 1 ml pipettes (Mettler-Toledo, Stockholm, Sweden) and 1 ml tips (Mettler-Toledo, Stockholm, Sweden) and mixed using a Dilushaker (labrobot, Stenungsund, Sweden) at a speed of 400 rpm for 3 seconds. Dilutions -5, -6 and -7 (10^5, 10^6 and 10^7) for the fermentation product and -4, -5 and -6 (10^4, 10^5 and 10^6) for the juice is then plated with a 0.1 ml pipette (Mettler-Toledo, Stockholm, Sweden) and 0.1 ml tips (Mettler-Toledo, Stockholm, Sweden) on MRS (de Man, Rogosa and Sharpe) agar plates (Biomérieux, Marcy I'Etoile, France) and spread with an inoculation spreader (Sarstedt, Nümbrecht, Germany). The plates are then put anaerobically using an anaerobic generator called GENbox anaer (BioMérieux, Marcy I'Etoile, France) and anaerobic indicator (Thermo Scientific, Basingstoke, United Kingdom or BioMérieux, Marcy I'Etoile, France) and incubated at 37°C for approximately three days. After the incubation, the plates are counted where the exact number of colonies are noted if >400, and the concentration of bacteria is calculated on the plates that have a colony count between 25 and 300. If two plates are within 25-300 colonies, the number of colonies is added and divided by 1.1, and the result corresponds to the lowest dilution. The result is then divided by 0.1 to take into account the added amount on the plates. An example of the calculation can be seen in the appendix, section 9.1. The error of the method of analysis is 20%.

4.4 Storage stability

The storage stability of the fermentation product and the mixture of fermentation product and juice was investigated. The stability in the fermentation product is considered unacceptable if the log CFU/ml have decreased below 7.0 upon its expiration date since that is the least amount of bacteria sufficient for a probiotic drink to be effective. The high number is because a many of the probiotic bacteria die during the passage through the body. (Nualkaekul & Charalampopoulos, 2011)
The criteria for the fermentation product to be put in juice is that the CFU/ml is $>5*10^8$ or 8.7 log CFU/ml. The stability of the fermentation product was evaluated at an average of 3.7°C, see Table 7 in the appendix, at time 0 and 7 days while the juice mixture was evaluated at time 0, 2 and 4 weeks. Unfortunately, three weeks into the lab work, the refrigerator in which the samples were stored broke over the weekend. This might have resulted in up to 48 hours of storage in room temperature (21.8°C). To evaluate possible effects of the storage in higher temperature, all samples were evaluated once more upon discovery of the broken refrigerator. This means that another data point other than the 0 and 7 days for the fermentation product and 0, 2 and 4 weeks for the juices can be observed for the samples affected by the breakage. All samples were after this point stored at an average of 4.3°C, see Table 8 in the appendix, during the entire experiment.

4.5 Sensory evaluation

A simple sensory evaluation was done with two participants to evaluate if the juices containing the fermentation product was considered acceptable to consumers. Since the aim of the sensory evaluation was to evaluate consumer acceptance, the test focused on three taste categories, good, bad and acceptable.

4.6 Statistics

Comparing the bacterial content in CFU/ml for the samples containing the different ingredients with the standard recipe, Table 1, is done by using statistics. The statistics is in turn done by using Kruskal-Wallis One Way ANOVA on Ranks, which is non-parametric and can therefore not be assumed to be normally distributed, when investigating if there is a significant difference between the ingredients used in the fermentation for all three bacteria respectively. If so, two ingredients must be compared with each other to investigate which ingredient was significantly different from the original recipe. This is done by using a multiple comparison procedure, Student-Newman-Keuls. When $p<0.05$, there is a significant difference.

5 Result and discussion

Ingredient 2 and 3 in the fermentation of probiotics may give off-flavours to the fruit drink. The following trials will investigate whether it is possible to either lower the amount or exchanging them for another ingredient and still get an acceptable growth and storage survival.

5.1 pH

The pH during the fermentation of *L. plantarum* in cultivation medium containing the different ingredients can be seen in Figure 3. The values are an average of fermenters done in duplicates for each of the ingredients. The fermentation of *L. plantarum* was done with different ratios of ingredient 2 and 3 as described in Table 2.

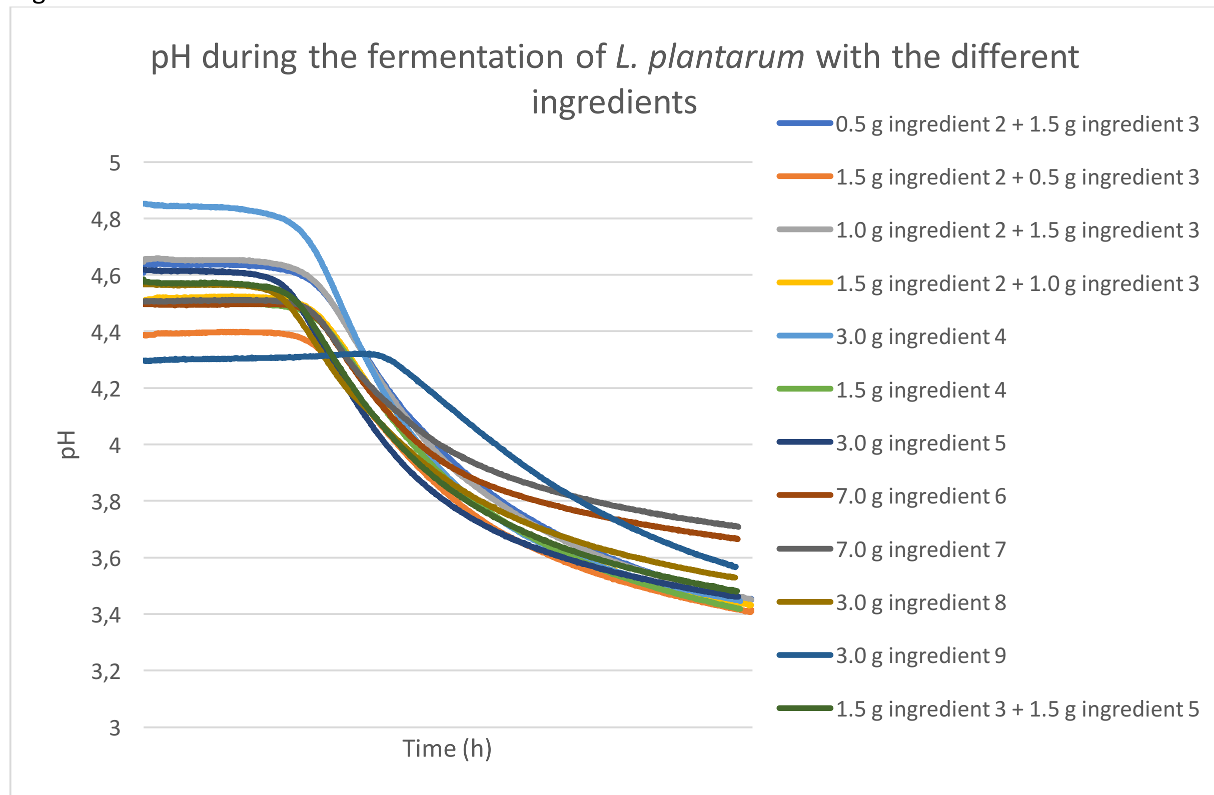

Figure 3. The pH during the fermentation of L. plantarum in cultivation medium containing different ingredients. The lines are an average of two fermenters done in duplicates for every ingredient.

From the figure, it can be seen that the ingredient 3 and ingredient 4 has a higher pH than e.g. the ingredient 2, resulting in a higher starting pH for the trials that contain higher contents of those ingredients. Despite the higher initial pH, the final pH is approximately the same (around 3.4), indicating that the initial pH does not matter that much. For the trials containing ingredient 6 or 7, the pH did not decrease to the same levels as for the other trials. This may be due to less lactic acid being produced which in turn indicates that there is a lower content of bacteria in the fermentation product. The lag-phase for the trial containing ingredient 9 is longer than for the other trials for *L. plantarum,* around 58% longer than the lag-phase of ingredient 2, 3, 4, 5, 6 and 7 and 91% longer for the lag-phase of ingredient 8. This may be due to the unavailability of nutrients in ingredient 9 or that the utilization of nutrients is more difficult.

The pH during the fermentation of *L. rhamnosus* in cultivation medium containing different ingredients can be seen in Figure 4. The values are averages which originates from duplicate fermenters. Since the bacterial content of *L. plantarum* in cultivation medium containing a combination of ingredient 2 and 3, was the highest for the trial with 0.5 g and 1.0 g ingredient 2 in combination with 1.5 g ingredient 3 (see section 5.2.1 below) and 3.0 g ingredient 4 (see section 5.2.2), those are the ones that are further tested on *L. rhamnosus.*

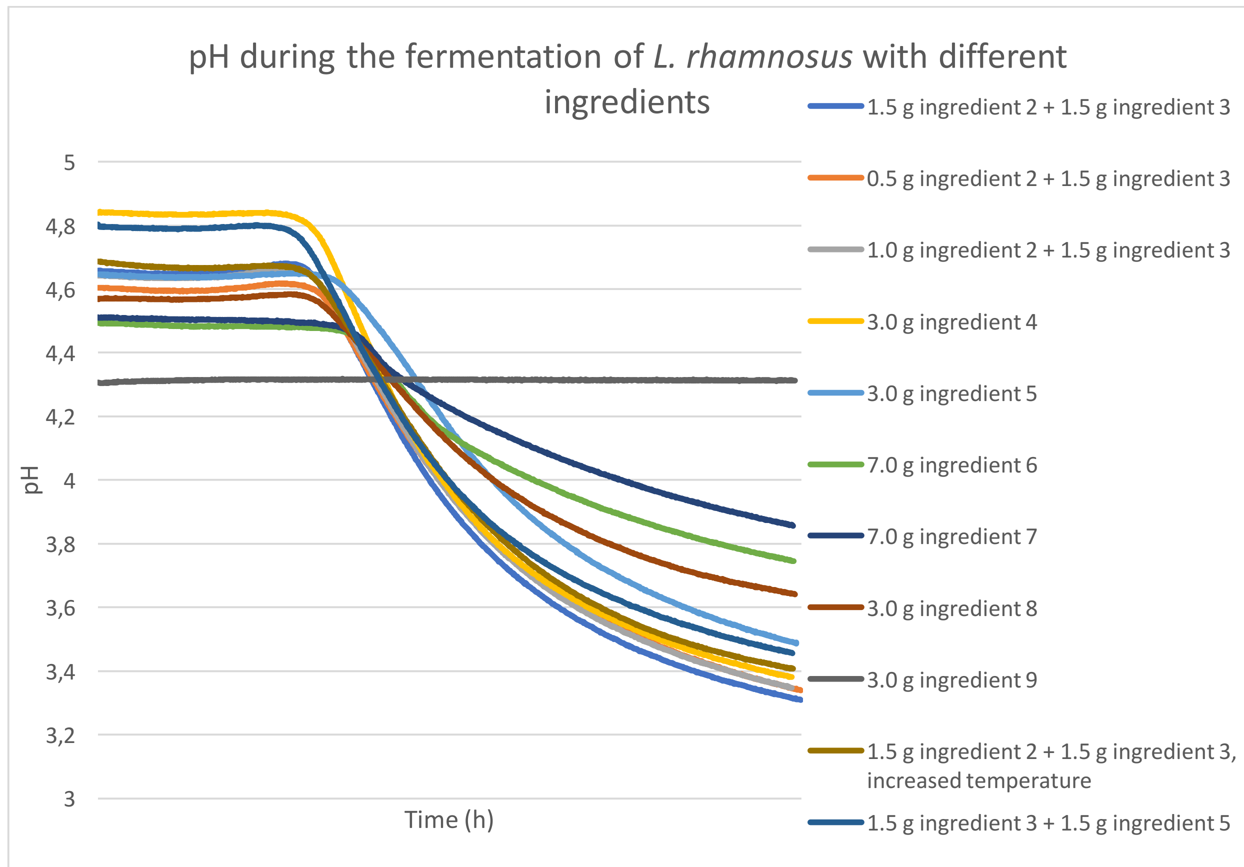

Figure 4. The pH during the fermentation of L. rhamnosus in cultivation medium containing different ingredients. The lines are an average of two fermenters done in duplicates for every ingredient.

For the trials containing a combination of ingredient 2 and 3, a small increase can be observed at the end of the lag-phase before the major decrease in pH. This might be due to the utilization of nutrients that have a low pH or the production of alkaline products by the bacteria. As previously discussed, the pH for the trials containing high concentrations of ingredient 3 is higher due to the more alkaline nature of these components but the pH will despite that be reduced to the same level as for the other trials containing ingredient 2, 3, 4 and 5. All the trials containing ingredients 2, 3, 4 and 5 reaches a final pH of around 3.3-3.4 while the trials containing ingredients 6, 7, 8 and 9 are a bit higher in final pH.

For the trials containing ingredient 6 and 7, the final pH did not decrease to the same level as for *L. plantarum*, indicating the growth and production of acids by *L. rhamnosus* was not as efficient in these ingredients for *L. plantarum.* The fermentation of *L. rhamnosus* in ingredient 7 had a slightly higher end pH (around 3.85) than the fermentation with ingredient 6 (around 3.75).

For the trial containing ingredient 9, there was no decrease in pH during the fermentation which means that the bacteria did not produce any acid. It was therefore assumed that there was no growth during the fermentation. However, to verify these results and make sure that there has not been a human error, the trial was redone. The second attempt on the fermentation of *L. rhamnosus* in cultivation medium containing ingredient 9 showed the same pattern for the pH as during the first fermentation which verifies the result that there was no production of acids.

The study described in the background, section 3.4, suggested that an increase in temperature to 42°C would increase the bacterial content for *L. rhamnosus*. However, looking at the pH, the curve

looks almost identical to the trial done with the same ingredients but at the standard temperature, indicating that the production of acids had not increased due to the increase in temperature.

The pH during the fermentation of *L. paracasei* in cultivation medium containing different ingredients can be seen in Figure 5. The values originate from two fermenters and are therefore averages. As for *L. rhamnosus*, the concentrations of ingredient 2 and 3 that are used for *L. paracasei* are the ones that gave the highest bacterial content for *L. plantarum,* 0.5 g and 1.0 g ingredient 2 in combination with 1.5 g ingredient 3 and 3.0 g ingredient 4.

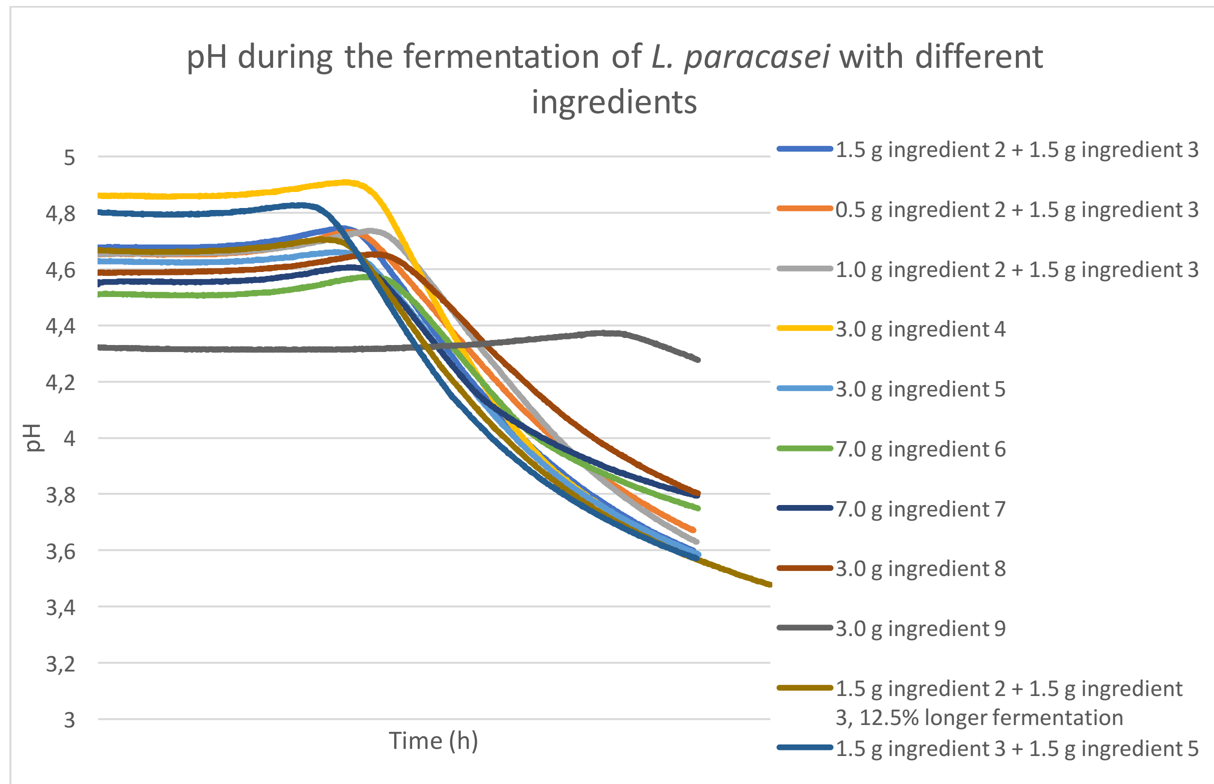

Figure 5. The pH during the fermentation of L. paracasei in cultivation medium containing different ingredients. The lines are an average of two fermenters done in duplicates for every ingredient.

The pH curves show a similar increase in pH at the end of the lag-phase and before the major decrease in pH as for *L. rhamnosus*. As discussed previously, this might be the result of the utilization of acidic nutrients or the production of alkaline products by the bacteria. In addition, the lag phase of *L. paracasei* seems to be longer compared to the other bacteria, around 40% longer than the lag-phase of the other bacteria and the final pH seems to be a bit higher. The final pH is around 3.6 for the trials containing ingredient 2, 3, 4 and 5 and around 3.8 for ingredient 6, 7 and 8. The lag-phase in the trial containing ingredient 9 was significantly longer than for the other trials, resulting in that the bacteria did not have time to grow for as long time as for the other trials.

As described previously, the lag-phase during the fermentation of *L. paracasei* was generally 40% longer than for the other bacteria and it was therefore of interest to try a total fermentation time that was 12.5% longer for *L. paracasei* than for the other bacteria. The profile of the curve looks the same as for the other fermentations with ingredient 2 and 3 and the end pH was slightly lower, indicating that there might have been an increase in bacterial number compared to the standard fermentation.

5.2 Growth and stability

The bacterial count for all the bacteria and the stability after one week of storage can be seen in Table 3. All trials were done in two fermenters in which the plating was done in duplicates, providing an average for each ingredient. The trial using 1.5 g ingredient 2 and 1.5 g ingredient 3, which is the original recipe, Table 1, for *L. plantarum* was previously done at Probi AB. The samples that are put in juice are written in bold as well as the samples that should have been put in juice but due to lack of time, was not. Some of the samples containing *L. plantarum* and *L. rhamnosus* were affected by the broken refrigerator, resulting in an additional measurement at day 4 and 3, respectively. Since the bacterial content of *L. rhamnosus* in the fermentation product containing ingredient 9 was 0 log CFU/ml, no stability measurement will be done for those samples. The stability of the sample containing *L. paracasei* and 1.5 g ingredient 3 and 1.5 g ingredient 5 after 7 days was measured after 6 days instead.

*Table 3. The average log CFU/ml with the standard deviation from the fermentation of L. plantarum, L. rhamnosus and L. paracasei for the different trials and the stability after one week of storage. The average is of two fermenters on which duplicate plates were counted, i.e. 4 values per combination. The measurements during day 3 and 4 was done due to the breakage of the refrigerator. The samples that are significantly different are marked with a star, *= $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$ compared to the original recipe. The samples that are put in juice as well as the samples that should have been put in juice if there was more time available are written in bold.*

Growth in log CFU/ml ± standard deviation	*L. plantarum*			*L. rhamnosus*			*L. paracasei*	
	Day 0	Day 4	Day 7	Day 0	Day 3	Day 7	Day 0	Day 7
Original recipe: 1.5 g ingredient 2 + 1.5 g ingredient 3	8.9	-	-	**8.8 ± 0.11**	8.7 ± 0.10	8.8 ± 0.09	8.4 ± 0.03	8.4 ± 0.05
0.5 g ingredient 2 + 1.5 g ingredient 3	**8.8 ± 0.07**	-	8.5 ± 0.13	8.7 ± 0.05*	8.8 ± 0.04	8.7 ± 0.11	8.3 ± 0.05***	8.3 ± 0.04
1.5 g ingredient 2 + 0.5 g ingredient 3	8.5 ± 0.05**	-	8.3 ± 0.04	-	-	-	-	-
1.0 g ingredient 2 + 1.5 g ingredient 3	**8.8 ± 0.06**	-	8.6 ± 0.04	**8.7 ± 0.13***	-	8.7 ± 0.08	8.3 ± 0.02	8.3 ± 0.10
1.5 g ingredient 2 + 1.0 g ingredient 3	8.7 ± 0.08**	-	8.5 ± 0.06	-	-		-	-
1.5 g ingredient 4	8.5 ± 0.05**	-	8.7 ± 0.01	-	-	-	-	-
3.0 g ingredient 4	**8.8 ± 0.14**	-	8.4 ± 0.03	**8.9 ± 0.07**	-	8.8 ± 0.06	8.4 ± 0.07	8.4 ± 0.01
3.0 g ingredient 5	**8.9 ± 0.06**	-	8.7 ± 0.09	**8.6 ± 0.13***	-	8.6 ± 0.10	8.4 ± 0.03	8.2 ± 0.11
1.5 g ingredient 3 + 1.5 g ingredient 5	**8.8 ± 0.08**	-	8.6 ± 0.13	**8.8 ± 0.10**	-	8.7 ± 0.08	8.5 ± 0.09	8.7 ± 0.17
7.0 g ingredient 6	8.4 ± 0.07**	8.4 ± 0.07	8.5 ± 0.12	8.1 ± 0.30*	-	8.2 ± 0.15	8.5 ± 0.07	8.6 ± 0.10
7.0 g ingredient 7	8.3 ± 0.10**	8.2 ± 0.53	8.5 ± 0.06	8.6 ± 0.13*	-	8.5 ± 0.21	8.6 ± 0.05	8.6 ± 0.10
3.0 g ingredient 8	**8.7 ± 0.15***	-	8.7 ± 0.11	8.4 ± 0.04*	-	8.3 ± 0.02	8.2 ± 0.01**	8.1 ± 0.06
3.0 g ingredient 9	8.6 ± 0.07**	-	8.6 ± 0.06	0 ± 0**	-	-	8.3 ± 0.04	8.7 ± 0.07

The highest cell count for *L. plantarum*, other than the trial with 1.5 g ingredient 2 and 1.5 g ingredient 3 which is the original recipe, was obtained with 1.0 g ingredient 2 and 1.5 g ingredient 3. When deciding which of the samples to put in juice, the two samples with the highest cell count were chosen, i.e. the trial with 0.5 g ingredient 2 and 1.5 g ingredient 3 and the one with 1.0 g ingredient 2 and 1.5 g ingredient 3, which are also the ones that are clearly >8.7 log CFU/ml which is the criteria for putting in juice. The trial with 1.5 g ingredient 2 and 1.0 g ingredient 3 seems to be above 8.7 log CFU/ml but was not. It appears as the content of ingredient 3 has the highest effect on the growth of the bacteria, as seen in Table 3 for *L. plantarum*. I.e. the concentration of ingredient 2 can be lowered while the concentration of ingredient 3 should stay the same. However, it can also be seen that a higher concentration of ingredient 2 will have a positive effect on the growth. The trials that gave the highest bacterial count was further tested with the other bacteria.

The stability of *L. plantarum* in all the fermentation products containing ingredient 2, 3, 4 and 5 is considered acceptable since the bacterial content is above 7.0 log CFU/ml, as described section 4.4. It seems like the bacteria in the first trial, i.e. the one with 0.5 g ingredient 2 and 1.5 g ingredient 3, had the lowest survival rate. However, one of the duplicate plates from this trial lost an unreasonably high amount, which can be seen at the high standard deviation and might suggest an error in either the initial or the final value for that sample. In addition, the two values for day 0 and day 7 from each duplicate sample originating from one fermenter came from different test tubes, which may indicate that there are some differences depending on which test tube that is used. A suggestion might therefore be to have a larger sample that all samples for measurements can be obtained from.

The trial with 1.5 g ingredient 2 and 1.5 g ingredient 3 for *L. rhamnosus* is high enough to be put in juice as well as one of the duplicate fermenters for trial 1.0 g ingredient 2 and 1.5 g ingredient 3. The values for day 3 have not increased that much, considering the standard deviation and the large error of the measuring method, which means that further measurements can be done on these samples and can therefore continue until day 7. The samples have stayed approximately the same in bacterial count during the storage week and as discussed previously, the storage stability is considered good.

As can be seen in the table, the trial with 3.0 g of ingredient 4 had higher concentration of *L. plantarum* than the trial with 1.5 g ingredient 4. Since 3.0 g ingredient 4 gave the best results, that was the concentration that was further used for the other bacteria. For the trial with 3.0 g ingredient 5, only one duplicate fermenter containing *L. rhamnosus* qualified to be put in juice while the other was below the limit, resulting in an average below the limit as well. Since none of the fermentation products have decreased one log, the samples can be considered stable during the storage for 7 days.

L. paracasei did not grow as well as expected, see the table above. It was hypothesized that the bacterial content would be in approximately the same range as for *L. plantarum* and *L. rhamnosus*. The longer lag-phase of *L. paracasei* might result in a lower bacterial content when fermented for as long time as for the other bacterial species. Since the lag phase is longer, a suggestion might be to increase the fermentation time with 12.5% to get a log-phase that is approximately the same as for the other two bacteria. This is to try to get the same bacterial count as for the other bacteria. Another alternative is that these results indicate that there are some nutrients essential for growth for *L. paracasei* that is missing in the culture medium. The content of *L. paracasei* was very low for all ingredients and none of the samples qualified to be put in juice. The storage stability of the fermentation product can be considered acceptable. The increase of the bacterial content in some of the samples can be considered negligible since the error of the measuring method is so large.

The study by Pathak et al. (2012) suggest that a cultivation medium containing high protein vegetal sources can be a substitute for MRS medium and it is therefore hypothesised that such sources can

be used instead of ingredient 2 and 3 in the medium currently used at Probi AB. However, as can be seen in the table, none of the samples containing ingredient 6 or 7 qualified to be put in juice since the bacterial content was too low. The reason for this might be that the nutrients in these ingredients are not as available for utilization for the bacteria as are the other nitrogen sources used in the experimental work, even though the protein content is high. In addition, as mentioned above, the lag-phase was longer which means that a higher cell count might be obtainable if the fermentation could go on for a longer amount of time. However, ingredient 6 and 7 provided the highest growth of *L. paracasei*, even higher than the medium containing ingredients 2, 3, 4 and 5. Generally, for *L. plantarum* and *L. rhamnosus,* the plant based ingredients, i.e. ingredient 6, 7, 8 and 9, seems to result in a slightly lower bacterial content. This can also be observed since these ingredients are significantly lower than the original recipe. The reason for that might be that there is some nutrient missing that is important for growth for *L. plantarum* and *L. rhamnosus* or that the biological availability of the nutrients is lower than for the other ingredients*.*

In the study by Pathak et al. (2012), the authors concluded that a medium containing vegetal seed powder provided better growth than conventional MRS medium. In this experiment, no comparison between MRS and the used ingredients were done. The study rather provide evidence that it would be possible to grow probiotics in medium containing ingredient 6 and 7, which it evidently is. However, the growth was not as good as hoped. This might be due to the many differences between the study and the experimental work. For example, the probiotic used in the study was *L. lactis* which most likely behaves differently than the bacteria used in this experiment and the ingredients in the medium used in the study contained additional ingredients, e.g. citrate and acetate which might influence the growth. In addition, the fermentation was done for 72 hours and the germination of the seeds prior to powdering was monitored in the study which was not the case with the ingredients in this experiment, providing different conditions right from the start.

All the samples containing ingredient 6 and 7 are considered stable since there is no sample that decrease more than one log-unit, and none are below 7.0 log CFU/ml. For *L.* plantarum in ingredient 7, the bacterial content during day 4 decreased, which was not expected. However, the standard deviation is very high, indicating that there might not have been a decrease at all. The reason for the high standard deviation could be that one of the plates for one of the fermenters had a significantly lower bacterial content, i.e. 7.3 log CFU/ml compared to 8.4, 8.4 and 8.7 for the other plates. The trial containing ingredient 6 and *L. rhamnosus* has also a high standard deviation, higher than is acceptable, i.e. >0.2. In general, the standard deviation of some of the values in the table are a bit high, which is a result of that the fermenters differed between one another despite containing the same ingredients. This might be explained by the addition the ingredients which, due to the accuracy of the scale, will differ slightly among the fermenters, providing different amounts of nutrients being added.

The study by Gao et al. (2008) showed that the production of lactic acid with 30 g/L acid-hydrolyzed rice bran gave as good results as with 8 g/L yeast extract. In this experiment, 3.0 g/600 ml ingredient 9 was used to investigate the possible impact of the ingredient on growth. The growth with ingredient 2, 3, 4 and 5 was slightly higher than ingredient 9 for *L. plantarum* when the same concentration of each ingredient was used. As suggested by Gao et al. (2008) it might be beneficial to combine ingredient 9 with a small amount of yeast to increase the growth, giving an even higher growth than when only yeast is used. However, there are some differences between the study and this experiment. The rice used in the study was acid-hydrolyzed rice bran at pH 0.5, 1 and 2, while ingredient 9 used in this experiment was not made of bran. In addition, the fermentation in the study were done at 42°C with addition of nitrogen gas and the focus of the study was the production of lactic acid and not the growth of probiotics. However, as the study concluded, it is possible to grow bacteria on rice but a higher bacterial count might be obtained when combined with yeast.

The reason for the lower number of bacteria in ingredient 9 might be that nutrients that are essential for growth was not present or that the biological availability of the proteins was not as high as in proteins from animal sources, as suggested by Han et al. (2015). However, the content of *L. rhamnosus* was unexpectedly low and the fermentation was therefore repeated to verify the results. The second fermentation did not show any colonies, as for the first fermentation, when diluted according to the dilution series explained in material and method. Theoretically, there should be as many bacteria present in the fermentation product as added before the fermentation if no growth occurred. Since there were less bacteria present, the bacteria have probably died off during the fermentation.

Despite the long lag phase, which was also observed in the study by Gao et al. (2008), and the short period of actual growth during the trial with *L. paracasei* and ingredient 9, the bacterial content was still in the same range as for ingredient 8 which did not have as long lag phase. This might suggest that the bacteria did grow but did not produce as much acid during that time to reduce the pH or that ingredient 8 was not as effective as ingredient 9 in supporting growth, resulting in as high growth for ingredient 9 as for 8 despite the long lag phase for ingredient 9.

The stability of the fermentation product containing both ingredient 8 and 9 is considered good. The sample containing ingredient 8 and *L. plantarum* stays approximately the same during the storage week while the samples containing *L. rhamnosus* and *L. paracasei* decreases. However, the decrease is very small considering the large error of the measuring method. The content of *L. paracasei* in the fermentation product containing ingredient 9 seems to increase, despite considering the standard deviation, suggesting that the bacteria might continue to grow during the storage or that a human error was made.

Comparing all the used ingredients with each other with ANOVA for each of the bacteria separately, there was a significant difference between the samples since $p<0.05$. For *L. plantarum,* the samples that had a significant difference regarding growth compared to the original recipe was 1.5 g ingredient 2 + 0.5 g ingredient 3, 1.5 g ingredient 2 + 1.0 g ingredient 3, 1.5 g ingredient 4, ingredient 6, 7, 8 and 9. When comparing these samples that showed no significant difference from the original recipe with each other, no significant difference could be found between them, indicating that these six samples can be considered equally successful in supporting growth. As previously discussed, it can also be seen from the statistics that lowering the concentration of ingredient 3 and 4 will result in a significantly lower growth compared to lowering the concentration of ingredient 2.

For *L. rhamnosus,* 3.0 g ingredient 4 and 1.5 g ingredient 3 + 1.5 g ingredient 5 gave no significant difference, i.e. these ingredients gave a growth that can be considered as good as the original recipe while the rest of the samples can be considered not as successful in supporting growth.

For *L. paracasei*, it can be observed that the original recipe was only significantly different from 0.5 g ingredient 2 + 1.5 g ingredient 3 and ingredient 8. That means that compared to the original recipe, all other ingredients were as good when considering growth. However, since the original recipe is not the ingredient that gave the highest bacterial count, it is also of interest to also investigate if a significant difference is obtained when the best ingredient, however not significantly the best, is compared with the other ingredients. The ingredient that gave the highest bacterial content, i.e. ingredient 7, had a significant difference with ingredient 5, 8, 9, 1.0 g ingredient 2 + 1.5 g ingredient 3 and 0.5 g ingredient 2 + 1.5 g ingredient 3. That means that ingredient 7 was significantly better than these ingredients. As for the other samples, no significant difference could be observed and the ingredients can therefore be assumed to be as good at supporting growth as ingredient 7.

The bacterial content and the stability after one week of storage in the fermentation product containing *L. rhamnosus,* fermented at the standard temperature or 42°C can be seen in Table 4. The cultivation medium contained 1.5 g ingredient 2 and 1.5 g ingredient 3 as according to the original recipe in Table 1.

*Table 4. The average log CFU/ml with the standard deviation and the storage stability for the fermentation product containing L. rhamnosus fermented at standard temperature and 42°C in cultivation medium containing 1.5 g ingredient 2 and 1.5 g ingredient 3. The trial was done in duplicates in two fermenters and in from which two plates were counted. The samples that are significantly different are marked with a star, *= p≤0.05, ** = p≤0.01 and *** = p≤0.001 compared to the original recipe at standard temperature.*

Conditions during the fermentation of *L. rhamnosus*	Average log CFU/ml ± standard deviation, day 0	Average log CFU/ml ± standard deviation, day 7
Standard temperature	8.8 ± 0.11	8.8 ± 0.09
42°C	8.3 ± 0.10**	8.4 ± 0.07

The bacterial content of the fermentation product was significantly lower when fermented at 42°C. It was therefore not advantageous to increase the temperature during the fermentation, despite the results obtained in the study by Cui et al. (2010) That might be due to the difference in behaviour of the strains, since the strain used in the study was not the same as the strain used in this experiment. In addition, the bacteria in the study grew on whey, which has a completely different nutritional content than the cultivation medium used in this experiment. Also, the difference in bacterial content between 37°C and 42°C in the study by Cui et al. (2010) was not more than around 0.24 log CFU/ml which is not a significant difference. In conclusion, the bacterial strain used in this experiment did not benefit from a raised temperature but gave a lower bacterial content. The stability is acceptable since the bacterial content does not decrease below 7.0 log CFU/ml. The increase, however, can be disregarded since there is an overlap between day 0 and 7 when the standard deviation of the values is considered.

The content of *L. paracasei* in medium containing 1.5 g ingredient 2 and 1.5 g ingredient 3 according to the original recipe in Table 1 that was fermented for 12.5% longer as well as for standard time can be seen in Table 5. The stability for the sample fermented for longer time was measured after 6 days instead of 7.

*Table 5. The average log CFU/ml with the standard deviation for the fermentation product containing L. paracasei fermented for standard time and 12.5% longer with 1.5 g ingredient 2 and 1.5 g ingredient 3. The trial was done in duplicates in two fermenters and the average CFU originated from two plates. The samples that are significantly different are marked with a star, *= p≤0.05, ** = p≤0.01 and *** = p≤0.001 compared to the original recipe.*

Conditions during the fermentation of *L. paracasei*	Average log CFU/ml ± standard deviation, day 0	Average log CFU/ml ± standard deviation, day 7/6
Standard time	8.4 ± 0.03	8.4 ± 0.05
12.5% longer fermentation	8.5 ± 0.09	8.6 ± 0.20

A small increase in bacterial content can be observed when the total fermentation time is increased with 12.5%. If the standard deviation is considered, there is no overlap between the samples, suggesting that the longer fermenting will increase the bacterial content. However, there is no significant difference between the samples, indicating that increasing the fermentation with 12.5% will not give a significantly higher bacterial content but a content in the same range as the standard fermentation. In addition, the increase is so small that it will not be beneficial to increase the fermentation time considering the extra time and cost that will be added during the production. The increase in bacterial content after 6 days of storage could in fact be a decrease due to the large standard deviation.

5.3 Stability in juice

5.3.1 Varying the concentration of ingredient 2 and 3

The survival of *L. plantarum* in the juice containing varying concentrations of ingredient 2 and 3 was effected by the breakage of the refrigerator, resulting in a higher value for week 2 compared to week 1, see Table 6. The survival of *L. plantarum* with ingredient 4 in the juice was also effected by the breakage of the refrigerator, resulting in an additional value at week 1.5. However, the increase is not as high, considering the big error in the measuring method, which means that the storage analysis can continue with the last measurement at week 4. The fermentation product containing *L. rhamnosus* containing varying concentrations of ingredient 2 and 3 will not be as effected by the broken refrigerator since the initial bacterial count does not matter as much since the storage stability will be evaluated relative to the initial value. Since one of the duplicate fermenters had a bacterial content below the limit to be put in juice, the values for trial 1.0 g ingredient 2 and 1.5 g ingredient 3 are based on one fermenter with duplicate plates. The stability of the trials containing *L. plantarum* or *L. rhamnosus* put in juice can be seen in Table 6.

Table 6. The stability of the fermentation product containing L. plantarum or L. rhamnosus and the different ingredients in LOKA and Mango juice during 4 weeks of storage, presented in average log CFU/ml with the standard deviation for duplicate fermenters on which duplicate plates were counted, except for the trial with 1.0 g ingredient 2 and 1.5 g ingredient 3 and ingredient 5 for L. rhamnosus on which only one fermenter with duplicate plates were put in juice. A standard deviation of 0 means that the two duplicate plates had the same number of colonies. The breakage of the refrigerator resulted in an additional measurement at week 1.5 for L. plantarum.

Sample put in juice	Average log CFU/ml ± standard deviation, week 0	Average log CFU/ml ± standard deviation, week 1.5	Average log CFU/ml ± standard deviation, week 2	Average log CFU/ml ± standard deviation, week 4
L. plantarum				
0.5 g ingredient 2 + 1.5 g ingredient 3 in LOKA	7.5 ± 0.06	-	8.0 ± 0.04	7.7 ± 0.41
1.0 g ingredient 2 + 1.5 g ingredient 3 in LOKA	7.6 ± 0.14	-	8.0 ± 0.13	8.0 ± 0.06
0.5 g ingredient 2 + 1.5 g ingredient 3 in Mango	7.9 ± 0.12	-	7.9 ± 0.24	8.1 ± 0.05
1.0 g ingredient 2 + 1.5 g ingredient 3 in Mango	8.0 ± 0.06	-	8.1 ± 0.09	8.3 ± 0.03
3.0 g ingredient 4 in LOKA	7.6 ± 0.04	7.9 ± 0.22	7.9 ± 0.06	7.9 ± 0.03
3.0 g ingredient 4 in Mango	8.2 ± 0.08	7.7 ± 0.25	7.8 ± 0.12	8.0 ± 0.12
3.0 g ingredient 5 in LOKA	7.6 ± 0.05	-	7.4 ± 0.03	7.2 ± 0.03
3.0 g ingredient 5 in Mango	8.0 ± 0.05	-	7.9 ± 0.03	7.6 ± 0.02
3.0 g ingredient 8 in LOKA	7.7 ± 0.03	-	7.7 ± 0.06	7.8 ± 0.04
3.0 g ingredient 8 in Mango	8.1 ± 0.08	-	8.1 ± 0.04	8.2 ± 0.02
L. rhamnosus				
1.5 g ingredient 2 + 1.5 g ingredient 3 in LOKA	7.7 ± 0.13	-	8.0 ± 0.36	7.5 ± 0.07
1.0 g ingredient 2 + 1.5 g ingredient 3 in LOKA	7.6 ± 0.13	-	7.5 ± 0.14	7.5 ± 0
1.5 g ingredient 2 + 1.5 g ingredient 3 in Mango	8.1 ± 0.13	-	8.2 ± 0.13	7.9 ± 0.07
1.0 g ingredient 2 + 1.5 g ingredient 3 in Mango	7.9 ± 0.06	-	7.8 ± 0.03	7.6 ± 0.04
3.0 g ingredient 4 in LOKA	7.3 ± 0.11	-	7.7 ± 0.20	7.3 ± 0.05
3.0 g ingredient 4 in Mango	7.9 ± 0.03	-	7.2 ± 0.07	7.0 ± 0.10
3.0 g ingredient 5 in LOKA	7.3 ± 0.08	-	7.5 ± 0.01	7.5 ± 0.03
3.0 g ingredient 5 in Mango	8.0 ± 0.03	-	8.0 ± 0.07	7.9 ± 0

The stability of the fermentation product containing *L. plantarum* and varying concentrations of ingredient 2 and 3 varies, but all are considered acceptable since they are all above 7.0 log CFU/ml. Since the samples at week 2 was evaluated after the breakage of the refrigerator, an increase in the bacterial content was expected, which was the case for all samples except for the trial containing 0.5

g ingredient 2 and 1.5 g ingredient 3 in Mango. Considering the standard deviation of that trial, it appears to overlap, suggesting that there might have been either a decrease or an increase during week 2. Looking at the survival of the bacteria in the juices after the breakage of the refrigerator, i.e. after week 2, some trials have increased (both trials in Mango) and some have decreased or stayed the same (both trials in LOKA) in bacterial content. However, considering the standard deviation, both trials in LOKA and the trial containing 0.5 g ingredient 2 and 1.5 g ingredient 3 in Mango have an overlap between week 2 and 4, indicating that the increase might not be an increase at all. The trial with 1.0 g ingredient 2 and 1.5 g ingredient 3 in Mango seems to have increased and the bacteria might have grown in the juice. The high standard deviations that can be seen in e.g. the fourth week of trial 0.5 g ingredient 2 and 1.5 g ingredient 3 in LOKA containing *L. plantarum* might be due to the difference between the samples originating from different fermenters.

The survival of *L. rhamnosus* in juice containing varying concentrations of ingredient 2 and 3 is considered good since none of the samples are below 7.0 log CFU/ml. Most of the samples decreased or stayed the same during the four weeks of storage while the trials with 1.5 g ingredient 2 and 1.5 g ingredient 3 in both LOKA and Mango seems to have increased. However, when considering the standard deviation of the values from the trials that have increased until week 2, both the LOKA and Mango have an overlap between the values for week 0 and 2, which indicates that there might not be an increase at all. In addition, the standard deviation of the trial containing 1.5 g ingredient 2 and 1.5 g ingredient 3 in LOKA during week 2 is large, which might be due to the large difference in bacterial content between the duplicate fermenters.

The stability of the fermentation products containing ingredient 4 and *L. plantarum* put in juice are all considered acceptable. For the trials put in LOKA, the breakage of the refrigerator resulted in an increase in bacterial content while for the trials with Mango juice, the breakage resulted in a decrease. Since the standard deviation does not overlap between week 0 and 1.5 for any of the trials, it seems like the increase and decrease might be true unless a human error has been made. The trial with *L. plantarum* put in Mango have increased during storage after week 1.5, suggesting that the bacteria can grow in the nutritious mango juice.

The trial containing *L. rhamnosus* and ingredient 4 put in LOKA seems to have increased during the second week of storage while the trials put in Mango seems to have decreased during the four weeks. When the standard deviation is considered, no overlap between the weeks could be found, suggesting that the increase and decrease is true.

All the samples containing ingredient 5 are considered stable considering that they are all above 7.0 log CFU/ml. The stability of *L. plantarum* does not seem to be as good as for *L. rhamnosus* when the values and their standard deviation is studied. However, the large error in the measuring method prevents from reaching that conclusion. The value of *L. rhamnosus* put in LOKA for week 0 seems to be a bit low which might be the result of some kind of error.

The juices containing ingredient 8 can be considered stable. The small increase that can be observed for the Mango juice after four weeks might not be an increase due to both the large error of the measuring method and the rounding off of the decimals.

5.4 Sensory evaluation

5.4.1 Varying the concentration of ingredient 2 and 3

The entire result from the small sensory evaluation of the juices containing *L. plantarum* can be seen in Table 9 in the appendix. Both trials in LOKA (0.5 g ingredient 2 + 1.5 g ingredient 3 and 1.0 g ingredient 2 + 1.5 g ingredient 3) tasted good with no umami off-flavours, although the strawberry flavour disappeared a bit when the fermentation product was added, giving a bland taste. However, LOKA is currently not adapted to having probiotics added to it and further development might remove the problem with the disappearing fruit flavour. The trial containing 0.5 g ingredient 2 and 1.5 g ingredient 3 in Mango did also taste good, but a slight umami off-flavour was noticed during week 2 and 4. For the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3 in Mango, the off-flavour seemed to have disappeared in week 4.

The table for the entire sensory evaluation for the fermentation product containing *L. rhamnosus* put in juice can be seen in Table 10 in the appendix. For the juices in week 0, the trial containing 1.5 g of both ingredient 2 and 3 was considered acceptable/bad since it had some meat and umami off-flavour while the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3 was considered good in both LOKA and Mango juice. For week 2, both trials were considered acceptable, where the trial with 1.5 g + 1.5 g had lost some of its off- flavour and the trial with 1.0 + 1.5 had gained some. After four weeks of storage, trial 1.5 + 1.5 was still considered acceptable due to its off-flavour while trial 1.0 + 1.5 in both LOKA and Mango juice was considered good due to that the off-flavour had disappeared.

Since the fermentation product containing *L. paracasei* was not put in juice, the fermentation product was tasted by itself. All fermentation products had a savoury and acidic taste and the off-flavour of ingredient 2 and 3 was clearly noticed.

5.4.2 Ingredient 4

The results from the sensory evaluation of the juices containing *L. plantarum* can be seen in its entirety in Table 12 in the appendix. The fermentation product that was put in LOKA was during the entire storage considered acceptable. The reason for this was that the strawberry flavour was lost and a savoury and salty off-flavour could be identified during the fourth week of storage. The fermentation product that was put in mango juice was well tasting.

The complete results from the sensory evaluation of *L. rhamnosus* put in juice can be seen in Table 13 in the appendix. The Mango juice was considered well tasting during the entire four weeks of storage, however, during the two first weeks an off-flavour of umami was noticed but the juice was considered good despite that. The trial put in LOKA was considered acceptable during week 0 due to off-flavours and bad during week 2 due to the lack of fruit flavour and a distinct taste of metal. After four weeks of storage, the trial put in LOKA was again considered acceptable due to that the metal taste had disappeared.

Since the fermentation product was not put in juice, it was tasted by itself. It had a strong savoury and salty flavour.

5.4.3 Ingredient 5

The entire result from the sensory evaluation of juice containing *L. plantarum* can be seen in Table 15 in the appendix. The sensory evaluation of the sample containing ingredient 5 showed an acceptable taste with an umami off-flavour during week 0 for both LOKA and Mango juice. After 2 weeks of storage, the off-flavour was not as apparent anymore and both samples were considered well tasting. However, after four weeks of storage the mango juice was still well tasting while an umami off-flavour had reappeared in the LOKA.

The sensory evaluation for the juice containing *L. rhamnosus* done on one of the duplicates can be seen in its entirety in Table 16 in the appendix. During week 0, both samples had a slight umami aftertaste but was still considered well tasting. After 2 weeks of storage, the sample in LOKA was considered acceptable due to a stronger umami/salty aftertaste which was still present after 4 weeks of storage. The sample containing Mango had a slight aftertaste of umami during the entire 4 weeks of storage but was still considered good.

Since the concentration of *L. paracasei* in the fermentation product was below the criteria to be put in juice, the fermentation product alone was tasted. It had a strong taste of umami and it was very acidic. One of the participants of the sensory evaluation said that these samples were the most disgusting thing she had ever tasted.

5.4.4 Combining ingredient 3 with ingredient 5

None of the samples were put in juice and the sensory evaluation will be on the fermentation products. The samples had an acidic taste with a clear sensation of umami and one other unidentified component. When comparing with the original recipe, this combination was not as good as the original.

5.4.5 Ingredient 6 and 7

Since none of the samples containing *L. plantarum* qualified to be put in juice, there will not be a sensory evaluation. However, the fermentation product was tasted and was quite nice. The sour taste that is usually present in the fermentation products was not there but the samples had rather a taste of vegetables. However, this might be more preferred when doing vegetable drinks containing probiotics. In addition, since the samples contained a large amount of the ingredients, it sank to the bottom of the containers fast, resulting in a not so pleasant appearance.

None of the samples contained high enough amount of *L. rhamnosus* to be put in juice. As for the taste of the fermentation product, it had the same characteristics as the fermentation product containing *L. plantarum*. The taste of the fermentation product containing *L. paracasei* had the same sensory characteristics as the fermentation product containing the other bacteria.

5.4.6 Ingredient 8

The entire table with the results from the sensory evaluation of juice containing *L. plantarum* and ingredient 8 can be seen in Table 18 in the appendix. All samples were considered good during all the weeks of storage except for one duplicate of LOKA during the fourth storage week that had a slight off-flavour of metal/minerals and was considered acceptable.

Since the content of *L. rhamnosus* in the fermentation product was not high enough to be put in juice, the fermentation product was tasted as it was. It had a sweet taste, suggesting that the sugar source in the fermentation product was not fully utilized. It was not as acidic and had a taste of vegetables. As for *L.* rhamnosus, the fermentation product containing *L. paracasei* was not acidic, as expected, and had a taste of cereals and soil. Since the acidic taste was avoided, these fermentation products might be easier to cover in a fruit drink.

5.4.7 Ingredient 9

Since the samples containing *L. plantarum* did not qualify to be put in juice, the fermentation product was tasted. The taste was mild and not as acidic as fermentation products usually are. It had a slight taste of vegetable.

No sensory evaluation will be done on the samples containing *L. rhamnosus* since the content of bacteria was non-existing in the fermentation product. The fermentation products containing *L.*

paracasei did not qualify to be put in juice and they were therefore tasted as they were. The taste profile was not acidic which was expected. It had a taste of grass and cereals which might be easy to cover when put in a fruit drink.

5.4.8 Increasing the fermentation temperature for *L. rhamnosus*

Due to lack of time, the fermentation product was not put in juice but was tasted as it was. The taste was acidic and reminded of Swedish sour milk (filmjölk). There was no clear off-flavour of umami.

6 Conclusion

The ingredients that gave the significantly highest bacterial growth for *L. plantarum* is 1.5 g ingredient 2 + 1.5 g ingredient 3, 3.0 g ingredient 5, 1.5 g ingredient 3 + 1.5 g ingredient 5, 1.0 g ingredient 2 + 1.5 g ingredient 3, 3.0 g ingredient 4 and 0.5 g ingredient 2 + 1.5 g ingredient 3. All these ingredients gave, according to the statistics, equally successful growth compared to the original recipe. In addition, they were also all good enough to be put in juice. However, due to lack of time, the trials containing 1.5 g ingredient 2 + 1.5 g ingredient 3 and 1.5 g ingredient 3 + 1.5 g ingredient 5 was not put in juice and can not be evaluated according to their sensory properties. The other ingredients put in juice were all considered either acceptable or good.

The trials that gave the significantly highest growth of *L. rhamnosus* are 1.5 g ingredient 2 + 1.5 g ingredient 3, 1.5 g ingredient 3 + 1.5 g ingredient 5 and 3.0 g ingredient 4. The trial containing 1.5 g ingredient 3 + 1.5 g ingredient 5 was not put in juice due to lack of time and was not subjected to sensory evaluation and could therefore not be considered further. The other two trials were put in juice but with less satisfactory results. The trial containing 1.5 g ingredient 2 and 1.5 g ingredient 3 in Mango and the trial containing 3.0 g ingredient 5 in LOKA was considered bad during one of the test weeks. In addition, for the trial with 1.5 g of both ingredient 2 and 3, no sample was ever considered good. None of the samples containing *L. rhamnosus* can therefore be considered good enough regarding the sensory aspect.

For *L. paracasei*, the trials that showed significantly the highest growth were 1.5 g ingredient 2 + 1.5 g ingredient 3, ingredient 6, ingredient 7, 1.5 g ingredient 3 + 1.5 g ingredient 5, 1.5 g ingredient 2 + 1.5 g ingredient 3 for +2 hours and 3.0 g ingredient 4. However, comparing the bacterial content of *L. paracasei* with the other two bacteria can not be considered a good idea since the growth is generally lower for *L. paracasei.* It was therefore not possible to put any sample in juice due to that the criteria for putting fermentation product in juice was set according to *L. plantarum* and should be reconsidered when fermenting *L. paracasei.*

In conclusion, the ingredients that gave the highest bacterial content and best results from the sensory evaluation would be the ingredients used with *L. plantarum*. Due to the insufficient growth of *L. paracasei* and the unsatisfactory result from the sensory evaluation of *L. rhamnosus*, neither of those bacteria are considered as good as the samples containing *L. plantarum*.

7 Future suggestions

It would be interesting to investigate what the difference is between *L. paracasei* and the other bacteria and why it does not grow as well as the other bacteria. There might be some essential nutrient missing in the cultivation medium that is important for growth and it would be beneficial to identify that component to be able to increase the growth.

Another suggestion would be to try other concentrations of ingredient 4 and 5, e.g. 2.0 g/600 ml, and investigate whether that will give a better growth than using 1.5 g/600 ml and a better taste than using 3.0 g/600 ml.

Due to the excellent sensory evaluation of the fermentation product containing *L. plantarum* and ingredient 8, it might be a good idea to develop this further. However, the growth was not that high when using ingredient 8. A suggestion might therefore be to combine this ingredient with a small amount of yeast extract or yeast peptone. In addition, one of the studies presented in the background suggested that adding a small amount of a yeast component to ingredient 9 will decrease the lag-phase and give a higher bacterial content for *L. plantarum* and *L. paracasei.*

8 References

Świątecka, D. et al., 2010. The impact of pea protein hydrolysates on bacterial physiological activity—An in vitro study. *International Journal of Food Microbiology,* 15 06, 140(2-3), pp. 263-270.
Axelsson, L., 2004. Lactic Acid Bacteria: Classification and Physiology. In: S. Salminen, A. von Wright & A. Ouwehand, eds. *Lactic Acid Bacteria Microbiological and Functional Aspects.* Ås: CRC Press.
Barać, M. B. et al., 2015. TECHNO-FUNCTIONAL PROPERTIES OF PEA (Pisum sativum) PROTEIN ISOLATES- A REVIEW. *Acta periodica technologica,* Issue 46.
Berry, A. R., Franco, C. M., Zhang, W. & Middelberg, A. P., 1999. Growth and lactic acid production in batch culture of Lactobacillus rhamnosus in a defined medium. *Biotachnology letters,* Volume 21, p. 163–167.
Butler, M., Sparling, R. & Court, D. A., 2010. Energy Metabolism of Cells Used for Industrial Production. In: M. C. Flickinger, ed. *In book: Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology.* s.l.:Wiley.
Cui, F. et al., 2010. Co-production of Lactic Acid and Lactobacillus rhamnosus Cells from Whey Permeate with Nutrient Supplements. *Food and Bioprocess Technology,* 05, 5(4), pp. 1278-1286.
FAO/WHO, 2002. *Guidelines for the Evaluation of Probiotics in Food,* London Ontario: FAO/WHO.
Filannino, P. et al., 2014. Metabolic Responses of Lactobacillus plantarum Strains during Fermentation and Storage of Vegetable and Fruit Juices. *Applied and Environmental Microbiology,* 04, 80(7), pp. 2206-2215.
Görke, B. & Stülke, J., 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nature Reviews Microbiology,* 08, Volume 6, pp. 613-624.
Gao, M.-T.et al., 2008 . Utilization of rice bran as nutrient source for fermentative lactic acid production. *Bioresource Technology,* 06, 99(9), p. 3659–3664.
Garcia Maia Costa, M., Vidal Fonteles, T., Tibério de Jesus, A. L. & Rodrigues, S., 2013. Sonicated pineapple juice as substrate for L. casei cultivation for probiotic beverage development: Process optimisation and product stability. *Food Chemistry,* 08, 139(1-4), pp. 261-266.
Han, S.-W., Chee, K.-M. & Cho, S.-J., 2015. Nutritional quality of rice bran protein in comparison to animal and vegetable protein. *Food Chemistry,* 01 04, Volume 172, p. 766–769.
Helland, M. H., Wicklund, T. & Harvhus, J. A., 2004. Growth and metabolism of selected strains of probiotic bacteria in milk- and water-based cereal puddings. *International Dairy Journal,* 11, 14(11), p. 957–965.
Holzapfel , W. H. & Wood, B. J., 2014. *Lactic Acid Bacteria: Biodiversity and Taxonomy.* Chichester: John Wiley & Sons.
Kleerebezem, M. et al., 2003. Complete genome sequence of Lactobacillus plantarum WCFS1. *PNAS,* 18 02, 100(4), pp. 1990-1995.
Ma, C. et al., 2016. Determination of the essential nutrients required for milk fermentation by Lactobacillus plantarum. *LWT - Food Science and Technology*, 01, Volume 65, pp. 884-889.
Makras, L., Van Acker, G. & De Vuyst, L., 2005. Lactobacillus paracasei subsp. paracasei 8700:2 Degrades Inulin-Type Fructans Exhibiting Different Degrees of Polymerization. *Applied and Environmental Microbiology,* 11, 71(11), pp. 6531-6537.
Molin, G., 2013. *LECTURES IN PROBIOTICS.* 2nd ed. Lund: s.n.
Nualkaekul, S. & Charalampopoulos, D., 2011. Survival of Lactobacillus plantarum in model solutions and fruit juices. *International Journal of Food Microbiology,* 30 03, 146(2), pp. 111-117.
Pathak, M. & Martirosyan, D., 2012. Optimization of an effective growth medium for culturing probiotic bacteria for applications in strict vegetarian food products. *Functional Foods in Health and Disease,* 2(10), pp. 369-378.
Probi AB, 2017. *Results from API.* Lund: s.n.

9 Appendix

9.1 Plate count analysis

Below is an example of a calculation of the bacterial concentration from the number of colonies.

One dilution within the interval 25-300:

Dilution	Number of colonies	Sum	Result
-4	104	104	$104*10^4/0.1 = 1.04*10^7$
-5	12		

Two dilutions within the interval 25-300:

Dilution	Number of colonies	Sum	Result
-4	256	304	$304*10^4/1.1 = 276*10^4 \rightarrow$
-5	48		$276*10^4/0.1 = 2.76*10^7$

9.2 Refrigerator temperature

The temperature of the refrigerator before the breakage can be seen in Table 7.

Table 7. The temperature of the refrigerator before the breakage.

Date	Temperature (°C)
2017-02-13	3.4
2017-02-15	4.1
2017-02-16	3.0
2017-02-17	3.8
2017-02-20	3.9
2017-02-22	3.4
2017-02-23	4.1
2017-02-24	3.5
Average	3.7

The 27th of February, the refrigerator broke, resulting in a measurement of 21.8°C after which a new refrigerator was being used and the temperature of that refrigerator was not noted until the 10th of March, as can be seen in Table 8. As can be seen, the thermometer used further on did not have any decimals.

Table 8. The temperature of the refrigerator after the breakage.

Date	Temperature (°C)	Date	Temperature (°C)	Date	Temperature (°C)
2017-03-10	4	2017-03-29	4	2017-04-20	4
2017-03-13	4	2017-03-30	5	2017-04-21	4
2017-03-15	3	2017-04-03	5	2017-04-24	4
2017-03-16	4	2017-04-05	4	2017-04-26	5
2017-03-17	4	2017-04-06	4	2017-04-27	5
2017-03-20	5	2017-04-07	4	2017-05-02	4
2017-03-22	5	2017-04-10	4	2017-05-04	5
2017-03-23	4	2017-04-12	4	2017-05-08	4
2017-03-24	4	2017-04-18	5	Average	4.3
2017-03-27	4	2017-04-19	5		

9.3 Varying the concentration of ingredient 2 and 3

The sensory evaluation of the juice containing *L. plantarum* and varying concentrations of ingredient 2 and 3 can be seen in Table 9 where 1 represents the trial containing 0.5 g ingredient 2 and 1.5 g ingredient 3 and 3 represents the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3.

Table 9. The results from the sensory evaluation where the fermentation product containing L. plantarum and varying yeast concentrations, where 1 represents the trial containing 0.5 g ingredient 2 and 1.5 g ingredient 3 and 3 represents the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3, were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
1 – LOKA	X			However, some strawberry flavour was lost
1 – Mango	X			
3 – LOKA	X			However, some strawberry flavour was lost
3 – Mango		X		An umami off-flavour was noticed
Week 2				
1 – LOKA		X		Did not have a fruit flavour anymore
1 – Mango	X	X		One of the duplicates had an umami off-flavour while the other did not
3 – LOKA	X			
3 – Mango		X		An umami off-flavour was noticed
Week 4				
1 – LOKA		X		
1 – Mango	X	X		One of the duplicates had an umami off-flavour while the other did not
3 – LOKA	X			
3 – Mango	X			

The entire result from the sensory evaluation of juice containing *L. rhamnosus* can be seen in Table 10, where 1 represents the trial containing 1.5 g ingredient 2 and 1.5 g ingredient 3 and 3 represents one of the duplicates from the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3.

Table 10. The results from the sensory evaluation where the fermentation product containing L. rhamnosus and varying yeast concentrations, where 1 represents the trial containing 1.5 g ingredient 2 and 1.5 g ingredient 3 and 3 represents one of the duplicates from the trial containing 1.0 g ingredient 2 and 1.5 g ingredient 3, were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
1 – LOKA		X		Some umami off-flavour
1 – Mango			X	Had a lot of umami off-flavour
3 – LOKA	X			Some strawberry flavour was lost
3 – Mango	X			
Week 2				
1 – LOKA		X		Lost strawberry flavour but the umami off-flavour was not as apparent anymore
1 – Mango		X		The umami off-flavour was not as apparent anymore
3 – LOKA		X		Some off-flavour was identified
3 – Mango		X		Some off-flavour was identified
Week 4				
1 – LOKA		X		Some off-flavour was identified (umami)
1 – Mango		X		Some off-flavour was identified (salty)

3 – LOKA	X			Very little strawberry flavour but the umami off-flavour was not present anymore
3 – Mango	X			The umami off-flavour was not present anymore

9.4 Ingredient 4

The composition of ingredient 4, can be seen in Table 11.

Table 11. The composition of ingredient 4 in % (g/g).

Component	Content in % (g/g)
Dry matter	96.4
Total nitrogen	11.13
Amino nitrogen	3.73
Sodium chloride	0.15

The results from the sensory evaluation of the fermentation product containing *L. plantarum* and 3.0 g ingredient 4 put in LOKA and Mango juice can be seen in Table 12.

Table 12. The results from the sensory evaluation where the fermentation product containing L. plantarum and ingredient 4, were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
LOKA		X		Some strawberry flavour is lost
Mango	X			A bit of a salty aftertaste
Week 2				
LOKA		X		The strawberry flavour was a bit stronger when the carbon dioxide had disappeared
Mango	X			
Week 4				
LOKA		X		Had some savoury and salty off-flavours
Mango	X			

The sensory evaluation in its entirety for *L. rhamnosus* put in juice can be seen in Table 13.

Table 13. The results from the sensory evaluation where the fermentation product containing L. rhamnosus and ingredient 4 were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
LOKA		X		Some strawberry flavour was lost and an aftertaste can be felt
Mango	X			Some aftertaste can be tasted
Week 2				
LOKA			X	No fruit flavour present and a taste of metal was noticed
Mango	X			An off-flavour was barely noticed
Week 4				
LOKA		X		No fruit flavour present and the metal taste disappeared
Mango	X			

9.5 Ingredient 5

The composition of ingredient 5 can be seen in Table 14.

Table 14. The composition of ingredient 5 in % (g/g).

Component	Content in % (g/g)
Dry matter	95.80
Total nitrogen	11.60
Amino nitrogen	3.70
Sodium chloride	0.27

The entire sensory evaluation for *L. plantarum* containing 3.0 g ingredient 5 in LOKA and Mango juice can be seen in Table 15 below.

Table 15. The results from the sensory evaluation where the fermentation product containing L. plantarum and ingredient 5 were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
LOKA		X		Some strawberry flavour was lost and an aftertaste can be felt
Mango		X		Some aftertaste can be felt
Week 2				
LOKA	X			
Mango	X			
Week 4				
LOKA		X		Some off-flavours can be felt, umami
Mango	X			

The sensory evaluation of *L. rhamnosus* put in LOKA or Mango juice can be seen in Table 16 below.

Table 16. The results from the sensory evaluation where the fermentation product containing L. rhamnosus and ingredient 5 were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
LOKA	X			A slight umami off- flavour was noticed
Mango	X			A slight umami off-flavour was noticed
Week 2				
LOKA		X		A clear umami/salty off-flavour was noticed
Mango	X			A slight umami off-flavour was noticed
Week 4				
LOKA		X		A clear umami/salty off-flavour was noticed
Mango	X			A slight umami off-flavour was noticed

9.6 Ingredient 6 and 7

The composition of ingredient 6 and 7 used in the fermentation can be seen in Table 17.

Table 17. The proximate composition in % of ingredient 6 and 7.

Component (%)	Ingredient 6	Ingredient 7
Moisture content	8.0	12.0
Protein	23.5	26.0
Ash	2.8	2.6
Fat	7.0	2.5
Carbohydrates	66.0	68.0

9.7 Ingredient 8

The sensory evaluation of *L. plantarum* and 7.0 g ingredient 8 put in either LOKA or Mango juice can be seen in Table 18.

Table 18. The results from the sensory evaluation where the fermentation product containing L. plantarum and ingredient 8 were put in either LOKA or mango juice. The sensory evaluation included two participants.

Sample	Good	Acceptable	Bad	Comment
Week 0				
LOKA	X			
Mango	X			
Week 2				
LOKA	X			
Mango	X			
Week 4				
LOKA	X	X		One of the duplicates had a slight off-taste of minerals and metal
Mango	X			