Weak acids' microbial and sensorial effect on marinated herring

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Degree Project in Applied Microbiology for Engineers

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Abstract

Marinated herring is a food product usually with a sauce containing acetic acid. In this project the weak acids malic acid, citric acid and lactic acid were evaluated for use in the sauce for marinated herring instead of using acetic acid. The microbial and sensorial effects of the acids were evaluated.

From the sensorial test, the marinated herring in a sauce with lactic acid was chosen as the favourite of the majority in the sensory panel since it was milder than the rest. The type with sauce containing malic acid was the least favoured due to high sourness and unbalanced flavours. It was concluded from the sensorial test that there were only small differences between the versions. The texture of the herrings was believed by the panel to differ between the acids but texture analysis showed no significant difference.

Accelerated test and microbial tests with the pickled herring jars to evaluate the shelf life indicated that there was no big difference in the presence of microorganisms depending on the acid. In the project accelerated tests using MRS growth medium inoculated with *Lactobacillus plantarum* (*L. plantarum*) CCUG 30503 T were performed. The MRS was modified with sodium chloride and weak acids to resemble the environment in a pickled herring jar. The results indicated that *L. plantarum* can grow well in the presence of acetic acid, malic acid, citric acid, and lactic acid. Malic acid and citric acid had least effect on the bacteria while the acetic acid had the highest effect resulting in a lower growth. In the samples with lactic acid the level of *L. plantarum* was stable on a higher concentration than in the rest of the samples when the highest concentration had been reached.

Key words: Marinated herring, lactic acid, citric acid, malic acid, acetic acid, *Lactobacillus plantarum*, sensorial test, accelerated test, MRS

Sammanfattning

Marinerad, även kallad inlagd, sill är en produkt som normalt marineras i en sås innehållande ättiksyra. I detta projekt utvärderades de svaga syrorna äppelsyra, citronsyra och mjölksyra för att användas istället för ättiksyra i såsen till marinerad sill. I försöken utvärderades de mikrobiella och sensoriska effekterna av syrorna.

I ett sensoriskt test med de olika sillsorterna valdes sillsorten med sås innehållande mjölksyra som favorit av majoriteten av försöksgruppen eftersom den var mildare än de andra. På grund av för hög syra och obalanserade smaker föredrog färre personer sillsorten med äppelsyra. Samtidigt kunde man dra slutsatsen att det bara var små skillnader mellan de olika versionerna. Enligt testgruppen var texturen på fisken den största skillnaden mellan de olika sillsorterna men enligt texturanalyserna var där ingen signifikant skillnad i textur.

För att undersöka den mikrobiella hållbarheten gjordes mikrobiella och accelererade mikrobiella tester med sillburkar. Testerna indikerade att val av syra i såsen inte påverkade mängden mikroorganismer i produkterna nämnvärt. I projektet gjordes även accelererade tester med MRS näringslösningar som *Lactobacillus plantarum* (*L. plantarum*) CCUG 30503 T inokulerades i. MRS-buljongerna var modifierade med natriumklorid och svaga syror för att likna miljön i en sillburk. Resultatet antyder att *L. plantarum* kan växa bra i närvaron av ättiksyra, äppelsyra, citronsyra eller mjölksyra. Äppelsyra och citronsyra hade minst effekt på bakterierna medan ättiksyra hade störst effekt vilket resulterade i en lägre tillväxt. I proverna med mjölksyra var nivån *L. plantarum* stabil vid en högre koncentration än de andra syrorna efter att högsta koncentrationen hade uppnåtts.

Nyckelord: Marinerad sill, mjölksyra, citronsyra, äppelsyra, ättiksyra, *Lactobacillus plantarum*, sensoriskt test, accelererat test, MRS

Preface

This master thesis project was performed from January to June 2016 at the department Applied Microbiology at Lund University and at the company Orkla Foods Sverige. The practical work took place at Applied Microbiology in Lund and at Orkla Foods Sverige in Kungshamn and Eslöv.

The aim in this degree project was to investigate if it is possible and desirable to use another weak acid in pickled/marinated herring instead of using acetic acid which is the most commonly used acid today in pickled herring products. Aspects that were taken in consideration were the microbial and the sensorial effects of the acids in the fish product.

I would like to thank Orkla Foods Sverige for the opportunity to do the project and to all employees in Eslöv, Malmö and Kungshamn for all your help. I would also like to thank my supervisors at Orkla Foods Sverige Pernilla Arinder and Richard Clerselius for your great knowledge, supporting help and for guiding me through this project. Also, I would like to thank my examiner Peter Rådström, and my supervisor at Applied Microbiology Jenny Schelin for your support and your fantastic will to always help everyone with a smile on your face.

Finally I would like to thank my family, friends, and all the people around me that I have forgot to mention for your support.

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List of abbreviations

BP = Baird Parker

CFU = Colony forming unit

DRBC = Dichloran-rosebengal agar

E. coli = *Escherichia coli*

HPLC = High Performance Liquid Chromatography

LAB = Lactic acid bacteria

L. monocytogenes = *Listeria monocytogenes*

L. plantarum = *Lactobacillus plantarum*

MO = Microorganism

MRS = de Man, Rogosa, Sharpe. Substrate selective for Lactic acid bacteria

NFA = National Food Administration (Livsmedelsverket)

NMKL = Nordic Committee on Food Analysis

PCA = Plate count agar

RPF = Rabbit Plasma Fibrinogen

S. aureus = *Staphylococcus aureus*

TSA = Trypton soya agar

VRBA = Violet red bile agar

VRBG = Violet red bile glucose

1 Introduction

1.1 Marinated herring

Marinated or pickled herring is a traditional food product that has been eaten in Sweden since about 1750 (Ullenius, 2010). It plays a big part in the Swedish food tradition since it is connected to many celebrations such as Easter, Midsummer, and Christmas.

The fish used for the pickled herring is Herring (*Clupea harengus*) which is a common fish that can be found along the Swedish seacoast, in the Atlantic Ocean and the White Sea. It has silvery skin and the size of 25-40 cm. (Swahn & Söderberg, 2016) Herring is a fat fish that contains a high level of unsaturated fat and omega 3 fatty acids, vitamin B12, vitamin D and selenium and is therefore considered as a healthy fish (Lindqvist, 2008). Herring that lives in the Baltic Sea north of the Swedish city Kalmar is called Baltic herring (strömming) which is a smaller fish of 15-20 cm with a lower degree of fat compared to herring. (Swahn & Söderberg, 2016)

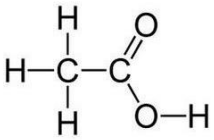
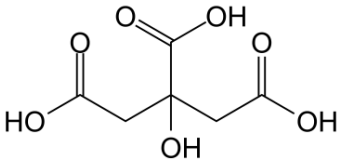
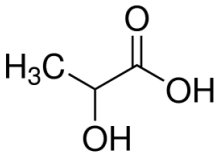
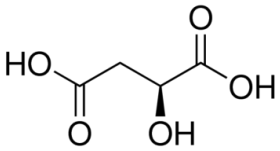
The food company Orkla Foods Sverige AB produces many types of pickled herring under the brand Abba in Kungshamn in Bohuslän in Sweden. The production of the marinated herring is done in two pickling steps but only the second step is made in Orkla's production. The herring are bought in a salt and acetic acid solution and arrives to the productions line in large barrels. (Abba, 2015) When the herring products are produced at Abba the herring from the barrels are mixed with a solution containing the traditional ingredients for pickled food which is water, acetic acid solution, salt, sugar and flavourings. The pickling solution also contains preservatives like sodium benzoate, and in some products also potassium sorbate (Abba, 2014).

Pickled herring is a chilled ready-to-eat-food that needs to be safe to eat without heating. Since the production of pickled herring do not include any heating step it is very important that the pH, temperature and salt concentration in the products are correct to achieve a safe product without spoiling microorganisms and pathogens. Dangerous pathogens that can be a risk in fish production can be the bacterium *Listeria monocytogenes* (Huss, 1994) and the spore and nerve toxin producing bacterium *Clostridium botulinum* (Livsmedelsverket, 2015). Quality degrading microorganisms (spoilage microorganisms) like lactic acid producing bacteria can also be a problem together with yeast and mould (Björkroth, Korkeala, & Lyhs, 2001).

1.2 Acids

Acetic acid (CH_3COOH) is normally used in the pickle solution but other weak acids like malic acid ($C_4H_6O_5$), citric acid ($C_6H_8O_7$) and lactic acid ($C_3H_6O_3$) should be possible to use instead as long as the correct pH is reached. The acids (Table 1) give a sour flavour and have antimicrobial effects at the same time which is two desired effects.

Table 1 The properties of the weak acids (acetic acid, citric acid, lactic acid and malic acid) used in the project.

	Acetic acid	**Citric acid**	**Lactic acid**	**Malic acid**
Chemical formula	CH_3COOH	$C_6H_8O_7$	$C_3H_6O_3$	$C_4H_6O_5$
Molecule structure	 (Ernest, 2016)	 (Nude Nicotine, 2016)	 (Sigma-Aldrich, 2016)	 (Nude Nicotine, 2016)
pK_a	pK_a = 4.76 (PubChem, 2016)	pK_{a1} = 3.09 pK_{a2} = 4.74 pK_{a3} = 5.41 (Belitz, Grosch, & Schieberle, 2009)	pK_a = 3.86 (Belitz, Grosch, & Schieberle, 2009)	pK_{a1} = 3.40 pK_{a2} = 5.05 (Belitz, Grosch, & Schieberle, 2009)
Molar mass	60.05 g/mole	192.124 g/mole	90.08 g/mole	134.087 g/mole

1.2.1 Acetic acid

Acetic acid is the acid that is most usually used in pickled herring and other pickled food products. Acetic acid can be produced by fermentation of wine (Livsmedelsverket, 2016) or by oxidation of liquid acetaldehyde in the presence of air and manganese acetate at 55-60°C. Another way to produce the acid is by oxidation of liquid butane at 200°C. (Nationalencyklopedin, 2016)

Acetic acid (E 260) can according to the Swedish National Food Administration (NFA) (Livsmedelsverket) be used without limits, but not more than needed, in food. It works as a preservative and to regulate the sourness of the food. (Livsmedelsverket, 2016)

1.2.2 Citric acid

Citric acid is a common weak organic acid that can be extracted from fruits like citric fruits and pineapple. In food it is often added as an antioxidant and preservative but also to give the food a sour taste. The preservation effect of the acid is to prevent rancidity of fat, preservation of vitamins and to prevent miscolouring of fruit product due to oxidation. Citric acid has the E-number E 330 and is commonly found in for example soft drinks, candy, and jam. Even though citric acid can be found naturally in fruit it is also common to produce it by fermentation of molasses or glucose by using the mould *Aspergillus niger*. (Livsmedelsverket, 2015) Citric acid can according to NFA be used in most food products without restrictions. The added amount of citric acid should not be more than needed. (Livsmedelsverket, 2016)

1.2.3 Lactic acid

Lactic acid is a weak organic acid that can be produced by fermentation of carbohydrates using lactic acid bacteria (LAB). Then sucrose/glucose is mixed with water and chalk/lime and left to ferment with LAB in a fermenter. In the fermentation crude calcium lactate is produced that have to be purified and separated from gypsum into crude lactic acid. After purification and concentration the end product is lactic acid which usually is sold in liquid form. (Corbion Purac, 2016) The chemical way of producing lactic acid is mainly by hydrolysis of lacto nitrile using strong acids. It is also possible to produce lactic acid in other ways for example base-catalysed degradation of sugars and oxidation of propylene glycol. (Rojan, Nampoothiri, & Pandey, 2007)

Lactic acid (E 270) is used in food to regulate the sourness and taste of the food and as a preserver. According to NFA there are no restrictions of how much lactic acid that can be added in food but one should not use more than is needed. (Livsmedelsverket, 2016)

1.2.4 Malic acid

Malic acid (E 296) can be found natural in most fruits like apples, nectarines, mangos, bananas etc. It is used in food products like soft drinks, candy, and ready-to-eat soups. In food it regulates the acidity and works as a preservative. (Äkta vara, 2008) (Acidpedia, 2016) The acid can according to NFA be used without limits in most food as long as not more than needed is added (Livsmedelsverket, 2016) Malic acid are produced synthetically from butane (Caldic, 2016).

1.3 The taste of acids

Taste is a very complexed area where the sensations of the flavours, temperatures, smells, and textures are combined to the experience of the food (Institute for Quality and Efficiency in Health Care, 2012). Usually it is declared that we have five basic tastes including sweetness, bitterness, saltiness, sourness and umami. The flavours are detected in the mouth especially by the tongue where lots of taste buds are found. The taste buds have taste pores with receptors that register the taste and then send a signal with different nerves to the brain where the sense is registered. Some of the taste pores are selective and can only register one kind of taste while other can register more than one of the basic tastes. (Förare Winbladh & Sandström, Lukt och Smak, 2011) Since most food contains more than one flavour the total taste of the food is a whole spectrum of flavours that give the experience of the food.

To notice the sour taste only a quite small amount is needed. To be able to notice the sour taste of citric acid a level of 0.003 % of acid in water is needed. It can be compared to that to notice sugar in water a level of 0.5 % is needed. (Förare Winbladh & Sandström, Lukt och Smak, 2011) Which parameters that determine the intensity of the sour taste have been debated. Food that has a low pH usually has a higher sourness compared to food with a higher pH. According to a study with pickled cabbage a suggested explanation of the sour taste is that what determines the taste is a combination of the molar concentration of the acids and the concentration of hydrogen ions (H^+) in the food.

“Sour taste intensity of a solution is linearly related to the sum of the molar concentration of all of organic acids containing at least one protonated carboxyl group (e.g. ethanoic acid in the form CH_3COOH, but not that as CH_3COO^-), plus the molar concentrations of hydrogen ions.” (Coulate, 2009)

The sour taste of acetic acid, citric acid, lactic acid and malic acid is not the same at the same pH. Acetic acid is regarded to have a sharp and penetrating taste and only a low concentration is needed to elicit the taste. The explanation for this might be that it is volatile and therefore it can irritate in the nose and on other mucous membranes. The taste of citric acid on the other hand is often regarded as a mild and fresh taste. Malic acid has a quite sharp but fruity taste compared to the much more round, mild and discrete taste of lactic acid. (Förare Winbladh & Sandström, 2011)

1.4 The microbial effect of weak acids

The anti-microbial effect of acids mainly depends on if the acids can enter the cells of the microorganisms or not. To enter the cell wall the acid molecules need to be undissociated (the molecule has not released its hydrogen) and to have an effect on the microorganisms the acid should dissociate inside the cell. The pH of the surrounding solution and the pK_a of the acids greatly affect the ability to enter the cell. (Ermen & Faruk Bozoglu, 2016)

When the pH in the surrounding is equal to the pK_a of the acid, 50 % of the molecules have dissociated. Then they are charged and cannot pass through the cell wall. Since pH is a logarithmic scale it means that when pH = pK_a + 1 the level of dissociated acid is 10 times higher than the undissociated molecules. (Adams & Moss, 2008) If the surrounding pH is higher than the pK_a up to 100 % of the acid are dissociated and when the pH is lower up to 100 % of the acid are undissociated. The principle of the level of dissociated acid with increased pH is shown in Figure 1 where the percentage of dissociated acid is increasing with the pH. In the figure the pK_a of the acid is marked.

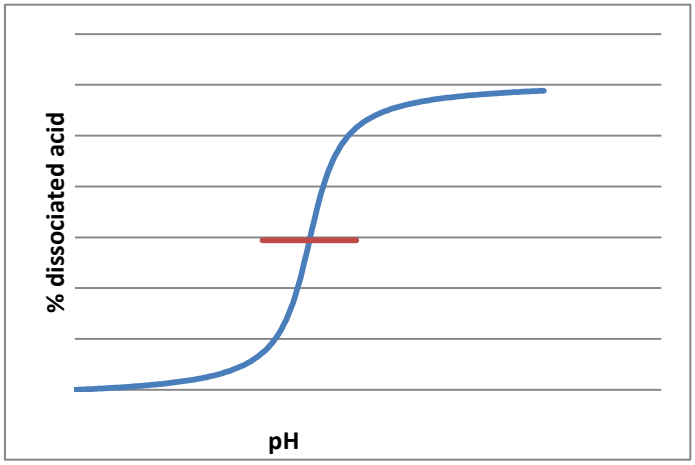


Figure 1 The principle of how much of an acid that is dissociated depending on the pH of the surrounding is shown in the figure. At the pK_a (marked in red) 50 % of the acid is dissociated (Adams, 2008). The picture is inspired by (Russell & Gould, 2003)

It can be concluded that the microbial effect of a weak acid is depending a lot on the pH of the surroundings. If the acid is too strong (low pK_a) it will be completely dissociated outside of the cells and it can therefore not go into the cell. Strong acids can on the other hand affect the microorganisms by denaturing proteins and enzymes of the surface of the cells. If the difference between the internal and the external pH of the cells is high the proton permeability can be increased due to increased pH gradient and this can lead to a decrease of the internal pH. (Ermen & Faruk Bozoglu, 2016)

Most organic acids are more or less hydrophobic when they are undissociated. Due to that, the acids can go through the wall by the concentration gradient. The principle of how a weak acid passes through the cell wall is shown in Figure 2. Inside the cells the higher pH in the cytoplasm makes the acid to dissociate and release protons (H^+) which will lower the pH in the cells. To be able to produce more cells the pH in the microorganism usually has to be around 7 (neutral). When the dissociated acid lowers the pH in the cells enzymes, proteins and other parts of the cell can be affected and

destroyed. To maintain the neutral pH the cell needs to pump out the protons and this demands a lot of energy (ATP) and therefore the growth of the microorganisms is inhibited. After some time of low production of new cells the level of microorganisms will decrease until all of them have died. (Ermen & Faruk Bozoglu, 2016)

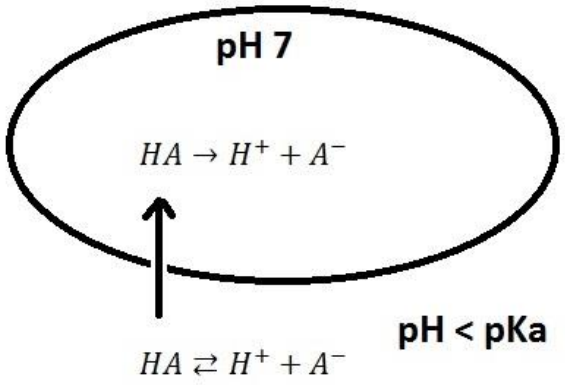


Figure 2 The picture shows the principle of how an undissociated weak acid (HA) can enter the cell wall of a microorganism. Inside the cell the pH is higher than the pK_a and the acid releases a proton (H^+). The cell needs to pump out (costs energy) the protons to maintain the pH neutral in the cell. The picture is inspired by (Russell & Gould, 2003)

1.5 Lactic acid bacteria and *L. plantarum*

L. plantarum is included in the genera LAB that produces lactic acid by fermentation of carbohydrates. LAB are Gram-positive and non-spore forming bacteria that can have the forms cocci, coccobacilli or rods. LAB usually live in oxygen free environments but they can also grow in the presence of oxygen. To be able to grow LAB need to have purines, pyrimidines, vitamins and many amino acids available. (Todar, 2012) Since LAB include many genera and species it is hard to set a general range in water activity, temperature, pH and other parameters where the LAB can grow or survive. Some species tolerate very low pH while other prefer more neutral pH. In general most LAB have their optimal start pH at 6-7 but due to the production of lactic acid the pH will be decreased until the pH has reached a level where the LAB will not produce lactic acid and therefore the pH will be stable. The optimum temperature for LAB is in general 20-40°C but some species like *Lactobacillus delbrueckerii-* spp *delbrueckerii* can grow in temperatures up to 55°C while other LAB like *Lactobacilli* can grow in temperature as low as 0°C. (Björkroth, Holzapfel, & Schillinger, 2006)

L. plantarum belongs to the genus Lactobacillus and is a facultative heterofermentative LAB. This means that they can either be heterofermentative or homofermentative depending on the environment. The heterofermentative fermentation of sugars results in lactic acid, carbon dioxide and ethanol or acetic acid while the only final product of homofermentative fermentation is lactic acid. (Dahlgren, Lindgren, Lärn-Nilsson, & Pedersen, 2016) *L. plantarum*'s optimal temperature is 15-45°C but it can survive in even lower and higher temperatures. The optimum start pH for the bacteria is in-between pH 5-6 but they can grow in pH as low as pH 3.2. (LaHaye & McIntyre, 2010) (Citizendium, 2010) (Fu & Mathews, 1999) (Cota & Stãnilã, 2013)

L. plantarum can cause both spoilage and desired effects in different food products. *L. plantarum* has together with other *Lactobacillus* species been found in spoiled marinated herring products. Other detected species of the genus *Lactobacillus* in spoiled semi-preserved fish products are *L. brevis, L.*

fermentum, and *L. leichmannii*. In semi-preserved marinated fish products like pickled herring the spoilage effect of LAB can be off-flavours, off-odours, low pH, and production of gas (CO_2). The gas production can be a result of so called “protein swell” which is decarboxylation of amino acids. (Björkroth, Holzapfel, & Schillinger, 2006)

L. plantarum is unwanted in marinated herring but in other products it can have a positive impact on the food by giving it probiotic properties. The fruit drinks of the brand Proviva contain the probiotic strain *L. plantarum 299v* that seems to have a positive effect on the stomach and the immune system. *L. plantarum* 299v is a suitable probiotic bacterium since it survives from mouth to colon through the human digestion system with varying pH from pH 1 in the stomach to pH 8 in the duodenum. (Naturligt om Hälsa, 2016) This was concluded in a study from 2005 where the faecal samples from people that had consumed *L. plantarum* 299v were examined (Goossensa, et al., 2005)
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2 Aim of study

The purpose in this project was to investigate how the use of citric acid, lactic acid, and malic acid instead of acetic acid in marinated herring would affect the flavours and the microbial effect of the fish. The aims were to:

1. Examine whether the acids could change the sensorial aspect of the herring and then achieve a changed product with new flavours and other characteristics that can attract more consumers at same time as the product is microbial safe.
2. Investigate how the growth of the pickled herring spoiling bacteria *L. plantarum* would be affected in a simulated herring product using MRS stock with citric acid, lactic acid, acetic acid, and malic acid.

3 Material and method

In this project pickled herrings with four weak acids were produced to evaluate the microbial effects and the sensorial effect of the acids. The four acids that were taken into consideration were acetic acid, lactic acid, malic acid, and citric acid. In the project two types of microbial tests were performed to measure the shelf life, with the real product and with MRS (de Man, Rogosa, Sharpe) growth medium. In Experiment 1 some herring jars were incubated in room temperature (accelerated shelf life evaluation) and some were stored in refrigerator (shelf life evaluation). A sensory evaluation with the four produced herring versions was performed to assess the fish products. In Experiment 2 the growth of *L. plantarum* inoculated in modified MRS growth medium was measured. The MRS was modified in pH and sodium chloride concentration to resemble the environment in a marinated herring jar.

3.1 Production of pickled herring samples for Experiment 1 and sensory evaluation

The production of the brand Abba's products takes place in the locality Kungshamn in Bohuslän in Sweden. In the factory four different types of pickled herring was prepared for this study. They were prepared close to the ordinary production line of pickled herring to get the same microflora as the regular pickled herring products from the factory contain. The recipe of the pickled herring was "Abba sill på 5 minuter" ("Abba herring in 5 minutes", also known as 5-minuterssill) which is a product of pickled herring with a neutral taste that one can use in a homemade sauce to get ready-to-eat herring in a fast way. The ingredients of "Abba sill på 5 minuter" are herring, sugar, salt, acetic acid solution, sodium benzoate and spice extracts (Abba, 2014).

Four versions of herring were produced and in all of them the same ingredients were used except for the acetic acid that was replaced by another weak acid in three of the versions. The weak acids that were used were liquid lactic acid (Galactic), crystalline citric acid (Jungbunzlauer), and crystalline malic acid (Polynt). The acids were added in a concentration so that the pH of the whole content of the jar including fish and pickling solution was pH 4.2 (± 0.1) which is the same pH as the original recipe with acetic acid. The version according to the original recipe with acetic acid was the control. The required amount of malic acid, citric acid and lactic acid to reach the correct pH was determined by using the K_{a1} values ($K_a = 10^{-pKa}$) of the acids and then calculate how much of the acids that was needed to get the same concentration of hydronium ions ($[H_30^+]$) as in the sauce with acetic acid. The buffering effect of the pickle solution was by mistake not taken into consideration and more than the calculated required amount of acids was used. For the calculations equation 1 was used. See calculations and discussion in Appendix 9.1.

The estimated reaction of the weak acid: $HA \leftrightarrows H^+ + A^-$

The dissociation constant: $K_a = \frac{[H^+]\times[A^-]}{[HA]}$ Eq. 1

HA = The weak acid in undissociated form
$H^+ = H_3O^+$, hydronium ions
A^- = The dissociated form of the weak acid
K_a = The dissociation constant for the weak acid

The four different pickles for the herring were prepared by weighting and mixing the ingredients by hand in the test kitchen and then the plastic containers with sauces were stored overnight in a cold room. All of the ingredients were added one by one with some stirring between each addition. The acids were added as the last ingredients to decrease the risk of reaction and flocculation which gives a turbid solution. Since the acids are not evenly strong and in different concentration not the same amount was added and therefore extra water was added to reach the same volume in all of the sauces. The malic acid and citric acid were in crystalline form and therefore the acids were dissolved into the extra water before addition to the rest of the ingredients.

A volume of sauce corresponding to 35 jars of herring was produced of each type by dividing the original recipe of 850 kg of sauce into smaller batches. The day after, the assembling of the herring jars were performed by weighting the fish (115 g ± 2 g) and then the sauce (125 g ± 2 g) to achieve 48 % fish and 52 % sauce in the jar containing 240 g. All the four versions (30 of each) were assembled at the same time. After assembling the jars were closed with lids and sealed with a machine that creates lower pressure in the jar. Due to problems with the machine only about 1/3 of the jars were sealed and the rest were store with lids in the cold room overnight and then sealed the day after. The jars were then stored again for a couple of days (up-side-down, to decrease the contact between air and fish) in the cold room until the jars were transported under cold conditions from Kungshamn to Eslöv. In Eslöv, the jars were store up-side-down in a refrigerator (7-8°C) up to 14 weeks.

3.2 Experiment 1: Accelerated shelf life evaluation with herring jars

The shelf life of the pickled herring jars produced in Kungshamn were examined in shelf life by performing an accelerated test where six jars of each type were placed in an incubator at 22.6°C for 15 days. In the test herring jars with the citric acid, acetic acid, malic acid, and lactic acid that had been stored in refrigerator for 19 days before the test started were used. Microbiological tests were performed with three jars of each acid after 7 and 15 days of incubation. The tests that were performed to show eventual contaminations of microorganisms were aerobic microorganisms, *Enterobacteriaceae*, thermostable coliform bacteria (especially *Escherichia coli*), coagulase positive *Staphylococcus (S. aureus*), LAB, yeast, and mould. In the first test, the herring that had only been stored in refrigerator since production were also examined for *Listeria monocytogenes* including the other mentioned microorganisms. The microbiological tests are the standardized methods based on Nordic Committee on Food Analysis' (NMKL) standards.

In the sampling for the microbial tests of *Enterobacteria*, thermostable coliform bacteria, coagulase positive *Staphylococcus*, LAB, moulds and yeasts three jars of each herring type were examined in every test. 10-15 g of herring without sauce were put into a sterile blender bag (Curved 400 180mm x 300mm x 70mu, VWR), peptone solution were added automatically to a concentration of 1:10 (herring: peptone solution) before the bag with content were mashed in a Stomacher machine (400 Circulator) for 30 seconds. The dissolved samples were pipetted into petri dishes with/without agar and selective or nonselective nutrition agar solutions for the analysed microorganisms were added. The concentrations of fish sample and dilutions solution were 1:10 or 1:100 and as diluent fluid peptone solution was used. The methods of the tests are described more accurate below. The substrates for the tests were prepared before by employees at the quality department at Orkla Foods Sverige in Eslöv where the tests took place.

The substrates used in the tests were: Tryptone soya agar (TSA) (CM0131, Oxoid), Violet red bile glucose agar (VRBG) (CM0485, Oxoid), Violet red bile agar (VRBA) (CM0107, Oxoid), Plate count agar (PCA) (CM0325, Oxoid), Dichloran-rosenbengal agar (DRBC) (CM0727, Oxoid) with antibiotic solution of Chloramphenicol and Chlortetracycline (0.5 %), MRS-aB agar (CM0361B, Oxoid), Half Fraser stock (3555797, ScanDiagnostics), and RAPID L. mono plates (3563964, ScanDiagnostics).

As dilution fluid a peptone solution containing 0.1 % neutralized bacterial peptone (LP0034B, Oxoid) and 0.85 % Sodium Chloride (1.06404.1000, Merck) dissolved in RO water was used.

3.2.1 *Enterobacteriaceae*

The family *Enterobacteriaceae* includes pathogens like *Escherichia coli*, *Salmonella*, and *Enterobacter*. They can be found in earth, in animals gut etc. and are non-spore forming and facultative anaerobic. (Christiansson, 2016) The method below is based on NMKL method No. 133, 3rd edition, 2005 and NMKL procedure No. 23, 2008.

1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were pipetted into an empty petri dish and then about 5 ml of melted TSA (45.0 ± 1.0°C) were added. The petri dish with lid on was circulated counter clockwise and clockwise to mix the solutions. The samples were left in room temperature (20-25 °C) for 1-2 hours. After this 10-15 ml of VRBG-agar (45.0 ± 1.0°C) were added to the now solidified TSA. When the VRBG-agar had solidified, the petri dishes were turned up-side-down and placed in an incubator (Termaks serie 6000) in 37 ± 1.0°C for 24 ± 2 hours before enumeration.

3.2.2 Coagulase positive *Staphylococcus* (*Staphylococcus aureus*)

This method analyses the presence of the coagulase (an enzyme) positive *Staphylococcus.* Baird Parker (BP) with rabbit plasma fibrinogen (RPF) agar plates (43531, Biomérieux) which are selective for *Staphylococcus aureus* were used. *S. aureus* can for example be found in the mouth and nose in humans and on the skin (Mandal, 2012). The method below is based on NMKL method No. 66, 5th edition, 2009 and NMKL procedure No. 23 2008.

0.1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were pipetted onto the petri dish with selective agar medium for *S. aureus*. The drop of sample was spread on the agar surface with a spreader. The petri dishes were incubated in 37 ± 1.0°C for 48 ± 4 hours before enumeration. When using the BF and RPF the colonies should be grey/black surrounded by an opaque zone.

3.2.3 Coliform bacteria and *Escherichia coli*

This method indicates presence of bacteria that are gram negative, non-spore forming, rods that ferment lactose and produces acid and gas if they are able to grow within 24 hours in 44°C. *E. coli* are included as possible bacterium. Coliform bacteria are found in the digestive track in humans and animals and can also be found in soil and on plants (New York State Department of Health, 2011). The method below is based on NMKL method No. 125, 4th edition, 2005 and NMKL procedure No. 23, 2008.

1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were pipetted into an empty petri dish and then about 5 ml of melted TSA (45.0 ± 1.0°C) were added. The petri dish with lid on was circulated counter clockwise and clockwise to mix the solutions. The samples were left in room temperature (20-25 °C) for 1-2 hours. After this 10-15 ml of VRBG-agar (45.0 ± 1.0°C) were added to

the now solidified TSA. When the VRBG-agar had solidified the petri dishes were turned up-side-down and placed in an incubator in 44 ± 0.5°C for 24 ± 2 hours before enumeration. The colonies of coliform bacteria should be dark purple colonies with a diameter of 0.5 mm with a reddish zone around.

3.2.4 Total aerobic microorganisms

This method gives an overview if the products are contaminated by any aerobic microorganisms that can grow at 30°C. The substrate for this analyse is Plate Count Agar (PCA). The method below is based on NMKL method No. 86, 5th edition, 2013 and NMKL procedure No 23, 2008.

1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were diluted in 9 ml of dilution fluid to the concentration 1:100 and then 1 ml of diluted sample was pipetted into an empty petri dish. 15-20 ml of PCA (45.0 ± 1.0°C) was added to each petri dish and the petri dishes were circulated (with lid on) counter clockwise and clockwise to mix the solutions. The petri dishes were places up-side down and incubated for 72 ± 6 hour in 30.0 ± 1.0°C before enumeration.

3.2.5 Yeast and mould

Yeast and mould can be found in the air, in soil etc. Mould can produce toxin and spoil the food while yeast only spoils food. (Frase, 2011)

In this method yeast and mould selective medium (DRBC) was used. Antibiotics are added to the medium to avoid growth of bacteria. The method below is based on NMKL method No. 98, 4th edition, 2005 and NMKL procedure No. 23, 2008.

1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were pipetted into an empty petri dish and then 15-20 ml of DRBC with antibiotic (45.0 ± 1.0°C) were added to each petri dishes. In some of the test the sample was diluted to 1:100 with peptone solution before pipetting 1 ml of the sample into the petri dish. In the measurements of the jars that had not been incubated the diluted fish sample was diluted again in 9 ml of dilution fluid to the concentration 1:100 before addition to the petri dish. The petri dishes were circulated (with lid on) counter clockwise and clockwise to mix the sample solution and agar. The samples were incubated (25.0 ± 1.0°C) for 5-7 days before enumeration.

3.2.6 Lactic acid bacteria

For analysis of presence of LAB a selective MRS-aB medium was used. LAB are bacteria that produce lactic acid by fermentation of carbohydrates. *Lactobacillus*, *Streptococcus,* and *Lactococcus* are examples of LAB. (Dahlgren, Lindgren, Lärn-Nilsson, & Pedersen, 2016) The method below is based on NMKL method No. 140, 2nd edition, 2007.

1 ml of the 1:10 (herring: peptone solution) mixture from the sampling were pipetted into an empty petri dish and then the MRS-aB agar (45.0 ± 1.0°C) was added. In some of the test the sample was diluted to 1:100 with peptone solution before pipetting 1 ml of the sample into the petri dish. The petri dishes were circulated (with lid on) counter clockwise and clockwise to mix the solutions and then left to solidify. The plates were incubated for 5 days in (25.0 ± 1.0°C) before enumeration.

3.2.7 *Listeria monocytogenes*

Listeria monocytogenes is a gram positive bacterium that can grow in low temperatures like in a refrigerator. It is a pathogen and can cause illness to humans. The method below is based on NMKL procedure No 23, 2008.

25 g of the fish were weighted in a sterile plastic bag. 225 ml of Half-Fraser stock were added and the bag was homogenized in a Stomacher for 30 seconds. The whole bag with content was then incubated in 25.0 ± 1.0°C for 24 ± 2 hours. 0.1 ml of the incubated mixture was pipetted to a petri dish with selective RAPID L. mono agar and the spread out with a spreader. The samples were incubated for 24 ± 2 hours in 37 ± 1.0°C followed by enumeration. The colonies of *L. monocytogenes* should be blue 1-2 mm in diameter colonies without a yellow zone. The result is given in positive or negative.

3.3 Shelf life evaluation of marinated herring jars

The herring jars with the four versions of sauces that were produced in Kungshamn were stored in refrigerator (7-8 °C) in Eslöv for up to fourteen weeks. To measure the concentration of microorganisms and thereby the shelf life of the pickled herring several microbial tests were performed. The tests that were performed were the same as mentioned in Accelerated shelf life evaluation with herring jars. The tests were made when the herrings had been stored for three, twelve and fourteen weeks. The tests after fourteen weeks were mainly performed as an extra control due to the results from the tests after twelve weeks of storage in refrigerator.

3.4 Measurements of pH

Connected to the microbial tests the pH of the pickled herring sample used in the bacterial analysis was measure. The fish and the sauce were mixed together with a stick blender into a homogenous mixture. The pH was determined with a pH meter (TIM 865 titration manager Radiometer) by putting the mixture into a cup with a magnet stirrer, measure the pH and then read the pH value when it was stable. Between each measurement the pH meter was sprayed with distilled water and then very gently dried with soft paper tissues.

3.5 Measurement of preservatives (benzoic acid)

The herring samples in the shelf life evaluation that had been stored for three weeks in refrigerator were also analysed for the content of the preservatives (benzoic acid). For this HPLC (high-performance liquid chromatography) (Varian ProStar UV-detector model 230) was used. Since all of the herring samples are from the same four batches, the content of preservatives was only measured once. The method to measure preservatives by using HPLC is based on NMKL method No. 124, 2nd edition, 1997.

The blended fish and sauce samples from the pH measurements were used and one sample from each jar was measured (in total 12 samples). 5 g of the fish mixture were weighted and mixed with 30 ml of MilliQ water in a 100 ml flask. The flask was shaken for 30 seconds and then 60 ml of methanol were added and the flask was shaken again. The flask was left in water to cool down and then another 100 ml of methanol was added. About 20-30 ml of the samples were poured through a filter paper (Whatman 40, d=15 cm). The first 10 ml of the filtrate was discarded and then 400 µL was injected through a membrane filter (45 µm) into viols with 1200 µL milliQ water. After this, the samples were ready to be analysed in the HPLC.

3.6 Texture analysis

The texture and firmness of the herring fillets were examined by measuring them with texture analyser (Stable Micro System) in Figure 3. The firmness was measured with a cylindrical pin P11 compression with pre-test speed 1.00 mm/sec, test speed 5.00 mm/sec and the post-test speed 10 mm/sec. The target mode was strain 50.00 % and the trigger type was auto (force) with a trigger force of 5.0 g.

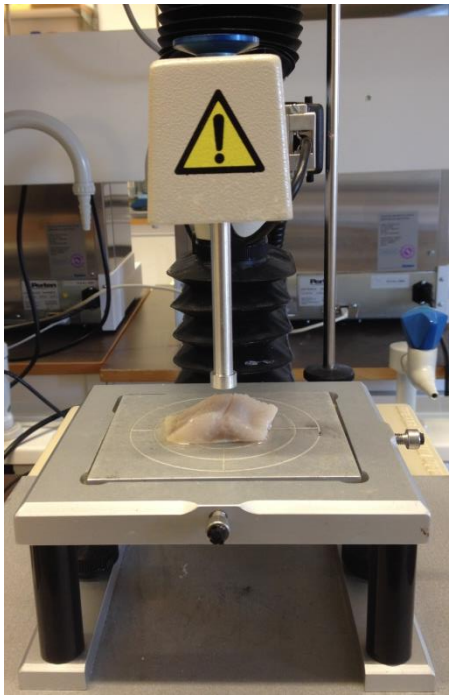

Figure 3 The texture analyser used to analyse the texture of the marinated herring. The fish was placed according to the figure with the skin side down.

Texture analysis of the pickled herring was performed two times. The first time was when the herring had been stored for four weeks in refrigerator and the second time was after thirteen weeks of storage. In the first analysis three jars of each type were used and three pieces of fish fillets from each jar were examined. In the second analysis only one jar of each type was used and eight pieces of fish fillets from each jar were measured.

The fish pieces were lying with the skin side of the fish turned down and the chosen pieces were as equal as possible in size between the jars. The principle of the measurements was that the cylinder went down towards the fish, the fish was partly penetrated and then the cylinder moved up again. The force (weight, kg) needed vs. time was recorded during the measurements and plotted against each other. In the measurements of the herring only the largest needed forces were taken into consideration. To determine eventual significant difference between the average results F-tests in Anova were used.

3.7 Sensory evaluation

To evaluate taste and texture of the pickled herring with different acids a sensory test was performed at Orkla Foods Sverige in Eslöv 5-6 weeks after production of the herring. The participants in the sensory panel were employees at Orkla Foods Sverige in Eslöv with or without experience of sensory evaluation. The total participants were 14 and the test was performed by letting the panel try the four herrings that were coded with three digits. The participants were asked to drink water between each sample and then fill out a questionnaire (see Appendix 9.2) where they should evaluate sweetness, sourness, saltiness, firmness and the general taste of the herring in a hedonic scale between 1 and 10. Also, the participants were asked to rank the versions 1 to 4 (from best to worst) and comment if there were a clear difference between the herrings. To evaluate the results F-tests in Anova were used.

A second sensory evaluation was planned to be held at Orkla Foods Sverige in Malmö 13 weeks after production of the pickled herring. Due to the results of the microbial test of the herring the second sensory evaluation was postponed to the future. New microbial tests (14 weeks after production) were performed as an extra control.

3.8 Experiment 2: Growth of *L. plantarum* in modified MRS nutrient medium

L. plantarum is a lactic acid bacterium that has been found in spoiled pickled herring (Björkroth, Korkeala, & Lyhs, 2001). To determine how the bacteria survive in a pickled herring jar, accelerated tests with modified MRS broth were performed. The samples with MRS were modified to have a salt concentration of 2.8 % which is the same concentration as in the pickled fish from Abba. The pH was modified by using acetic acid, citric acid, malic acid, and lactic acid to pH 4.2 and 5.0. The pH in the pickled herring should be less than 4.5 (McLay, 2001) and the pH 5.0 was used to see if there were any differences in the survival of the bacteria or if it would be possible to use a higher pH in the product when focusing on the LAB. Two independent experiments were performed with one day in between. The results from the experiments were evaluated with one-tailed t-test.

For the trials strain *Lactobacillus plantarum* CCUG 30503 T was used. The other used substances were MRS broth (1106610500, Merck KGaA), MRS agar (1106600500, Merck KGaA), Sodium chloride (1064041000, Merck KGaA), citric acid, malic acid, lactic acid 80 % food (LAFCL80, Galactic), and 24 % acetic vinegar (ättika) for pickling (Perstorp).

3.8.1 Preparations

The MRS broth for *L. plantarum* starter cultures was prepared by mixing 250 ml of MilliQ water with 13.05 g of MRS broth granules in a 0.5 L bottle. The granules were dissolved in the water by using a magnet stirrer.

The modified MRS broth for the experiments was prepared by adding 28 g/L of NaCl and 52.2 g/L of MRS broth granules to MilliQ water. The MRS broth was mixed in 500 ml bottle with 0.5 L in each. The granules were dissolved in the MilliQ water by using a magnet stirrer.

The MRS agar for the viable count was prepared by mixing 68.2 g/L of MRS agar granules with MilliQ water. After autoclaving of the MRS agar it was cooled down a bit before it was poured into petri dishes and then left to solidify and then stored up-side-down in a cold room (6-8°C).

All of the mentioned solutions above were autoclaved for 15 minutes at 0.5 bars together with the 1.5 ml Eppendorf tubes used for serial dilutions of the samples in the experiments.

3.8.2 Starter culture of *L. plantarum*

A pre-trial was performed to find out how much the bacteria would grow over night. 20 ml of MRS broth (without added NaCl) was poured into a 50 ml Falcon tube and one colony of *L. plantarum* was inoculated. The inoculated Falcon tube was placed in 37°C incubator overnight with the lid loose but secured using tape. The next coming day (around 20 hours later) the OD of the start culture was measured by dilution with MRS to the concentrations 1:10, 1:50 and 1:100.

The preparations of starter culture for the accelerated experiments were performed as above. The OD values obtained from the pre-trial was used to estimate the volume of pre-culture to use for inoculation in each experimental set-up to reach a starting concentration of 10^5 CFU/ml. One pre-culture was made for each of the two independent experiments.

3.8.3 Preparation of sample solutions with acids and assembling of the samples

For the experiments MRS stock modified to resemble the environment in the pickled herring jar was used. The MRS stock with added salt was modified in pH from around 5.45 to 5.0 and 4.2 by using crystalline citric acid, crystalline malic acid, liquid lactic acid, and 24 % liquid acetic acid. The acids were added to the flasks with MRS stock and mixed with a magnetic stirrer. Since it was unknown how large amount of acids that was needed MilliQ water solutions with 1:100 diluted acids was prepared. These were only used in a small amount in the MRS stock with citric acid and pH 5.0 before it was noticed that the concentrated acid was needed to adjust the pH more effectively. When the pH 5.0 and 4.2 was reached the solutions was filter sterilized directly into the 50 ml Falcon tubes used for the trials. Two tubes of each acid and pH solution was filled with 40 ml of MRS stock for each trial (in total 8 + 8 tubes/trial), one for inoculation and one kept as control (see Figure 4). 70 µL of pre-culture with *L. plantarum* was added to each of the inoculum tubes to reach a level of 10^5 CFU/ml. After inoculation, the tubes and the control tubes were closed tightly and placed in the 25°C incubator.

3.8.4 Sampling and determination of growth of *L. plantarum*

Two independent experiments with MRS stock were started with one day in-between. To determine the growth of *L. plantarum* in the samples total aerobic plate count was used and sampling was performed after 3, 7, 10 and 14 days of incubation. The sample tubes were carefully turned back and forth until the sedimentation on the bottom of the tubes was mixed and the sample was homogenous. 100 µL of the sample was pipetted into a 1.5 ml Eppendorf tube with 900 µL of 0.9% NaCl solution and then mixed with a vortex. The sample was diluted in a serial 10X dilution using Eppendorf tubes containing 900 µL of NaCl solution to obtain dilutions of 10^{-1}, 10^{-2}, 10^{-3} etc. 100 µL of from appropriate dilutions were plated on MRS agar plates (duplicate). The dilution series were stored in a cold room (6-8°C) in case additional dilutions should be plated. The sampling, dilution and plating were performed in a LAF safety bench.

The petri dishes with sample were closed, placed in up-side-down in a stack in a plastic bag and incubated in a 37°C incubator for 36-48 hours. The day after the incubation the growth was evaluated to get an impression if the number of colonies would be around 30-300 in one plate. If the number of colonies seemed to be too high or too low more diluted samples from the dilution series were plated on new agar plates. After incubation the number of colonies of *L. plantarum* was determined and CFU/ml sample was calculated.

Figure 4 shows an overview of the experiments with the modified MRS inoculated with *L. plantarum*.

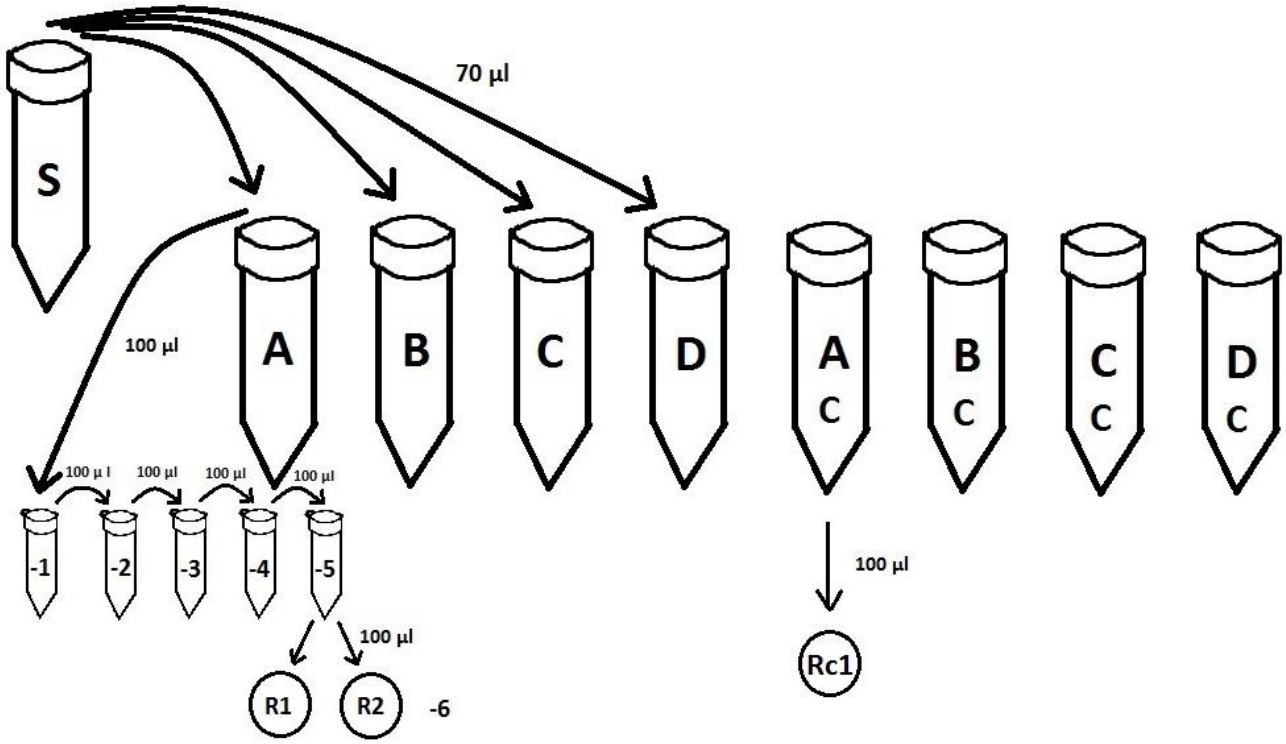

Figure 4 The picture shows an overview of the experiments with modified MRS inoculated with *L. plantarum*. The tube marked with S is the starter culture with MRS where one colony of *L. plantarum* was inoculated and then the tube was incubated overnight in 37°C. 70 µL of the starter culture (S) with *L. plantarum* were inoculated in the tubes A, B, C and D with MRS modified in salt concentration (2.8 %) and in pH with acetic acid (A), malic acid (B), lactic acid (C) or citric acid (D). One session with pH 4.2 and one with pH 5.0 was performed. The control tubes Ac, Bc, Cc and Dc contained the same MRS as tubes A, B, C and D except for that they were not inoculated with starter culture (S). Both the inoculated samples and the control samples were incubated in 25°C. At each sampling after 0, 3, 7, 10, and 14 days, 100 µL from the samples (A, B, C and D) were diluted in a serial 10X dilution. 100 µL from appropriate dilutions were plated on duplicate MRS agar plates (R1 and R2) and incubated in 37°C for 48 h before enumeration. Sampling of all the control samples was performed after 0, 7 and 14 days. Then 100 µL were plated (without dilution series) on MRS agar plates (Rc1) that were incubated in 37°C for 48 h before enumeration. The experiment described was performed twice.

4 Results

4.1 Experiment 1: Accelerated shelf life evaluation with herring jars

In the accelerated test with the pickled herring that were incubated in 22.6°C for 15 days samples were examined before incubation, after 7 days of incubation and after 15 days of incubation. The incubated herring versions were pickled herring in a sauce with acetic acid, citric acid, lactic acid or malic acid. Before the pickled herrings were incubated all the jars had been stored in refrigerator for around three weeks. At each sampling three jars of herring of each version were examined. The tests that were performed with the herring were total aerobic microorganisms, *Enterobacteriaceae*, thermostable coliform bacteria including *E. coli*, coagulase positive *Staphylococcus*, LAB, yeasts, and moulds. Prior to the start of incubation the presence of *Listeria monocytogenes* was also controlled.

In the initial examination before incubation the blended pickled herring and sauce was analysed with HPLC to measure the level of preservatives (benzoic acid) to see that the level was the same in all four types of pickled herring. In all samples the pH of the mixture of herring and sauce was measured to see any differences between the herring versions.

4.1.1 Microbial tests in accelerated shelf life evaluation

The purpose of the microbial examination was to detect possible natural contamination and measure the eventual concentration of the microorganisms mentioned above. A complete presentation of all results from the tests is shown in Table 9, Table 10, and Table 11 that are found in Appendix 9.3.

In the first sampling (Table 9) the herring that had not been incubated was examined. Those herring jars had only been stored in refrigerator since production of the herring jars (about three weeks). All of the tests were negative with no microbial growth on the agar plates leading to a concentration of < 2 log CFU/g or < 1 log CFU/g for all tested microorganisms.

In the second sampling (Table 10) the jars that had been incubated for seven days were examined the obtained results showed concentrations below the detection concentration in most of the jars. The tests for coliform bacteria, coagulase positive *Staphylococcus*, LAB, yeast, and mould were negative for all jars leading to concentration < 2 log CFU/g or < 1 log CFU/g of those microorganisms. In two of the jars with citric acid there was a small indication of contamination of aerobic microorganisms and the concentrations were determined to 2.0 log CFU/g in those jars. In one of the jars with malic acid the concentration of *Enterobacteriaceae* was determined to > 4.4 log CFU/g in the product. Since there was no presence of aerobic microorganisms it was assumed that the presence of *Enterobacteriaceae* was due to contamination when taking the sample.

With the herring samples that had been incubated for 15 days (Table 11) the results were below the detection concentration of *Enterobacteriaceae*, coliform bacteria, coagulase positive *Staphylococcus,* LAB, yeast or mould in all of the samples. This means a concentration of < 2 log CFU/g or < 1 log CFU/g of those microorganisms. There was a detected concentration of aerobic microorganisms in at least one of the samples from each type of acid. In two out of three of the jars with acetic acid the concentrations of aerobic microorganisms were determined to 2.0 log CFU/g and 2.3 log CFU/g respectively. One of the jars with lactic acid had a concentration of 2.3 log CFU/g and one of the citric acid jars had the concentration 3.0 log CFU/g of aerobic microorganisms. In the samples with malic acid only one of the jars showed detected concentration of aerobic microorganisms and the concentration was determined to 2.0 log CFU/g.

4.1.2 pH measurements of pickled herring

In each microbial examination of the pickled herring the herring and sauce was blended into a homogenous mixture and then the pH of the mixture was measured. The purpose was to see if there were any differences between the four types with citric acid, acetic acid, lactic acid or malic acid in the sauce. If there is a change of pH this could be a reason or an indication of eventual contamination of microorganisms due to for example production of lactic acid by LAB.

The results of the pH measurements are shown in Figure 5. There, the results from the jars that had been stored in refrigerator for three weeks (until the start of the accelerated shelf life evaluation test) are presented together with the samples that and been incubated in room temperature (the accelerated shelf life test) for 7 and 15 days. The bar diagram also shows the pH of the herring jars from the microbial tests that were performed with herring jars that had been stored in refrigerator for 12 weeks.

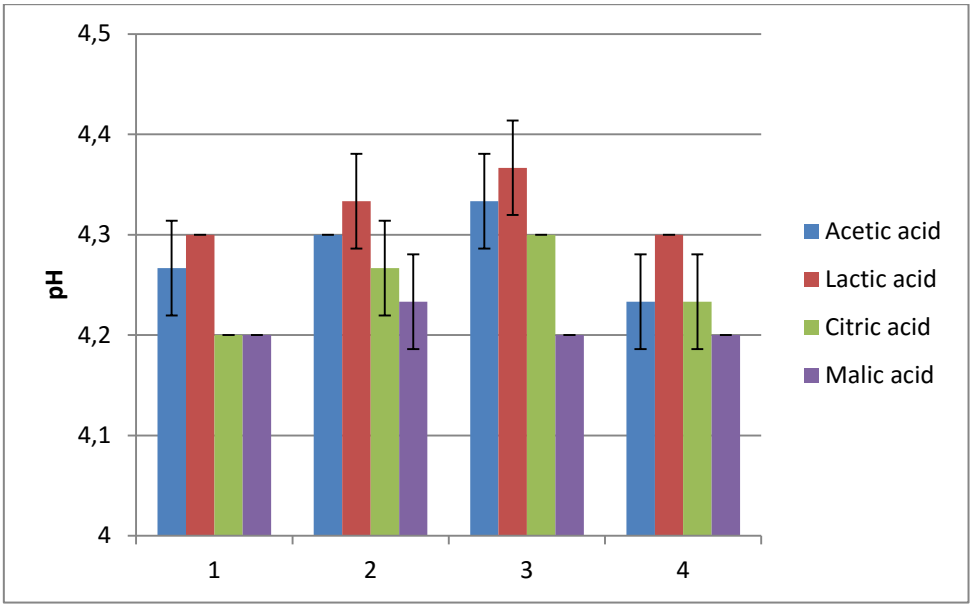

Figure 5 pH of the marinated herring jars. The pickled herring including sauce with acetic acid, lactic acid, citric acid or malic acid were blended and the pH was measured. In the bar diagram group 1 indicates herring jars that have been stored for 3 weeks in refrigerator and the measurements were performed just before the start of the accelerated shelf life test. Group 2 and 3 show the results of the pH measurements with herring jars that had been incubated in 7 and 15 days respectively (the accelerated shelf life evaluation). Number 4 is the results of the pH measurement with the herring jars that had been stored in refrigerator for 12 weeks. Each bar is an average of the pH in 3 jars.

Three weeks after production the pH of the herring jars (stored in refrigerator) was determined to 4.20-4.30 for the different samples. The herring jars that had been incubated for seven days had pH 4.23-4.33 and the herring jars that had been incubated for fifteen days had pH 4.20-4.36. The herring jars that had been stored in refrigerator for twelve weeks had pH 4.20-4.30. The obtained results demonstrate that the pH of the herring does not change very much over time in normal storage and in the higher temperature in the accelerated shelf life test. There was a small increase with time in the accelerated shelf life test. This might be because of temperature differences between the samples. From all pH measurements it can be concluded that the herring samples with malic acid and citric acid has a slightly lower pH than the rest while the herring with lactic acid has a bit higher pH.

4.1.3 Measurement of preservatives in pickled herring

The level of preservative (sodium benzoate) in the pickled herring (three weeks after production) was measured in three jars of each acid type that had not been incubated. The purpose was to confirm that the concentration was the same in all of the samples. In the herring samples with acetic acid the concentration of sodium benzoate was 1.48 g/kg, in the lactic acid samples 1.52 g/kg, in the citric acid samples 1.41 g/kg, and in the malic acid samples 1.41 g/kg. It can be concluded that the initial amount of preservative was almost equal in all samples. The amount of added preservative in each batch of the four versions of sauces was the same but if there had been a mistake it could be a reason for eventual presence of microorganisms. The results, the average concentration and the standard deviation are shown in Figure 6.

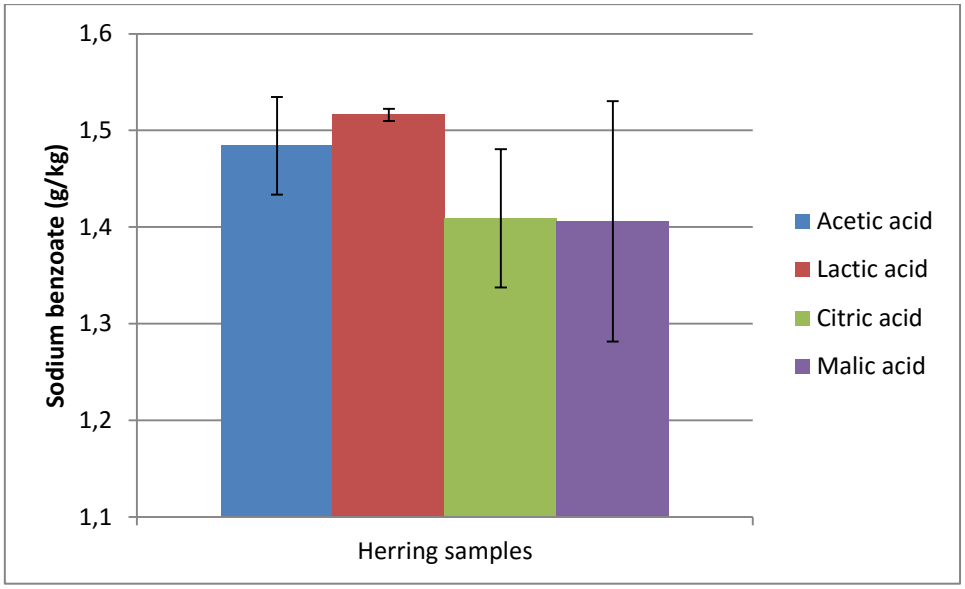

Figure 6 Concentration of benzoic acid. The concentration of preservative was measured by using HPLC. Three samples of each herring type were measured and the results are presented in the figure.

4.2 Texture analysis of the pickled herring

The texture of the pickled herrings was measured by using a texture analyser. The textures were measured two times, after four weeks of storage in refrigerator and after thirteen weeks of storage. In the first measurements the herring with lactic acid showed a little harder texture with a force of 1.73 kg. The force for the other acid herring versions were 1.47 kg (acetic acid), 1.38 kg (citric acid), and 1.44 kg (malic acid). In the second measurements all of the acid herring versions showed forces around 1.22-1.28 kg with very small differences. The results of the texture analysis are shown in Figure 7 and Figure 8. The bars in the figures represent the average force of all herring pieces of the same kind of acid sauce.

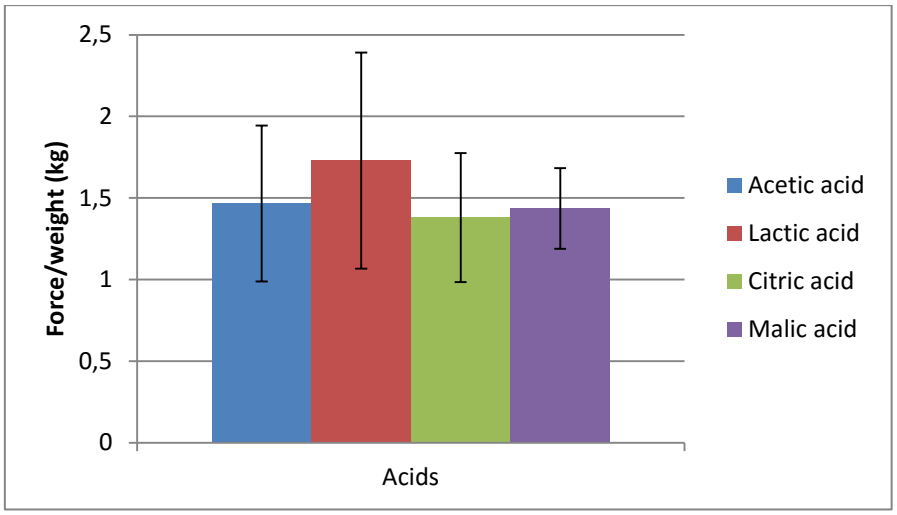

Figure 7 Texture after 4 weeks. The texture of the herrings was measured after four weeks of storage in refrigerator. The result are presented in the bar diagram. The firmness was measured with a SMS texture analyser and a cylindrical pin P11 compression that partly penetrated the fish fillet and measured the required force/time.

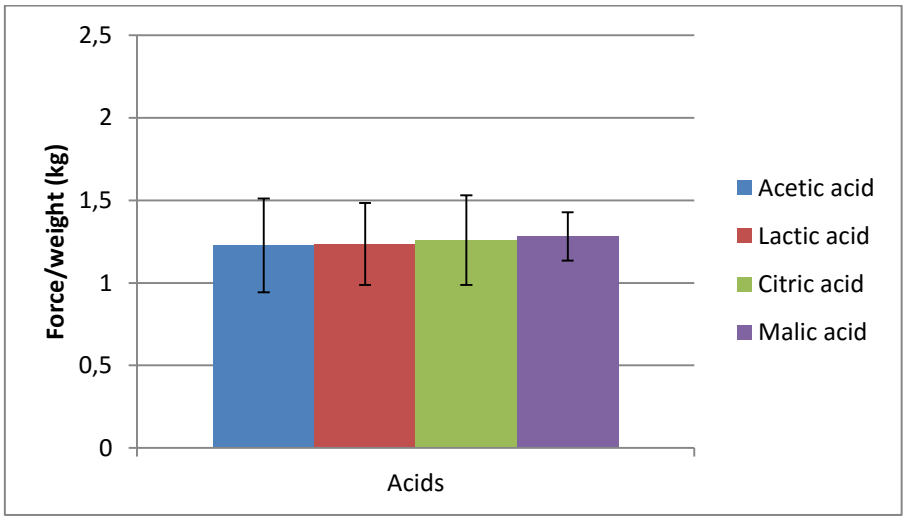

Figure 8 Texture after 13 weeks The texture of the herrings was measured after thirteen weeks of storage in refrigerator in the same way as after 4 weeks. The result are presented in the bar diagram. The measurements were performed with a SMS texture analyser and a cylindrical pin P11 compression that partly penetrated the fish fillet and measured the required force/time.

Based on the results it can be observed that in the texture analysis after four weeks of storage there was a greater difference between the samples. In the measurements after thirteen weeks of storage the texture was quite even with a lower standard deviation. In both texture analysis there were no significant difference between the different herrings at the significant level α=0.05. In the first analysis the values were F = 0.973682 and F-crit = 2.90112 and for the second measurements the values were F = 0.079193 and F-crit = 2.946685. With F-values lower than the F-crit values the null hypothesis cannot be rejected and thereby there was not any significant difference in texture.

4.3 Sensorial evaluation of the pickled herring

In this project four different versions of pickled herring were produced in Kungshamn. The different versions were all produced manually in the same way and the only difference between the samples was the weak acid used in the sauce for the herring. The acids used in the sauces are acetic acid (like in the original recipe), citric acid, malic acid, and lactic acid. When the herring jars had been stored in refrigerator for 5-6 weeks a sensorial test was performed to evaluate the four acids effect on the flavours, texture etc.

In the sensorial test the panel were asked to evaluate the following parameters: sourness, salinity, sweetness firmness and taste on a hedonic scale from 1-10. The participants in the test were also asked to rank the samples in preference. 13 out of the 14 participants did the ranking.

All of the results from the sensorial evaluation were analysed with Anova to validate if there is a significant difference between the herrings.

4.3.1 Sensorial test

The sourness of the herring was evaluated from 1 to 10 by the panel where 1 implied not sour at all while 10 implied very sour. In Figure 9 the results of the perceived sourness including standard deviation are presented. From anova, the F-values were F = 1.007136 and F-crit = 2.7826 with α=0.05. Since the F-value was lower than the F-crit value the null hypothesis cannot be rejected and therefore there was no significant difference between the samples. The P-value was 0.397089.

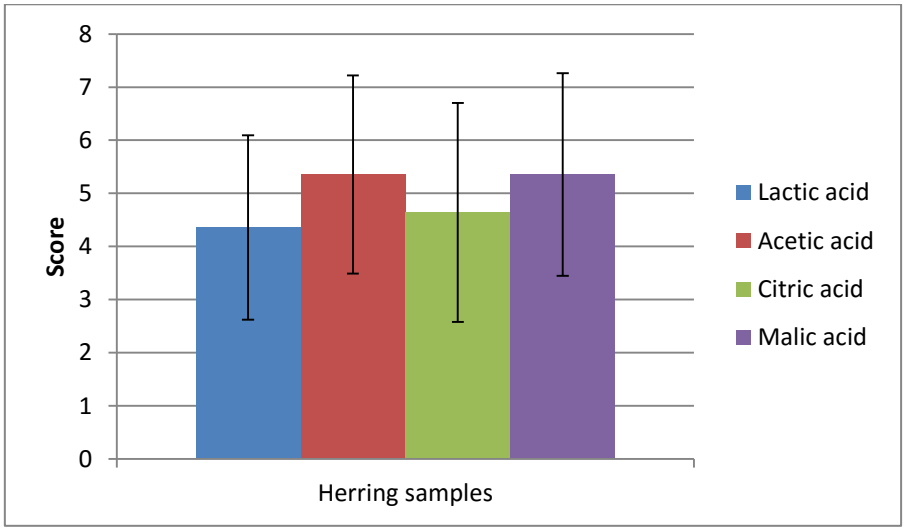

Figure 9 Sourness The bar diagram shows the results of sourness of the pickled herring in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the herrings in sourness from 1-10 and a higher score indicated a more sour perceived taste of the herring.

The salinity of the herring was evaluated from 1 to 10 by the panel where 1 implied not salty at all while score 10 implied a very salty taste. In Figure 10 the results of the salinity including standard deviation are presented. The results from Anova with α=0.05 was F= 0.839543 and F-crit= 2.7826 giving no significant difference between the samples. The P-value was 0.478389.

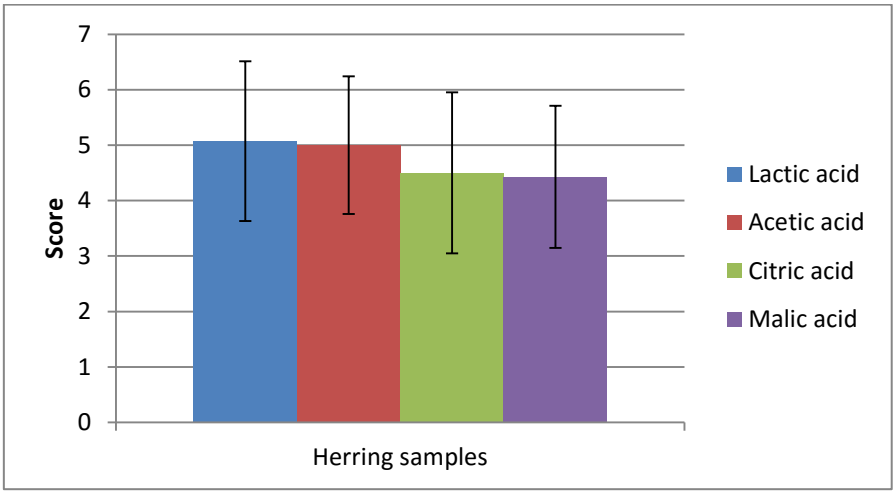

Figure 10 Salinity The bar diagram shows the results of salinity of the pickled herring in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the herrings in salinity from 1-10 and a higher score indicated a more salt perceived flavour of the herring.

The sweetness of the herring was evaluated from 1 to 10 by the panel where 1 implied not sweet at all while 10 implied a very sweet taste. In Figure 11 the results of the sweetness of the herring including standard deviation are presented. When using Anova (α=0.05) on the results the values were F= 0.471299 and F-crit= 2.7826. This means that there was no significant difference in sweetness of the herrings. The P-value was 0.703592.

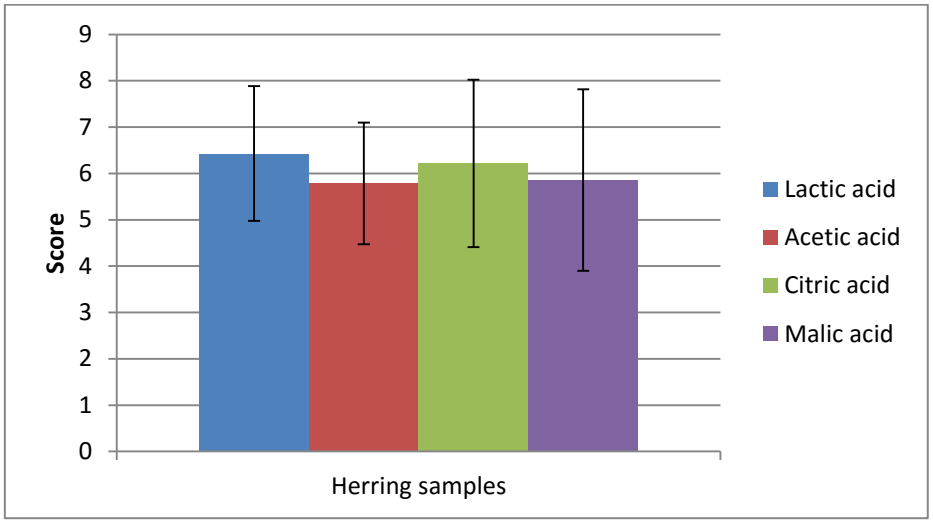

Figure 11 Sweetness The bar diagram shows the results of sweetness of the pickled herring in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the herrings in sweetness from 1-10 and a higher score indicated a more sweet perceived flavour of the herring.

The firmness of the herring was evaluated from 1 to 10 by the panel where 1 implied very soft and loose texture while 10 implied very firm and hard texture. In Figure 12 the results of the firmness including standard deviation are presented. Anova gave F=0.448819 and F-crit= 2.7826 with the significant level α=0.05 which means that there was no significant difference between the samples in firmness. The P-value was 0.719191.

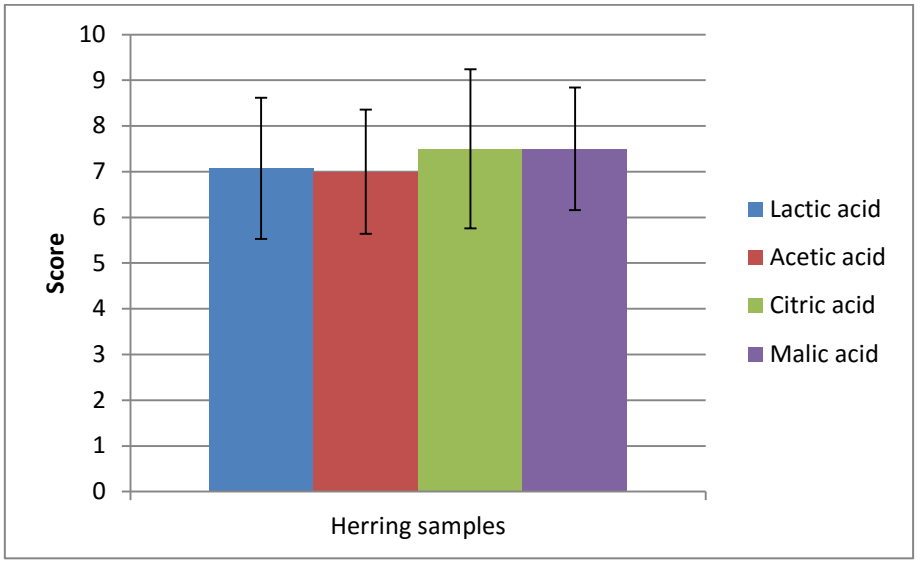

Figure 12 Firmness The bar diagram shows the results of firmness of the pickled herring in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the herrings in firmness from 1-10 and a higher score indicated a more firm perceived texture of the herring.

The taste of the herring was evaluated from 1 to 10 by the panel, where 1 implied to dislike the taste and 10 implied to like the taste of it. In Figure 13 the results of the taste including standard deviation are presented. The results from Anova with the significance level α=0.05 was F= 0.760814 and F-crit= 2.7826 meaning no significant difference between the samples. The P-value was 0.521196.

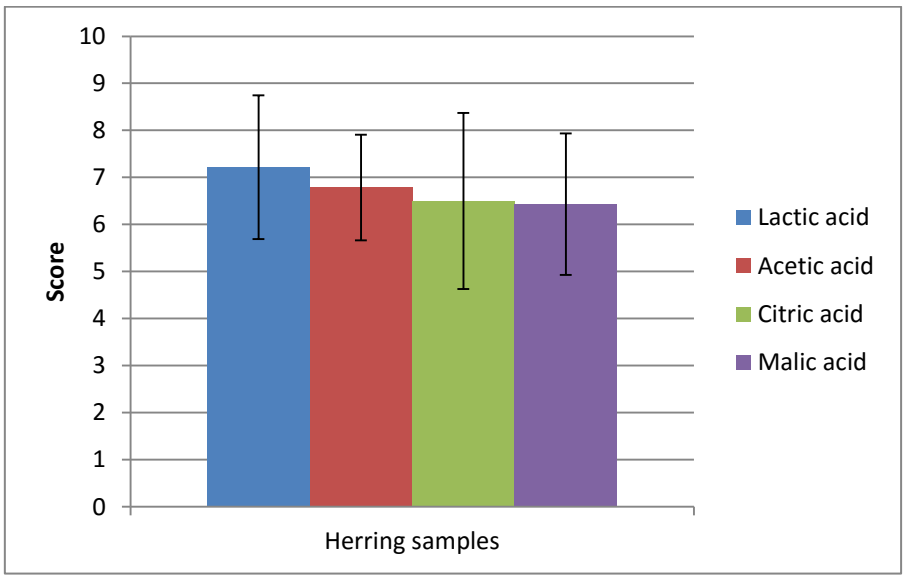

Figure 13 Taste The bar diagram shows the results of overall taste of the pickled herring in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the herrings in overall taste from 1-10 and a higher score indicated a more perceived taste of the herring.

The participants in the sensory evaluation were asked to rank the different types of herring according to which one they preferred where 1 was the favourite and 4 was the least preferred. In Figure 14 the results of the ranking are presented. If a person gave the grade 1 (favourite) to a herring type the score of the herring from that person was 4 and it was thereby giving a higher score in the bar diagram. Grade 1 = score 4, grade 2 = score 3, grade 3 = score 2 and grade 4 = score 1 in the diagram. In the diagram the average of the scores are presented. This means that the higher bar the more preferred herring.

In the analysis when using Anova the significance level α=0.075 was chosen and the results were F= 2.510264 and F-crit= 2.44849. Since the F-value was higher than the F-crit value it can be concluded that there was a significant difference in preference between the pickled herring samples. The P-value from Anova was 0.069801.

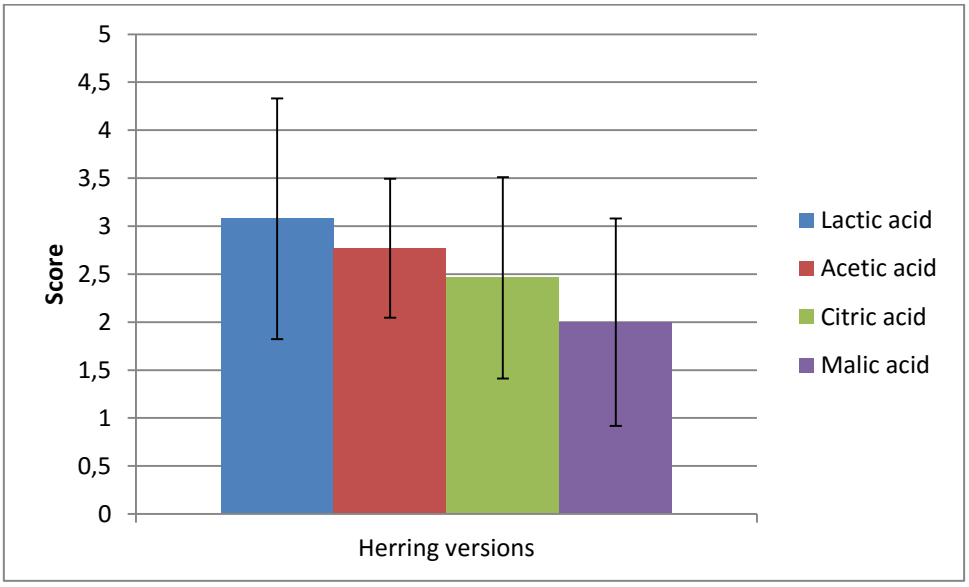

Figure 14 Total ranking The bar diagram shows the average results of the total ranking in the sensorial test with the four herring versions that had been stored in refrigerator for 5-6 weeks. The panellists were asked to rank the pickled herrings from 1-4 where 1 indicates the favourite and 4 the least favoured version. In the diagram the scores are turned to the opposite, 4 indicated the favourite and 1 is the least favoured type. The bars present the average of the panel's scores and a higher score indicates a more preferred pickled herring version.

The results of how the panel has given the scores can be seen in Table 2. From the results of that it can be concluded that the pickled herring containing lactic acid was the most preferred type of herring.

Table 2 The table presents the results of the distribution of how the participants in the sensorial evaluation ranked the pickled herring version with different acids in the sauce. Rank 1 implies the favourite version while rank 4 implies the least favourite.

Acid	No. of rank 1	No. of rank 2	No. of rank 3	No. of rank 4
Lactic acid	8	0	3	2
Acetic acid	1	9	2	1
Citric acid	2	3	3	5
Malic acid	2	1	5	5

In the sensorial test the participants were also asked to comment if it was challenging to notice any difference between the pickled herrings. They were also asked to give general comments regarding the herrings. Below is a selection of the comments that has been translated by the author. In the test the samples were given a three digits code and the participants did not know which code was connected to which sample. In the comments, the acid in the samples has been added by the author. The codes are 367 (lactic acid), 792 (acetic acid), 954 (citric acid), and 185 (malic acid).

- "It was almost too firm texture" (about malic acid)
- "I like the firmer texture" (about malic acid)
- "Quite similar in taste, no big differences but it was mild and tasty pickled herrings"
- "367 (lactic acid) was the most balanced. 185 (malic acid) had a bad sourness and it tasted too much fish + had an off-flavour. 792 (acetic acid) was interesting but the sourness was a bit too sharp. 954 (citric acid) was too firm."
- "185 (malic acid) was a little bit bitter at the end of the flavour. It has too low sourness."
- "I thought 367 (lactic acid) was the least sour and it was therefore my favourite. I think all of the samples were tasty."
- "954 (citric acid) and 185 (malic acid) had a too weak sourness. It was not the correct balance between sourness, salinity and sugar. 367 and 792 (lactic acid) had a quite good balance while 954 and 185 did not have the balance in flavours."
- "In some of the samples the sweetness was more prominent"
- "It was very hard to feel any differences between the samples."
- "There were only small differences. I think the texture was the parameter that varied the most."

4.4 Shelf life evaluation of marinated herring jars

A second sensorial test was planned to be held 15 weeks after production of the herring but due to a high number of aerobic bacteria (> 4.4 log CFU/g) in a jar of malic acid pickled herring it was cancelled. It was suspected to be contamination caused when performing the microbial analyses rather than a true contamination in the herring since the sample did not show positive results for the other microbial tests: *Enterobacteriaceae*, coagulase positive *Staphylococcus*, LAB, yeasts, and moulds. The second sensorial test was postponed while waiting for the results of repeated microbial tests. The results from the microbial test after twelve weeks of storage in refrigerator are presented in Table 12 in Appendix 9.3. The pH of the herring samples was also measured and the results are presented in Figure 5.

The extra microbial tests performed after 14 weeks of storage in refrigerator are presented in Table 13 in Appendix 9.3. They only show a low concentration of microorganisms. In two jars with acetic acid a low concentration (2.0 log CFU/g) of aerobic microorganisms was found but the other microbial tests showed concentrations of < 1 log CFU/g or < 2 log CFU/g. The samples with lactic acid showed concentrations < 1 log CFU/g or < 2 log CFU/g for all microorganisms. In the samples with citric acid there was a concentration of 1.3 log CFU/g for mould in one jar and < 1 log CFU/g or < 2 log CFU/g for the other microorganisms. There was a concentration of 1.0 log CFU/g for mould in one jar with malic acid but the other tests showed concentrations < 1 log CFU/g or < 2 log CFU/g.

4.5 Experiment 2: Growth of *L. plantarum* in modified MRS nutrient medium

To evaluate the effect of the acids against microorganisms MRS mediums that had been modified were inoculated with *L. plantarum*. In these experiments no herring were included but the MRS nutrient mediums were modified to resemble the environment in the herring product by adjusting the sodium chloride concentration to 2.8 % and the pH to 4.2 and 5.0. The pH was regulated by using acetic acid, citric acid, malic acid, and lactic acid. The inoculated bacterium in the MRS was *L. plantarum* that is a common LAB that have been found in spoiled pickled herring. The experiments were performed in tightly sealed plastic tubes that were incubated in 25°C to get a more suitable environment for the bacteria to easier evaluate the acids' effect. A level of about 10^5 CFU/ml of the bacterium *L. plantarum* was added to each acid set-up to follow growth over time. The samples were incubated in 25°C for 14 days and every third or fourth days samples were collected for examining the level of *L.* plantarum.

4.5.1 Microbial growth

Two independent experiments (two biological replicates) were started with one day in between and the results from the average of the two experiments are shown in Figure 15 for the experiments with pH 4.2. The average results from the experiments with pH 5.0 are presented in Figure 16. Individual plate count results are also presented in Table 14 and Table 15 in Appendix 9.4.

4.5.1.1 Experiments with pH 4.2 samples

In the samples with pH 4.2 it can be seen in the graph that in all samples the initial *L. plantarum* level was about 5.3 log CFU/ml. In the second sampling (after three days of incubation) the growth in the samples differed slightly. There was a significant difference between the samples with malic acid and acetic acid and the samples with malic acid and lactic acid. In the samples with malic acid and citric acid the level of bacteria seemed to have reached its maximum after three days of incubation. The samples with acetic acid and the lactic acid on the other hand seemed to reach their top level not until after seven days of incubation. The highest concentration of the sample with malic acid was 8.9 log CFU/ml, 8.5 log CFU/ml in the citric acid sample, 8.7 log CFU/ml in the lactic acid samples, and 8.2 log CFU/ml in the acetic acid samples.

When the maximum concentrations had been reached the amount of bacteria in the samples was decreasing slowly to almost become stabilized. The level of bacteria was decreasing faster in the sample with malic acid than for the other acids. For the sample with lactic acid the level of bacteria was decreasing slightly after the maximum after seven days of incubation but the level remained overall stable. In the sample with citric acid the maximum was reached after three days of incubation but after this the level of *L. plantarum* was quite stable. The sample containing acetic acid was somewhat slower in growth initially, it had the lowest maximum level and showed a noticeable decrease in growth over time.

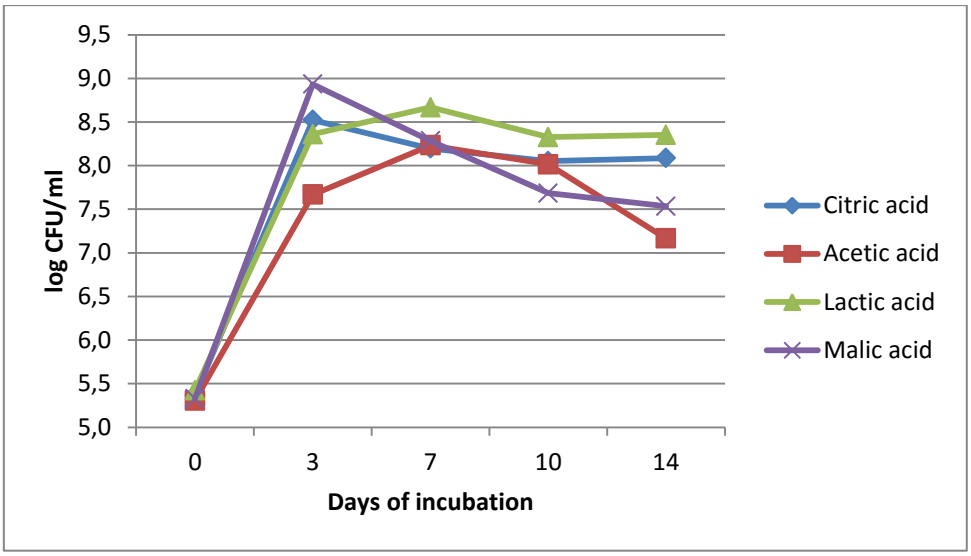

Figure 15 Average concentration of *L. plantarum* in pH 4.2 In the experiment *L. plantarum* was inoculated in salt and acid modified MRS and then concentrations of the bacteria were determined by viable count every third or fourth day. Two independent biological replicate were performed and the average concentration (log CFU/ml) of *L. plantarum* at each measurement are presented in the figure. In these experiments the pH was adjusted to 4.2 with citric acid, acetic acid, lactic acid or malic acid.

In the graph with the growth of *L. plantarum* in the MRS there are some visual differences between the concentrations of *L. plantarum* in the difference acid samples. At each sampling after 0, 3, 7, 10, and 14 days the concentration results of the acid samples were compared with one-tailed t-tests (α=0.05) between the samples. The p-values of the results and if there was a significant difference between the samples in pair (higher calculated t-value than the t-crit value) are presented in Table 3. It can be concluded as mentioned that there was significant difference between malic acid MRS and acetic acid MRS and between malic acid MRS and lactic acid MRS after three days of incubation. The significant difference between the lactic acid MRS and malic acid MRS was kept to the end of the test period.

Table 3 In Experiment 2 the growth of *L. plantarum* in salt and pH modified MRS was examined. The pH was adjusted to 4.2 in all samples by using citric acid, acetic acid, lactic acid, and malic acid. At each sampling after 0, 3, 7, 10, and 14 days of incubation one-tailed t-tests with α=0.05 were used between the results to determine significant differences. In the table the p-values of from t-test are presented. If the value is marked in bold with an S there is a significant difference between the acid sample in the row and the acid sample in the column. There are no p-values connected to acetic acid after 10 days of incubation due to no concentration result for acetic acid in that sampling.

Days of incubation	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
0	Citric acid	-	-	-	-
	Acetic acid	**P=0,026^{S}**	-	-	-
	Lactic acid	P=0,275	P=0,149	-	-
	Malic acid	P=0,163	P=0,223	P=0,129	-
3	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	P=0,091	-	-	-
	Lactic acid	P=0,216	P=0,054	-	-
	Malic acid	P=0,075	**P=0,038^{S}**	**P=0,018^{S}**	-
7	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	P=0,469	-	-	-
	Lactic acid	P=0,164	P=0,074	-	-
	Malic acid	P=0,225	P=0,444	P=0,147	-
10	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	-	-	-	-
	Lactic acid	P=0,178	-		-
	Malic acid	P=0,074	-	**P=0,042^{S}**	-
14	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	**P=0,017^{S}**	-	-	-
	Lactic acid	P=0,070	**P=0,007^{S}**	-	-
	Malic acid	P=0,078	**P=0,007^{S}**	**P=0,024^{S}**	-

4.5.1.2 Experiments with pH 5.0 samples

In the experiments with modified MRS to pH 5.0 the growth of the bacteria was more similar between the different acids. All samples started with a bacterium level of about 5.3 log CFU/ml and in the second measurement the bacteria had grown to a level of around 9.2 log CFU/ml in all samples. After three days of incubation the highest concentrations were reached and then the bacteria level started to decrease. The amount of bacteria in the samples containing acetic acid, malic acid, and citric acid was very similar while the number of bacteria in the sample with lactic acid did not follow the same trend. The lactic acid sample had a much slower decrease compared to the other samples.

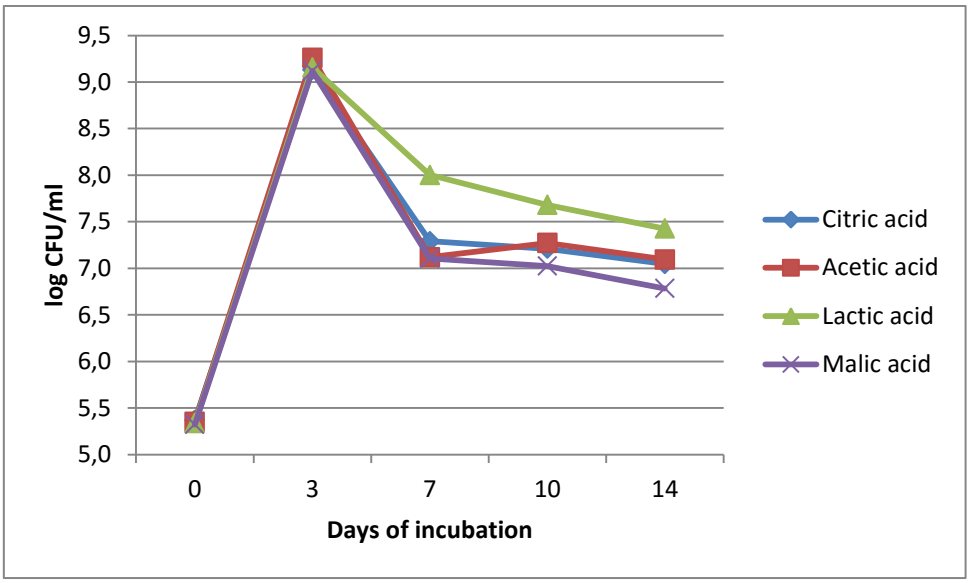

Figure 16 Average concentration of *L. plantarum* in pH 5.0 In Experiment 2 *L. plantarum* was inoculated in salt and acid modified MRS and then concentrations of the bacteria were determined by viable count every third or fourth day. Two independent biological replicate were performed and the average concentration (log CFU/ml) of *L. plantarum* at each measurement are presented in the figure. In these experiments the pH was adjusted to 5.0 with citric acid, acetic acid, lactic acid or malic acid.

According to the one-tailed t-tests there was no significant difference in concentration between the samples in the first and second sampling which also can be assumed from the graph. In the third sampling after seven days of incubation there was a clear difference in the graph between the lactic acid sample and the other samples. The t-tests also indicated that there was a significant difference between the lactic acid sample and acetic acid sample and citric acid sample respectively from the third sampling and to the end of the test period. The results of the one-tailed t-tests with $\alpha=0.05$ are presented in Table 4.

Table 4 In Experiment 2 the growth of *L. plantarum* in salt and pH modified MRS was examined. The pH was adjusted to 5.0 in all samples by using citric acid, acetic acid, lactic acid and malic acid. To determine whether there was significant difference between the average concentrations of *L. plantarum* t-test with α=0.05 was used. At each sampling after 0, 3, 7, 10 and 14 days of incubation one-tailed t-tests were used between the results. In the table the p-values of from t-test are presented. If the value is marked in bold with an S there is a significant difference between the acid sample in the row and the acid sample in the column.

Days of incubation	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
0	Citric acid	-	-	-	-
	Acetic acid	P=0.418	-	-	-
	Lactic acid	P=0.220	P=0.445	-	-
	Malic acid	P=0.097	P=0.331	P=0.404	-
3	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	P=0.267	-	-	-
	Lactic acid	P=0.406	P=0.169	-	-
	Malic acid	P=0.370	P=0.282	P=0.396	-
7	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	**P=0.025^{S}**	-	-	-
	Lactic acid	**P=0.014^{S}**	**P=0.006^{S}**	-	-
	Malic acid	P=0.218	P=0.475	P=0.064	-
10	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	P=0.075	-	-	-
	Lactic acid	**P=0.023^{S}**	**P=0.015^{S}**	-	-
	Malic acid	P=0.233	P=0.202	P=0.094	-
14	**Acid sample**	Citric acid	Acetic acid	Lactic acid	Malic acid
	Citric acid	-	-	-	-
	Acetic acid	P=0.153	-	-	-
	Lactic acid	**P=0.001^{S}**	**P=0.026^{S}**	-	-
	Malic acid	**P=0.033^{S}**	P=0.053	**P=0.013^{S}**	-

In each experiment eight tubes with the same modified MRS but without inoculated bacteria were incubated together with the samples with bacteria. Samples were taken in the start, after seven days and after fourteen days of incubation. All the tests were negative except for the control sample with pH 5.0 using lactic acid in the first trial. After fourteen days of incubation the concentration in the control sample was determined to 10 CFU/ml but since there was only one colony on the plate (concentration 10^{-1}) it was considered as no growth.

4.5.2 pH of the samples

The pH of the samples was measured before inoculum of *L. plantarum* and after the samples had been incubated for 14 days. The pH before and after incubation are presented in Table 5.

The pH in all samples where bacteria had been inoculated had changed a lot from the start pH of 4.2 and 5.0 respectively. This indicated that the bacteria have grown and produced lactic acid which lowers the pH. In the samples where the start pH was 4.2 the pH had decreased to about pH 3.2 - 3.5 in all samples. In the samples with acetic acid the pH after the incubation time was a little bit higher than in the other samples in both experiments with pH 3.5. The pH of the pH 4.2 samples with citric acid on the other hand had an end pH of 3.2 and 3.4 in trial one and two respectively.

In the samples with start pH 5.00 the end pH for all of the acids was around 3.4 – 3.5 and there was no noticeable difference between the different acid samples.

Table 5 In Experiment 2 L. plantarum was inoculated in salt and acid modified MRS in Falcon tubes and then incubated for 14 days in 25°C. The pH was modified from start to 4.2 or 5.0 with acetic acid, citric acid, malic acid or lactic acid and the pH of the MRS was measure before incubation and after the test period (14 days). The pH of the control tubes with the same modified MRS without inoculum was also determined before and after the test period.

Acid	pH before inoculum of *L. plantarum* and incubation	pH after 14 days of incubation with inoculum			pH after 14 days of incubation without inoculum (control tubes)		
		Replica 1	Replica 2	Average	Replica 1	Replica 2	Average
Acetic acid	4.2	3.4	3.5	3.5	4.1	4.3	4.2
	5.0	3.4	3.5	3.5	4.9	5.0	5.0
Citric acid	4.2	3.2	3.3	3.2	4.1	4.2	4.2
	5.0	3.3	3.5	3.4	4.9	5.0	5.0
Malic acid	4.2	3.3	3.4	3.4	4.1	4.3	4.2
	5.0	3.4	3.5	3.4	4.9	5.1	5.0
Lactic acid	4.2	3.2	3.4	3.3	4.1	4.2	4.2
	5.0	3.3	3.5	3.4	4.9	5.0	5.0

The pH of the control samples that were not inoculated with *L. plantarum* was also measured at the end of the experiment after 14 days. The pH remained the same as before incubation, as expected, which indicates that there were no contamination of LAB in the control tubes.

5 Discussion

In this project experiments have been performed to evaluate the microbial and sensorial effects of weak acids in marinated/pickled herring. Normally, flavours and the defence against microorganisms are achieved with acetic acid in marinated herring but in this project also lactic acid, malic acid and citric acid have been evaluated. Different acids have different flavours meaning that a change in acid composition can create a new kind of product that can attract more customers which is a desired effect. Since marinated herring is a semi-preserved food product that is not heated before serving the product has to be safe to eat when you take it from the refrigerator. Therefore it is very important that the ingredients including the acid not only give a tasty product but also a safe product.

Marinated herring is a rather safe product with its high salt concentration, low pH and usually including preservatives. There is still a risk, however, of contamination of spoiling microorganisms like LAB and yeast or microorganisms that can cause illness like *Listeria* and *Clostridium*. The reason for spoilage of a fish product can be difficult to find out but since acetic acid tolerant LAB have been found in pickled herring those have been more focused on in this work.

Other studies with marinated herring with especially LAB have been done before. In a study from 2000 spoiled marinated herring jars (with acetic acid) where there had been gas formation were examined to find out the spoiling microorganisms in the products. The results showed that there were various species of the genus *Lactobacillus* including *Lactobacillus alimentarius* that was suggested as the reason for the gas production. (Björkroth, Korkeala, & Lyhs, 2001) In another study spoiled marinated herring jars with slime and gas formation were examined. The herring jars did not include preservatives like in this master thesis project but they included herring, onion, carrots, water, sugar (18 %), sodium chloride (2.4 %), acetic acid and all-spice and the concentration of LAB in the spoiled products was found to be up to 2.4 x 10^9 CFU/g. The bacteria that were assumed to be the reason for the gas and slime production in the marinated herring were the LAB *Leuconostoc gelidum* and *Leuconostoc gasicomitatum*. (Björkroth, Koort, Lundström, & Lyhs, 2004) Also other LAB like *L. plantarum L. brevis, L. fermentum* and *L. leichmannii* have been found in spoiled marinated herring (Björkroth, Holzapfel, & Schillinger, 2006).

In the accelerated shelf life test with herring jars incubated in 22.6°C for 14 days there were only low concentrations of microorganism (aerobic microorganisms and mould). There were no presence of LAB and there were no big differences between the acid samples in concentration of microorganisms. In all of the samples where there was a low concentration of microorganisms the concentration of the microorganism was still lower than Orkla Foods Sverige's internal guidelines meaning that they were still regarded as safe and not spoiled. Also in the test where the herring jars had been stored in refrigerator there were only low concentrations of microorganisms.

There is no legislation of limits for the microorganisms that were tested in the experiments except for presence of *L. monocytogenes*. Within EU the legislation is that for ready-to-eat-meals just after production *L. monocytogenes* should not be present in 25 g in five out of five samples in food where the environment is favourable for *L. monocytogenes*. When the product is examined after it has been released in the store but before the expiration day the concentration should not be more than 100 CFU/g in five out of five samples. (EUR-lex, 2005) For the other microorganisms there are only guidelines of limits for microbial quality tests. The company ALcontrol Laboratories provides

guidelines that are based on experience and NFA's literature. In 2016 they suggest that a non-heat treated ready-to-eat-meal like marinated herring is satisfying if the concentrations of microorganisms are maximum 7.5 log CFU/g aerobic microorganisms, 1.0 log CFU/g *E. coli*, 2.0 log CFU/g coagulase positive *Staphylococcus*, 3.0 log CFU/g yeast, and 4.0 log CFU/g mould. They also suggest that the product is not satisfying if the concentrations of microorganisms are 2.0 log CFU/g for *E. coli* and 4.0 log CFU/g for coagulase positive *Staphylococcus*. (ALcontrol Laboratories, 2016) All these criteria are fulfilled in the experiments with the marinated herring.

Accelerated testing to incubate the herring in a higher temperature than recommended such as room temperature (20-25°C) is a way of checking the quality of the product faster compared to storing it in refrigerator for a long time. Since the pickled herring is not in its right storage temperature the microorganisms that possible can grow in the pickled herring might be slightly changed. In low storage temperatures bacteria such as *Listeria* can grow while other microorganisms are inhibited. In the higher temperature mesophilic microorganism like again *Listeria* but also some LAB and *Enterobacteriaceae* are more favoured. (Ahlström, et al., 2007) (Christiansson, 2016) Still many bacteria can grow or survive in a temperature range including both higher and lower temperatures. To incubate in a higher temperature is a faster method but it might not give the complete or fully realistic picture. Also the samples of pickled herring in the tests contained preservatives that should affect the possible growth of microorganism.

In the accelerated tests with MRS, *L. plantarum* was inoculated into MRS that had been modified with sodium chloride and acid to resemble the environment in marinated herring. No pieces of herring were included in these experiments. There was a visual difference between the experiments with pH 4.2 and 5.0 and there were larger differences in growth of *L. plantarum* in pH 4.2 to compare to the samples with pH 5.0. In the 4.2 experiments the concentration of *L. plantarum* increased faster in the samples with citric acid and malic acid than the samples with lactic acid and acetic acid. Also, their highest concentration (around 9 log CFU/ml) was reached earlier, after three vs. seven days, in the samples with citric acid and malic acid. This should be due to that malic acid (pK_{a1} = 3.40) and citric acid (pK_{a1} = 3.09) have lower pK_a values than lactic acid (pK_a = 3.86) and acetic acid (pK_a = 4.76) and therefore they are more dissociated than the other acids at pH 4.2. The pK_a values should also explain why the curve for lactic acid and acetic acid is kind of following each other in growth except for that the curve for lactic acid shows a slightly higher concentration of *L. plantarum* during the whole experiment except for the start concentration. The acetic acid (pK_a = 4.76) at pH 4.2 have a higher degree of undissociated molecules than lactic acid (pK_a = 3.86).

For malic acid the highest concentration of *L. plantarum* was 9 log CFU/ml in the 4.2 samples just like in the trial with start pH 5.0 while the other acid samples (pH 4.2) had a lower maximal concentration. The most logical case, due to the pK_a of the acids, would be that the citric acid would be the sample with the highest concentration of bacteria. Since the samples of the MRS was only taken every third or fourth day it could be that the highest concentration has already been reached and the result after three days of incubation is when the concentration is going down.

In the MRS experiments with pH 5.0 the decrease of concentration of *L. plantarum* in the lactic acid samples did not follow the other acid samples after seven days from start and onward. In the sample with lactic acid the decrease of *L. plantarum* was slower and the level was stable on a higher concentration than in the other samples. A possible explanation for this is that *L. plantarum*'s optimal

initial pH is 5-6 (Fu & Mathews, 1999) and therefore they will grow in the presence of all of the acids from the beginning. Since the initial pH 5.0 is higher than all of the acids' pK_a a high level of the acid molecules will be dissociated and then they have lower impact on the *L. plantarum* since they cannot pass through the cell wall of the bacteria. When *L. plantarum* is producing lactic acid the pH of the MRS will decrease and approach the pK_a of the acids leading to a higher level of undissociated acid molecules that can pass through the cell wall of the bacteria and inhibit them. A hypothesis why the concentration of *L. plantarum* is higher in the samples with lactic acid is that that the *L. plantarum* can take more of the lactic acid rather than other acids since it is producing it by itself under normal conditions. No studies that confirm or disagree to this hypothesis have been found.

In the experiment with modified MRS no herring was included and the samples were incubated in 25°C which is not the normal storage temperature of pickled herring. This means that the result can not completely be applied on marinated herring stored in 8°C or colder. LAB seem to be able to grow in pickled herring since they have been found in spoiled pickled herring (Björkroth, Korkeala, & Lyhs, 2001) but when the temperature is lower the growth is slower. In the trials all of the acid samples showed a large growth of *L. plantarum* before the acids had an effect and the concentration of bacteria decreased. The pH of the MRS stock was only determined before and after the trial and therefore it is unknown if the pH was as low as around pH 3.5 (end pH) already e.g. after seven days of incubation or if it decreased later in the experiment. It could be that it was mainly the lowered pH (due to lactic acid production) that had an impact on the acids inhibiting effect and decreasing effect of *L. plantarum*. If that is the case then the acids only have an effect when it already is "too late" since the concentration of microorganisms is high and the spoilage of the fish has begun. If the bacteria can reach a high concentration they are able to spoil the food. To improve the shelf life the lag phase before the microorganism starts to increase in concentration needs to be extended.

Since LAB are quite tolerant to acid and pH they perhaps manage the acid better than other microorganisms. In these experiments with MRS, and with no herring included, only a monoculture was used but in normal cases the herring can be exposed to various microorganisms from the natural flora in the production. Still, the principle of how well the *L. plantarum* could grow in the different acids can be applied on other spoiling microorganisms. Other microorganisms that can spoil pickled herring are e.g. moulds and yeasts. Mould can grow from pH 2-8.5 while yeast manage the pH range 4-4.5 (Battcock & Azam-Ali, 1998) and in the experiments the lactic acid seemed to have smaller impact on the lactic acid producing *L. plantarum*. It might be that if another microorganism like yeast or mould was inoculated the result would have been different.

L. plantarum and other LAB are rather salt tolerant bacteria that can grow and produce lactic acid even in high salt concentrations. In 2004 it was concluded in a study that the strains *Lactobacillus plantarum 541* and *Lactobacillus plantarum A6* can grow and produce lactic acid in salt concentrations (sodium chloride) up to 8 %. In this study the MRS was modified with sodium chloride and phosphate-citrate buffer containing di-sodium hydrogen phosphate and citric acid to adjust the pH. In the study it was concluded that at initial pH 6.0-6.6 and salt concentration 0-4 % none of the strains had any lag phase. When that salt concentration was 6 % and 8 % the lag phases were determined to 2 and 4-5 hour respectively. (Guyot, Pintado, Rao, & Stevens, 2004) In this study only one acid (citric acid) was included to adjust the pH of the MRS. In the results (Figure 15 and Figure 16) in the accelerated experiments with MRS both modified to pH 4.2 and 5.0 there were no lag phases. This can also be due to that the first samplings after start were not until after three days of

incubation meaning that the lag phase could have been before that. The study by Guyot *et al.* also concluded that for *L. plantarum 541* and *L. plantarum A6* out of pH and salt concentration pH is the most important parameter (Guyot, Pintado, Rao, & Stevens, 2004).

Based on the results of the sensorial test of the herring marinated in four different acid sauces it can be concluded that there is not a profound difference between the different types in flavours. Instead it appeared that the acids had an effect on the texture and where malic acid was found to perform the herring with the firmest texture. At the same time, the texture analysis of the herring shown that there was no significant difference in texture between the samples. When fresh fish is put into a solution with a low pH the proteins will denature due to the low pH and this will have the same effect as cooking the fish in texture (Wolke, 2004). This is what happens in pickled herring and also the dish ceviche where fresh and raw fish pieces are put into a marinade with lots of lemon juice (mostly citric acid) with a low pH. The texture is as "cooked" fish but the fish is still kind of raw (Kenji López-Alt, 2011). The flesh of a herring includes many proteins and a protein is composed of many amino acids that have many side chains that can be positively or negatively charged. If the pH is higher or lower than the isoelectric points of the numerous amino acids in the herring the net charge of the protein will not be zero. Depending on the pH around the protein the sidechain in the protein can be charged or uncharged. If the protein chains are equally charged they will repel and the structure of the protein will be more "stretched out" and the texture of the fish will changed. (Coupland & Ettelaie, 2014) A hypothesis is that if the texture of the fish is different it depends mainly on the pH and not on which the acid is. According to the sensory evaluation the panel thought as mentioned that the malic acid herring was a bit firmer than the other. The reason for this might be that the pH in the malic acid sample was slightly lower than in the other due to miscalculations. See Table 8 in Appendix 9.1.

In flavour there were only small differences according to the panel. Some commented that it was difficult to notice any difference at all between the samples but that the samples with malic acid and the citric acid was a bit unbalance in sourness, sweetness and salinity. Since the salt and sugar concentration was the same in all samples the only parameter that could have affected those flavours is the acid. The acids might also affect the taste of salt since it is believed that the receptors for sour taste (H^+) in the taste buds in the mouth also can register the salt taste (Na^+) due to that the ions are quite similar (Heady, 2006).

The taste of acids is not only sour but rather sour in different ways. Acetic acid has a sharp and penetrating sourness, and malic acid is also a rather sharp compared to the lactic acid which has a much more round and milder flavour. (Förare Winbladh & Sandström, 2011) This is also what can be suggested from the results of the sensory evaluation where the panel thought that the sourness of the samples with acetic acid and the malic acid sample stronger than in the rest.

One interesting results from the sensory evaluation was that the herring that for most of the panel was chosen as the favourite was not the regular pickled herring with acetic acid but rather the type with lactic acid. Reasons for why the lactic acid pickled herring was the best were that it was milder, not as sour and had balanced flavours of salt, sourness and sweetness. Since the acids have different flavours and the recipe of "Abba sill på 5 minuter" is made to balance the flavours with acetic acid it can be expected that this is the reason for an unbalanced flavours. The samples with lactic acid and

acetic acid were considered to be more balanced which indicates that when it comes to flavour the used amount of lactic acid in the samples could replace the acetic acid.

The pickled herring with different acids in these experiments were manually produced according to the “Abba sill på 5 minuter” recipe in the factory at Abba in Kungshamn. The fish raw material was the pre-pickled fish that is usually used in the pickled/marinated herring products produced by Abba. The marinade the fish is stored in when it is delivered to Abbas production is containing salt, water and acetic acid solution. (Abba, 2015) Since the raw material for the marinated herring samples with the other acids already is marinated in a solution containing acetic acid the produced products in this study are containing an amount of acetic acid. The amount of acetic acid in the different versions is unknown but it could have had an impact in both the sensorial test and the microbial tests.

To avoid the acetic acid and to be able to evaluate the effect of using another kind of acid than acetic acid more correct the raw material has to be fresh fish that has not been pre-marinated. Since Abba today purchases the fish pre-marinated it would be a question of cost and production possibilities if it is a wish to in the future be able to produce pickled herring by using another weak acid like lactic acid, malic acid or citric acid.

6 Conclusions

By changing the weak acid in the sauce of marinated herring from the usually used acetic acid to citric acid, malic acid or lactic acid the result is four versions of marinated herring with only small differences.

Both the accelerated shelf life test and the microbial tests after 14 weeks of normal storage in refrigerator with the marinated herring versions indicate that all four herring versions were safe products without spoilage. There were no big differences in shelf life for this period but more investigations are needed to determine the shelf life of the products for a longer time period.

The sensorial evaluation conclude that the favourite of the four produced pickled herring versions was the type with lactic acid since it was milder, not as sour and it had a balance between sweetness, sourness and saltiness. The panellists commented that there were only small differences in flavours between then samples and that the texture was rather the parameter that differed. The texture analysis on the other hand showed that there was no significant difference between the marinated herring versions.

L. plantarum that has before been found in spoiled marinated herring is perfectly able to grow in MRS with 2.8 % sodium chloride and pH adjusted to 4.2 and 5.0 with acetic acid, citric acid, malic acid or lactic acid. A lower initial pH is affecting the growth and it can be concluded that malic acid and citric acid have lower impact on *L. plantarum* compared to lactic acid and acetic acid. In both MRS with pH 4.2 and 5.0 with lactic acid the concentration of *L. plantarum* was stable (stationary phase) on a higher level than in the other acids. Since *L. plantarum* and other LAB are producing lactic acid and thereby regulate the pH the result would perhaps be different with other microorganisms.

7 Future Perspectives

The sensorial test of the marinated herring indicated that consumers prefer when the herring is a little less sour which can be achieved by using lactic acid that has a milder taste compared to acetic acid. To really evaluate the taste of pickled herring with malic acid, citric acid, and lactic acid, fish that has not first been marinated in a solution containing acetic acid has to be used. For a production of pickled herring containing only the weak acid (not acetic acid) the question of if it is possible in the production has to be considered. Because of the comments of the results of the sensorial evaluation it can be discussed whether there is a future in changing acetic acid to another weak acid when using fish pre-marinated with acetic acid before reaching Orkla's production. The majority of members in the panel believed that there were only small changes in taste of the herring versions. The next step in the sensorial aspect would be to try to produce marinated herring with raw fish as raw material to evaluate the effect of the acids on the characteristics of the product. If using the fish that is pre-marinated in acetic acid (the raw material in Orkla's production), then the recipe of the sauce should be regulated for each acid to achieve balance of the flavours sweetness, sourness and salinity of the fish. At the same time the correct pH of the solution has to be reached. To do that investigation for each acid for every sauce has to be made because of the buffering effect of the other ingredients.

In the safety aspect more studies with the pickled herring is recommended. In this project the herrings were only stored in refrigerator for about three month due to the time frame of the Master thesis study and the normal shelf life of marinated herring is more than that. In the project accelerated tests in around room temperature were performed to simulate a longer storage time but in a higher temperature microorganisms that prefer higher temperatures rather than lower temperatures would be favoured. The next step would be to store the pickled herring for a longer time in refrigerator and do regularly microbial tests to measure eventual growth and thereby determine the different shelf lives of the products. The produced pickled herring in this project contained preservatives to make the safer for the sensorial test and to further evaluate the effect of acids in shelf life the pickled herring should have been without preservatives.

In the accelerated test with modified MRS the samples were incubated in 25°C but it could have been stored in a refrigerator to be more correct as the storage of marinated herring. More microorganisms like yeast and mould could also have been examined. In the future it would be interesting and valuable to inoculate microorganism in the real pickled herring, store it in refrigerator and regularly measure the concentration of the microorganism. Still the results of this project can be used to estimate the microbial effect of the acids.

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9 Appendix

9.1 Calculations for amount of acid in the marinated herring jars

The amount of acetic acid is fixed according to the original recipe from Abba for the pickle solution. To determine the hydronium ions concentration in the acetic acid pickle solution the equation for the dissociation constant (K_a) was used.

$$K_a = \frac{[H^+]\times[A^-]}{[HA]} \rightarrow [H^+] \times [A^-] = K_a \times [HA]$$

HA = The weak acid in undissociated form
$H^+ = H_3O^+$, hydronium ions
A^- = The dissociated form of the weak acid
K_a = The dissociation constant for the weak acid

It is estimated that all of the pickle solutions contain the same (none) amount of hydronium ions before addition of acid, $[H^+] = [A^-] = x$

$$x^2 = K_a \times [HA] \rightarrow x = \sqrt{K_a \times [HA]}$$
$$= hydronium\ ions\ and\ the\ concentration\ of\ dissociated\ acetic\ acid\ molecules$$

The concentration of hydronium ions should be the same in all of the four pickle solution using the acetic acid recipe as a reference. This means that the x-value for the pickle solution with malic acid, citric acid and lactic acid should be the same as for the acetic acid solution.

$$x = \sqrt{K_a \times [HA]} \rightarrow \frac{x^2}{K_a} = [HA]$$

The acetic acid pickle solution contains in total 2 % of acetic acid solution (containing 12 % acetic acid) and the molar mass of acetic acid is 60.05 g/mole. This gives a concentration of acetic acid of 0.040 mole/L

$$\frac{0.02 \times 0.12 \times 1000}{60.05} = 0.039967 \frac{mole}{L} = [HA]$$

The K_a value of acetic acid is 1.7378 x 10^{-5} giving the x-value 0.000833391

$$x = \sqrt{K_a \times [HA]} = \sqrt{1.7378 \times 10^{-5} \times 0.039967} = 8.33391 \times 10^{-4}$$

To determine the amount of needed acid the equation below was used and the numbers for the acids in Table 6 was used

$$\frac{x^2}{K_a} = [HA]$$

Table 6 The table shows the dissociation constant (K_a), molar mass (M) and density of the acids that was used to produce the sauces for the four different pickled herring versions.

Acid	K_a	Molar mass (M) (g/mole)	Density (g/cm^3)
Acetic acid	1.74×10^{-5}	60.05	Was estimated to 1.00
Citric acid	7.04×10^{-4}	192.124	1.66
Lactic acid	8.32×10^{-4}	90.08	1.20
Malic acid	3.98×10^{-4}	134.087	1.60

Citric acid: $\frac{x^2}{K_a} = \frac{8.33391 \times 10^{-4\,2}}{7.04 \times 10^{-4}} = 0.00099\ mole/L$

$$C \times M = 0.00099 \times 192.124 = 0.18954\ g/L$$

Malic acid: $\frac{x^2}{K_a} = \frac{8.33391 \times 10^{-4\,2}}{3.98 \times 10^{-4}} = 0.00175\ mole/L$

$$C \times M = 0.00175 \times 134.0874 = 0.23399\ g/L$$

Lactic acid: $\frac{x^2}{K_a} = \frac{8.33391 \times 10^{-4\,2}}{8.32 \times 10^{-4}} = 0.00083\ mole/L$

$$C \times M = 0.00083 \times 90.08 = 0.0752\ g/L$$

Since the lactic acid is in 80% liquid solution needed amount is $\frac{0.0752}{0.8} = 0.094\ g/L$

The volume of sauce corresponding to 35 jars of pickled herring was produced. One jar contains 240 g herring (48 %) and sauce (52 %) which means that 4 368 g of sauce for each acid was produced.

$$35 \times 240 \times 0.52 = 4\,368\ g$$

For acetic acid 87.36 g of acetic acid solution (12 %) was added according to:

Acetic acid: $240 \times 0.52 \times 0.02 \times 35 = 87.36\ g\ acetic\ acid\ solution$

By accident the calculated numbers were used in a wrong way, the concentration was thought as the needed amount of acid in each jar, and therefore a larger amount of acid was added to the solutions. The added amount of acids for sauce to 35 jars for each acid is presented in Table 7.

Table 7 The amount of acids that was added to the sauces to the volume of 35 jars (a' 124.8 g).

Acid	Added amount for 35 jars
Acetic acid (12% solution)	87.36 g
Citric acid (crystalline form)	6.640 g
Malic acid (crystalline form)	8.190 g
Lactic acid (80% solution)	3.290 g

To reach the same volume of all of the sauces extra water was added in the sauces containing citric acid, malic acid and lactic acid. The extra amount of water was calculated by using the density of the acids. For example:

Citric acid 6.640 g of acid was added which is equal to 11.01 cm^3 then 76.35 ml of extra was added to the sauce.

$$V_{water} = V_{Acetic\ acid} - V_{Citric\ acid} = 87.36 - (6.640 \times 1.66) = 76.35\ cm^3$$

It was noticed after the production of the herring that it is not possible to calculate the needed amount of acid in the pickled herring sauce since the other ingredients like the preservatives might buffer the acids differently. These calculations above only give a guideline of how much acid that should be added to reach the same pH in all of the solutions.

The general reaction of an acid in water is

$$HA + H_2O \leftrightarrows H_3O^+ + A^-$$

This means that if the conjugated acid (A^-) of the acid (HA) is present in the solution the reaction will go more to the left and the acid will be undissociated again. (Buffer Solutions, 2015)

In the production of the pickled herring the pH of blended fish and sauce in two jars of each version was measured. Also the pH of the bare sauce was measured using a pHmeter (Mettler Toledo). The results of the pH measurements are presented in Table 8.

Table 8 Four different versions of pickled herring were produced with different acids in the sauce. The pH of the blended fish and sauce are presented in the table.

Acid	pH (fish + sauce)	pH (sauce)
Acetic acid	4.2	4.1
	4.2	
Citric acid	4.2	4.2
	4.2	
Lactic acid	4.3	4.7
	4.2	
Malic acid	4.1	4.0
	4.2	

A larger amount of acid than intended (according to the calculations) was added in the sauces with citric acid, lactic acid, and malic acid. The pH of the sauces is somewhat various but still the pH of the blended fish and sauce is around 4.2 as intended. It can be concluded that the pH of the fish, which was the same for all pickled herring types, has a large impact of the final pH of the mixture. In the defence against microorganisms there might be some effects due the slightly higher or lower pH but since the pH is below 4.5 all versions should be safe products. The pH should be lower than 4.5 to avoid for example the very dangerous pathogen *Clostridium botulinum* that can grow in anaerobic environments with pH higher than 4.5 (Livsmedelsverket, 2015).

9.2 Sensory evaluation questionnaire

The questionnaire used in the sensory evaluation was in Swedish but the used questionnaire below has been translated into English by the author afterwards.

Sensorial evaluation of pickled herring

Do you in general enjoy eating pickled herring?

Yes No It tastes ok I don't know

Placed in front of you have a plate with four different pickled herring versions. Taste the herrings and evaluate them in the form below. Rinse your mouth with water after each herring sample.

Sample 367

Evaluate the sourness of the herring. Make a circle around the correct number! (10 = Very sour, 1 = Low sourness).

1 2 3 4 5 6 7 8 9 10

Evaluate the salinity of the herring. Make a circle around the correct number! (10 = Very salty, 1 = Low salinity)

1 2 3 4 5 6 7 8 9 10

Evaluate the sweetness of the herring. Make a circle around the correct number! (10 = Very sweet, 1 = Low sweetness)

1 2 3 4 5 6 7 8 9 10

Evaluate the firmness of the herring. Make a circle around the correct number! (10 = Very firm, 1 = Soft/loose texture)

1 2 3 4 5 6 7 8 9 10

Evaluate the taste of the herring. Make a circle around the correct number! (10 = Enjoy it very much, 1 = Dislike it very much)

1 2 3 4 5 6 7 8 9 10

Sample 792

Evaluate the sourness of the herring. Make a circle around the correct number! (10 = Very sour, 1 = Low sourness).

1 2 3 4 5 6 7 8 9 10

Evaluate the salinity of the herring. Make a circle around the correct number! (10 = Very salty, 1 = Low salinity)

1 2 3 4 5 6 7 8 9 10

Evaluate the sweetness of the herring. Make a circle around the correct number! (10 = Very sweet, 1 = Low sweetness)

1 2 3 4 5 6 7 8 9 10

Evaluate the firmness of the herring. Make a circle around the correct number! (10 = Very firm, 1 = Soft/loose texture)

1 2 3 4 5 6 7 8 9 10

Evaluate the taste of the herring. Make a circle around the correct number! (10 = Enjoy it very much, 1 = Dislike it very much)

1 2 3 4 5 6 7 8 9 10

Sample 954

Evaluate the sourness of the herring. Make a circle around the correct number! (10 = Very sour, 1 = Low sourness).

1 2 3 4 5 6 7 8 9 10

Evaluate the salinity of the herring. Make a circle around the correct number! (10 = Very salty, 1 = Low salinity)

1 2 3 4 5 6 7 8 9 10

Evaluate the sweetness of the herring. Make a circle around the correct number! (10 = Very sweet, 1 = Low sweetness)

1 2 3 4 5 6 7 8 9 10

Evaluate the firmness of the herring. Make a circle around the correct number! (10 = Very firm, 1 = Soft/loose texture)

1 2 3 4 5 6 7 8 9 10

Evaluate the taste of the herring. Make a circle around the correct number! (10 = Enjoy it very much, 1 = Dislike it very much)

1 2 3 4 5 6 7 8 9 10

Sample 185

Evaluate the sourness of the herring. Make a circle around the correct number! (10 = Very sour, 1 = Low sourness).

1 2 3 4 5 6 7 8 9 10

Evaluate the salinity of the herring. Make a circle around the correct number! (10 = Very salty, 1 = Low salinity)

1 2 3 4 5 6 7 8 9 10

Evaluate the sweetness of the herring. Make a circle around the correct number! (10 = Very sweet, 1 = Low sweetness)

1 2 3 4 5 6 7 8 9 10

Evaluate the firmness of the herring. Make a circle around the correct number! (10 = Very firm, 1 = Soft/loose texture)

1 2 3 4 5 6 7 8 9 10

Evaluate the taste of the herring. Make a circle around the correct number! (10 = Enjoy it very much, 1 = Dislike it very much)

1 2 3 4 5 6 7 8 9 10

Rank the pickled herrings types (1 = The best version, 4 = The worst version)

1.

2.

3.

4.

Comments:_____
_____
_____

Was there a clear difference between the four pickled herrings?

Yes No

Comments:_____
_____
_____

Other comments (sourness, flavours etc.):
_____
_____
_____

Thank you!

9.3 Microbial tests in accelerated and not accelerated shelf life evaluation with pickled herring jars in Experiment 1

Table 9 Four versions of pickled herring with different acids: acetic acid, lactic acid, citric acid or malic acid, in the sauce were produced and microbial tests were performed to evaluate the shelf life of the herrings. At each microbial test one sample from three jars of herring of each version were examined. The table shows the results of the microbial tests with herring jars that had been stored in refrigerator for three weeks after production.

		Acetic acid			**Lactic acid**			**Citric acid**			**Malic acid**		
	No.	486	487	488	489	490	491	492	493	494	495	496	497
	Conc	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Aerobic MO	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Enterobacteriaceae	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coliform bacteria*	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase positive *Staphylococcus*	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
LAB	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Yeasts	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Moulds	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
L. monocytogenes	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2

* Negative results of coliform bacteria indicate no presence of *E. coli* as well.

Table 10 In Experiment 1 four versions of pickled herring with different acids: acetic acid, lactic acid, citric acid or malic acid, in the sauce were incubated in 22.6°C for 15 days. Microbial tests with the herrings were performed after seven and fifteen days and the table shows the results of the microbial tests after seven days of incubation. At each sampling three jars of each type and one sample in each jar were examined.

		Acetic acid			**Lactic acid**			**Citric acid**			**Malic acid**		
	No.	524	525	526	527	528	529	530	531	532	533	534	535
	Conc	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Aerobic MO	-2	<2	<2	<2	<2	<2	<2	2.0	2.0	<2	<2	<2	<2
Enterobacteriaceae	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	>4.4*	<1
Coliform bacteria**	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase positive *Staphylococcus*	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
LAB	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Yeasts	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Moulds	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	1.0

* Since there was no presence of aerobic microorganisms it was assumed that the high concentration of *Enterobacteriaceae* was due to contamination when preparing the sample for the microbial tests.

** Negative results of coliform bacteria indicate no presence of *E. coli* as well.

Table 11 In Experiment 1 for versions of pickled herring with different acids: acetic acid, lactic acid, citric acid or malic acid, in the sauce were incubated in 22.6°C for 15 days. Microbial tests with the herrings were performed after seven and fifteen days and the table shows the results of the microbial tests after fifteen days of incubation. At each sampling three jars of each type and one sample in each jar were examined.

		Acetic acid			**Lactic acid**			**Citric acid**			**Malic acid**		
	No.	546	547	548	549	550	551	552	553	554	555	556	557
	Conc	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Aerobic MO	-2	2.0	<2	2.3	<2	<2	2.3	<2	3.0	<2	<2	<2	2.0
Enterobacteriaceae	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coliform bacteria*	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase positive *Staphylococcus*	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
LAB	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Yeasts	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Moulds	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

* Negative results of coliform bacteria indicate no presence of *E. coli* as well.

Table 12 Four versions of pickled herring with different acids: acetic acid, lactic acid, citric acid or malic acid, in the sauce were produced and microbial tests were performed to evaluate the shelf life of the herrings. At each microbial test one sample from three jars of herring of each version were examined. The table shows the results of the microbial tests with herring jars that had been stored in refrigerator for twelve weeks after production.

		Acetic acid			**Lactic acid**			**Citric acid**			**Malic acid**		
	No.	674	675	676	677	678	679	680	681	682	683	684	685
	Conc	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Aerobic MO	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	>4.4	<2	<2
Enterobacteriaceae	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coliform bacteria*	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase positive *Staphylococcus*	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
LAB	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Yeasts	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Moulds	-1	<1	<1	<1	<1	<1	<1	<1	1.0	<1	<1	<1	1.0

* Negative results of coliform bacteria indicate no presence of *E. coli* as well.

Table 13 Four versions of pickled herring with different acids: acetic acid, lactic acid, citric acid or malic acid, in the sauce were produced and microbial tests were performed to evaluate the shelf life of the herrings. At each microbial test one sample from three jars of herring of each version were examined. The table shows the results of the microbial tests with herring jars that had been stored in refrigerator for fourteen weeks after production. These extra tests were performed as an extra control due to the results of the microbial tests after twelve weeks.

		Acetic acid			**Lactic acid**			**Citric acid**			**Malic acid**		
	Name	790	791	792	793	794	795	796	797	798	799	800	801
	Conc	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Aerobic MO	-2	2.0	<2	2.0	<2	<2	<2	<2	<2	<2	<2	<2	<2
Enterobacteriaceae	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coliform bacteria*	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase positive *Staphylococcus*	-2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
LAB	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Yeasts	-1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Moulds	-1	<1	<1	<1	<1	<1	<1	<1	1.3	<1	<1	<1	1.0

* Negative results of coliform bacteria indicate no presence of *E. coli* as well.

9.4 Experiment 2: Growth of L. plantarum in modified MRS nutrient medium

Table 14 In Experiment 2 *L. plantarum* was inoculated in salt and acid modified MRS medium. Two identical biological replicated were performed and in the experiments the pH in the MRS was adjusted to 4.2. The concentration of *L. plantarum* was determined with viable count and samples were taken every third or fourth day. The table shows the concentration (log CFU/ml) of *L. plantarum* in the biological replicate 1 (blue), biological replicate 2 (white) and the average of the replicates (orange) at each sampling.

Acid (pH 4.2)	Start 1	Start 2	Average start 1 & 2	1.2	2.2	Average 1.2 & 2.2	1.3	2.3	Average 1.3 & 2.3	1.4	2.4	Average 1.4 & 2.4	1.5	2.5	Average 1.5 & 2.5
Citric acid	5.342	5.396	5.369	8.498*	8.547	8.522	7.908*	8.485	8.196	7.703*	8.399	8.051	8.000	8.174	8.087
Acetic acid	5.275	5.339	5.307	7.393	7.943	7.668	8.313	8.152	8.233	**	8.015	8.015	7.128	7.204	7.166
Lactic acid	5.333	5.515	5.424	8.203	8.515	8.359	8.645	8.690	8.668	8.152*	8.502	8.327	8.342	8.363	8.352
Malic acid	5.279	5.378	5.328	8.810*	9.056	8.933	8.072*	8.497	8.285	7.423*	7.945	7.684	7.585	7.483	7.534
Days of incubation	0	0	0	3	3	3	7	7	7	10	10	10	14	14	14

* The results from the viable count come from a diluted sample that has been stored overnight in refrigerator before spreading out on an MRS plate.

** There were problems with the dilutions of the sample and therefore a result from that sample could not be achieved.

Table 15 In Experiment 2 *L. plantarum* was inoculated in salt and acid modified MRS medium. Two identical biological replicated were performed and in the experiments the pH in the MRS was adjusted to 5.0. The concentration of *L. plantarum* was determined with viable count and samples were taken every third or fourth day. The table shows the concentration (log CFU/ml) of *L. plantarum* in the biological replicate 1 (blue), biological replicate 2 (white) and the average of the replicates (orange) at each sampling

Acid (pH 5.0)	Start 1	Start 2	Average start 1 & 2	1.2	2.2	Average 1.2 & 2.2	1.3	2.3	Average 1.3 & 2.3	1.4	2.4	Average 1.4 & 2.4	1.5	2.5	Average 1.5 & 2.5
Citric acid	5.312	5.427	5.370	9.068*	9.198	9.133	7.130*	7.452	7.291	7.036*	7.383	7.210	7.018	7.075	7.046
Acetic acid	5.360	5.345	5.352	9.332	9.184	9.258	6.945*	7.293	7.119	7.083*	7.459	7.271	7.041	7.149	7.095
Lactic acid	5.251	5.421	5.336	9.173*	9.142	9.158	7.811*	8.194	8.002	7.473*	7.888	7.681	7.400	7.453	7.426
Malic acid	5.280	5.366	5.323	9.026*	9.215	9.120	7.097*	7.115*	7.106	7.017*	7.031	7.024	6.783	6.785	6.784
Days of incubation	0	0	0	3	3	3	7	7	7	10	10	10	14	14	14

* The results from the viable count come from a diluted sample that has been stored overnight in refrigerator before spreading out on an MRS plate.