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Growth kinetics and spoilage potential of Aeromonas salmonicida strain SU2 in pre-rigor filleted back loins of salmon in vacuum and modified atmosphere packaging during cold storage

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Master in Food and Technology
Submission date: August 2018
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Preface

This thesis covers 30 ECTS points and was carried out as the final part of the MSc programme for Food Science and Technology at the Department of Food Science, faculty of Science at the University of Copenhagen. This master thesis was completed at the institute of Biotechnology and Food Science, faculty of natural sciences at the Norwegian University of Science and Technology in Trondheim in spring 2018.

Great thanks to Associate professor Anita Nordeng Jakobsen and PhD Assistant Professor Sunniva Hoel and scientist Elena Shumilina for excellent supervision and guidance throughout the project. Furthermore, appreciations to the lab technicians and all employees at NTNU answering questions and providing exceptional help in laboratory, thereby making the experiments feasible. I would also like to thank my fellow students in Copenhagen and Trondheim for motivating me during the long hours of lab work and writing. Finally, a great thanks to my friends and family for always supporting and listening, despite not always understanding the context of my studies.

15th of August 2018

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Abstract

Aeromonas spp. are ubiquitous bacteria in all environments and can easily be isolated from aquatic habitats. They have frequently been isolated from seafood and present a potentially food spoilage and food safety hazard in ready-to-eat seafood because of their ability to grow in a relatively wide range of temperatures including refrigerated storage. However, there is a limited knowledge about the growth and spoilage potential of *Aeromonas salmonicida* in cold water fish species and under various environmental conditions.

The aim of this study was to assess growth kinetics and the spoilage potential of *A. salmonicida* SU2 in vacuum packaging and modified atmosphere packaging (CO_2:N_2 40:60) on *pre-rigor* filleted back loins of salmon stored at 4 and 8°C. A challenge study was conducted to examine growth kinetics of *A. salmonicida.* Eleven fish metabolites including amino acids, sugars, biogenic amines as well as different products of the ATP degradation process were quantified by nuclear magnetic resonance (NMR).

The results showed that the inoculated strain of *A. salmonicida* was capable of growth in both vacuum packaging and in MAP at both storage temperatures. The maximum growth rate of *A. salmonicida* was approximately 3x higher at 8°C than at 4°C in both vacuum and MAP salmon. However, the lag-phases were generally longer in modified atmosphere. In addition, the results demonstrated the effect of MAP, resulting in a 2-fold decrease in the growth rate of *A. salmonicida* at 4°C compared to vacuum. Generally, the growth rate increased under vacuum packaged conditions for all samples. The data from NMR analysis showed a general increase in the formation of undesired biogenic amines at higher temperatures and at vacuum packaging compared to MAP.

The overall results showed that growth of *A. salmonicida* is highly affected by temperature, and it demonstrated that refrigeration (4°C) and MAP are hurdles to inhibit growth of *A. salmonicida* and to reduce spoilage on *pre- rigor* fileted back loins of salmon. However, more studies are needed to elucidate the actual role of *A. salmonicida* as a spoilage organism in vacuum packed and MAP salmon.

Sammendrag

Aeromonas spp. er vidt utbredt i naturen og kan enkelt isoleres fra akvatiske miljøer. Arten har flere ganger blitt isolert fra sjømat, og med tanke på dens evne til å vokse i ulike temperaturer (inkludert kjølelagring) utgjør den en reell risiko med tanke på forringelse og mattrygghet i spiseklar sjømat. Det finnes lite kunnskap om veksten og forringelsespotensialet til *Aeromonas salmonicida* i kaldtvannsfisk og under ulike miljøforhold.

Målet med denne oppgaven var å undersøke veksthastigheten og forringelsespotensialet til *A. salmonicida* SU2 i vakuum pakket og modifisert atmosfære pakket (CO_2: N_2 40:60) *pre- rigor* filetert laks ved 4 og 8°C. Det ble utført et lagringsforsøk for å undersøke veksthastigheten til *A. salmonicida* og ved bruk av NMR ble elleve metabolitter kvantifisert i laksen derav aminosyrer, biogene aminer, i tillegg til ATP nedbrytningsprosesser.

Resultatene viste at den inokulerte stammen av *A. salmonicida* kunne vokse i vakuum og modifisert atmosfære ved 4 og 8°C. Den maksimale veksthastigheten til *A. salmonicida* var omtrent 3 ganger høyere ved 8°C enn ved 4°C i både vakuum pakket og modifisert atmosfære pakket laks. Lag fasen var generelt lenger i modifisert atmosfære. Resultatene viste at MAP hadde en effekt, som resulterte i en 2 ganger reduksjon i veksthastighet av *A. salmonicida* ved 4°C sammenlignet med vakuum. Generelt økte veksthastigheten i prøvene som var pakket i vakuum. Resultatene fra NMR analysene viste en generell økning av uønskede biogene aminer ved 8°C og ved laks pakket i vakuum sammenlignet med laks pakket i MAP.

De samlede resultatene viste at vekst av *A. salmonicida* påvirkes av temperatur og pakkemetode. Resultatene viser at kjølelagring (4°C) og MAP er viktige barrierer for å hemme vekst av *A. salmonicida* og redusere forringelse på *pre-rigor* filetert laks. Flere studier er nødvendig for å belyse den faktiske rollen til *A. salmonicida* som en forringelsesorganisme i vakuum pakket og MAP laks.

Table of contents

1. Introduction

Over the past 30 years, the aquaculture sector has become the fastest growing sector of the global food market (Little et al., 2015). Norway, with a coastline of more than 83. 000 km, is one of the leading nations in production of marine fisheries and aquaculture. The Norwegian aquaculture industry has gone through an astounding development in recent years. The last ten years, total revenue growth has increased because of the increasing prices and burgeoning demand in both existing and new markets (Liu et al., 2011). This sector has played an important economic and social role nationally and regionally, and the fish industry is the basis for settlement and employment along the entire Norwegian coast (Richardsen and Bull-Berg, 2016).

There is an increasing demand for seafood products that are both easy to prepare and have a long shelf life. Because such products are not further processed, the microbiological risks are of increasing interest for the industry (Jaroni et al., 2010). Minimally processed seafood has been defined by Rosnes et al. (2011) as "*a category that has been exposed to a thermal process, typically to a final processing temperature from a low pasteurization at 60°C to a high pasteurization at 95°C for 10 to 30 min, with a water activity >0.85 and pH >4.6"*. The increasing demand for fresh chilled fish is a result of a general increase in consumption of food eaten raw. Sushi and sashimi are examples of foods contributing to this trend. The current knowledge and technology allow us to produce food that extends shelf life and reduce microbial growth by applying hurdles such as salting, mild heat treatment, vacuum- and or modified atmosphere packaging (MAP) (Gram and Huss, 1996). However, when these hurdles are reduced in ready to eat food, increased attention to factors that affect microbial growth in a product are of high importance. For instance, temperature control for controlling microbial growth throughout the value chain (Koutsoumanis, 2001, Mercier et al., 2017). Understanding the dynamics of microbial behavior along the entire food chain is fundamental to correctly determine the shelf life of a product, control microbial spoilage as well as limit the growth of pathogenic bacteria. Some pathogenic bacteria can enumerate during refrigerated storage, such as *Listeria monocytogenes* and *Aeromonas spp*. (Ferreira et al., 2014). Special attention must be paid to these microbial hazards in seafood production since *Aeromonas spp*. are mostly associated with aquatic environments, evidenced by seafood often being contaminated by these bacteria (Ullmann et al., 2005, Hoel et al., 2015). Aeromonads

are particularly capable of adaption to different environments, and the bacteria possess properties which enable them to survive at low temperatures (Karem et al., 1994).

To date, the knowledge about growth of *Aeromonas spp.* in ready to eat seafood is very limited. Generally, a limited amount of studies have been published regarding growth potential of *Aeromonas salmonicida* in salmon (Hoel et al., 2018). Studies are done regarding growth potential of *Aeromonas spp.* in rainbow trout (González-Rodríguez et al., 2002), *Aeromonas hydrophila* in oysters (Birkenhauer and Oliver, 2002) and squid (Park and Ha, 2014) in normal atmospheres. Thus, the increased consumption of ready to eat seafood requires more data about the composition and behavior of the microbial communities, both at different temperatures and packaging conditions to assess microbial spoilage and shelf life of seafood. The aim of this study was to assess growth kinetics and the spoilage potential of *A. salmonicida* in vacuum packaging and modified atmosphere packaging (CO_2: N_2 40:60) on *pre-rigor* filleted back loins of salmon stored at 4 and 8°C.

2. Background

2.1 The role of *Aeromonas spp*. as food pathogen and spoiler organism

Aeromonas spp. are ubiquitous in all environments and can easily be isolated from aquatic habitats, fish, foods and natural soils (Janda and Abbott, 2010). They are gram-negative rods of the family Aeromonadaceae. They are oxidase positive, glucose-fermenting, facultative anaerobic and most of them are motile by polar flagella. The cells are typically 1,0 to 4,4 µm in diameter. Most aeromonads can grow at a pH range of 5,5- 9,0 (Isonhood and Drake, 2002). The optimum growth temperature is around 28°C, but a wide temperature growth range have been observed (Kirov, 1993, Kirov et al., 1990, Mateos et al., 1993, Pavan et al., 2000), whereas the maximum temperature is believed to be around 38-41°C. Palumbo et al. (1985) established that several isolates were capable of growth at temperatures down to 4°C. This makes aeromonads particularly significant in refrigerated foods. For example, *A. hydrophila* has been reported to have an optimal growth temperature of 28°C, although it can tolerate and grow from 1- 42°C. However, this is not the only species that can grow at cool temperatures (González-Rodríguez et al., 2002). The genus Aeromonas can be classified into two main groups. First, the psychrophilic non-motile strains, primarily *A. salmonicida* that infects fish. The second and larger group is motile mesophilic aeromonads associated with human diseases such as gastroenteritis disease, septicemia and wound infections (Isonhood and Drake, 2002, Parker and Shaw, 2011). There are approximately 30 recognized species of Aeromonas where *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii biovar sobria,* and *Aeromonas dhakensis* are more frequently implicated in human infections (Martínez-Murcia et al., 2016).

Aeromonas spp. have received increased attention as human pathogens because of their widespread occurrence in food such as in fresh vegetables, red meat, poultry, raw milk and seafood products (Neyts et al., 2000, Osman et al., 2012, Odeyemi and Ahmad, 2017, Ventura et al., 2015, Yucel et al., 2005, Topic Popovic et al., 2015). Water is considered as the main source of food contamination. A selection of different virulence factors enables these bacteria to colonize, invade and infect different hosts. The most studied virulence factors are the pore-forming hemolytic toxins, hemolysin, and aerolysin (Howard et al., 1987, Parker and Shaw, 2011), and three different enterotoxins, including cytotoxic (Act), heat-labile cytotonic (Alt), and heat-stable cytotonic (Ast) enterotoxins. All these toxins have been linked to cases of diarrhea (Galindo et al., 2006). Furthermore, the expression of lateral or peritrichous flagella

is associated with enhanced adherence and invasiveness in addition to the ability to biofilm formation (Gavín et al., 2003).

Aeromonas are recognized as a spoilage organism in fish, mainly in fish species from tropical and warmer waters (Ryder et al., 2014), however the role of Aeromonas as a spoiler in Atlantic fish such as Atlantic salmon is not widely explored. Several species of Aeromonas are identified as potential food spoilers, and there is most likely differences in spoilage potential at species and strain level (Beaz-Hidalgo et al., 2015). *A. salmonicida* was identified as part of the spoilage microbiota in ice stored sea bream, however the actual spoilage potential was not elucidated (Parlapani et al., 2013). In addition, *Aeromonas spp*. has been identified as part of the dominating microbiota in spoiled grass carp (Huang et al., 2018). Additionally, high levels of *Aeromonas spp.* (8 log cfu/ml) have previously been detected in milk (Kirov, 1993). It has been proven by Kirov (1993) that *Aeromonas spp*. are not very sensitive to MAP, and can grow to high levels when the competitive microbiota is low. Thus, refrigerated MAP food and vacuum packaged food could be a great niche for some Aeromonas species. For instance, growth of *A. caviae* in fresh salads was stimulated by MAP (Allende et al., 2002). Moreover, Aeromonas isolates associated with spoilage (in fish and meat) has been characterized as slime producers (Arslan and Küçüksari, 2015).

A study by Hoel et al. (2015) detected *Aeromonas spp*. in 71% of fresh retail ready-to-eat (RTE) sushi boxes purchased in Norwegian supermarkets. The sushi meals typically consist of various components of vegetables and fish, which are excellent substrates for growth. *A. salmonicida* was found to be the most prevalent (74%) in fresh retail sushi. However, *A. hydrophila* is by far the most studied species and is generally not thought to constitute a risk in refrigerated food. However, the genus Aeromonas consist of more than 30 recognized species and the spoilage potential under various conditions that are relevant for food are not fully explored for all food relevant species.

2.2 *Post- mortem* changes in fish

Sensory changes, autolytic changes, bacteriological changes and lipid oxidation and hydrolysis are the common *post- mortem* changes in fish. Knowledge about *post-mortem* changes is important to maintain quality and safety of fish products. Figure 1 illustrates a characteristic pattern of the deterioration of cod. The deterioration is shown by quality score of absolute freshness, and days during storage. While 10 indicate absolute freshness and 4 is the rejection level. During first phase, the fish is fresh and tastes normal. In phase two the fish loses the characteristic smell and taste, however with no off odors and still having a nice texture. For both these phases the major changes are caused by autolysis. In the third phase there are tendencies to spoilage and a range of volatile compounds are growing making unpleasant smelling substances. In the last phase, the fish is characterized as spoiled and putrid mainly because of bacterial activity (Huss, 1988).

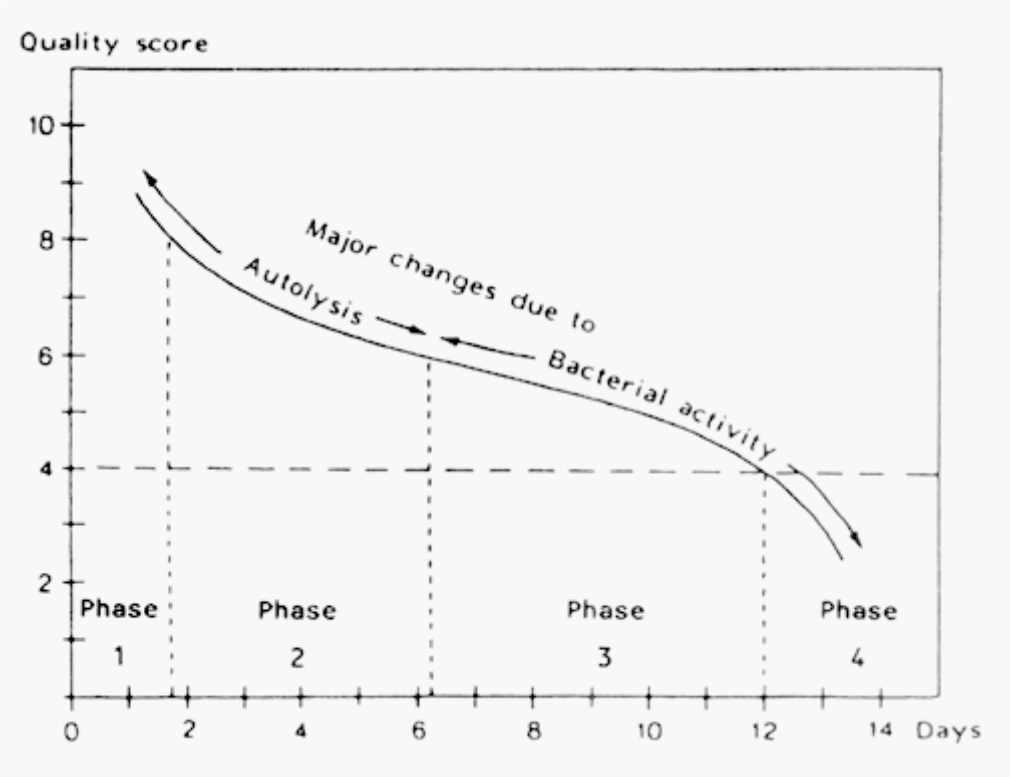

Figure 1: Four phases of changes in determination of eating quality of iced cod due to autolysis and bacterial activity. The quality score 10 indicates absolute freshness and quality score 4 is the rejection level (Huss, 1988).

At the point of death, autolysis is responsible for the degradation of fish, figure 2. This means that the enzymes from the fish starts the degradation itself chemically. When the fish dies, a lot of different reactions starts, which in the end leads to death stiffening (*rigor mortis*). The supply of oxygen to the muscle makes it possible to release energy as ATP. However, under anaerobic conditions blood is no longer pumped into the muscle. The autolytic reaction will then start by glycolysis. This *post-mortem* glycolysis results in accumulation of lactic acid which in turn lowers the pH in the muscle (Huss, 1988). At the same time ATP- degradation will happen. This reaction ceases when the creatine or arginine phosphates are depleted. This reaction will contribute to both wanted and unwanted aromatic substances. Both glycogen and ATP content are depleted before the fish is in rigor mortis. The cells in the fish will then use the vitamins and antioxidants available at death stiffening, and after a period of time this will cause the fish to rancid. When the autolytic degradation is complete, the bacteriological degradation starts (Hong et al., 2017).

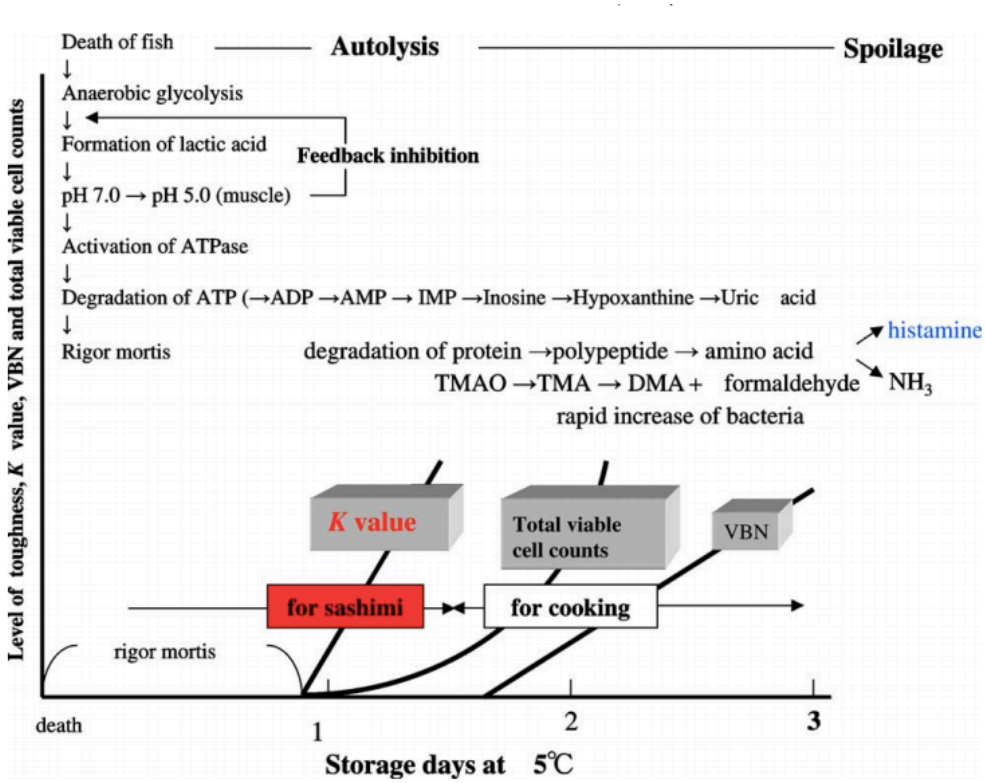

Figure 2: Post-mortem changes in fish meat (Hamada-Sato et al., 2005)

2.3 Spoilage of seafood

Food spoilage is any change in the appearance, smell or taste of a food product. These changes make it unacceptable to eat for the consumer. Spoiled food may be safe to eat but generally not acceptable mainly because of reduced quality. However, spoilage causes losses to producers and distributors as well as consumers (Montville et al., 2012).

Microbial spoilage is a consequence of microbial growth and/or activity, which contributes to changes in sensory characteristics in the food. The spoilage organisms are those that gain access to the food and uses the available nutrients to grow. Microbial control during processing and storage is essential when the quality and shelf life of the food is determined. However, spoilage is usually only observed for the consumer when the microbial population density is high. Since food spoilage is a result of microbiological growth there is a direct relationship between the original total number of microorganisms and degree of spoilage. However, in the same type of product, spoilage may develop differently. Geographical origin, and other unknown factors can also affect the microbial development in the raw material (Gram and Huss, 1996).

Salmon contains high amounts of unsaturated fatty acids. These acids can be classified into omega- 3(ω-3), omega- 6 (ω-6) and omega-9 (ω-9) based on the chemical structure of the fatty acid (Sun et al., 2011). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is two types of ω-3 which is the most abundant unsaturated fatty acids in oily fish (Gordon Bell et al., 2002). These acids has shown beneficial health effects, whilst being essential they cannot synthesize in the body and have to be added through diet (Kris-Etherton et al., 2002).

Oxidation of unsaturated fatty acids can happen through photooxidation, autoxidation, and enzyme- catalyzed oxidation, whereas autoxidation is the main process among them (Sun et al., 2011). Rancidity is reactions that causes off odors and taste on food products that contains fats and oils. The risk of oxidation increases with the number of double bonds in a fatty acid, because of the double bonds that are more exposed to free radical's attack. For example, EPA with five double bonds are more exposed to rancidity than linoleic acid which contains three double bonds (Blokhina et al., 2003). For the industry, this can be challenging in thoughts of shelf life, distribution and storage of fish.
In autoxidation, the free radical reaction of organic compounds with oxygen is a natural and spontaneous process. In this reaction, unstable hydrogen peroxides without any smell or taste

are formed. These peroxides will then further react to new radicals such as aldehydes, ketones and alcohols. These peroxides will then give bad smell and taste. During this reaction, the longer acids are cleaved to molecules which easier attracts microorganisms. Autoxidation is a free radical chain process consisting of chain initiation, propagation and termination (Sevanian and Hochstein, 1985). The reactions between fat and oxygen will go faster if increased temperature, light or metal ions are present. If metal ions are present, peroxides will easily degrade to radicals. Not only can they contaminate from the processing environment, but also from equipment or packaging. Rancidity can therefore often be minimized by proper hygienic production, packaging and sufficient cooling of the product (Gutteridge and Halliwell, 1990).

In the initiation step, the key event is the formation of a lipid radical. Initiation is introduced by free radicals, which are produced by breaking up of the hydroperoxides that are present in food that contains unsaturated lipids. The rate of reaction depends on the structure of the fatty acid. As isomers of unsaturated fatty acid hydroperoxides undergo isomerization the reaction is complex. Hydroperoxide as primary product is unstable, and it breaks up into secondary compounds, complex interaction and fragmentation. These reactions results in the production of volatile compounds such as aldehydes, ketones, organic acids or polymers giving a strong and pungent taste (Porter et al., 1995).

Because of high content of soluble substances in the flesh, fish is very perishable because of the many polyunsaturated fatty acids specially in fatty fish such as salmon, clupeids and mackerel (Strobel et al., 2012). Fish flesh naturally contains very low levels of carbohydrates which are further depleted during the death struggle of the fish. This has two important consequences for spoilage. It limits the degree of post mortem acidification, so the ultimate pH is higher (pH 6,2-6,5) than in mammalian muscle (pH 5,5). Second, the low levels of carbohydrate mean that bacteria present will immediately resort to using soluble nitrogenous materials, producing off odors far sooner (Adams and Moss, 2007). This makes it possible for the specific microorganisms to grow in the fish and produce malodorous components such as hydrogensulphide (H_2S) and trimethylamine (TMA) making the product unattractive for the consumer (Serio et al., 2014). In addition, fish is characterized by a high level of enzymatic activity, which plays a major role in the early stages of spoilage (Rossi, 2016).

H_2S producing bacteria can be an indicator on a bacteria's ability to spoil fish and fish products (Serio et al., 2011). At aerobic respiration sulfate will be reduced to hydrogen sulfide which bacteria can use in their metabolism. Detection of these bacteria can happen on iron agar, and black colonies will be formed when H_2S produced from thiosulphate reacts with metal ions in presence of L-cysteine (Gram et al., 1987).

2.3.1 Specific spoilage organisms

In spoiled fish, there are normally 10^7- 10^8 cfu/g bacteria. However, not all bacteria in the microflora will contribute to spoilage (Gram et al., 1987). Only those that has the ability to produce metabolites in adequate quantities are considered as the main cause of spoilage, and those are called specific spoilage organisms (SSO). At the beginning of spoilage, the fish contains SSO (Gram and Huss, 1996) which are typically present in low numbers and compose a very small fraction in seafood microflora. Identification of these SSO happens when analyzing their ability to produce off odors and metabolites such as formation of TMA and production of H_2S (Gram and Huss, 2000). The seafood SSO also produce ammonia, biogenic amines, organic acids and sulfur compounds from amino acids, hypoxanthine from ATP degradation products, and acetate from lactate. During storage, these organisms grow faster than the remaining microbiota whereas they are responsible for the off-flavors in spoilage of seafood (Gram and Dalgaard, 2002).

Different factors affect the selection of SSO, for example the initial microflora (also dependent upon fish origination and contamination after catch), applied preservation hurdles, temperature and atmospheric conditions during storage and distribution. The microflora of fish from temperate waters is dominated by psychotropic aerobic or facultative anaerobic Gram- negative, rod-shaped bacteria, and in particular by *Pseudomonas, Moraxella, Acineobacter, Schewanella putrefaciens, Flavobacterium, Vibrio, Photobacterium* and *Aeromonas* (Gram and Huss, 1996). During chilled storage, there is a shift in bacterial types. Gram negative, psychrotolerant bacteria such as *Pseudomonas spp*. and *Shewanella spp.* are the main SSO that will dominate on chilled fish. Whereas *Aeromonas spp*. are normally linked to spoilage of fish from tropical regions (Ryder et al., 2014). In addition, the prevalence of SSO is greatly influenced by packaging conditions. The elevated CO_2 and reduced O_2 of MAP prolongs shelf life, but also affects the synthesis of spoilage microbiota and the selection of SSO (Noseda et al., 2014). *Photobacterium phosphoreum*, various lactic acid bacteria, including *Carnobacterium*, and *Brochothrix thermosphacta* usually

predominate under MAP fish from the Atlantic Ocean (Macé et al., 2012, Hovda et al., 2007, Dalgaard et al., 1993, Dalgaard, 2003). In vacuum packaging the microflora typically becomes dominated by lactic acid bacteria (LAB) and G-negative fermentative bacteria, including psychrotrophic *Enterobacteriaceae*, in addition to some species of *Shewanella* capable of anaerobic respiration (Gram and Huss, 1996).

2.4 Packaging methods

Fishery products are highly perishable food products because of close to neutral pH and high levels of soluble nitrogen compounds in the tissue (Gram and Huss, 1996). Two methods that are used a lot are vacuum and modified atmosphere packaging to prolong shelf life and prevent microbial growth through storage. This has become more important as the use of additives are reduced and the increased consumption of ready to eat seafood (Noseda et al., 2014).

2.4.1 Vacuum packaging

In vacuum packaging the air is removed from the package prior to sealing. Preventing the presence of oxygen during storage, prolongs the shelf life of a food product. Vacuum packaging is a simple method to achieve this (Fellows, 2009). Vacuum packaging prolongs the growth of aerobic spoilage bacteria and limits the oxidative reactions in the fish. Since the fish is very perishable, the fish should be kept at low temperatures, whereas the storage life normally being 5-7 days (Rossi, 2016). Also, vacuum decreases the packaging volume compared to modified atmosphere packaging (Hansen et al., 2007).

2.4.2 Modified Atmosphere Packaging

The principle of MAP is to replace the air in the package with a different fixed gas mixture. A modified atmosphere can be obtained by changing the carbon dioxide (CO_2), oxygen (O_2) or nitrogen (N_2) content inside the package. It has been recommended that gas mixtures for fat fish should be 60% CO_2 and 40% N_2 (Randell et al., 1999). CO_2 is the most important in packaging of fish, because of the bacteriostatic effect. It is used to inhibit growth of spoilage bacteria, and increased concentrations of CO_2, leads to increased inhibition of bacteriological growth. However, some bacteria harbor high CO_2 resistance. The solubility of CO_2 increases

with decreasing temperatures; therefore, it is important that a sufficient level of CO_2 with a suitable gas/product ratio is used in the headspace of the package to achieve wanted antimicrobial effect. N_2 is an inert and tasteless gas and mostly used as a filler gas to complement the desired CO_2 and O_2 levels. At high CO_2 levels, the package has a tendency to collapse. To avoid this "snug-down" effect, N_2 are used in the mixture because of its low solubility properties in water and fat (Sivertsvik et al., 2002).

2.5 Detection of metabolites by the use of NMR

The monitored fish metabolites include amino acids, dipeptides, sugars, vitamins, biogenic amines, as well as different products of the ATP degradation as well as amount of all metabolites that are necessary for the calculation of the K- value and H- index used to express fish freshness (Shumilina et al., 2015).

Fermentation involves various types of bacteria that ferment sugars and produce different products, such as 2,3-butanediol, ethanol, acetic acid and others. Ethanol are formed by yeast or by *Zynomonas* species. In both cases, ethanol is formed by fermentation of glucose by different pathways. This anaerobic process provides energy (along with lactic acid fermentation) when oxygen is scares. 2,3-butanediol is anaerobic fermentation of glucose with 2,3-butandiol as one of the end products. Butanediol fermentation is typical for the facultative anaerobes *Klebsiella* and *Enterobacter* (Mûller, 2001).

Glycogen is another compound found in salmon. Once the supply of oxygen to the muscle is depleted, glycogen, the main carbohydrate in fish muscle undergoes anaerobic glycolysis to lactic acid (Eskin and Shahidi, 2012). From glycolysis glycogen levels tends to decrease during fish storage, which results in decreasing concentrations of sugars in the muscle. This contribute to a gradual loss of sweet flavor of fresh fish (Shumilina et al., 2015).

Amino acids, peptides, nucleotides and organic acids are some of the most important classes of metabolites influencing flavor. The distribution of these compounds varies with fish species and may be different for farmed and wild fish (Aoki et al., 1991). Farmed fish tend to contain less free amino acids than wild fish (Haard, 1992). The most important nucleotide in living organisms is adenosine 5′-triphosphate (ATP). ATP transfers energy within the cell and is considered as the main energy source. The ATP is degraded by endogenous enzymes

causing the formation of adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), inosine-5′-monophosphate (IMP), inosine (Ino or HxR) and hypoxanthine (Hx) that degrades to xanthine (X) and uric acid (U) (Massa et al., 2005). It is known that nucleotides affect taste and flavor in fish, for example IMP contributes to a pleasant and sweet taste. However, these properties are decreased with the degradation of IMP to Ino and Hx and bitter flavors are presented (Tejada, 2009). By measuring products of ATP degradation, freshness of fish during storage can be determined by the use of calculating K- value which is most used, figure 3. K-value is a good technique to grade fish freshness and it is defined after Saito et al. (1959). Another freshness indicator is the use of H-index. Higher values correspond to a less fresh fish. The difference between K-value and H-value is that K-values are used as quality criteria to determine the consumption limit for very raw, chilled fish (Tejada, 2009).

$$K - value\ (\%) = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx} x\ 100$$

Figure 3: Calculation of K-value to determine fish quality (Tejada, 2009).

Higher values correspond to a less fresh fish. Previous studies by (Erikson et al., 1997) showed that maximum level of rejection of Atlantic salmon ranged from 70- 80% in K- value. In contrast, a K- value of 20% is the limit for raw fish (high quality) consumption (Hamada-Sato et al., 2005). However, these values ranges in thoughts of fish species, and also within the species dependent on size and storage temperatures (Tejada et al., 2007).

Trimethylamine N-oxide (TMAO) is a compound primarily found in fish and shellfish of marine origin. Also, it is an important protecting osmolyte which allows organisms to endure extreme environments (Yancey, 2005). Amount of TMAO varies between fish species, also other factors such as season, size and age of the fish varies (Hebard et al., 1982). Trimethylamine (TMA) is a compound formed by bacterial reduction at anaerobic respiration of TMAO or by breakdown of choline during the spoilage of fish (Adams and Moss, 2007). Therefore, this reaction can be reduced by applying oxygen in MAP in white fish. The reduction of TMAO to TMA is mainly performed by specific spoilage microorganisms, and the fishy odor that develops through storage is to a large extent, due to the formation of TMA. Therefore, this measure can be an indicator of freshness in fish (Malle et al., 1986).

Biogenic amines are found in all food that contain proteins or free amino acids. In raw salmon, biogenic amines are undesirable and can be used as an indication of microbial

spoilage (Al Bulushi et al., 2009). They are non-volatile and formed by decarboxylation of amino acids. Tyramine, cadaverine and histamine are example that has been found to be useful indexes for evaluating fish microbial spoilage (Al Bulushi et al., 2009, Ruiz-Capillas and Moral, 2004, Suyama and Yoshizawa, 1973, Emborg et al., 2002, Paleologos et al., 2004).

Nuclear magnetic resonance (NMR) technique can be used to study post-mortem changes in Atlantic salmon. It is one of the mostly used analytical tool to study metabolites in an organism, and a great amount of information can be given in a very short period of time (Pan and Raftery, 2007). The spectrum consists of a series of sharp signals where the frequencies and peeks can be related to the chemical nature of the different hydrogen atoms. Also, the intensity is directly related to the number of hydrogens producing the signal. Therefore, all hydrogen atoms produce signals at the same frequency if they have the same chemical surroundings (Guille and Ruiz, 2001, Sacchi et al., 1993). The technique provides a high throughput of data; however the method also has some disadvantages in thoughts of sensitivity compared to other analytical methods (Mannina et al., 2012). Continuous development in hardware has allowed the sensitivity to be much increased (Styles et al., 1984). Nevertheless, it is one of the best suitable techniques in thoughts of food quality control and assurance. It is non-invasive and non-destructive, reproducible and it can analyze with very little sample preparation (Erikson et al., 2012, Miyakawa et al., 2015).

NMR spectroscopy uses radiofrequency waves to discover magnetic nuclei. The signal can only detect nuclei that have a magnetic movement, in other words a non-zero spin by applying the electromagnetic radiation (Tomassini et al., 2013). The chemical shifts in NMR can be split in two, three or more sub-peaks, these subpeaks are called doublets, triplets and multiplets respectively. One of the most used nuclei are ^{1}H (proton) which cover a range of signals of 10 ppm (Lenz and Wilson, 2007). Because of the small chemical range, ^{1}H NMR can be more difficult to analyze because the signals more easily overlap in comparison to ^{1}C NMR which cover a range of 200 ppm range (Markley et al., 2017). To be able to quantify the absolute concentration of metabolites, trimethylsilyl propanoic acid (TSP) is added in the sample as a reference in the NMR spectrum. The chemical shift of TSP is defined as 0 ppm (Dona et al., 2016).

3. Materials and methods

3.1 Challenge study preparation

Salmon back loin was collected in March- April 2018 from SalMar at Frøya. The salmon was fresh in order to eliminate any background flora as much as possible. All equipment and surfaces were sprayed with 70% ethanol before use. The salmon for round one was cut aseptically into 100 gram pieces 2 days post-harvest. The fish was placed in black plastic trays (Faerc plast, Denmark) covered with plastic and kept in storage at 4°C before packaging the next day. The MAP packed salmon was cut into 90 gram pieces 3 days post-harvest and packed in MAP the same day.

One *A. salmonicida* strain which is previously characterized by Hoel et al. (2017) was used in this challenge test. The strain was grown in shake flasks containing TSB at 37°C at 100 rpm for 1 day. The next day, when the strain had reached late exponential phase, 1 ml of the culture was transferred to a new shake flask, which contained 100 ml TSB. The incubation temperature was lowered to 8°C for 24 hours reaching late exponential phase. Temperature was lowered to allow the bacteria to adapt to cold storage. The bacteria culture was diluted with saltwater (0,9%) to a obtain the appropriate optical density (OD) corresponding to a bacterial concentration of ~ 3 log cfu/ml (Hoel et al., 2018). OD of the culture was measured to approximately 0,01, using a UV spectrophotometer (600nm, Shimadzu UV spectrophotometer, Japan).

In the vacuum-packed salmon, 1 ml from inoculum was transferred to already cut pieces of 100 gram fish using a sterile pipette. In MAP salmon, 800 µl was used. With the use of a plastic spreader the inoculum was spread evenly on the fish surface, and then dried for 30 minutes covered by aluminum foil.

Each piece of both control and inoculated fish was randomly placed in separate vacuum bags and packed in a vacuum machine (Webomatic, Germany). For MA packaging the fish was randomly placed in small plastic trays (Faerc plast, Denmark) with an absorbent underneath. 40% CO_2 and 60% N_2 was used for packaging in the MAP machine (Webomatic, Germany). For both vacuum and MA packaging, three parallels for each sample were incubated at 4 and 8°C for storage.

3.1.1 Sampling and quantification

By the use of MAP controller (PBI Dansensor, Denmark), all parallels for MAP fish were controlled before sampling to ensure the package contained the correct atmosphere. 10 grams of fish was cut and transferred aseptically into a stomacher bag. Furthermore, off-odors of all samples were detected and evaluated just prior to microbial analysis. Additionally, approximately 10 grams was cut and placed in a freezer maintaining -80°C for later NMR analysis. Both the surface and interior of the fish were represented in the sample. 90 grams of peptone water (8,5 g NaCl [VWR] and 1,0 neutralized bacteriological peptone [Oxoid England]) was then poured into the stomacher bag making a 1:10 dilution. The sample was homogenized (1 min, IUL Masticator, Spain) and after 10 ml was transferred to a dilution tube. Serial dilution of the 10^1 dilution was then prepared and spread on their respective agar plates. *Aeromonas spp.* were quantified to the NMKL method described by Nordic committee of food analysis (NMKL, 2004) using Starch Ampicillin Agar (SAA) (peptone (10 g/L, Oxoid, England), BBL beef extract powder (1 g/L, BL BBL, USA), NaCl (5 g/L, VWR, Belgium), soluble starch (10 g/L, BD Difco, USA), phenol red (25 mg/L, Difco laboratories, USA), and agar (15 g/L [bacterial agar no.1], Oxoid, France)). After the agar solution had cooled down to approximately 50 °C, it was supplemented with Ampicillin (10 mg/L, Sigma, China). Each sample was spread evenly on the surface by the use of plastic spreader before incubation (37°C for 24 h) upside down. The aerobic plate count (APC) and H_2S- producing bacteria were quantified to the NMKL method described by Nordic committee of food analysis (NMKL, 2006) as total and black colonies on Lyngby`s iron agar (43,6 g/L, Oxoid, England). Before pour plating, the iron agar was supplemented with L-cysteine (5 g/100 ml, Sigma- Aldrich, China) for enhancing and stabilizing the blackening of the colonies. The plates were incubated (22 °C for 72 h ± 6 h) upside down.

Salmon packed in vacuum sampling was done every second day for 14 days at 8°C and 16 days at 4°C. For salmon packed in MA, sampling was done every third day for 21 days since MAP is considered to have a longer shelf life than vacuum packed foods (Goulas and Kontominas, 2007, Taylor et al., 1990). On SAA plates, all colonies were counted. On Lyngby`s iron agar black colonies (H_2S- producing bacteria) and white colonies (aerobic count) were recorded.

3.2 Chemical analysis by NMR

5 grams of fish was cut into smaller pieces and placed into a 45 ml Falcon tube with a lid. 18 iron balls (3mm) were put into each tube, and afterwards homogenized (5 min; 30/s, Retsch MM400, Germany). 30 ml TCA of 7,5% (75g/L) chilled to 4°C were added, and the mixture again homogenized (4 min; 20/s, Retsch MM400, Germany). The sample was centrifuged (10 min, 8°C, 6500 rcf, Eppendorf centrifuge 5430R, Germany). The supernatant was then filtered and placed into a new 50 ml Falcon tube. The mixture was adjusted to pH 7 ± 0,03 adding 9 M NaOH. Final volume of the extract was used for further calculations. 1000 µl was pipetted into three Eppendorf tubes and placed in a freezer maintaining -20°C for later preparation of NMR samples. The NMR tubes were prepared adding 55 µl of 10 mM TSP (3-(trimethylsilyl) propionate-2,2,3,3-d4) (in 20 mM phosphate buffer in D_2O, pH 7,0) and 495 µl of the TCA extract were put into a new Eppendorf tube, vortex and centrifuged (5 min; 8°C, 20000xg, Kubota 3500, Japan). 530 µl of the supernatant were then put into a 5-mm NMR tube. ID ^{1}H NMR spectra were acquired at 300 K with a Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe at the NMR center of the Faculty of Natural Sciences and Technology at the Norwegian University of Science and Technology in Trondheim. The ^{1}H experiment (Bruker, noesygppr1d sequence) had the following settings: NS=48, RG=144, SW=20 ppm. Phase and baseline corrections of the spectra were performed using the TopSpin 3.5 pl7 (Bruker, Germany). Concentration of the metabolites was calculated using internal TSP standard.

3.2.1 Principal component analysis (PCA)

All ^{1}H NMR spectra were binned into unregular 95 buckets from 9.0 to 0.75 ppm using AMIX software (version 2.5, Bruker GmbH, Germany). The integral values of each bucket were subsequently used as an input for PCA analysis carried out by Unscrambler software (version 10.4, Camo ASA, Oslo, Norway).

3.3 Statistical analysis

Three replicates of each sample were analyzed, with the exception of time zero (n=4). Presented data are shown as average (n3) ± standard error on log transformed data. Average log cfu/g ± SE were calculated in excel. The statistical software SPSS statistics was used for statistical analysis. All statistical analysis was done on log transformed data. For microbial

statistical analysis, the general liner model (Duncan post-hoc p=0,05) was incorporated by a two-way ANOVA. The predictive model growth curve of (Baranyi and Roberts, 1994) was used to estimate maximum specific growth rates (μ_{max}) and duration of lag phase. One-way ANOVA was used to compare the different metabolites in vacuum packaging and modified atmosphere packaging.

4. Results

Pre- rigor fileted back loins of salmon were inoculated with *A. salmonicida* strain SU2 to assess the bacterial growth at two different temperatures and packaging conditions. Using vacuum packaging, sampling was done every other day until day 14. With MA packaging, the storage conditions were the same as for vacuum packaging. However, sampling was done every third day for 21 days.

4.1 Growth in vacuum packaging

4.1.1 *Growth of A. salmonicida* (SU2) on Starch-Ampicillin- Agar

The growth curves of mesophilic *A. salmonicida* (SU2) and presumptive *Aeromonas spp*. quantified on Starch-Ampicillin- Agar (SAA) are illustrated in figure 4. Concentrations of *Aeromonas spp*. in the inoculated samples at 4 and 8°C start at 4,87 ± 0,3 log cfu/g. The exponential phase of 8°C is shorter than 4°C. However, both samples reached 8,0 log cfu/g. In the beginning of storage, no aeromonads were detected in the control samples. During storage at day 6, growth of presumptive *Aeromonas spp.* was detected at 8°C (4,80 ± 0,2 log cfu/g), and after day 10 at 4°C (3,43 ± 0,3 log cfu/g). There were statistically significant differences (general linear model (GLM), $p<0,05$) in microbial count of presumptive *Aeromonas spp.* on SAA between all four groups under vacuum packaging conditions. In general, samples at 8°C reached a higher log cfu/g than samples stored at 4°C.

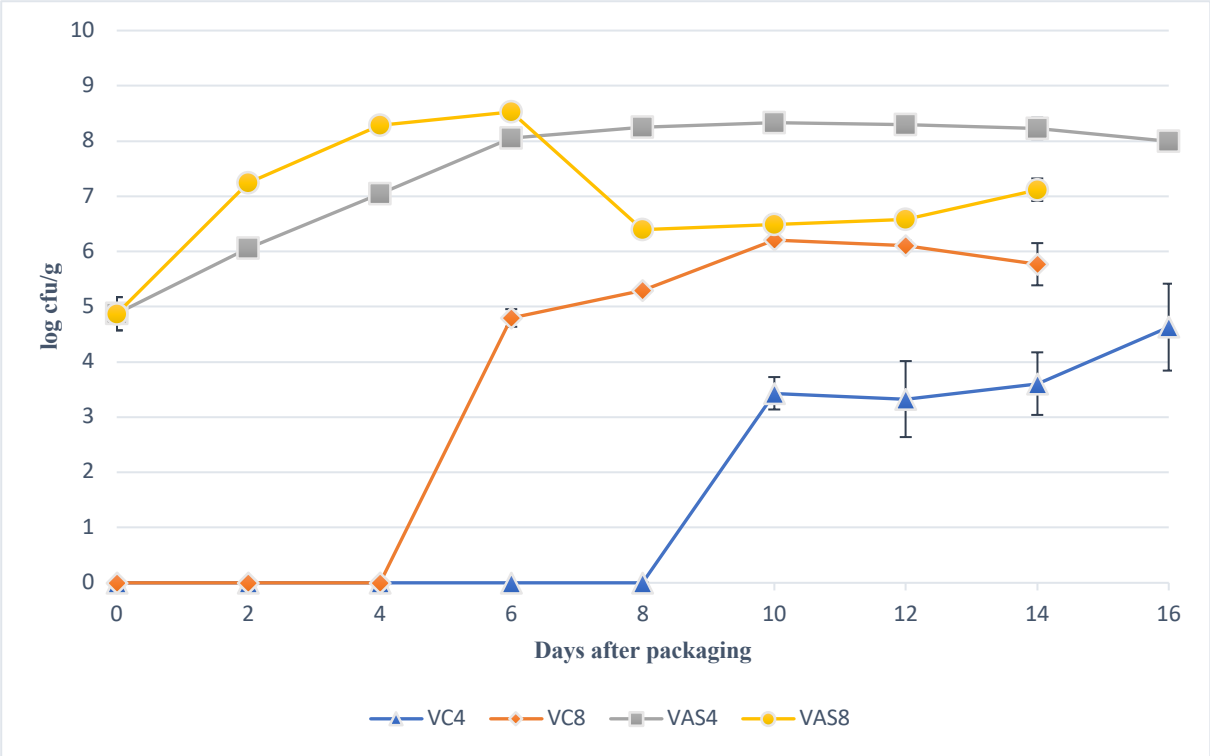

Figure 4: Growth curves of mesophilic Aeromonas spp. and presumptive Aeromonas spp. in vacuum packaging after 14-16 days of storage. Vacuum control at 4 ℃ (VC4), vacuum control at 8 ℃ (VC8), vacuum inoculated with A. salmonicida at 4 ℃ (VAS4) and vacuum inoculated with A. salmonicida at 8 ℃ (VAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4)

The maximum growth rate of *A. salmonicida*, estimated by fitting to the primary model of Baranyi and Roberts (1994), was approximately 3x higher at 8°C than at 4°C in control samples. In inoculated samples µmax was approximately 2x higher at 8°C than at 4°C. The control at 8°C showed a lag phase of approximately 3 days ± 1,6, whereas at 4°C the lag phase was approximately 6 days ± 2,1, indicating that the bacteria stored at 4°C required more time to adapt to the growth conditions, see table 1.

Table 1: Growth kinetic parameters of A. salmonicida SU2 and presumptive Aeromonas spp. in vacuum packaging at 4 ℃ and 8 ℃ estimated from primary model of Barnyi and Roberts. Maximum growth rate (µmax 1/days), lag phase duration (day), carrying capacity (Ymax log cfu/g). R2 is the fit of the model, SE the standard error of R2. NA: No asymptote. NL: no lag

Sample	µmax (1/day)	Lag phase (day)	Ymax (log cfu/g)	R^2	SE
VC4	0.49 ± 0.12	5.8 ± 2.1	NA	0.850	0.78
VC8	1.31 ± 0.51	3.3 ± 1.6	NA	0.784	1,29
VAS4	0.56 ± 0.04	NL	8.23 ± 0.05	0.991	0.11
VAS8	1.19 ± 0.12	NL	8.42 ± 0.12	0.991	0.16

4.1.2 Hydrogen sulphide producing bacteria on iron agar

Black colonies on iron agar, representing H_2S- producing bacteria, were observed in vacuum packaged salmon in both inoculated and non- inoculated samples (figure 5). H_2S- producing bacteria had a similar growth pattern to the inoculated *A. salmonicida* strain on SAA. Inoculated samples started at 4,71± 0,4 log cfu/g. After day 6 of storage, inoculated samples reached approximately 8,45 log cfu/g and followed the same pattern until the last day of storage. For the control samples, H_2S producing bacteria were not detected until day 8 and 10 in storage for 8°C and 4°C respectively. However, some growth was observed after two days of storage at 8°C. GLM analysis showed that the two inoculated groups were significantly different ($p<0,05$) from both control samples, and that the control samples were significantly different from each other ($p<0,05$).

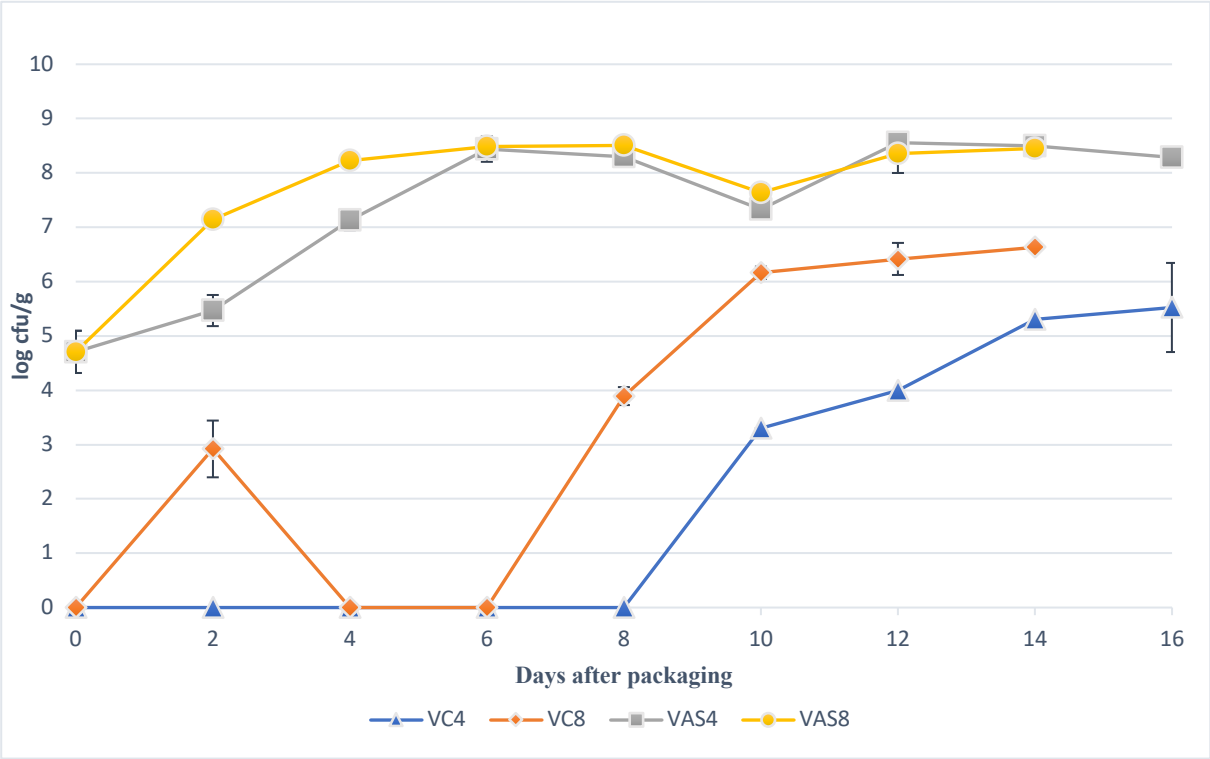

Figure 5: Growth curves of hydrogen sulphide producing bacteria detected on iron agar in vacuum packaging after 14 days of storage. Vacuum control at 4 ℃ (VC4), vacuum control at 8 ℃ (VC8), vacuum inoculated with A. salmonicida at 4 ℃ (VAS4) and vacuum inoculated with A. salmonicida at 8 ℃ (VAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4)

4.1.3 Total aerobic count on iron agar

In figure 6 the initial concentration of total aerobic bacteria including H_2S- producing bacteria was 4,71± 0,4 log cfu/g in the inoculated samples. Statistical analysis revealed that there were differences among the four groups regarding aerobic growth on iron agar. Growth of aerobic bacteria was detected in control samples after the second and fourth day of storage at 8 and 4°C respectively. After 10 days of storage, all four groups reached similar concentrations, and no statistical difference (p=0,109) was observed.

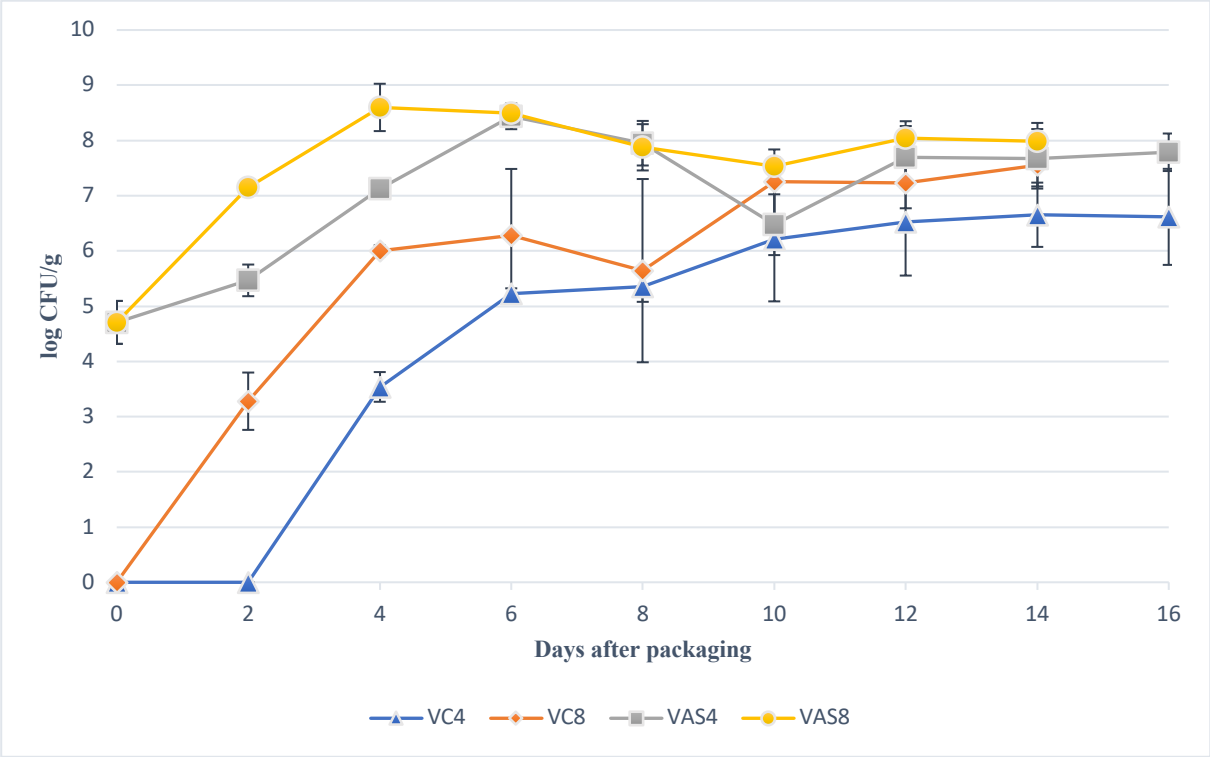

Figure 6: Growth curves of aerobic count in vacuum packaging after 14-16 days of storage. Vacuum control at 4 ℃ (VC4), vacuum control at 8 ℃ (VC8), vacuum inoculated with A. salmonicida at 4 ℃ (VAS4) and vacuum inoculated with A. salmonicida at 8 ℃ (VAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4).

4.2 Growth in modified atmosphere packaging

Atmospheric composition was measured at each time point illustrated in figure 7. Initial values were set to 60% N_2 and 40% CO_2. Initially, the average N_2 concentration for all four groups (MC4, MC8, MAS4, MAS8) was measured to 56,15% ± 0,5. At the end of storage, N_2 concentration was measured to 54,33% ± 2,8. The starting concentration for CO_2 in all four groups was 43,7% ± 0,5. On the last day of storage the concentration was 45,62% ± 2,8.

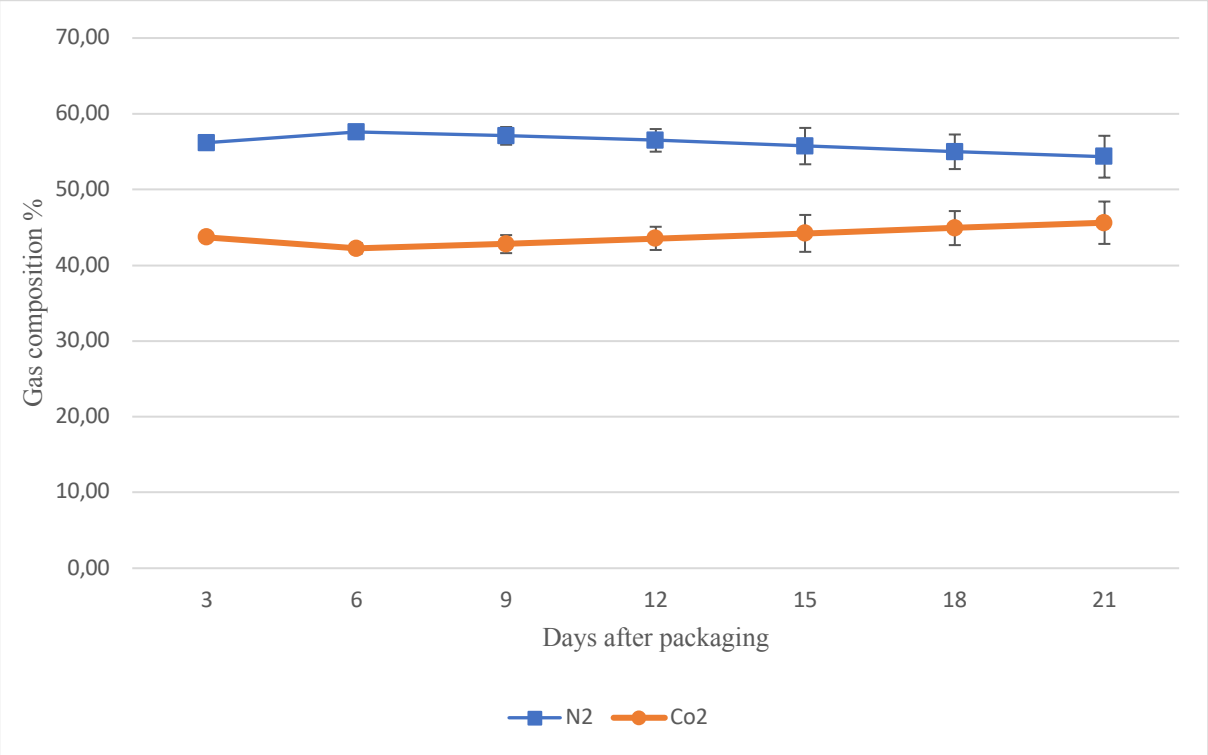

Figure 7: Composition of N_2 and CO_2 in % every third day after packaging. The average of all four groups (MC4, MC8, MAS4, MAS8) was calculated. Error bars indicate ± 1 SE and n=3 for each sampling point.

4.2.1 Growth of *A. salmonicida* (SU2) on Starch-Ampicillin- Agar

The results from fish packaged in MA demonstrated that *A. salmonicida* (SU2) were capable of growth in an atmosphere consisting of approximately 40% CO_2 and 60% N_2, at 4 and 8°C. Figure 8 illustrate the microbial growth pattern of presumptive *Aeromonas spp*. in non-inoculated and inoculated samples at 4 and 8°C for 21 days of storage. At time zero in inoculated samples, the initial concentration of presumptive *Aeromonas spp.* was 4,48 ± 0,11 log cfu/g. Microbial counts in inoculated samples at 8°C was higher than in the other groups. At day 21 both inoculated samples at 8°C (8,26 ± 0,01 log cfu/g) and control samples at 8°C (7,49 ± 0,33 log cfu/g) almost reached the same microbial count. After 21 days the microbial count in inoculated samples at 4°C was 6,00 ± 0,91 log cfu/g). After 21 days the microbial count in control samples at 4°C was 3,45 ± 0,00 log cfu/g). Significant differences were detected among the four groups ($p<0,01$ (GLM) regarding microbial count of presumptive *Aeromonas spp.* at different temperatures.

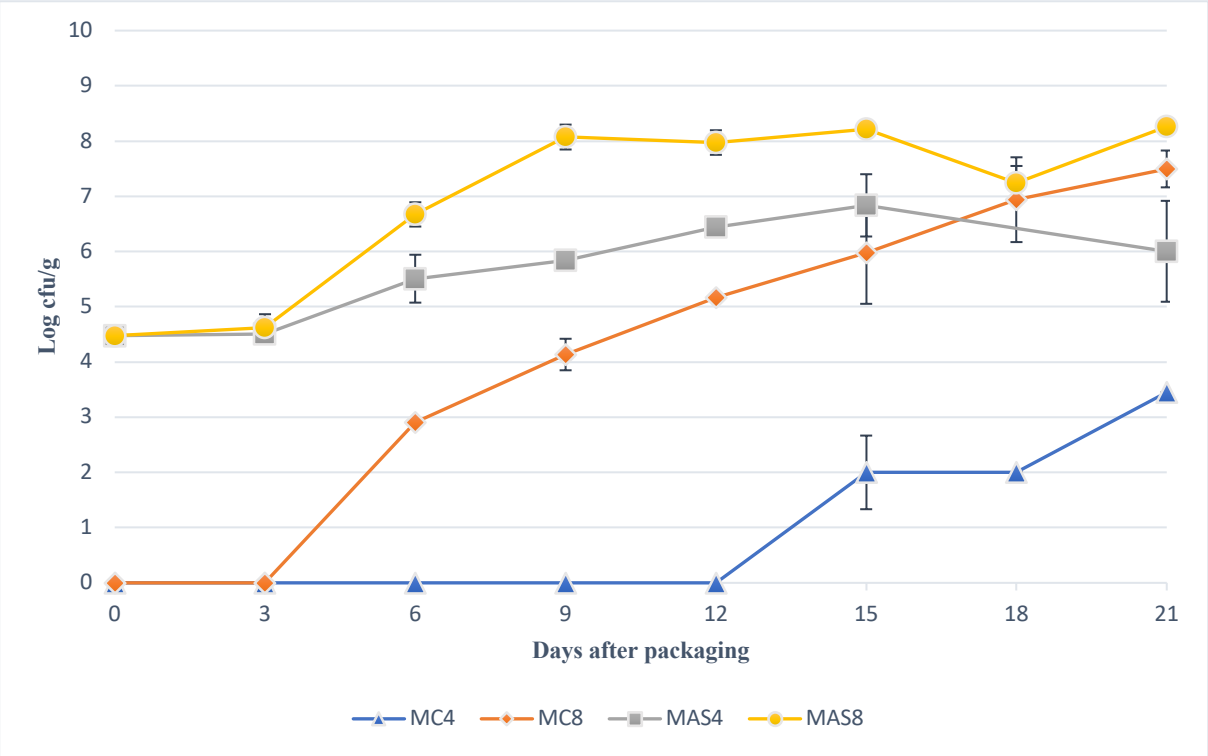

Figure 8: Growth curves of mesophilic Aeromonas spp. and presumptive Aeromonas spp. after 21 days of storage. MAP control at 4 ℃ (MC4), MAP control at 8 ℃ (MC8), MAP inoculated with A. salmonicida at 4 ℃ (MAS4) and MAP inoculated with A. salmonicida at 8 ℃ (MAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4).

The control sample in MAP at optimal storage (4°C) showed a lag phase of 11 days, in comparison to the control sample at 8°C which showed a lag phase of approximately 2 days average, table 2. When comparing control samples at 4°C and control samples at 8°C, μmax is 2x higher at 8°C. In inoculated samples, the lag phase at 4°C was 2.4 days ± 3.4 which is a bit shorter than inoculated at 8°C (3.5 days ± 2.2). μmax is approximately 3x higher at 8°C than at 4°C. In general, temperatures at 4°C has a shorter maximum growth rate than samples stored at 8°C.

Table 2: Growth kinetic parameters of A. salmonicida SU2 and presumptive Aeromonas spp. in modified atmosphere packaging at 4℃ and 8℃ estimated from primary model of Barnyi and Roberts. Maximum growth rate (μmax 1/days), lag phase duration (day), carrying capacity (Ymax log cfu/g). R2 is the fit of the model, SE the standard error of R2. NA: No asymptote.

Sample	μmax (1/day)	Lag phase (day)	Ymax (log cfu/g)	R^2	SE
MC4	0.35 ± 0.07	11.0 ± 1.6	NA	0.918	0.39
MC8	0.69 ± 0.21	2.4 ± 2.0	5.69 ± 0.57	0.933	0.67
MAS4	0.24 ± 0.13	2.4 ± 3.4	6.43 ± 0.25	0.814	0.39
MAS8	0.88 ± 0.69	3.5 ± 2.2	7.95 ± 0.19	0.931	0.41

4.2.2 Hydrogen sulphide producing bacteria on iron agar

The initial concentration of H_2S- producing bacteria was 4,53 ± 0,11 log cfu/g (figure 9). Microbial count in inoculated samples was 8,26 ± 0, 22 log cfu/g after 9 days of storage at 8°C. Microbial count in inoculated sample at 4°C was 6,03 ± 0,01 log cfu/g after 9 days of storage. Microbial count in control samples at 8°C was 4,52 ± 0,22 log cfu/g. Growth in control samples at 4°C was detected first after 15 days of storage. Control samples stored at 4 °C showed a longer lag phase, and less H_2S-producing bacteria than the control samples at 8°C.

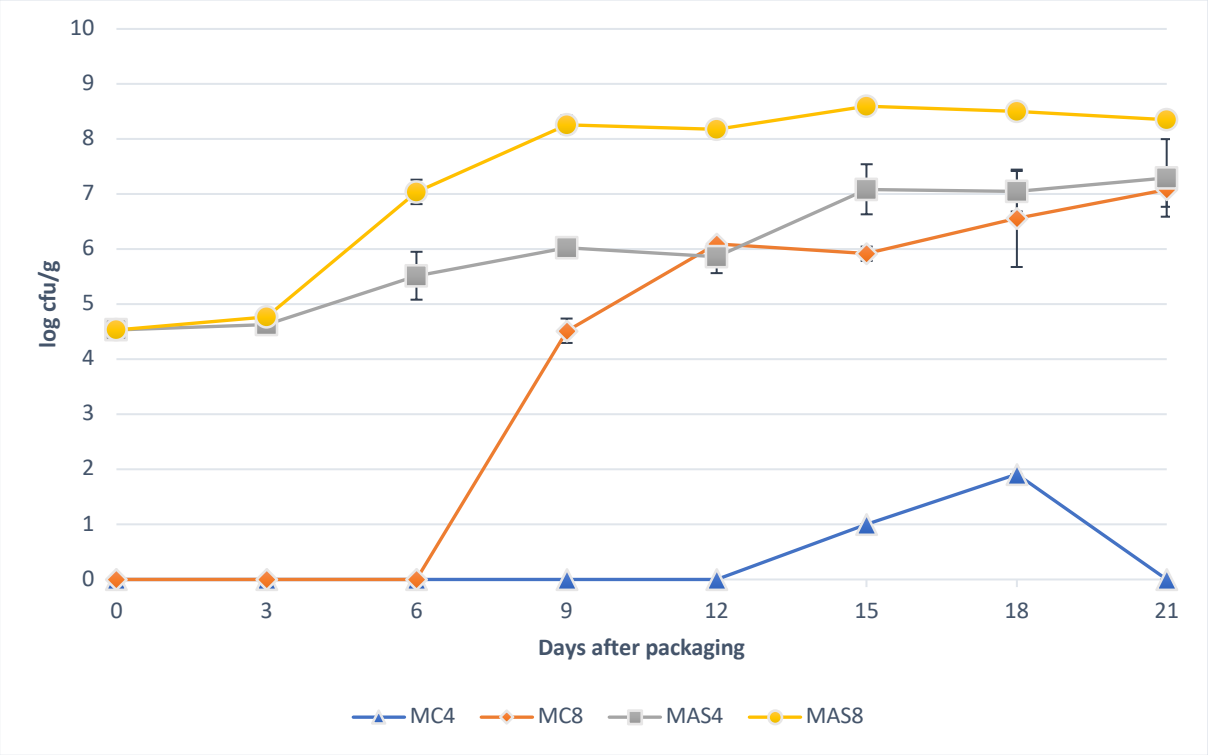

Figure 9: Growth curves of H_2S- producing bacteria detected on iron agar after 21 days of storage. MAP control at 4 ℃ (MC4), MAP control at 8 ℃ (MC8), MAP inoculated with A. salmonicida at 4 ℃ (MAS4) and MAP inoculated with A. salmonicida at 8 ℃ (MAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4).

4.2.3 Total aerobic count on iron agar

The initial concentration of the aerobic bacteria was 4,53 ± 0,11 log cfu/g in inoculated samples. In control samples, microbial count of aerobic bacteria was detected after 3 days at 8°C (1,42 ± 0,06 log cfu/g). In control samples at 4°C, microbial count was detected after 9 days (2,01 ± 0,47 log cfu/g). Samples stored at 8°C (inoculated and non-inoculated) approximately reached the same microbial count after 21 days of storage. These samples were significant different from non-inoculated and inoculated samples stored at 4°C (p=0,441). Control samples and inoculated samples stored at 4°C reached a microbial count of 5,29 ± 0,18 log cfu/g and 6,42 ±0,89 log cfu/g respectively (figure 10).

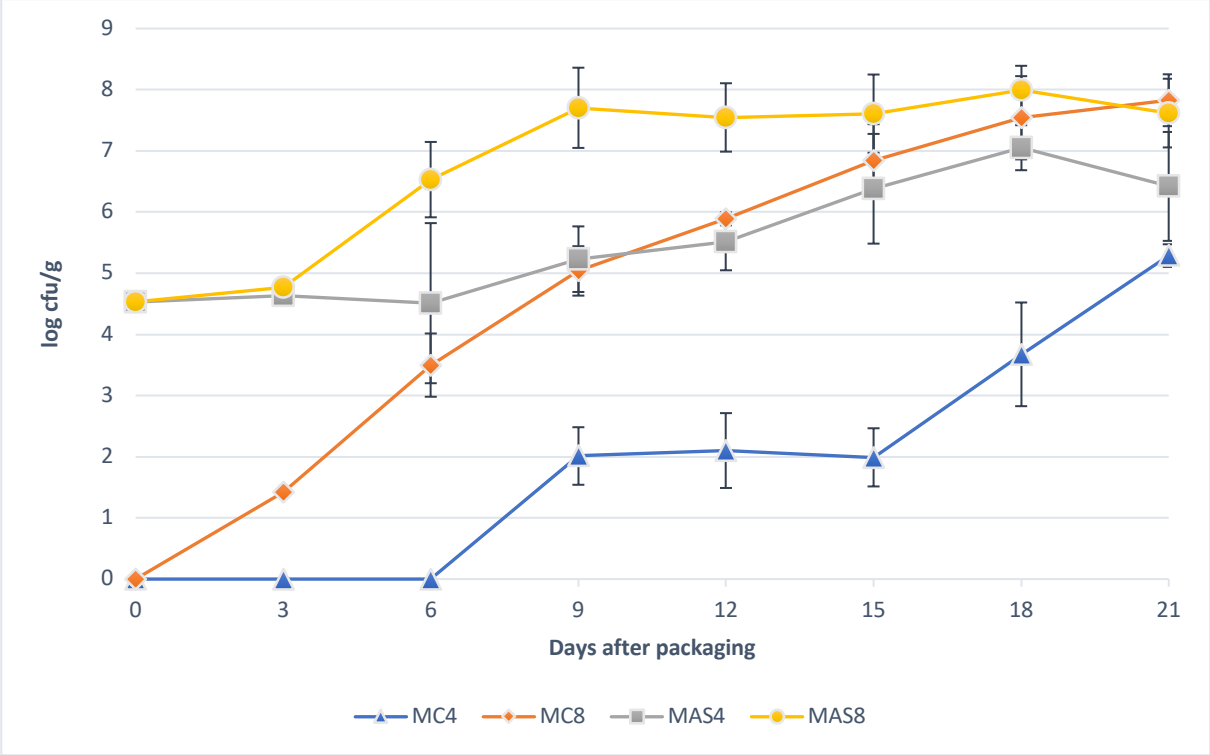

Figure 10: Growth curves for aerobic count at different time points. MAP control at 4 ℃ (MC4), MAP control at 8 ℃ (MC8), MAP inoculated with A. salmonicida at 4 ℃ (MAS4) and MAP inoculated with A. salmonicida at 8 ℃ (MAS8). Error bars indicate ± 1 SE and n=3 for each sampling point, except for time 0 (n=4).

4.3 Metabolite production by NMR

Some of the most important polar fish metabolites were monitored by ^{1}H NMR in samples inoculated with *A. salmonicida* strain SU2 and in-inoculated samples stored at 4 and 8°C. Metabolite profiling was carried out using methodology from previous studies by Shumilina et al. (2015). In this experiment, post-mortem changes were analyzed at day 0, day 14 (vacuum) and day 15 (MAP) after packaging, table 3-6. Significant differences were observed for all metabolites among salmon stored in MAP and vacuum packaging, except for ethanol ($p=0,062$).

Significant differences in glucose concentration was observed among samples stored in vacuum ($p<0,05$). The initial average concentration of glucose detected in non-inoculated samples was 36,3 ± 9,1 mg/100g. Initial average concentration in inoculated samples was 47,9 ± 4,5 mg/100g. A rapid decrease was observed at samples vacuum packed at 8°C after 14 days of storage for both inoculated (3,4 ± 1,8 mg/100g) and non-inoculated samples (0,7 ± 0,7 mg/100g), see table 3. It was not observed significant differences in glucose concentrations among samples stored in MAP ($p=0,060$). Average glucose concentrations for inoculated samples at 8°C was 31,1 ± 12,2 mg/100g, and non-inoculated samples 48,7 ± 0,6 mg/100g. In general, higher concentrations of glucose was observed in salmon packed in MAP compared to vacuum after 15 days of storage.

Concentrations of maltose also follows the same rapid decrease as for glucose at vacuum packed samples at 8°C after 14 days of storage of both non- inoculated (0 ± 0 mg/100 g) and inoculated (0,7 ± 0,7 mg/100g) samples. However, no significant difference is observed among samples stored in vacuum ($p=0,065$). Nevertheless, samples at 8°C contains lower concentrations of maltose compared to samples stored at 4°C. No significant difference was observed in samples stored in MAP ($p=1,158$). Neither temperature or inoculation seemed to have an effect on the concentration of maltose.

A significant difference was observed in concentration of 2,3-butanediol among vacuum packed samples ($p<0,05$). The initial concentration of 2,3- butanediol in inoculated samples was 0,1 ±0,04 mg/100g. In control samples the initial concentration was zero. Table 3 shows the concentrations of 2,3- butanediol at each sampling point. The concentration seems to be a bit affected by temperature, with increasing concentrations at 8°C. However, it does not seem

to be affected by inoculation. Salmon packed in MAP had a significant lower concentration of 2,3- butanediol in non-inoculated and inoculated samples than of salmon packed in vacuum($p<0,05$). However, the results suggest that concentration of 2,3- butanediol are not affected by temperature or inoculation in MAP samples.

No significant difference was observed in concentration of ethanol among samples stored in vacuum packaging and MAP ($p=0,062$). The initial concentration of ethanol in non-inoculated and inoculated samples was 8,1 ± 5,7 mg/100g and 24,3 ± 8,3 mg/100g respectively. The concentration of ethanol increased in both vacuum packaging and MAP throughout storage. The increase seems to be slightly affected by inoculation in vacuum packaging, see table 3. A higher increase in concentration are observed at 8°C in MAP than at 4°C for both non-inoculated and inoculated samples.

Significant differences are observed in concentration of acetate in vacuum packed samples and in MAP samples ($p<0,05$). Initial concentration of acetate in non-inoculated and inoculated samples was 1,1 ±0,2 mg/100 g and 1,5 ±0,2 mg/100g respectively. For both vacuum packaging and MAP, the concentration of acetate increases more in samples stored at 8°C than samples stored at 4°C. In vacuum packaging the concentration of acetate at 4°C for non-inoculated and inoculated samples are 12,4 ± 1,6 mg/100g and 22,3 ± 0,7 mg/100g respectively. In MAP the concentration of acetate at 4°C for non-inoculated and inoculated samples was 4,7 ± 3,7 mg/100g and 2,6 ± 0,8 mg/100g respectively. In both vacuum and MAP, concentration of acetate does not seem to be affected by inoculation.

Significant differences were observed among vacuum packed and MAP samples in concentration of TMAO and TMA ($p<0,05$). The initial average concentration of TMA was 0,03 ± 0 mg/100g in non-inoculated samples, and 0,1 ± 0,01 mg/100g in inoculated samples. There is a slower formation of TMA in MAP at 4 °C than for packaging in vacuum at 4°C, except inoculated samples in MAP at 8°C which contained 25,2 ± 2,3 mg/100g. For example, non- inoculated samples in MAP at 4°C, contained 3,8 ± 3,5 mg/100g after 14 days, in comparison to non-inoculated samples in vacuum at 4°C contained 11,8 ± 3,7 mg/100g after 15 days, see table 5. Formation of TMA is affected by packaging method, temperature and inoculation. A significant difference in the concentration of TMA is observed between non-inoculated and inoculated samples at 4°C in vacuum packaging ($p<0,05$), (table 3). In MAP a

significant difference in TMA is observed between non-inoculated and inoculated samples at 8°C ($p<0,05$) (table 5).

Significant differences were observed in concentration of leucine in vacuum packaging and modified atmosphere packaging ($p<0,05$). Start concentration of leucine in non-inoculated and inoculated samples are 8,3 ± 1,6 mg/100g and 10,3 ± 0,7 mg/100g. For both vacuum packaging and MAP, the concentration of leucine increases more in samples stored at 8°C than samples stored at 4°C. The concentration of leucine seems to be unaffected by inoculation in both vacuum and MAP samples.

As an indicator of freshness, some of the ATP degradation products were quantified in samples packed in vacuum (table 5) and MAP (table 6). Significant differences were observed in the formation of Hx among vacuum and MAP samples ($p<0,05$). Initially, concentration of Hx was 5,8 ± 0,6 mg/100g in non-inoculated samples and 7,3 ± 1,2 mg/100g in inoculated samples. Formation of hypoxanthine was highest in vacuum packed samples compared to samples stored in MAP. In both vacuum packaging and MAP, salmon stored at 4°C had a lower formation of Hx than salmon stored at 8°C after 14 and 15 days of storage respectively. The concentration of Hx does not seem to be affected by inoculation.

Initial concentration of IMP was 63,0 ± 9,7 mg/100g in non-inoculated samples and 113,8 ± 7,0 mg/100g in inoculated samples. The concentration had drastically decreased from day 0 to day 14 and 15 for both MAP and vacuum, and significant differences in concentration of IMP were observed from day 0 to day 14 and 15 of storage ($p<0,05$). However, no statistical difference was observed among vacuum packaging and MAP. The results do not seem to be affected by packaging conditions, temperature nor inoculation.

Significant differences were observed among vacuum packed and MAP samples ($p<0,05$) concerning H-index. Both vacuum packed and MAP samples at day 14 and 15 respectively was significant different from the control samples ($p<0,05$). Samples from day 0 both non-inoculated and inoculated samples had an H-index of 7,5 ± 1,3% and 9,0 ± 0,9% respectively. An increase was observed for both vacuum-packed and MAP salmon. However, salmon packed in vacuum had a higher H-index than fish packed in MAP. Inoculation does not seem to affect the samples; however, temperature seems to have an effect on the H-index.

Statistical differences were observed in concentration of cadaverine, in vacuum packed and MAP samples ($p<0,05$). Vacuum packed samples showed a significant difference in content of tyramine($p<0,05$) whereas no significant difference was observed in vacuum packed samples for concentration of putrescine($p=0,058$). MAP salmon did not contain any putrescine. In addition, no significant difference was observed in MAP in concentration of tyramine ($p=0,052$). The biogenic amines seem to be affected by packaging conditions and temperature, and not affected by inoculation.

Table 3: Concentration of analyzed metabolites (mg/100g) in inoculated and non-inoculated salmon during storage at 4 and 8 ℃ in vacuum packaging. Also, samples at day 0 of both inoculated and non-inoculated samples. The average (n3) of each metabolite is presented. V=Vacuum, AS= A. Salmonicida, C=Control, d=day of storage. SE and n=3 for each sampling point.

Sample	Leucine	2,3-butanediol	Ethanol	Acetate	TMA	TMAO	Glucose	Maltose	Cadaverine	Putrescine	Tyramine
C_d0	8,3±1,3^{a}	0,00±0,0^{a}	8,1±5,7^{a}	1,1±0,2^{a}	0,03±0,0^{a}	39,6±7,1^{b}	36,3±9,1^{c,d}	18,8±7,1^{a}	0,0±0^{a}	0,00±0^{a}	0,0±0^{a}
AS_d0	10,3±0,7^{a}	0,1±0,04^{a}	16,7±9,6^{a}	1,5±0,2^{b}	0,05±0,01^{a}	59,6±6,1^{c}	47,9±5,0^{d}	20,3±5,4^{a}	0,0±0^{a}	0,00±0^{a}	0,0±0^{a}
VC4_d14	34,0±1,3^{b,c}	24,1±11,7^{a,b}	23,6±9,3^{a}	12,4±1,6^{b}	11,8±3,7^{b}	38,9±6,0^{b}	41,4±8,6^{d}	21,1±12,4^{a}	30,8±5,4^{b}	0,00±0^{a}	0,4±0,4^{a}
VC8_d14	47,0±12,3^{c,d}	46,4±11,3^{b}	21,0±4,5^{a}	30,2±2,6^{d}	24,7±2,9^{c}	11,4±3,4^{a}	0,7±0,7^{a}	0,0±0^{a}	47,1±6,8^{c}	32,2±24,0^{a}	4,6±1,3^{b}
VAS4_d 14	24,3±6,7^{a,b}	27,9±3,3^{a,b}	30,0±4,5^{a}	22,2±0,7^{c}	27,0±1,7^{c}	9,8±2,0^{a}	20,3±5,9^{b,c}	27,0±14,1^{a}	32,5±4,9^{b}	2,1±1,1^{a}	0,4±0,4^{a}
VAS8_d 14	56,2±8,8^{d}	42,8±12,6^{b}	29,3±3,4^{a}	36,8±2,7^{c}	27,2±2,7^{c}	5,7±2,0^{a}	3,4±1,8^{a,b}	0,7±0,7^{a}	50,0±2,4^{c}	9,3±1,0^{a}	2,2±1,1^{a}

^{a,b,c,d} Different superscript letters in each column indicate significant difference between groups ($p<0,05$).

Table 4:Hx, IMP, H-index in vacuum packaging

Sample	Hx	IMP	H-index
VC_d0	5,8±0,6^{a}	63,0±9,7^{b}	9,0±0,9^{a}
VAS_d0	7,4±1,2^{a}	113,8±6,8^{c}	7,5±1,3^{a}
VC4_d14	31,3±5,5^{b}	4,2±1,4^{a}	36,8±6,4^{b}
VC8_d14	62,6±3,1^{c}	0,9±0,5^{a}	79,6±6,3^{c}
VAS4_d14	42,2±2,5^{b}	3,5±1,1^{a}	49,1±2,6^{b}
VAS8_d14	65,0±8,8^{c}	1,5±0,9^{a}	79,4±6,8^{c}

^{a,b,c,d} Different superscript letters in each column indicate significant difference between groups ($p<0,05$).

Table 5: Concentration of analyzed metabolites (mg/100g) in inoculated and non-inoculated salmon during storage at 4 and 8 ℃ in modified atmosphere packaging. Also, samples at day 0 of both inoculated and non-inoculated samples. The average (n3) of each metabolite is presented. M=MAP, AS= A. Salmonicida, C=Control, d=day of storage. SE and n=3 for each sampling point.

Sample	Leucine	2,3-butanediol	Ethanol	Acetate	TMA	TMAO	Glucose	Maltose	Cadaverine	Putrescine	Tyramine
C_d0	8,3±1,3^{a}	0,0±0^{a}	8,1±5,7^{a}	1,1±0,2^{a}	0,03±0^{a}	39,6±7,1^{b,c}	36,3±9,1^{a}	18,8±7,1^{a}	0,0±0^{a}	0,0±0	0,0±0^{a}
AS_d0	10,3±0,7^{a}	0,1±0,04^{a}	16,7±9,6^{a}	1,5±0,2^{a}	0,1±0,01^{a}	59,6±6,1^{c}	47,9±5,0^{a}	20,3±5,4^{a}	0,0±0^{a}	0,0±0	0,0±0^{a}
MC4_d15	23,2±3,4^{b}	1,3±0,5^{a,b}	10,3±2,0^{a}	4,7±3,7^{a}	3,8±3,5^{a}	30,7±10,6^{b}	43,5±3,3^{a}	27,0±8,7^{a}	3,3±3,3^{a}	0,0±0	0,0±0^{a}
MC8_d15	54,3±5,0^{d}	2,4±0,8^{b}	26,1±11,4^{a}	11,7±0,7^{b}	2,5±0,9^{a}	30,9±11,5^{b}	48,7±0,6^{a}	16,1±2,6^{a}	0,0±0^{a}	0,0±0	2,0±1,3^{a}
MAS4_d15	30,1±4,2^{b}	0,9±0,04^{a,b}	15,9±3,1^{a}	2,6±0,8^{a}	1,5±0,6^{a}	47,6±4,8^{b,c}	55,4±8,3^{a}	37,9±9,1^{a}	0,0±0^{a}	0,0±0	0,0±0^{a}
MAS8_d15	41,1±2,9^{c}	8,7±0,8^{c}	28,3±5,2^{a}	32,4±3,6^{c}	25,2±2,3^{b}	4,7±1,1^{a}	31,1±12,2^{a}	22,3±16,3^{a}	14,4±2,5^{b}	0,0±0	0,6±0,6^{a}

^{a,b,c,d} Different superscript letters in each column indicate significant difference between groups ($p<0,05$).

Table 6: Hx, IMP, H-index in modified atmosphere packaging

Sample	Hx	IMP	H-index
VC_d0	5,8±0,6^{a}	63,0±9,7^{b}	9,0±0,9^{a}
VAS_d0	7,4±1,2^{a}	113,8±7,0^{c}	7,5±1,3^{a}
MC4_d15	17,3±1,4^{b}	3,1±0,5^{a}	26,8±3,3^{b}
MC8_d15	30,2±3,6^{c}	3,5±0,6^{a}	38,1±2,7^{c}
MAS4_d15	24,6±3,7^{c}	3,9±0,4^{a}	28,9±3,5^{b}
MAS8_d15	39,9±0,6^{d}	4,1±1,6^{a}	51,3±0,7^{d}

^{a,b,c,d} Different superscript letters in each column indicate significant difference between groups ($p<0,05$).

4.4 Principal component analysis (PCA)

Principal component analysis (PCA) was carried out to see the general sample distribution and grouping of metabolites in *pre- rigor* fileted back loins of salmon. These data allow to identify the main trends in the post-mortem processes within differently packed samples. Score plots of PCA of both package types shows good separation between samples at day 0 of packaging, samples that were stored at 4°C and 8°C. In addition, the storage of the samples at 8°C allows to see separation of the inoculated and non-inoculated samples.

The PCA of vacuum packed samples (PC1/PC2) is shown in figure 11. Score plot show a good group separation and explains 73% of variables. The distribution of the samples along horizontal axes is caused by an increase in storage temperature. Storage temperature of 4°C does not allow to identify the differences in post-mortem processes within inoculated and non-inoculated samples (overlapping of the samples in 4°C group). Metabolites composition for non-inoculated and inoculated samples was detected from beginning of storage and after 14 days of storage at 4 and 8°C. The correlation plot (figure 11) shows that the samples at the first day of storage contained more glucose, maltose, IMP, TMAO and inosine. At the same time, samples from the left side of loading plot contained more metabolites that had developed during storage (TMA, formic acid, biogenic amines), figure 11.

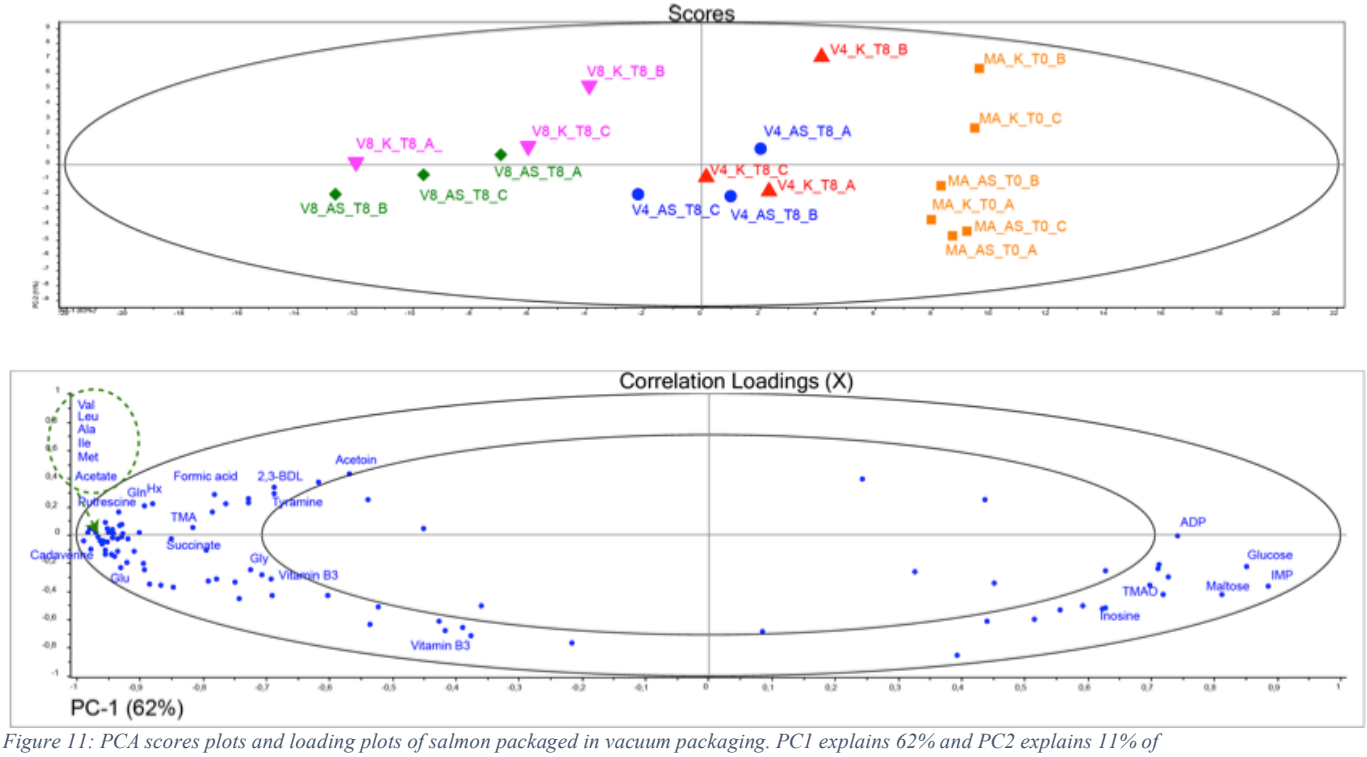

Figure 11: PCA scores plots and loading plots of salmon packaged in vacuum packaging. PC1 explains 62% and PC2 explains 11% of variables. Non-inoculated samples day 0 of storage (MA_K_T0 A,B,C), non-inoculated samples at 4 ℃ day 14 of storage (V4_K_T8 A,B,C), inoculated samples at 4 ℃ day 14 of storage (V4_AS_T8 A,B,C), non-inoculated samples at 8 ℃ day 14 of storage (V8_K_T8 A,B,C), inoculated samples at84 ℃ day 14 of storage (V8_AS_T8 A,B,C).

The PCA of MAP samples (PC1/PC2) is shown in figure 12. Score plot show a good group separation and explains 74% of variables. The distribution of the samples along horizontal axes is caused by an increase in storage temperature. Storage temperature of 4°C does not allow to identify the differences in post-mortem processes within inoculated and non-inoculatd samples (overlapping of the samples in 4°C group). However, quite good separation of the inoculated and non-inoculated samples can be detected for the samples that were stored at 8°C for 15 days MAP. Therefore, in these groups the bacteria inoculation causes the differences in metabolite composition. Metabolites composition for non-inoculated and inoculated samples was detected from beginning of storage and after 15 days of storage at 4 and 8°C. The correlation plot (figure 12) shows that the samples at the first day of storage contained more ADP and IMP. At the same time, samples from the right side of loading plot contained more metabolites that had developed during storage (TMA, biogenic amines), see figure 12.

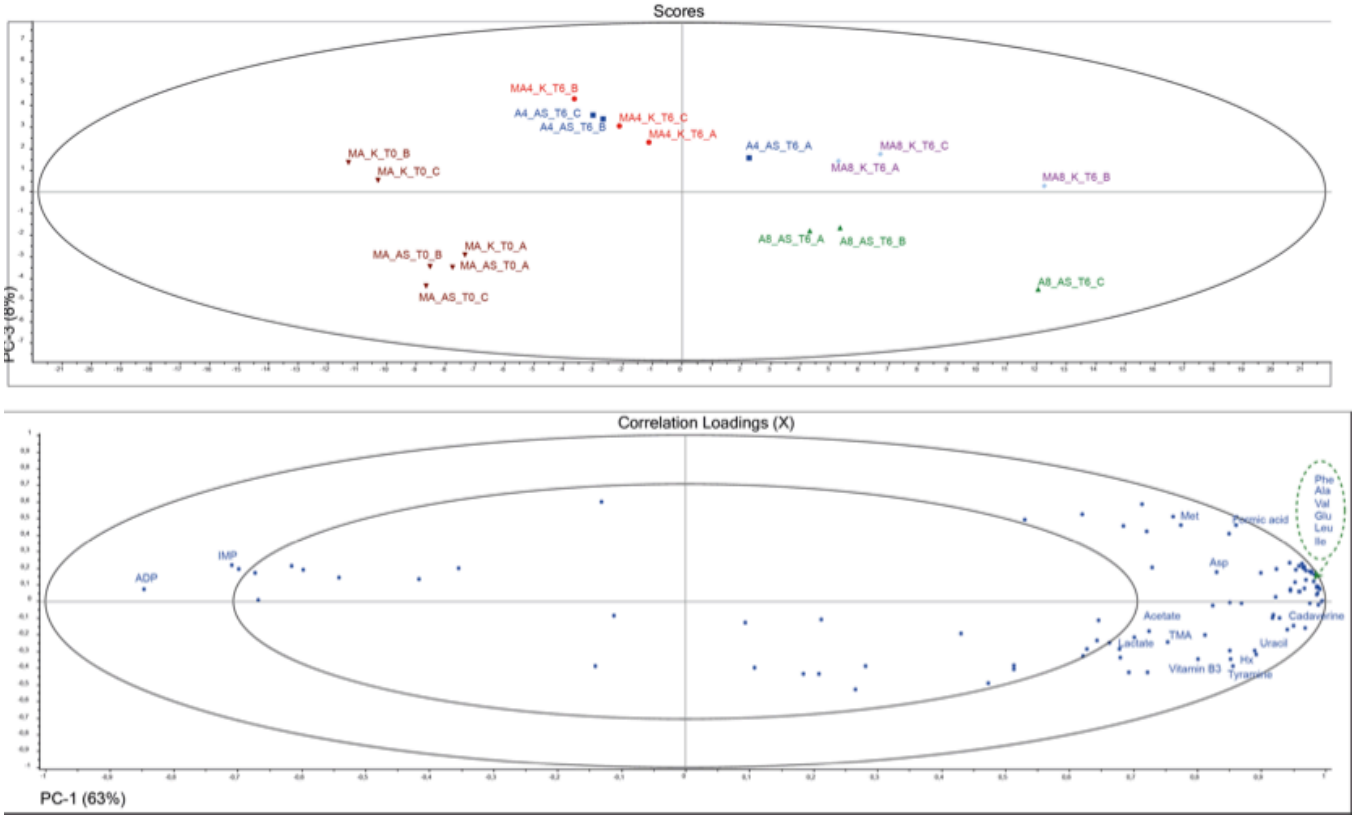

Figure 12: PCA scores plots and loading plots of salmon packaged in modified atmosphere packaging. PC1 explains 62% and PC2 explains 11% of variables. Non-inoculated samples day 0 of storage (MA_K_T0 A,B,C), non-inoculated samples at 4℃ day 14 of storage (MA4_K_T8 A,B,C), inoculated samples at 4℃ day 14 of storage MA4_AS_T8 A,B,C), non-inoculated samples at 8℃ day 14 of storage (MA8_K_T8 A,B,C), inoculated samples at 8℃ day 14 of storage (MA8_AS_T8 A,B,C).

5 Discussion

In this study, growth kinetics of an environmental strain of Aeromonas (*A. salmonicida* strain SU2) was investigated in pre- rigor fileted back loins of salmon inoculated and packed in vacuum and modified atmosphere at 4 and 8°C. In addition, ^{1}H NMR was used to asses various metabolites in samples from packaging, both at day 0 and day 14 and 15 in vacuum packaging and MA samples respectively. An understanding of the bacterial growth rate as well as metabolic production of *A. salmonicida*, both at different temperatures and packaging methods is important to assess how this organism contributes to spoilage of salmon.

Growth of *Aeromonas spp*. in fish are commonly reported (Joffraud et al., 2001, di Pinto et al., 2012, Abd-El-Malek, 2017, Liu et al., 2017). Previous studies by Hoel et al. (2015) have detected growth of mesophilic *Aeromonas spp*. in a substantial proportion of fresh retail sushi. In a follow up study, mesophilic *A. salmonicida* was most prevalent (74%) in salmon, followed by *Aeromonas bestiarum* (9%), *A. dhakensis* (5%), *A. caviae* (5%), *Aeromonas media* (4%), *A. hydrophila* (2%), and *Aeromonas piscicola* (1%) (Hoel et al., 2017). Because of its prevalence in salmon intended for raw consumption and its ability to grow at refrigerated temperatures *A. salmonicida* was chosen for further studies in this master thesis.

In this study the growth potential of *A. salmonicida* SU2 was examined under optimal storage temperature (4°C) and at slightly elevated temperature (8°C). Normally, food is exposed to temperature fluctuations during distribution, which may expedite spoiling (Huis In't Veld, 1996). In addition, it is reasonable to assume that temperatures in grocery stores fluctuate during cold storage (Laguerre et al., 2012). In order to examine how temperature affects growth and metabolic production, salmon was stored at two different temperatures in this study. The spoilage rate of fish is highly temperature dependent and the use of cold storage can reduce bacterial growth by increasing the bacterial lag-phase and improve shelf life of fish (Sivertsvik et al., 2002). Vacuum packaging and MAP are the two most common packaging methods for fresh salmon (Noseda et al., 2014). For this reason, these two packaging technologies were chosen to investigate if they had an effect on spoilage of salmon.

5.1 Growth in vacuum packaging

The results from fish packaged in vacuum suggest that *A. salmonicida* (SU2) are capable of growth under vacuum conditions and at 4 and 8°C. It has also been proven that the same *A. salmonicida* strain (SU2) can grow in salmon packaged in air at 4 and 8°C (Hoel et al., 2018). There is a significant difference ($p<0,01$) among the four groups regarding growth of mesophilic *Aeromonads* in two different temperatures and under vacuum conditions. The maximum specific growth rate of *A. salmonicida* was found to be 2x higher in vacuum at 8°C than at 4°C. These results correspond well with findings by Hoel et al. (2018). They found the maximum specific growth rates of *A. salmonicida* (SU2) to be approximately 2x higher at 8°C than at 4°C in both salmon and nigiri sushi samples, packaged in normal aerobic atmosphere.

It has been demonstrated that *Aeromonas spp*. in addition to other bacteria such as psychrotrophs and *LAB* are the predominant microorganisms of vacuum-packaged fish (Gui et al., 2014, Macé et al., 2012). *A. hydrophila* has been reported to be the most studied species in the genus *Aeromonas* (Adams and Moss, 2007, Kirov, 2001), primarily because of its ability to grow at low temperatures. Initially, no *Aeromonas spp*. were detected in control samples. However, growth of presumptive *Aeromonas spp*. was observed from day 6 and 10 and throughout the storage for 8°C and 4°C respectively. This may indicate that it is the inoculated strain *A. salmonicida* (SU2) which has grown in the inoculated samples from beginning of storage. Most likely, *Aeromonas spp.* have been present in the control samples during the entire time of storage, however not in high enough concentrations to be detected.

Due to the nature of the growth curve of vacuum packaged inoculated salmon (a drop in the detected concentration of *A. salmonicida* at 8 °C (VAS8) after 6 days), only the sampling points up to 6 days were used to fit the data to the primary model of Baranyi and Roberts (1994). Additionally, this could have led to a minor overestimation of the μmax for this group.

5.2 Growth in modified atmosphere packaging

Modified atmosphere packaging with different ratios of oxygen, nitrogen and carbon dioxide has been used extensively the last years to increase the shelf life of food products (Milne and Powell, 2014, Hansen et al., 2009). However, fish products with a high fat content such as salmon should be packaged in an O_2 free environment to avoid enhancing oxidative rancidity. In fatty fish, an extended shelf life is mainly achieved by the addition of an atmosphere consisting of 60% CO_2 and 40% N_2 (Randell et al., 1999, Noseda et al., 2014, Pettersen et al., 2011, Schirmer et al., 2009). In this study the concentrations of CO_2 were reduced because tolerance against CO_2 in *Aeromonas spp.* was unknown. Studies show that in high CO_2 concentrations *Aeromonas spp*. do not grow (García-Gimeno et al., 1996, Doherty et al., 1996, Wang et al., 2014). Using high concentrations of CO_2 may have given too much effect which could have inhibited the growth making it difficult to study growth kinetics and metabolite production. Therefore, milder gas-concentrations were chosen in this study. However, the combination of low temperature (4°C) and an atmosphere consisting of 40% CO_2 and 60% N_2 was not enough to inhibit presumptive *Aeromonas spp.* in this study. Further studies using the atmosphere normally used and recommended by the industry is needed. Moreover, there might be differences in CO_2 tolerance between different species of Aeromonas.

A certain amount of CO_2 has to be dissolved into the food in order to inhibit bacterial growth, and it has been found that the inhibition obtained is proportional to the concentration of dissolved CO_2 (Devlieghere et al., 1998). Several factors such as pH, lipid content, and water content may influence CO_2 uptake, and temperature may be one of the most important factor (Jakobsen and Bertelsen, 2004). Previous studies generally agree that increasing temperatures will decrease the solubility of CO_2 in muscle tissue. Therefore, the concentration of CO_2 was expected to increase in packages stored at 8°C. Results obtained from this study indicate that the concentration is higher in packages stored at 8°C than at 4°C, although stable during storage. Earlier studies by Sivertsvik et al. (2002) have shown that higher temperatures inevitably lead to less dissolved CO_2 in the product and consequently loss of inhibitory effect, which may result in higher microbial and enzymatic activity. Another study by Abel et al. (2018) showed that the amount of dissolved CO_2 was influenced by temperature.

In this study, mesophilic aeromonads were detected in both inoculated and control samples and at both temperatures. Since *Aeromonas spp*. were detected in non- inoculated samples, one can assume that there are more than the inoculated strain present in the samples. It may have been of interest to investigate which Aeromonas species that naturally was present in the samples. However, at 4°C, growth of presumptive *Aeromonas spp*. was detected at day 15 of storage. This may indicate that the *Aeromonas spp.* detected throughout the entire storage period was the inoculated strain. Most likely, other aeromonads have been present during the entire time of storage, however not in high enough concentrations to be detected.

The maximum growth rate (μmax) of *A. salmonicida* in the MAP inoculated sample, was close to 3x higher at 8°C than at 4°C, which demonstrates that proper temperature control is essential to prevent growth of *A. salmonicida* in salmon. The lag phase in control samples in MAP at 4°C was close to 2x longer than that of control samples at 4°C packaged in vacuum. The maximum growth rate (μmax) of *A. salmonicida* in MAP inoculated samples, was close to 3x higher at 8°C than at 4°C. As expected, μmax was generally shorter at 4°C than at 8°C for both vacuum and modified atmosphere packaged samples, which was expected.

Absence of H_2S-producing bacteria (black colonies on iron agar) in the non-inoculated samples during the first 9 days of storage (8°C) and 15 days of storage (4°C) may indicate that the black colonies in inoculated samples were the inoculated *A. salmonicida* strain prior to these time points.

5.3 Conditions that inhibited growth during storage time

The lag phase of aerobic count was considerably shorter in vacuum packaging than in MAP, especially non-inoculated samples stored at 4°C. In addition, the non-inoculated samples at 4°C reached higher concentrations after 16 days of storage (6,61 log cfu/g) in comparison to non-inoculated samples stored in MAP after 16 days of storage (2,5 log cfu/g). This corresponds well with findings by Hansen et al. (2009) which suggested that vacuum packaged samples had the highest bacterial count throughout the whole time of storage in comparison to MAP. The same study investigated the quality changes of *pre-rigor* filleted Atlantic salmon packaged in modified atmosphere using CO_2 emitter, traditional MAP (60% CO_2, 40% N_2) and vacuum. Based on microbial count, sensorial analysis, and color of salmon, this study concluded that salmon stored in MA packaging preserved the quality better than salmon stored in vacuum. They found that MA packaging with a CO_2 emitter gave similar or better results compared to traditional MAP, thus CO_2 emitters are well suited for reduction of volume of MA packaged farmed salmon filets. The results from this study demonstrated that refrigeration (4°C) and packaging technologies such as vacuum or MA gas mixture have a major effect on growth. In this study, the microbiota is not known, however dominating microbiota normally present in MAP chilled salmon are LAB such as *P. phosphoreum* and *B. thermosphacta* (FAO, 2014).

Black colonies formed on iron agar are often identified as *Shewanella* species by 16s rRNA sequencing in marine fish (Vogel et al., 2005). However, they may also include other H_2S producing bacteria identified as *Aeromonas spp., Enterobacter* and *Citrobacter* farm reared freshwater prawn (Lalitha and Surendran, 2006, NMKL, 2006). In the present study, the presence of these bacteria was more pronounced in non-inoculated samples stored in vacuum than in MAP, especially at lower temperatures (4°C). These results correspond to others showing that H_2S-producing bacteria are inhibited by CO_2 gas on farmed halibut (Hovda et al., 2007), farmed cod (Hansen et al., 2007), salmon (Schirmer et al., 2009) and on model substrates (Dalgaard, 1995). Additionally, H_2S-producing bacteria have been shown to be capable of growth under anaerobic conditions due to the utilization of TMAO as an electron acceptor (Ravn Jørgensen et al., 1988).

Initially, the *A. salmonicida* inoculum used in this study was supposed to obtain an initial density of approximately 3 log cfu/g. Unexpectedly, the inoculated samples started at

approximately log 5 cfu/g, which may have been a bit too high. Consequently, concentrations of *Aeromonas spp.* were certainly high enough to examine. One may speculate whether it would have been possible to reach disease- causing concentrations during storage if the inoculated samples had started on 3 log cfu/g. Inoculated samples stored in vacuum packaging at 4°C displayed a 3-fold increase reaching concentrations of 8 log cfu/g of presumptive aeromonads. Inoculated samples stored in modified atmosphere at 4°C displayed a 2-fold increase, reaching concentrations of approximately 7 log cfu/g of presumptive aeromonads. Data from this study suggest that the inoculated samples in vacuum packaging and MAP would have reached concentrations given foodborne disease. However, if the starting point had been 3 log cfu/g one may question if salmon packaged in MAP at 4°C would have reached disease- causing concentrations at >6 log cfu/g. Also, studies of food that on purpose have been inoculated with *Listeria spp*. have shown that bacterial growth in naturally contaminated food often appears slower than food that has been inoculated, that is possibly because of the presence of competing species (Ross et al., 2000).

After approximately 7 days, amine and sour odors were detected in vacuum packaged salmon inoculated samples at 4 and 8°C by sensory analysis, corresponding to results obtained in previous studies (Emborg et al., 2002). Figure 6 showing total aerobic count of approximately 8 log cfu/g, which is characterized as spoiled fish (Gram et al., 1987). In MAP the inoculated samples at 4 and 8°C delivers off odors such as ammonia after 15 days of storage. The increase in ammonia odor can be related to bacterial development of TMA, although the TMA level in salmon is relatively low in comparison to for example cod (Hebard et al., 1982). These findings can be reinforced by figure 10 which approximates an aerobic count of 7-8 log cfu/g. This corresponds well with a study by Hansen et al. (2009) which detected sour odor, pungent odor, sulfur odor, ammonia odor, off-odor, rancid odor, and the total odor intensity with an increase after 15 days of storage in MAP.

5.4 Metabolite production by NMR

Eleven different fish metabolites influencing freshness and taste properties were investigated simultaneously. This method has advantages in terms of simplicity of quantification and minimal sample preparation. However, one of the limitations is the narrow range of chemical shifts (10 ppm), which leads to overlapping signals causing uncertainty in the spectral assignments. Another drawback is the low sensitivity. There were some limitations regarding the quantitative results obtained in this study, granted the large standard error observed in some of the metabolites (Emwas, 2015).

The concentration of glucose especially decreased in vacuum packaging at 8°C after 14 days of storage compared to samples from day 0 of storage. In MAP, the concentration of glucose did not differ significantly among samples from day 0 and day 15 of storage for temperatures 4 and 8°C, which indicates a small amount of glucose fermentation. The changes in the concentration of sugar/and or sugar phosphates in the muscle contribute to the gradual loss of the sweet flavor of fresh fish (Sikorski, 1990). The decrease in carbohydrates may be due to alcoholic fermentation processes which lead to the production of the organic acid acetate. The concentration of acetate positively correlates with the amount of glucose in vacuum packaging at 8°C after 14 days of storage. The concentration of acetate is considerably higher at 8°C than at 4°C. Concentration of acetate in MAP was also higher at 8°C than at 4°C. However, the concentration of acetate was considerably lower at 4°C in MAP than in vacuum packaging at the same temperature. Alternatively, the decrease in carbohydrates may be due to another fermentation process which leads to the production of alcohols such as ethanol and 2,3-butanediol. A higher production rate of 2,3-butanediol was observed in vacuum packaging compared to MAP. No statistical significance was observed in the concentration of ethanol among control samples at day 0 as well as among all samples at day 14 and day 15 of storage in vacuum packaging ($p=0,063$) and MAP ($p=0,090$), respectively. However, tendencies toward increased concentrations at higher temperature (8°C) in both vacuum packaging and MAP were observed, in addition to a higher production of these substances with increasing storage time. These results correspond well with results done by Shumilina et al. (2015) which monitored post-mortem changes in Atlantic salmon filets stored at 0 and 4°C by the use of NMR. They also found that concentration of ethanol and 2,3-butanediol increased during storage time, with a higher rate at 4°C in comparison to 0°C.

Fermentation involves various types of bacteria which ferment sugars and produce different products, such as 2,3-butanediol, ethanol, acetic acid and others. Among these fermentation processes the concentration of 2,3- butanediol, ethanol and acetate increase with increasing storage time. Additionally, the increase in concentration was observed with higher rate at 8 °C, which may indicate that some bacteria was involved in the fermentation process. A study by Shumilina et al. (2015) suggested that the production of alcohols (i.e. ethanol and 2,3-butanediol) increased at higher temperatures and storage time. According to (Mûller, 2001) in production of ethanol, *Zymonomas spp*., LAB, *Enterobacteria, Clostridia, Escherichia* are involved. In production of acetate, *Moorella thermoacetica*, *Acetobacterium woodii* may be involved. Enteric bacteria including *Klebsiella*, *Enterobacter*, *Serratia*, *Erwinia* and *Hafnia* are typically involved in fermentation of 2,3-butanediol. In addition, *Aeromonas spp*. is found to be able to produce 2,3-butanediol (Stanier and Adams, 1944, Van Houdt et al., 2007). Enteric bacteria not participating in 2,3-butanediol fermentation, are instead involved in mixed acid fermentation which then produces acetate, ethanol and lactate (Madigan et al., 2015).

During spoilage of fish, TMAO is converted to TMA which is a volatile substance with an unpleasant specific “fishy” odor. TMA further degrades to formaldehyde (Van Waarde, 1988). The formation of TMA was detected in all samples since the sample were extracted after 3 days of slaughter, and not the day of slaughter. The amount of TMAO was highest in samples from day 0 of storage. In MAP, the concentrations of TMA were considerably lower in comparison to vacuum, except for inoculated samples at 8°C. In vacuum packaging there was no significant difference in concentrations of TMA among the control samples at 8°C and the inoculated samples at the end of storage ($p=0,479$). However, a significant difference is observed between non-inoculated and inoculated samples at 4°C ($p<0,05$), (table 3). In vacuum packaging an effect of inoculation is observed, suggesting that *A. salmonicida* can be a TMA producer and thereby a spoilage organism in vacuum packed salmon. However, further studies are necessary to elucidate the actual contribution from the inoculated strain. Others have reported that *Aeromonas spp.* are capable of producing TMA in fish (Liu et al., 2018). In MAP, a significant difference is observed in non-inoculated and inoculated samples at 8°C ($p<0,05$), (table 5). In MAP the high levels of TMA are achieved at 8°C and inoculation, which means that in storage at 4°C formation of TMA is inhibited. The increase in TMA concentration correlated with the decrease in TMAO concentration. The amount of TMAO depends on the species, season, fishing ground etc. In general, the highest amount of

TMAO is found in elasmobranchs, squid and cod while salmon, flatfish and pelagic fish have the least amount of TMA. The amount of TMAO increases with the age and size. In a study by Dalgaard et al. (1993) the spoilage criterion was found to be 30 mg/100g for fresh MAP cod. However, due to the low content of TMAO in salmon, these numbers are unlikely to be useful in fresh MAP salmon. The amount of TMA is often used as a spoilage quality indicator. However, there is no regulation concerning the TMA level. But beyond 12 mg/100g the product quality is generally considered as spoiled in salmon (Macé et al., 2012). Therefore, control samples at day 0 and salmon packed in modified atmosphere after 15 days at 4°C are the only samples which may be considered as not spoiled according to Macé et al. (2012).

Amino acids, nucleotides and organic acids are some of the most important classes of metabolites influencing flavor. The distribution of these compounds varies with fish species (Aoki et al., 1991). In addition it varies in farmed and wild fish where farmed fish tend to contain less free amino acids than wild fish (Haard, 1992). The concentration of leucine increased during storage at both packaging conditions. However, the amount of leucine generally increased more at vacuum conditions than at MAP for both 4°C and 8°C. According to Shumilina et al. (2015) bacterial spoilage becomes relevant after fourteen days of storage when salmon is stored in ice, and the bacteria accelerates the rate of protein hydrolysis with further release of free amino acids in the fish muscle. This corresponds well with results from this study showing an increase especially at higher temperatures, which makes sense since the bacteria thrives at higher temperatures.

As an indicator of freshness, some of the ATP degradation products were quantified in samples packed in vacuum and MAP. Hx is a metabolite indicating loss of freshness with increasing concentrations during storage, which means the higher number, the less fresh fish. In contrast, high concentrations of IMP are related to good quality fish. The overall results showed that vacuum packed salmon had a higher Hx-level than MAP salmon. The results from IMP did not show any significant difference among vacuum packed and MAP samples ($p=0,599$). However, there was a significant difference from day 0 and among non-inoculated and inoculated samples at day 0 ($p<0,05$). Shumilina et al. (2015) found the same pattern, with decreasing content of IMP and increasing Hx-level after 14 days of storage in air.

By measuring products of ATP degradation, freshness of fish during storage can be determined by the use of K- value and/ or H- index. K- value is a good parameter to grade fish freshness. However, since the fish used in this experiment was not fresh enough (samples not taken the same day of harvesting), all ATP was already degraded. A study by Erikson et al. (1997), found that the influence of the K-value was only observed during the first two days post mortem. Therefore, these results correspond well with findings in this study since the fish was packed three days after harvest. By the use of NMR, a K- value in non-inoculated samples from day 0 and inoculated samples from day 0 was of 61,61± 3,80% and 53,96± 2,58% which is close to the maximum level of rejection. Samples in both vacuum and MAP after 14 and 15 days of storage, respectively, was close to a K- value of approximately 100%. The H- index was used in this experiment since the fish had already been stored (not yet packed) for 3 days post- harvest. H- values were lower in MAP at 4°C than in vacuum packaging at 4°C after 15 and 14 days of storage respectively. This indicates that the freshness of salmon stored in MAP after 15 days is better than salmon stored in vacuum after 14 days.

High number of biogenic amines are produced during processing and storage of seafood as a result of microbial contamination and inadequate storage conditions. Some of the most important biogenic amines in seafood are histamine, tyramine, tryptamine, putrescine and cadaverine which are formed from their respective amino acids histidine, tyrosine, tryptophan, ornithine and lysine (Lee et al., 2015). By utilizing NMR, the formation of cadaverine, putrescine and tyramine was observed in higher concentrations after 14 and 15 days of storage in vacuum packaging than in MAP. Özogul and Özogul (2006) studied the biogenic amine content of sardines stored in vacuum packaging and MAP at 4°C. It was found that fish stored in vacuum packaging exhibited higher biogenic amine values than in MAP, which corresponds well with findings from this study.

5.5 Limitations of the layout

One cannot assume that spoilage is attributed to one specific species. Jørgensen et al. (2000) introduced the term 'metabolic spoilage association' to describe situations where two or more microbial species contribute to spoilage in a product, this by exchange of nutrients and/or metabolites. This scenario could be covered by the SSO concept. However, one must keep in mind that a community of different microorganisms interact to spoil a food product (Gram et al., 2002). In this layout, there is a background flora and therefore one cannot be certain that changes in metabolic composition is only caused by *Aeromonas spp.* alone. One might speculate if it is a result of growth of other microorganisms through storage as well.

6 Conclusion

This study assessed growth kinetics and the spoilage potential of *A. salmonicida* strain SU2 stored at 4 and 8°C in vacuum packaging and modified atmosphere (gasmix) in *pre- rigor* fileted back loins of salmon.

It was found that *A. salmonicida* are capable of growth in vacuum packaging and in modified atmosphere packaging (40% CO_2 and 60% N_2) down to refrigeration temperature (4°C). The overall results showed that growth of *A. salmonicida* is highly affected by temperature, and it demonstrated that refrigeration (4°C) and MAP are hurdles to inhibit growth of *A. salmonicida* and to reduce spoilage on *pre- rigor* fileted back loins of salmon. In addition, results indicate that MAP reduce the growth rate of A. *salmonicida.* The concentration of eleven metabolites was measured simultaneously. Results showed an increase in formation of undesired biogenic amines at higher temperatures and at vacuum packaging compared to MAP. However, more studies are needed to elucidate the actual role of *A. salmonicida* as a spoilage organism in MAP salmon.

7 Future perspectives

In this study, 40% CO_2 and 60% N_2 was used in modified atmosphere packaging. Limited studies were found regarding CO_2 tolerance of *Aeromonas spp*., therefore tests should be carried out to investigate CO_2 tolerance in environmental strains of *Aeromonas spp*. Furthermore, a CO_2 emitter has been demonstrated to compensate for the CO_2 gas that is absorbed by the product (Hansen et al., 2007). It could have been interesting to see how this would affect growth.

To obtain a better knowledge about growth kinetics and spoilage potential in the Aeromonas genus, other strains of *Aeromonas spp*. could be chosen for further studies. For example, *A. bestiarum*, *A. dhakensis*, *A. caviae*, *A. media*, *A. hydrophila*, and *A. piscicola* which already have been assessed by Hoel et al. (2017) from fresh retail sushi. In the Aeromonas genus, there can be various tolerance for CO_2, temperature, packaging conditions and so on. Therefore, it could be interesting to test more strains and also, a mix of different strains. Additionally, there is a background flora which is not characterized. It could have been interesting to see which other microorganisms that were present during storage.

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