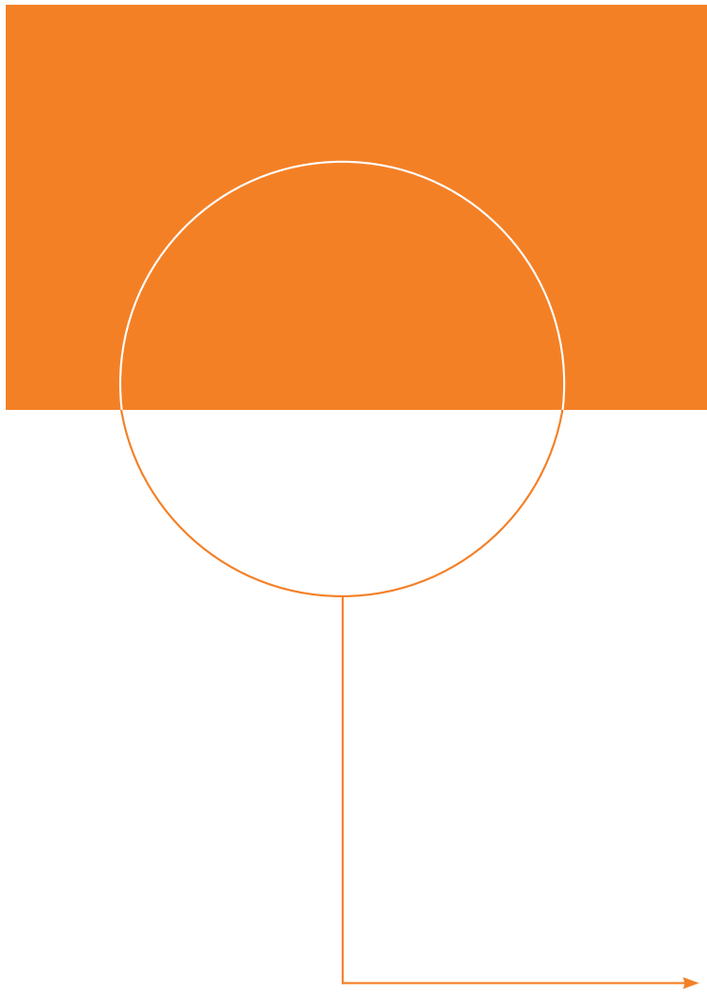

Master's thesis

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Biotechnology, 5 years
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Preface

Thank you to all my supervisors, Turid Rustad, Jørgen Lerfall, and Nanna Abel, for allowing me to be a part of your project. My gratitude for the many hours you spent in the “kitchen“ and the lab to help me is immense.

Thanks a lot to all of the regulars in the labs at Kalvskinnet for all help in the day-to-day goings, sorry for hogging the autoclaves and producing absurd amounts of garbage.

Abstract

An increasing demand for convenient, delicate, and healthy foodstuffs has been identified in an increasingly time-strained and health-conscious consumer base. Atlantic salmon (*Salmo salar*) has long been available in ready-to-eat forms such as cold-smoked salmon or gravlax, but the fish is not commonly sold as a minimally processed fillet-type product. Seafoods are highly perishable due to their high post-mortem pH and high concentration non-protein nitrogen, so the utilisation of Atlantic salmon in such a product poses unique challenges to microbiological safety. Notably, *Listeria monocytogenes* is a high-risk infective pathogen in ready-to-eat seafood. A shelf-life study over 24 days at 4°C was conducted to analyse the growth of spoilage microorganisms and *Listeria innocua*, as an analogue to provide an indication to *L. monocytogenes* growth. Preservative hurdles were implemented in the form of heat treatments by sous-vide (40, 50, or 60°C) combined with packaging technologies (modified atmosphere packaging (MAP) with or without soluble gas stabilisation (SGS)). The study was conducted in two parts, where part 1 involved inoculation with *L. innocua* and part 2 involved injection of salt brine (10% sodium chloride, with and without carbonation), to produce a more attractive product. The implementation of SGS significantly increased the microbial stability in part 1, with the combination of the 50°C heat treatment and SGS inhibiting all growth across the microbiological tests. The addition of an extra processing step in part 2 significantly increased microbial growth in all tests, allowing growth even of the heat sensitive hydrogen sulphide producing bacteria types. This was attributed to the brine injection method contaminating the otherwise sterile interior of the flesh, where the heating-process was less severe. Other ways of imparting salt or other flavour into the product is suggested, to preserve the microbial stability achieved in part 1. A collaborator determined that the usage of SGS had no significant effect on tested quality parameters, including drip loss and surface colour.

Sammendrag

Det er identifisert en økende etterspørsel etter raske, delikate, og sunne matvarer hos dagens forbrukere grunnet stadig økende tidspress og helsefokus. Atlantisk laks (*Salmo salar*) har lenge vært tilgjengelig i «ready-to-eat» former som kaldrøkt laks og gravlaks, men fisken er ikke vanligvis solgt som et minimalt prosessert filet-type produkt. Sjømat er kjennetegnet av rask fordervelse grunnet deres høye post mortem pH og høye konsentrasjon av «non-protein nitrogen», så benyttelsen av atlantisk laks i slike produkter medbringer en rekke utfordringer til mikrobiell sikkerhet. *Listeria monocytogenes* er nevneverdig som en høyrisiko infiserende patogen i «ready-to-eat» sjømat. En holdbarhetsstudie ble gjennomført gjennom 24 dager ved 4°C for å analysere veksten av fordervelses-mikroorganismer og *Listeria innocua*, som ble benyttet som en analog til *L. monocytogenes* for å gi en indikasjon på vekst. Preserveringshindre ble implementert i form av varmebehandling med sous-vide (40, 50, eller 60°C) kombinert med emballeringsteknologi (modifisert atmosfære innpakking (MAP) med og uten soluble gas stabilisation (SGS)). Studiet ble utført i to deler, hvor del 1 involverte inokulering med *L. innocua* og del 2 involverte injeksjon med saltlake (10% natriumklorid, med og uten karbonisering) med hensikten å produsere et mer attraktivt produkt. Implementeringen av SGS førte til signifikant økt mikrobiell stabilitet i del 1, hvor kombinasjonen av varmebehandling til 50°C og SGS inhiberte all vekst gjennom alle mikrobiologiske tester. Tilføringen av et ekstra prosesseringssteg i del 2 økte mikrobiell vekst signifikant i alle tester, og tillot t.o.m de meget varmesensitive hydrogensulfitt produserende bakteriene å vokse. Injeksjonsmetoden var antatt å være skyldig i denne tendensen, ved å kontaminere det ellers sterile interiøret i fiskekjøttet, som førte til at disse bakteriene mottok en svakere varmebehandling. Andre metoder for å tilføre salt eller annen smak anbefalt, for å bevare den mikrobielle stabiliteten som ble oppnådd i del 1. En samarbeidspartner fant ut at bruken av SGS ikke hadde noen signifikant effekt på kvalitetsparameterne som ble testet, inkludert drypptap og overflatefarge.

Table of contents

Abbreviations

ANOVA - analysis of variance

APC - aerobic plate count

ATP - adenosine triphosphate

BHI - brain heart infusion

BLA - Brilliance Listeria Agar

CFU = colony forming unit

CO2 - carbon dioxide

CPE - *Clostridium perfringens* enterotoxin

DF - degree of filling

GHP - good hygienic practice

GLM - general linear modelling

H^+ - proton

H_2CO_3 - carbonic acid

HACCP - hazard analysis and critical control point

HCO_3^- - bicarbonate

IA - iron agar

IA - iron agar

ID_{50} – infective dose for pathology for 50% of consumers

LC-PUFA - long chain polyunsaturated fatty acids

MA - modified atmosphere

MAP - modified atmosphere packaging

MID - minimum infective dose

MRS - de Man, Rogosa and Sharpe

N_2 - nitrogen gas

NaCl - natrium chloride

NaCl - sodium chloride

NBF - natural bacterial flora

NPN - non-protein nitrogen

O_2 - oxygen gas

OD - optical density

OTR - oxygen transmission rate

PCR - polymerase chain reaction

SD - standard deviation

SFP - Shahidi Ferguson Perfringens

SGS - soluble gas stabilisation

SSO - specific spoilage organism

TMA - trimethylamine

TMAO - trimethylamine oxide

α = carbonated 10% NaCl (w/v) brine + MAP,

β = 10% NaCl (w/v) brine + MAP,

γ = 10% NaCl (w/v) brine + SGS

1. Introduction

Changing societal structures due to rapid urbanization, an increasing number of small households, and increasing female participation in the workforce has led to an increased demand for convenient food products that require minimal preparation on the consumers end (Kearney, 2010; Speranza et al., 2009). Restaurants, and fast-food dining, as well as ready-to-cook and ready-to-eat foods has filled this demand in developing and industrialized nations alike, but many of the affordable alternatives are typically dense in calories and lacking in nutrients. The prevalence of information technology is making the consequences of poor nutrition apparent to increasing parts of the population, raising health awareness in the general consumer base. While there is a disconnect between some consumers attitude towards healthy foods and their consumption pattern (Kearney, 2010), further development of convenient and healthy foods will remove some of the perceived obstacles to healthy eating. Due to its high content in protein and long-chain polyunsaturated fatty acids (LC-PUFA), Atlantic salmon (*Salmo salar*) is commonly seen by consumers as a healthy and nutritious foodstuff, and are marketed as such (European Commission, n.d.; Sidhu, 2003). Atlantic salmon is currently a major aquaculture species, allowing a stable supply of fresh raw material to both consumers and the food processing industry (Liu et al., 2011; SSB, 2019).

The ready-to-eat Atlantic salmon products that are widely available in grocery stores consists of raw, lightly heat-processed, and lightly-preserved products, such as sushi, hot- and cold smoked Atlantic salmon, and “gravlax” (Ryder, 2014a). To the knowledge of the author, no minimally processed fillet-type Atlantic salmon with long shelf life are currently sold as ready-to-eat products. The aim of this study will be to determine the microbiological quality of such a product over a storage period of 24 days, by enumeration of natural spoilage organisms and pathogenic bacteria under different modified atmosphere, heat treatment, and injection salting permutations. As *Listeria monocytogenes* growth is a major risk for lightly heat-processed fish products (Ryder, 2014a, 2014b), samples can be inoculated with *Listeria innocua* to provide a suitable analogue(Abel et al., 2019a; Hudecová et al., 2010; Hugas et al., 1998; Lorentzen et al., 2010; McLaughlin et al., 2011).

1.1 Ready-to-eat foods

Ready-to-eat foods are a class of products that can be consumed directly from the package without further preparation, with heating to preferred serving temperature as an optional step. Along with ready-to-cook products, they serve as a convenient alternative to home-cooking for the private consumer (Speranza et al., 2009), as well as having the potential to provide instant single serving meals to sectors like healthcare (Lund and O'Brien, 2009). A long shelf life is a desirable characteristic for the bulk-users and producers of these products, as it alleviates logistical concerns and reduces food waste.

Within the seafood category these products have largely consisted of lightly-preserved and semi-preserved foods, where microbial growth has been limited by preservative measures such as smoking, curing, brining, fermenting, and marinating. Any growth of pathogenic microorganisms post processing in ready-to-eat products are unlikely to be inactivated before consumption and may cause disease if growth or initial contamination was sufficient, depending on infective dose of the pathogen in question (Huss and Ryder, 2004). For long shelf lives to be attainable, it is important to formulate products where significant growth of notable pathogens cannot occur during storage. The traditional preservative measures commonly found in ready-to-eat Atlantic salmon alters sensory characteristics and cannot be relied on to produce a fillet-type meal with a delicate flavour. Such a product will have to rely on heat inactivation during processing as a suitable packaging technology and cold storage to prevent sensory and microbial degradation (Abel et al., 2019a; Speranza et al., 2009).

In a hospital or other healthcare setting, demands towards microbiologically safe products are magnified due to the high fraction of immunocompromised consumers (Lund and O'Brien, 2009). The infective dose for a pathogen, like *L. monocytogenes,* is generally lower for the sick, elderly, and pregnant consumers, or patients receiving otherwise immunocompromising treatment (Ryder, 2014b). Food-borne disease outbreaks in hospitals have thus historically been characterised by high rates of serious illness or mortality, such as the case study on a listeriosis outbreak from a Brazilian hospital conducted by Martins et al. (2010) which resulted in a mortality rate of 83%.

1.2 Microbial flora in seafood

Compared to meat from land animals, fish and seafood products are considered highly perishable foodstuffs. The effect of three individual mechanisms as well as their interactions results in quick quality deterioration; enzymatic reactions, microbial activity and chemical oxidation of lipids. The activity of endogenous enzymes leads to the consumption of adenosine triphosphate (ATP) and other bioactive molecules, leading to a loss of desirable taste and odour (Boziaris and Parlapani, 2017) . Further activity by proteolytic endogenous enzymes partially degrades connective- and muscle tissue proteins, leading to softening of the flesh and gaping (Hultmann and Rustad, 2004).

1.2.1 Microbiological spoilage

At the same time as autolytic degradation is starting, microorganisms are spreading to the flesh. Fish flesh exhibit low acidity compared to meat, since their lower carbohydrate content leads to a lesser production of lactic acid, generally resulting in a final pH>6.0 (Gram and Huss, 1996). This low acidity, combined with large amounts of small non-protein nitrogen (NPN) compounds usually being present in fish muscle, favours the growth of spoilage bacteria. The by-products and metabolites produced by spoilage bacteria are considered to have undesirable and foul flavour and odour. Sensory rejection by consumers usually occurs when spoilage bacteria becomes numerous enough to produce notable off-odour and -flavour, but microbial spoilage is also characterized by formation of slime due to production of extracellular polysaccharide and in some advanced cases, visible colonies (more prevalent with mould-spoilage).

The initial microbiota of fish flesh is usually diverse, consisting of a low abundance of several bacterial genera. The indigenous microbiota, the bacterial populations naturally found on the skin, gills, and digestive tract of the fish, will be largely be determined by the waters where the fish live (Boziaris and Parlapani, 2017). While Norwegian aquaculture Atlantic salmon have their juvenile phase in land-based recirculating aquaculture systems (Ebeling and Timmons, 2012), they almost exclusively face on-growing in sea-cage systems along the coast (Liu et al., 2011). The Atlantic salmon are thus exposed to cold temperate marine waters, dominated by mainly psychrophilic and psychrotrophic bacteria such as *Photobacterium, Shewanella, Psychrobacter* and *Pseudomonas* (Boziaris and Parlapani,

2017). The other organisms found on fish products are the result of contamination from the terrestrial environment and from product processing and handling, defined as the exogenous microbiota. Typical viable counts of the initial microbiota of fish flesh falls in the 3-4 log colony forming units (CFU)/g (Boziaris and Parlapani, 2017).

While the initial microbial load is diverse, different processing methods, preservative measures, and storage conditions will give some bacterial genera a competitive edge, and altogether inactivate or prevent the growth of others. The ultimate result of these conditions is that a few favoured microorganisms outcompeting the rest, eventually dominating the product and making up the spoilage microbiota (Boziaris and Parlapani, 2017). There are many parameters in play for deciding the makeup of the spoilage microbiota. Seasonal variation, pre-slaughter stress, and origin location will all lead to differing initial microbiotas between batches of raw material, even before any processing has occurred (Hovda et al., 2012; Mørkøre and Rørvik, 2001). Processing steps such as heat treatment, freezing, and high-pressure treatment will have different rates of inactivation on different bacteria types, limiting competition for survivors. Said survivor's growth may then be retarded or fully inhibited by altering product characteristics such as acidity and water activity, or by controlling atmospheric conditions and temperature during storage. Removal of O_2 through vacuum- or modified atmosphere packaging (MAP) retards the growth of aerobically respirating bacteria, and the addition of high concentrations of carbon dioxide (CO_2) retards the growth of several types of bacteria, such as *Photobacterium phosphoreum* and *Brochothrix thermosphacta* (Abel et al., 2019a; Boziaris and Parlapani, 2017; Church and Parsons, 1995).

1.2.2 Specific spoilage organisms

Out of the prevailing dominant microbiota, only some microorganisms have the ability produce adequate quantities of undesirable metabolites to induce organoleptic rejection by consumers. These microorganisms that exhibit *spoilage activity* are considered the main cause of spoilage in a given product and are referred to as specific spoilage organisms (SSOs) (Boziaris and Parlapani, 2017; Gram and Huss, 1996). The SSOs in a given product often consist of a single genus or species of bacteria. Determination and accurate enumeration of these microorganisms for a given product is an important tool for determination of shelf life and further development of product parameters to extend said shelf life (Gram and Dalgaard, 2002). The spoilage effect of a SSO is illustrated in figure 1.1.

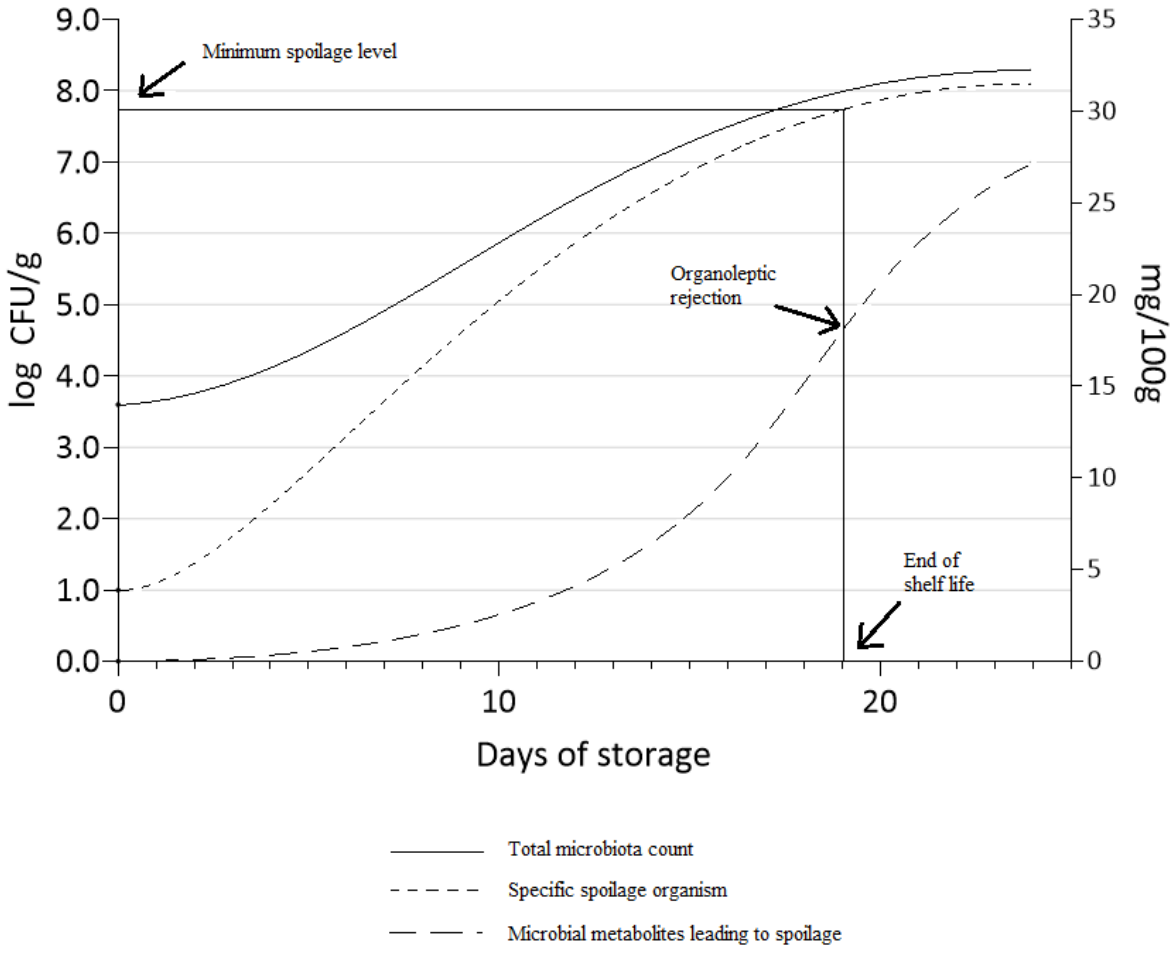

Figure 1. 1 Microbiological spoilage by metabolites produced by a specific spoilage organism (adapted from Boziaris and Parlapani, 2017).

Identification of SSO's can be carried out by determination of phenotypical traits by classical microbiological techniques (Boziaris and Parlapani, 2017). These traits are determined by plating samples on different types of media, isolating colonies, and performing tests such as the Gram-reaction, oxidase test, determining metabolic products, determining motility and sensitivities and so forth. The determination of phenotypical traits combined with knowledge of raw materials can often limit the SSO candidates to a few or even a single bacteria type. Methods such as 16S ribosomal RNA sequencing can be used for phylogenetic determination of SSOs, but requires extraction (and amplification) of DNA directly from the fish flesh if the SSOs escapes growth on laboratory media. This can be a challenging task that requires advanced machinery, but recent advances in sequencing technology has produced high-throughput platforms that makes sequencing of entire microbiotas possible within hours (Boziaris and Parlapani, 2017).

Enumeration of spoilage organisms is commonly carried out by plating out diluted product samples on various selective media plates and counting the bacterial colonies. These culture techniques rely on simple machinery and robust procedures, such as those published by the Nordic Committee on Food Analysis (NMKL, n.d.). They have the downside of being slow in producing colony counts, with incubation times from 1 to 5 days being required to produce accurate counts, depending on the media type (Boziaris and Parlapani, 2017; NMKL, 2008). For a microorganism to be enumerated by these methods, there must also be a selective media developed for them to grow on. Most of the common SSOs can be enumerated by these methods, but its not always possible to differentiate between genera without further phenotypic or phylogenetic testing: Such is the case with the prevalent SSOs *Shewanella* and *P. phosphoreum* enumerated on Lyngby iron agar (IA). Some real-time polymerase chain reaction (PCR) techniques has been developed to allow for faster enumeration of SSO's than for the plate count techniques, allowing for results within hours. Macé et al. (2013) developed such a method that allowed enumeration of *P. phosphoreum* in fresh Atlantic salmon in 6 hours. A downside to these culture-independent methods is that intact DNA in dead cells are enumerated as well as DNA in living cells. Processing of the product that leads to inactivation of the SSO being enumerated will produce inaccurate counts, but some methods provides counts that correlate well to traditional plate counts when performed on raw materials (Boziaris and Parlapani, 2017). Methods have been developed that bypass this problem by blocking the amplification of DNA from dead cells by addition of propidium monoazide (Mamlouk et al., 2012) or ethidium bromide monoazide (Lee and Levin, 2007).

Microbial spoilage in fish products typically occurs when SSO's reaches counts of 10^7-10^9 CFU/g, dependent on which SSO are dominating the spoilage microbiota (Boziaris and Parlapani, 2017). Both of the notable spoilage organisms *Shewanella putrefaciens* and *P. phosphoreum* causes spoilage by similar mechanisms (Gram and Huss, 1996). In oxygen-excluding conditions such as in vacuum- and modified atmosphere packaging, both will use trimethylamine oxide (TMAO) as a final electron acceptor for anaerobic respiration, producing substantial amounts of the spoilage metabolite trimethylamine (TMA). While these SSOs have some metabolic similarities, *P. phosphoreum* is able to cause spoilage at lower counts, typically at ~10^7 CFU/g, whereas *S. putrefaciens* needs to grow to concentrations of 10^8-10^9 CFU/g. This is attributed to the large size of individual *P. phosphoreum* cells of 5 µm, allowing them to produce higher amounts of metabolites per cell; *P. phosphoreum* is able to produce 30 times more TMA than *S. putrefaciens*, on average (Boziaris and Parlapani,

2017; Dalgaard, 1995). Regardless, off-odour and flavour from *Shewanella putrefaciens* spoilage is notably fouler than spoilage brought by *P. phosphoreum* as a SSO, due to a high production of volatile sulphur compounds (Gram and Huss, 1996).

1.2.3 Spoilage in modified atmosphere packed seafood

The psychrotolerant *P. phosphoreum* is resistant to high CO_2 concentrations, and is the SSO most commonly found in chilled MAP seafood (Gram and Dalgaard, 2002) . Yet it is an unlikely cause of microbial spoilage in heat treated products, as it has been shown to be inactivated even by gentle heat-processing (Abel et al., 2019a). *Brochothrix thermosphacta* has been found to be a dominant spoilage organism in such products, due to its ability to grow at low temperatures and resistance to high temperatures (D_{50}= 180 sec) (Abel et al., 2019a; Baranyi et al., 1996). While this bacteria has been found to grow in foods that have received prolonged heat treatment, Abel et al. (2019) showed that high concentrations of dissolved CO_2 were effective in significantly increasing heat inactivation of *B. thermosphacta*. This was achieved through soluble gas stabilisation (SGS), and a suggested reason for the reduction in heat resistance was due to the increased acidity caused by dissolved CO_2. A prevalent class of spoilage microorganisms in lightly preserved seafoods is lactic acid bacteria (LAB), notably *Lactobacillus* and *Carnobacterium* (Gram and Dalgaard, 2002). Several LAB species are capable of anaerobic growth and can thrive in low pH and high CO_2 conditions, but their growth is generally found to be several log units lower than other notable seafood SSOs in fresh MAP and vacuum-packed fish. They may become dominant in the spoilage microbiota if their competition is inhibited or eliminated by heat-processing and SGS (Abel et al., 2019a; Leroi, 2010). In cold-smoked Atlantic salmon, a product type where LAB is commonly found as the SSO, bacterial loads of 10^7-10^9 CFU/g is often detected at the point of organoleptic rejection. Conversely, 10^7-10^8 CFU/g has been shown to sometimes be present in such products several weeks before spoilage leads to organoleptic rejection (Gram and Huss, 1996). These variations are a result of a lack of selectivity in agar media used for enumeration, which does not allow for differentiation of LAB based upon spoilage activity (Gram and Dalgaard, 2002).

1.2.4 Causes of foodborne disease

Foodborne disease is caused by contamination of food by pathogenic bacteria, viruses, parasites, and in some species accumulation of toxins from the environment (Ryder, 2014c, p. 2). The flesh of Norwegian farmed Atlantic salmon is shown to be absent of the nematode parasites associated with foodborne disease in humans, due to it being absent in their feed (Lunestad, 2003). The major concern in Atlantic salmon products is therefor pathogenic bacteria, which can be classified as infective or toxin producing. The infective pathogenic bacteria cause disease by infecting tissues in their host after consumption, and the number of viable pathogenic cells needed to inflict such disease varies between bacterial species (and even strains) and is referred to as minimum infective dose (MID). While some infective pathogens are considered to be of lesser importance in products that are expected to undergo heating by consumers, they are highly relevant in ready-to-eat products. Since any growth of infective pathogenic bacteria after processing is likely to be consumed in these products, they face more stringent regulation in terms of microbiological criteria from regulatory authorities (European Commission, 2005; Ryder, 2014c). Some toxin producing pathogens have also been associated with ready-to-eat foods due to being resistant to inactivation, as well as being capable of growth through commonly imposed preservative hurdles (Ryder, 2014c). Growth limiting factors of the pathogenic bacteria that are noteworthy within the confines of this study are showed in table 1.1

Table 1. 1 Growth limiting factors of pathogenic bacteria of interest in a lightly heat-processed ready-to-eat Atlantic salmon product, stored chilled under modified atmosphere (adapted from Huss and Gram (2003)).

Pathogenic bacteria	**Temperature, ˚C**		**Acidity, pH**	**Water activity, a_w**	**Envitomental [NaCl] (%)**
	Minimum	Optimum	Minimum	Minimum	Maximum
Listeria monocytogenes	0-2	30-37	4.6	0.92	10
Non-proteolytic *Clostridium botulinum*, type B, E, F	3.3	25-28	5.0	0.97	3-5
Clostridium perfringens	12	43-47	5,5	0.93	10

As the concept of D-values (the time required for a 1-log reduction in cells at a given temperature, and circumstance) is well established in food microbiology, D-values for pathogens of note in this study is given in table 1.2.

Table 1. 2 Heat resistance of pathogenic bacteria of interest in a lightly heat-processed ready-to-eat Atlantic salmon product, stored chilled under modified atmosphere (adapted from Huss and Gram, (2003), and Ryder (2014b)).

Pathogenic Bacteria	**Heat resistance**
Listeria monocytogenes	D_{55} = 1-12 min D_{60} = 0.2-0.5 min
Non-proteolytic *Clostridium botulinum*, type B, E, F	D_{100} (spores) = 0.1 min; $D_{82.2}$ = 0.5 - 2.0 min (broth); D_{80} (spores) = 4.5 - 10.5 min in products with high fat content
Clostridium perfringens	D_{90} (spores) = 0.015 - 4.93 min (buffer) D_{100} (spores) = 0.31-13.0 min (broth)

1.2.5 *Listeria*

Listeria monocytogenes is a highly relevant infective pathogenic species due to its ability to grow significantly in ready-to-eat seafood as well as the severity of listeriosis, the disease it causes (Abel et al., 2019a; Martins et al., 2010; Ryder, 2014b). The species are prevalent in soil and decaying vegetation in the general environment and regularly finds its way to aquaculture locations by coastal runoff. Contamination of processing facilities, such as slaughter- and smokehouses, is also common (Rørvik et al., 1995). As a foodborne pathogen, *L. monocytogenes* has the unusual ability to grow significantly at refrigerated temperatures, and growth has been detected as low as 0˚C. It is a facultative anaerobe which grows with similar speeds in aerobic and anaerobic conditions, and is capable of growth in atmospheres consisting of 100% CO_2 (Fernández et al., 1997). Salinity must also be high to have an inhibitive effect on *L. monocytogenes*, and the bacteria is well suited for growth at the pH levels general seafood products (table 1.1). Its resistance to heat processing has been found to be higher in lipid rich products, such as salmon (Huss and Gram, 2003; Ryder, 2014b)

Since contamination in the processing facilities are the common source of *L. monocytogenes* in foodstuffs, control of listeriosis are mainly achieved by adherence to Hazard Analysis and Critical Control Point (HACCP) and Good Hygienic Practice (GHP) (Ropkins and Beck, 2000; Ryder, 2014a). If listericidal processing can be applied while the product is in its final packaging, it can serve as a critical control point (Huss and Gram, 2003). This is not a viable measure for MAP products, due to the packages being highly insulated and unsuitable for heat treatment. Inhibitory product formulation and adherence to GHP is therefore the only viable controls of *L. monocytogenes* in many ready-to eat products.

Listeria monocytogenes is suggested to have an ID_{50} (the number of cells that must be consumed for infection to occur in 50% of cases) in the millions of cells for healthy and immunocompromised alike. Due to the relative virulence of the bacterial strain and the susceptibility of the subpopulation exposed to the pathogen, estimates of infective varyies by 5 to 6 log units in studies of different listeriosis outbreaks (Ryder, 2014b). Infective listeriosis leads to fatality 20-40% of cases (Ryder, 2014b), which combined with the highly uncertain dose-infection ratio have led regulatory lawmakers to set strict microbiological criteria in ready-to-eat products. The microbiological criteria of *L. monocytogenes* for foodstuffs to be sold in the European Union is given in table 1.3. If it can be scientifically justified that the product formulation is unable to support growth of *L. monocytogenes*, it may be subject to less stringent microbiological criteria (European Commission, 2005).

Table 1. 3 The microbiological criteria that applies to *L. monocytogenes* in foodstuffs to be sold in the European Union. n= number of units comprising the sample, c= number of sample units giving values between m and M. m=M for the food categories in this table (adapted from European Commission, 2005).

Food category	**Microorganism**	**Sampling plan**		**Limits**		**Stage where criterion applies**
		n	**c**	**Vm**	**M**	
Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes	*Listeria monocytogenes*	10	0	Absence in 25 g		Products placed on the market during their shelf life
Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 CFU/g		Products placed on the market during their shelf life
		5	0	Absence in 25 g		Before the food has left the immediate control of the food business operator, who has produced it
Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 CFU/g		Products placed on the market during their shelf life

Listeria innocua is a non-pathogenic species in the *Listeria* genus. *Listeria monocytogenes* and *L. innocua* are phenotypically similar and exhibit similar growth patterns in MAP food. *Listeria innocua* is sometimes shown to be more resistant to heat-processing, dependent on the strain. *Listeria innocua* is thus considered a suitable analogue for studying the growth of *L. monocytogenes* within the experiment parameters, and expected to produce conservative results (Abel et al., 2019a; Hudecová et al., 2010; Hugas et al., 1998; Lorentzen et al., 2010; McLaughlin et al., 2011).

1.2.6 *Clostridium*

Clostridium botulinum is a notable pathogenic toxin producer in ready-to-eat MAP products due to its obligate-anaerobic nature and ability to produce extremely heat-resistant spores, which may later become vegetative pathogenic cells (Huss and Gram, 2003). The different types of human-pathogenic *C. botulinum* are classified by the toxin they produce, namely the proteolytic types A/B/F and the non-proteolytic types B/E/F. The non-proteolytic types have a higher sensitivity to heat and sodium chloride (NaCl) concentrations, but are more prevalent in seafood products due to having aquatic environments as natural habitats and being psychrotrophic (Huss and Gram, 2003). Non-proteolytic type E toxins are most commonly found to be the cause of food borne outbreaks from seafood products, partially because it is ubiquitous in the aquatic environment of cold temperate waters and the arctic zones. Proteolytic toxin producing *C. botulinum* may find their way into aquatic products, by land runoff to raw materials or processing contamination, but does not pose a significant threat in chilled seafood products due to their mesophilic nature preventing growth in chilled storage.

Botulinum toxin has a very low lethal dose at 0.1-1 μg/kg when consumed and acts as a potent neurotoxin. Lethality is typically brought on by respiratory failure, but lethality-rates has decreased in the latter parts of the twentieth century due to the availability of antiserum and respiratory support systems in hospitals (Ryder, 2014c). Botulinum toxin is largely inactivated by heat during pre-consumption cooking, but this is not a factor in ready-to-eat product design. Non-proteolytic toxin producing *C. botulinum* spores are less heat resistant than the proteolytic types, but mild heat-processing is not enough to significantly inactivate spores (recommended heat treatments for 6-log reduction are 90°C for 10 minutes) (Huss and Gram, 2003). Control of *C. botulinum* is therefor achieved by inhibition of growth by storage conditions and/or altering product characteristics such as acidity, water action, and salinity (see table 1. 1 for growth limiting factors). In this study, growth and toxin formation is expected to be inhibited for the storage duration by storage at less than 3.0°C and modified atmosphere (Ryder, 2014c).

Clostridium perfringens is another spore-forming pathogenic species of the *Clostridium* genus which is capable of anaerobic growth (Huss and Gram, 2003). It is ubiquitous in the general environment and may find its way into seafoods by the same vehicles as proteolytic type *C. botulinum* species; land runoff, faeces, and contamination of processing plants. Vegetative cells that are consumed can survive and sporulate in the intestine. The sporulating

cells produces several toxins, notably CPE (*Clostridium perfringens* enterotoxin) which can cause gastroenteritis (Petit et al., 1999). Proliferation of *C. perfringens* is controlled by chilled storage and grows slowly in temperatures <20°C (Huss and Gram, 2003), due to its mesophilic nature (table 1.1). It does pose a food safety threat when subjected to temperature abuse. Its optimal growth temperature is 43-47°C (Huss and Gram, 2003), combined with its anaerobic nature and short generation time (Labbé et al., 2014), means that a light heat treatment in a vacuum pack would serve as an incubation chamber for this species. Brynestad and Granum (2002) states that that the minimal infective dose for CPE to lead to foodborne disease is at least 10^7 vegetative cells.

1.3 Hurdle technology

Hurdle technology is a concept in food processing where several preservative methods, or hurdles, are combined to lengthen the shelf life of a product while maintaining its organoleptic qualities (Leistner, 2000). Important hurdles that are widely used in the food processing industry are temperature (heat processing and chilled storage/freezing), packaging (vacuum/MAP), water activity (by drying or adding salt), acidity, competitive microorganisms (mainly LAB-types), and preservatives such as nitrite. While intense application of a single of these hurdles may produce a microbiologically stable product, it will often have a detrimental effect on organoleptic quality. While the so called "botulinum cook" could be employed on a ready-to-eat Atlantic salmon product to inflict a 12-log reduction in *C. botulinum*, it would not make for a product a consumer finds desirable (Huss and Gram, 2003). A reason for the synergistic effects observed by the combination of hurdles are the disruption of homeostasis in microorganisms. Cells strive to keep their internal conditions (pH, solutes) at an optimum at all time. Preservative hurdles often work by altering these conditions. Before the cells can initiate growth, they must once again attain homeostasis. By low-intensity application of several of these hurdles, product safety may be drastically increased with minimal impact to quality (Leistner, 2000). The application of such stress may lead some types of bacteria to become more virulent or resistant to further stress, as they have the ability to produce stress shock proteins.

1.3.1 Heat treatment

Heat treatment serves two purposes in food processing: correct employment of it beneficially alters product quality (taste, aroma, and texture) in many products, and pathogenic microorganisms as well as spoilage microorganisms may be inactivated. Fish is generally considered to be cooked when its connective tissues (collagen) are sufficiently denaturated, allowing the flesh to come apart in flakes (Baldwin, 2012). This occurs at temperatures of 46-49°C, but Atlantic salmon is often heated to rare at temperatures of 42°C. Without combination with additional hurdles, these cooking temperatures are insufficient to produce microbiologically safe products unless the treatment is prolonged. Unless standard pasteurisation recommendations are to be used, a product will benefit by having preservative hurdles present during the heat processing step (Leistner, 2000).

Sous-vide is a cooking technique where the product is sealed in a vacuum pouch and poached in a temperature-controlled water-bath. This method benefits from precise temperature control of the water-bath and swift heat transfer from water to product, allowing low temperatures to be employed for even distribution of heat across the product surface (Baldwin, 2012). Sous-vide is notable for maintaining a high degree of juiciness and flavour in the product, and sous-vide-cooked fish have been found to retain greater amounts of LC-PUFAs and nutrients than traditionally cooked fish. The need to vacuum pack the product before heat processing is beneficial in products intended for distribution in the same packaging, eliminating the risk of recontamination, but it introduces a work-intensive extra step when the products are to be repacked into MAP (Baldwin, 2012; Ohlsson and Bengtsson, 2002a). Strong vacuums (10-15mbar) have been shown to reduce the organoleptic quality in fish and poultry products (Baldwin, 2012), so determining correct vacuum strength is critical. Low vacuum strength may lead to ballooning of the pouches and provide local insulation where gas is present, reducing the effect of heat treatment.

1.3.2 Salting

The addition of salt to muscle foods has long been used to improve texture and impart a desirable flavour, as well as having a retarding effect on microbial growth. The perceived juiciness of a muscle-based product is based on the water bound within it, the majority of which is held by capillary forces within the spaces between myofibrils, myofilaments, and outside muscle fibres (Strasburg et al., 2007). Addition of monovalent salts such as NaCl

increases the electrostatic repulsion between myofibrillar proteins. This leads to expansion of the myofibrils, allowing more free water to be held within. Other protein interactions at elevated NaCl concentrations >2.5% also leads to higher water binding. Salt is a crucial component in the flavour profile of many seafoods, but the high amounts commonly used in processed foods are considered detrimental to health (Kearney, 2010). The preservative effect of salt addition comes from reducing the availability of water to the microorganisms, expressed as water activity (a_w). If the a_w becomes too low as a result of increased solute (NaCl) concentration, water will diffuse out of bacterial cells, causing dehydration and preventing growth. Marine microorganisms are generally adapted so saline conditions, exhibiting ideal growth at 1-4% NaCl (Madigan et al., 1950). Minimal a_w values and maximal NaCl concentrations for relevant pathogenic species are showed in table 1.1

Addition of salt may be performed by several methods. Dry salting and marinating with brine are traditional methods but are slow and may not be suitable for all products (Lynum, 2011). Dry salting draws water out of the product for instance. Injection with salt brine is a quick way of getting salt into the product, with the added benefit of increasing product weight. Excessive brine injection will negatively affect the texture of the product and are seen as unethical by consumers (Lynum, 2011). The many needles that penetrate the flesh to deliver the brine may shove microorganisms present on the surface into the flesh, contaminating the otherwise sterile flesh-interior. The circulating brine may also serve as a reservoir for pathogenic bacteria (Gill et al., 2005a).

1.3.3 Modified atmosphere packaging

Modified atmosphere (MA) packaging is a preservative hurdle that combined with chilled storage retard the growth of microorganisms in general. In traditional MAP, two parameters are adjusted to cause this inhibition of growth (Church and Parsons, 1995; Ohlsson and Bengtsson, 2002a, 2002b). The availability to oxygen (O_2) can be limited to prevent aerobic growth or enriched to have a toxic effect. Limited presence of some oxygen may be desirable in meat products due to maintain colour, but the high amounts of unsaturated lipids in fatty fish such as Atlantic salmon will cause oxidative spoilage and rancidity if oxygen is present (Church and Parsons, 1995). Many notable food spoilers are capable of significant anaerobic growth, so the absence of oxygen may in some cases only alter the type of spoilage instead of

delaying it. Removal of oxygen will also allow obligate anaerobic organisms to grow, such as pathogenic *Clostridium* spp. (Farber, 1991).

The inclusion of high amounts of CO_2 in packaging has an inhibitory effect on the growth on many microorganisms (Church and Parsons, 1995). The inhibitory effect increases linearly up to 50-60% headspace CO_2, after which minimal additional inhibition occurs for most microorganisms. Carbon dioxide is highly soluble in water and liquid (but not solid) lipids compared to O_2 and nitrogen (N_2), readily dissolving as CO_2 and carbonic acid (H_2CO_3) (Abel et al., 2018; Farber, 1991). The solubility of CO_2 is inversely proportional with temperature, further increasing the growth retardant effect of chilled storage in some organisms. There is currently no consensus on the complete inhibitory functions of CO_2, but it's apparent that its growth inhibitive effect is reliant on its dissolution into the product. Farber (1991) summarizes some theories to its bacteriostatic effect as:

- Influencing nutrient uptake and absorption by alteration of bacterial cell membrane
- Inhibiting enzymes or reducing their reaction-rate
- Intracellular pH changes after penetrating bacterial cell membrane
- Changing the physio-chemical properties of proteins

There is also a small increase in acidity (<0.1 pH) due to dissociation of carbonic acid into bicarbonate (HCO_3^-) and protons (H^+) in MA-packed muscle foods with pH=6 or higher, this effect is however too small to have a significant inhibitory effect (Farber, 1991).

Since the lipid composition, water content, and initial microbiota varies widely between products, ideal gas composition must be determined on a case-to-case basis. For example, in products where *P. phosphoreum* is a notable SSO (such as Atlantic salmon), chilled MAP storage may not increase shelf life notably due to its resistance to CO_2 inhibition. Emborg et al. (2002) implemented a freezing-hurdle as a pre-step to MAP to inactivate *P. phosphoreum* and extend the shelf life of their Atlantic salmon product in such a scenario. This illustrates how knowledge of the spoilage microbiota in a given material may be utilized to employ the ideal hurdles for a given SSO.

Modified atmosphere packaging can be used to produce long shelf life products that are presentably showed in the packaging, but the technology has some notable disadvantages. It is comparatively expensive to implement, partially due to equipment requirements and training of personnel (Farber, 1991). To alleviate logistic demands, a producer would ideally want a high degree-of-filling (DF, volume of product vs. volume of package) in each MAP-

tray, but high DF will reduce the amount of dissolved CO_2 in the product (Rotabakk et al., 2008; Rotabakk and Sivertsvik, 2012). The packaging will therefore be bulky compared with vacuum-packaging or plastic bags, and the large gas volume slows down chilling by acting as insulation. Due to how readily CO_2 dissolves into water, the gas volume of a MAP product will decrease as the product reach equilibrium. With high CO_2 ratios or high DF, this can lead to a collapse of the packaging. This effect is counteracted by balancing the atmosphere with N_2, an inert gas which dissolves in water to a lesser degree than CO_2, allowing it to maintain the gas volume (Farber, 1991; Rotabakk and Sivertsvik, 2012).

1.3.4 Soluble gas stabilisation

Novel technologies have been developed to achieve higher equilibriums of dissolved CO_2 and higher DF in MAP products. One notable enhancement of MA-packaging is soluble gas stabilisation (Fletcher, 2012; Sivertsvik and Birkeland, 2006). This technology involves dissolving CO_2 into the product before packaging it, typically in MAP. This pre-step allows the product to reach CO_2-concentrations close to the targeted equilibrium in advance of packaging, meaning less CO_2 from the packaging dissolving into the product. This results in a lower loss of gas volume, allowing higher CO_2 ratios, and consequentially a greater inhibitory effect, or higher DF without package-collapse. The smaller packaging produces less waste, is logistically beneficial, and allows faster chilling of the product.

Soluble gas stabilisation also presents an opportunity to act synergistically as a hurdle during other processing steps. If CO_2 is dissolved into the product before heat processing, it may reduce certain microorganisms' resistance to heat inactivation. This effect was demonstrated by Abel et al. (2019) *B. thermosphacta* inoculated fish patties, where samples treated with SGS prior to heat processing showed significantly higher inactivation than other samples.

Soluble gas stabilisation have produced promising results in several product classes. Rotabakk et al. (2008) demonstrated a growth retarding effect of SGS on aerobic plate count (APC) and psychrotrophic count (PC) in Atlantic halibut (*Hippoglossus Hippoglossus*), but no effect on *B. thermosphacta* or hydrogen sulphide producing bacteria (HSPB). The microbial quality (determined by APC, PC, and LAB) of ready-to-eat deep-water shrimp

(*Pandalus borealis*) was shown to be improved by Sivertsvik and Birkeland (2006) over 20 days of storage; LAB growth were not significantly retarded by SGS in this study.

2. Materials and methods

A two-part study was conducted to determine the effect of heat processing, packaging technology, and injection brining on the growth of microorganisms during storage. The experiments were conducted with minimally processed Atlantic salmon fillets to be consumed without further cooking. The experimental factors in part 1 were heat processing (to core temperature of 40, 50, or 60°C), packaging technology (MAP or SGS into MAP), microbial flora (natural or *L. innocua*-inoculated), and storage time (0, 6, 10, 13, 17, or 24 days) (table 2.1). Part 1 was conducted in two rounds, separated by packaging technology (henceforth referred to as part 1.1 and part 1.2 for the MAP round and the SGS round, respectively).

The experimental factors in part 2 were heat processing (to core temperature of 40 or 50°C), brine composition (10% NaCl or 10% NaCl with carbonation, w/v), packaging technology (MAP or SGS into MAP), and storage time (0, 7, 10, 14, 17, or 24 days) (table 2.2). Heat processing to 60°C were not performed in part 2 based on results from part 1.

A shorthand was created for the three different brine and packaging technology combinations in part 2 of the study, referred to as treatments. The treatments consist of α, β, and γ:

- α = carbonated 10% NaCl (w/v) brine + MAP,
- β = 10% NaCl (w/v) brine + MAP,
- γ = 10% NaCl (w/v) brine + SGS

A general overview of the execution is presented in figure 2.1.

Table 2. 1 The experimental design and responses of part 1 of the study.

Design parameters	**Levels**
Processing temperature	40 / 50 / 60 °C
Packaging technology	MAP / SGS
Microbial flora	Natural flora / Inoculated with *Listeria innocua*
Total groups	12
Storage time	0 / 6 / 10 / 13 / 17 / 24 days
Response variables	**Analysis**
Microbiological	Aerobic plate count (including H_2S-producing bacteria), lactic acid bacteria, H_2S-reducing *Clostridium* spores, *Listeria* spp.

Table 2. 2 The experimental design and responses of part 2 of the study.

Design parameters	**Levels**
Processing temperature	40 / 50 °C
Packaging technology and brine composition (treatment)	MAP + carbonated 10% NaCl brine (α) / MAP + 10% NaCl brine (β) / SGS + 10% NaCl brine (γ)
Microbial flora	Natural flora
Total groups	6
Storage time	0 / 7 / 10 / 14 / 17 / 24 days
Response variables	**Analysis**
Microbiological	Aerobic plate count (including H_2S-producing bacteria), lactic acid bacteria, H_2S-reducing *Clostridium* spores, *Listeria* spp.
Quality	Acidity (pH)

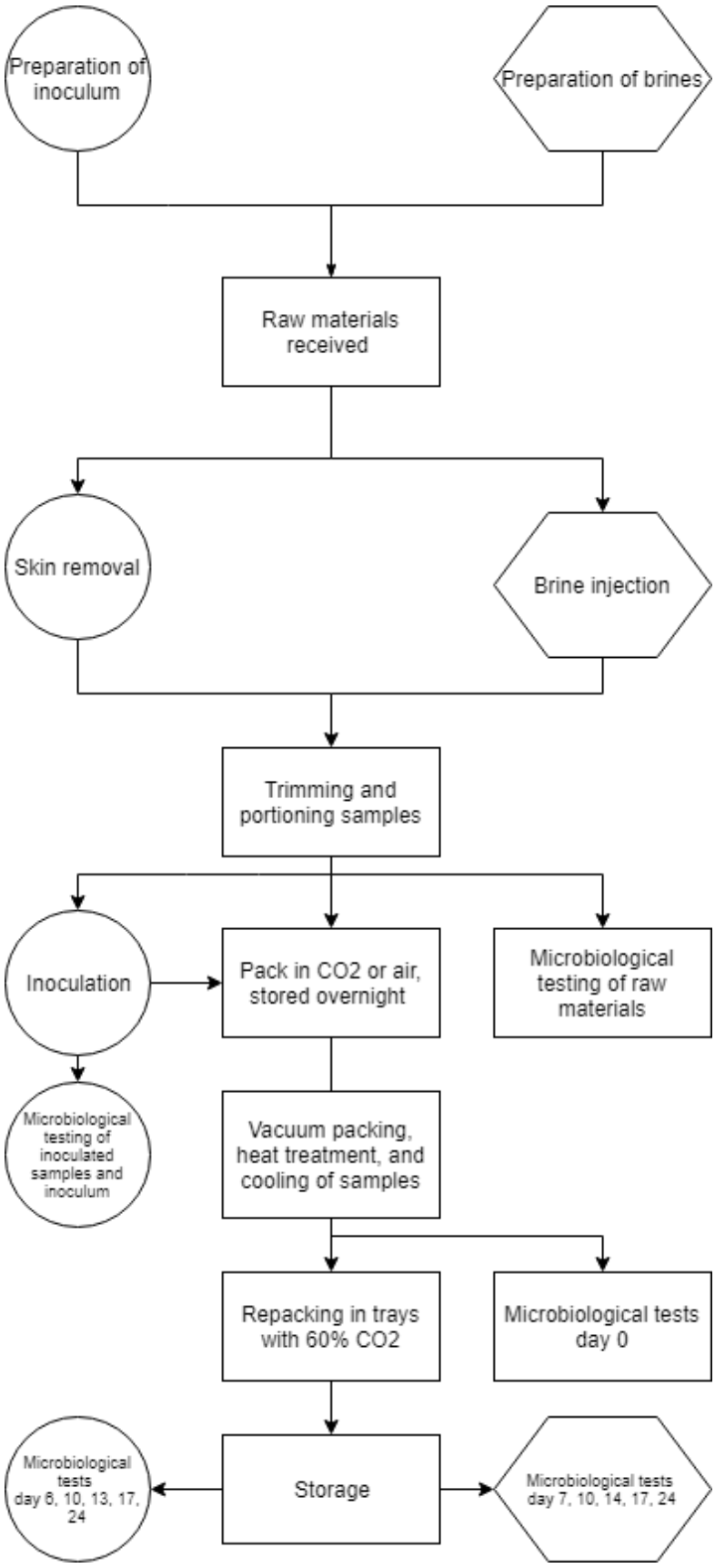

Figure 2. 1 Flowchart of the execution of the study. Rectangular components were conducted in both parts of the study, while circular and hexagonal components were conducted only in part 1 or part 2, respectively.

2.1 Raw materials

Atlantic salmon (*S. salar*) were procured from Salmar (Frøya, Norway) in the form of 1-1.4kg pre-rigor filleted. Raw materials for part 1 were obtained on day of slaughter and stored on ice in fridge (4.2±2.4°C) for 3 days before processing to ensure post rigor state. Tail, belly- and backflaps were trimmed and skin removed before fillets were portioned into equal sizes (79.8±2.3g) with heights lower than approximately 3cm (figure 2.2).

Raw materials for part 2 were obtained 5 days after slaughter (kept at 0-2°C per Salmar, post rigor assumed) and stored on ice in fridge (4.2±2.4°C) overnight before processing. Tail, belly- and backflaps were trimmed (figure 2.2).

Figure 2. 2 Schematic illustration of flap/tail trimming and subsequent sample portioning (adapted from Europe Epic-Cure, 2019).

2.2 Bacterial strain

For part 1, a frozen culture of *L. innocua* (-80°C, ATCC 33090) were obtained from the University of Gothenburg culture collection. The culture was transferred to brain heart infusion (BHI) agar (Oxoid CM1136, Oxoid Ltd., Basingstoke, UK), prepared according to manufacturer directions, and incubated for 24 hours at 37°C. Single colonies were isolated and transferred to BHI broth (Oxoid CM1032, Oxoid Ltd., Basingstoke, UK), prepared according to manufacturer directions, and incubated at 8°C for 5 days to produce cold-adapted cultures in early stationary phase. Samples of culture were diluted to OD_{600} (optical density, at 600 nm) of approximately 0.1 (0.104-0.110) to obtain a cell density of

approximately $1x10^5$ CFU/ml ($2.7x10^5$ CFU/mL and $2.9x10^5$ CFU/mL for MAP and SGS samples, respectively).

2.3 Brine preparation

For part 2, a surplus of brine was prepared by adding tap water (Trondheim, Norway) to NaCl to a 10% NaCl ratio (w/v). A portion was transferred to an airtight stainless-steel keg connected to a CO_2-filled gas cannister and pressurised to 2bar. The keg was vigorously shaken for approximately 20 minutes. Both the brines were stored in fridge overnight (4.2±2.4°C).

2.4 Inoculation, brine injection and pre-treatment.

Half of the samples in part 1 were drip inoculated by dispersing 100µL of inoculum across the top surface of the samples with a pipette (figure 2.3). The samples were then left to dry in a fume hood for 10 min. All samples were put on trays (C2325-1C, Færch Plast, Holstebro, Denmark) with vacuum pouches (425x650 mm PA/PE sous-vide pouch, Maske AS, Trondheim, Norway) in batches of 13. The packages were filled with atmospheric air or 100% food grade CO_2 for MAP and SGS samples, respectively. The samples were left to dry and reach CO_2-saturation in fridge (3.7°C) for 16-18 h. Four replicates of each samples were prepared.

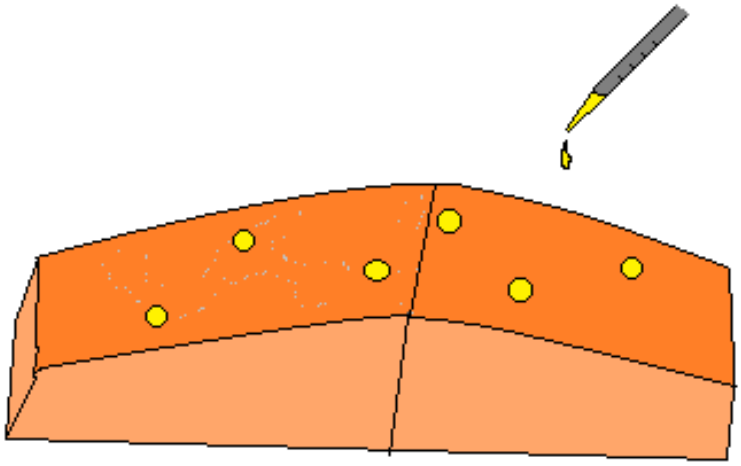

Figure 2. 3 Schematic illustration of drip inoculation with 100 µL *Listeria innocua* culture.

The trimmed fillets in part 2 to be injected with 10% NaCl brine were individually fed into the brine injector (PSM-57-2.5-ZD, Dorit DFT Fleischereimaschinen GmbH, Ellwangen, Germany) (filled with 10% NaCl brine) and injected. They were then portioned into equal sizes (81.0±2.0g, height <3cm) (figure 2.2). All samples were put on trays with vacuum pouches in batches of 13. The packages were filled with atmospheric air or 100% food grade CO_2 for MAP and SGS samples, respectively. The samples were left to dry and reach CO_2-saturation in fridge (3.7°C) for 16-18 h. In the following day, the fillets to be injected with carbonated brine were injected with carbonated 10% NaCl brine and portioned to equally sizes (81.0±2.0g, height <3cm) (figure 2.2). These samples were immediately heat treated, as described in section 2.5, to limit the dissipation of CO_2 from the carbonated brine. Four replicates of each samples were prepared.

All sampling and inoculation were performed aseptically.

2.5 Heat processing and packaging in modified atmosphere

All samples to be heat processed were packed in vacuum pouches (135x180mm PA/PE sous-vide pouch, Maske AS, Trondheim, Norway). The heat processing was performed in a sous-vide cooker (Diamond M, Fusionchef by Julaba, Seelbach, Germany). The processing times required to attain core temperatures 5°C lower than processing temperatures were decided by pre-experiments, described in appendix A. These corresponded to 15 min at 45°C, 18 min at

55°C, and 21 min at 65°C. All samples were immediately transferred to ice water to stop further heating.

After cooling, all samples were repacked into semi rigid crystalline polyethylene terephthalate trays (300mL, C2125-1B, Færsch Plast, Holstebro, Denmark) by tray sealing packaging machine (TL250, Webomatic, Bochum, Germany), with a drip absorbent in each tray. The samples obtained a DF of approximately 1:3 (v/v). A gas mixture of 60% CO_2 balanced with N_2 was produced by a gas mixer (MAP Mix 9000, Dansensor, Ringsted, Denmark) using food grade CO_2 and N_2. The atmosphere of the trays was evacuated by a vacuum pressure of 25mbar and flushed with the gas mixture. The trays were sealed with a 40µm top film (Topaz B-440 AF, Plastopil, Almere, Netherlands). Oxygen transmission rate (OTR) of the tray was 66-78cm^3 x 25µm/m^2 x 24h^{-1} at 23°C, and 2.5cm^3 x 40µm/m^2 x 24h^{-1} at 23°C for the film cover. Repackaging after heat treatment was performed aseptically to limit recontamination.

For part 1, negative samples were prepared to microbiological testing for days 0, 6, 10, 13, and 24. They received no heat treatment or inoculation with *L. innocua*, but otherwise followed identical processing to other part 1 samples. Four replicates were produced for MAP samples and three replicates were produced for SGS samples (due to lack of raw materials).

For part 2, negative samples were prepared only for day 0 microbiological testing (due to workload constraints), receiving no heat treatment but otherwise following identical processing to other part 2 samples. Three replicates were produced for each combination of packaging technology and brine composition.

The samples were stored for 24 days at 2.4±1.0 °C.

2.6 Headspace gas analysis

Analysis of the headspace gas composition (% CO_2 and O_2) was performed prior to microbiological analysis on all samples (days 6, 10, 13, 17, 24 or 7, 10, 14, 17, 24 for part 1 and part 2, respectively) except for day 0 samples (as CO_2-equilibriums would not have been reached). The measurement was performed with an oxygen and carbon dioxide analyser (Checkmate 9900 analyser, PBI-Dansensor, Ringsted, Denmark) by collecting 20ml of headspace gas with a syringe after perforating the film of the packages. Single-use foam pads

were placed on the film prior to sampling to prevent atmosphere leakage. Gas composition measurements of empty trays were performed immediately after packaging on each of the packaging days to control correct gas composition.

2.7 Microbiological analysis

All laboratory culture mediums used in this section was prepared according to the manufacturer's directions, with notification and elaboration given in case of deviation from description. All procedures performed in this section was performed aseptically. Table 2.3 has been created for ease of reference for the various procedures.

10 g of each sample were taken from the surface where inoculation was performed and homogenized for 60 sec in 90 ml of sterile peptone water (0.85% NaCl and 0.1% neutralised bacteriological peptone (LP0034, Oxoid Ltd, Basingstoke, UK), by w/v). The homogenized samples were diluted in appropriate decimal dilution series in sterile peptone water (0.85% NaCl and 0.1% neutralised bacteriological peptone (LP0034, Oxoid Ltd, Basingstoke, UK), by w/v).

All samples (including negative) were analysed for APC, LAB, *Clostridium* spores, and *Listeria* spp. on days 0 and 24. The remaining days (table 2.1 and 2.2) natural bacterial flora (NBF) samples were analysed for APC and LAB, while inoculated samples were analysed for APC and *Listeria* spp. Negative samples were tested for APC, LAB, and *Listeria* spp. the remaining days (table 2.1).

The 10^{-1} dilution of every sample were kept frozen (-80°C) in case further testing was required (i.e. the dilutions did not produce viable counts). In the cases where further testing was performed, the samples were thawed in a room temperature water bath (approximately 22°C), before dilution series were created and the relevant procedures were applied.

Aerobic plate count analysis was performed in accordance with NMKL-184 (NMKL, 2006). Iron agar (Lyngby) (IA) without L-cysteine (CM0964, Oxoid Ltd., Basingstoke, UK) was used in the described pour plate with top layer method, adding L-cysteine before pouring. The plates were incubated aerobically at 22°C for 3 days.

Lactic acid bacteria enumeration was performed in accordance with NMKL-140 (NMKL, 2007). De Man, Rogosa and Sharpe (MRS) agar (CM0361, Oxoid Ltd, Basingstoke, UK)

with 10mg/L amphotericin-B was used, and the plates were anaerobically incubated in at 25°C for 5 days.

Sulphite reducing *Clostridium* spores were analysed in accordance with NMKL-56 (NMKL, 2008). Shahidi Ferguson Perfringens (SFP) agar base (DIFCO28110, Thermo Fisher Scientific, Waltham MA, USA) was used, without addition of egg yolk. The diluted samples were heated at 80°C in water bath to inactivate vegetative cells before plating and top layer application. The samples were incubated anaerobically at 15°C for 5 days.

Listeria spp. analysis was performed with chromogenic *Brilliance* Listeria Agar (BLA) (CM1080, Oxoid Ltd, Basingstoke, UK) with *Brilliance* Listeria Selective Supplement (SR0227, Oxoid Ltd, Basingstoke, UK). Plates were incubated aerobically at 37°C for 24±2 h. Plates with blue/green colony-counts in the 30-300 interval were enumerated. *Listeria monocytogenes* were enumerated as blue/green colonies with haloes.

Table 2. 3 Overview of the microbial analysis procedures. IA= iron agar (Lyngby) without L-cysteine, MRS= de Man, Rogosa and Sharpe, SFP= Shahidi Ferguson Perfringens, BLA= *Brilliance* Listeria Agar

Microbial analysis	**Agar medium**	**Incubation conditions**			**Procedure**
		Atmosphere	**Temp., °C**	**Time**	
Aerobic plate count	IA + L-cysteine	Aerobic	22	72±6 h	NMKL-184 (2006)
H_2S-producing bacteria					
Lactic acid bacteria	MRS + amphotericin-B	Anaerobic	25	5 days	NMKL-140 (2007)
H_2S reducing *Clostridium*	SFP without added egg yolk	Anaerobic	15	5 days	NMKL-56 (2008)
Listeria spp.	BLA + Brilliance Listeria Selective Supplement	Aerobic	37	24±2 h	See text

2.8 pH Measurement

Single measurements of pH values were performed directly in all samples of part 2 with a spear tip pH electrode (Testo 206-pH2, Testo, Lenzkirch, Germany), as well as the pH of both brines.

2.9 Statistical analysis of results

All statistical analysis was performed using Minitab 18.1 (Minitab, Coventry, UK). All microbiological data was log-transformed before statistical analysis to ensure normality and equal variance. Outliers were detected and removed with Grubbs outlier test at significance level $p<0.05$. Significant differences were detected by analysis of variance (ANOVA) through general linear modelling (GLM) with a significance level $p<0.05$. Tukey's test was performed to detect which groups were statistically different at significance level $p<0.05$. Unless otherwise stated, data is given as mean ± standard deviation (SD).

A summary of the values employed to determine statistically significant differences is given in appendix B.

3. Results and discussion

The study was performed in collaboration with a fellow researcher at NTNU, who performed analysis of chemical quality parameters as well as being part of the microbiological analysis. Soluble gas stabilisation had no significant impact upon colour, water holding capacity, drip loss, or formation of ATP-degradation products when compared to only MAP (Abel et al., 2019b, pending review).

As the target of this study consist largely of the natural spoilage microbiota, some notes on the initial bacterial load of the raw material is in order. Across the three batches of raw materials, significant differences ($p<00.1$) in the initial bacterial load was found by all the microbial analysis which produced viable counts, with the exception of APC in part 1.2 and part 2. The results of this analysis are showed in figure 3.1. Such differences in microbial loads of raw materials are to be expected, since the raw material batches was obtained at three different time instances; January, March, and September for part 1.1, 1.2 and 2, respectively. Differences in the microbial composition of aquatic environment based on seasonal variations (such as temperature and precipitation) are well established (Delille, 1993; Suh et al., 2015), which in turn affects the composition of microbes that colonize the surfaces of the fish (Hovda et al., 2012). The feeding pattern and activity of the poikilothermic Atlantic salmon is also dependent on seasonal variability (temperature, day length), characterised by lower feed uptake and growth in the autumn and winter months (Mørkøre and Rørvik, 2001). The faster growth during the warmer summer and spring months appears to result in softer flesh and increased gaping (Mørkøre and Rørvik, 2001),

indicating a quality difference that may impact both initial bacterial load of the raw materials, and bacterial growth during storage.

Hydrogen sulphide reducing bacteria were below detection level in the raw materials (figure 3.1) but were detected in negative MAP samples after growth (day 6 and onwards).

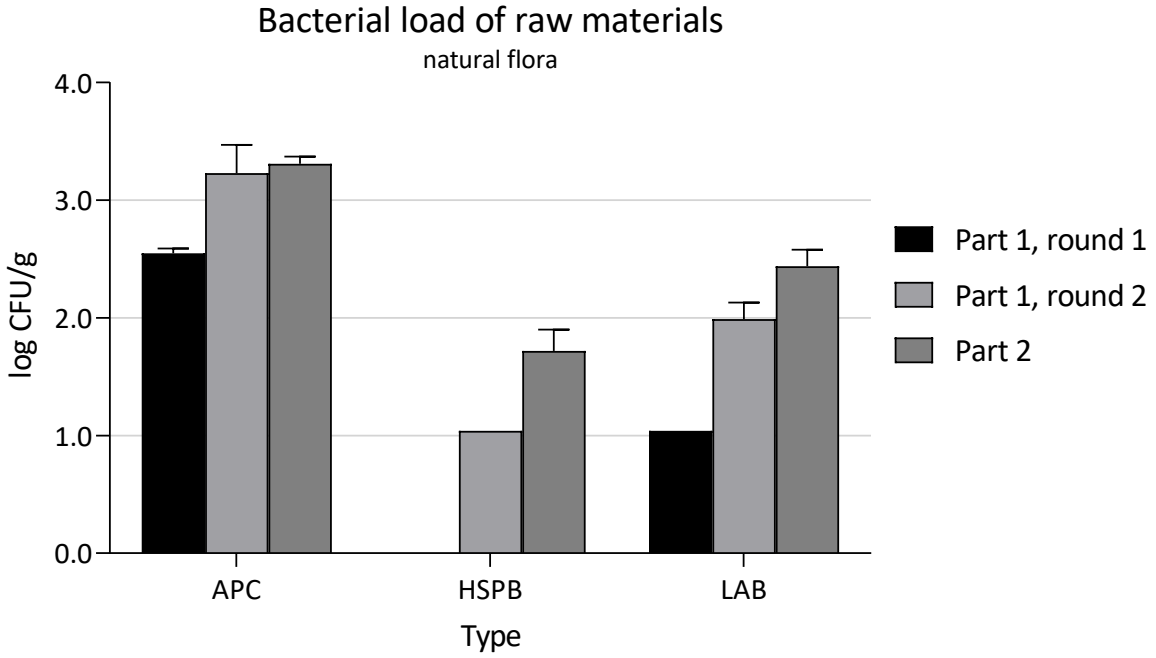

Figure 3. 1 The initial bacterial load of each batch of raw material. Presence of *Listeria* spp and *Clostridium* spp. not detected. Given as mean CFU/g + 1 standard deviation. APC = aerobic plate count, HSPB = hydrogen sulphide producing bacteria, LAB = lactic acid bacteria.

A general trend throughout this study was that the effect of the heat treatment in all microbial analysis, was always significantly inhibitive for microbial growth ($p<0.001$ in all cases). All samples heated to a core temperature of 60°C in part 1 produced no viable counts by any analysis throughout the storage period. This data (combined with workload considerations) led to the researchers omitting this treatment for part 2 of the study. While such a heat treatment may have led to high stability when considering spoilage microorganisms and *Listeria* spp., it does not result in a product that consumers consider attractive. Baldwin (2012) stated that a heating to as little as 42°C is common for Atlantic salmon, but this value is assumed to be ideal when the product is to be consumed immediately after heating. Consumers may find a stronger heat treatment more attractive in a long shelf life ready-to-eat product. The heat process had a notable impact on the presentation of the product: stronger

heat treatments resulted in increasingly paler colour and coagulation of sarcoplasmic protein. Appearance is an important factor in the consumers decision-making process when purchasing food. Anderson (2001) states that colour is one of the most important criteria for selection of salmon products by consumers, with more vibrant red/oranges being preferred. Anderson is assumedly describing the consumers criteria for selecting *fresh* salmon. Colour might be a less important selection criteria in a pre-heated product.

The complete absence of microbial growth at 60°C was unexpected. The heat-processes used in this study were designed by pre-trials (included in appendix A), and microbiological testing produced viable APC after all heat treatments. The pre-trial samples were not packed in MA, so the growth may be attributed to aerobic microorganisms with a higher heat-tolerance than encountered in part 1 of this study.

Viable cell counts were below the detection threshold for all NBF-samples heated to a core temperature of 50°C in the day 0 analysis (figure 3.1-3.6), regardless of treatment or packaging technology. This was not the case for *L. innocua* inoculated samples, where the 50°C samples packed with SGS displayed lower heat inactivation than those packed under normal MA (figure 3.8). This was determined to be a result of ballooning of the vacuum pouches that were observed during heat treatment. Since the ability of CO_2 to dissolve in water/lipids is inversely proportional to temperature (Farber, 1991), gaseous CO_2 dissipated from the samples and into the vacuum pouch. A similar issue is well established in sous-vide-cooking when water starts to form steam at high temperatures (Baldwin, 2012), and the impact on heating will be similar. The gas pockets provides insulation to the samples and reduces the conductance between water-bath and sample. This have presumably led to the SGS samples on average receiving a milder heat treatment than the MAP samples. The effect is assumed to be more visible in the *L. inoculated* samples than the NBF samples due to its relatively high heat tolerance compared to a large portion of the psychrotrophic natural flora (such as *P. phosphoreum* and *S. putrefaciens*) (Boziaris and Parlapani, 2017; Ryder, 2014b).

Headspace gas measurements showed that significantly higher CO_2-equilibriums were established in packages which had been treated with SGS ($p<0.001$), compared to only MAP. In part 2 of the study, samples with carbonated brine injected had significantly higher CO_2-concentrations ($p<0.001$) than those injected with normal brine (when SGS samples were omitted), suggesting an increased bacteriostatic effect may be achieved by this method compared to normal NaCl brine. This increase was lesser than that observed by employing

SGS. When comparing part 1 to part 2, significantly lower CO_2 equilibriums ($p<0.001$) were detected in samples with brine injected (carbonated brine omitted), particularly in MAP samples. This may be a result of altering the product characteristics by brine injection, or a result of seasonal variations between raw materials. Higher core temperature samples had significantly lower CO_2-concentrations ($p<0.001$) in the package headspace across the study. Mean values of these CO_2-measurements can be found in table 3.1.

Table 3. 1 The amount of carbon dioxide in the packaging headspace of samples used for microbiological analysis. As no growth were detected in samples heated to 60°C, their values were omitted. All values are given as mean ± 1 standard deviation. MAP = modified atmosphere packaging, SGS = soluble gas stabilisation, brine = 10% NaCl, carb. brine = carbonated 10% NaCl brine

Part of study	**Variable**			**CO_2 (%) in headspace**
	Core temperature, °C	**Packaging technology**	**Brine**	
1 and 2	40			48.09±6.47
	50			47.32±6.28
1		MAP		44.33±3.42
		SGS		55.17±2.08
2		MAP[I]		40.57±1.03
		SGS		54.00±1.20
2			brine[II]	40.57±1.03
			carb. brine	41.56±1.21
1 and 2			no brine	49.55±5.79
			brine[III]	47.28±6.85

I = carbonated brine samples omitted

II = SGS samples omitted

III = carbonated brine samples omitted

The pH of samples used in part 2 of the study was measured to determine if the injection of carbonated brine would lead to an increase in acidity. There was no significant difference in the measurements between treatments α (pH= 6.301±0.065) and β (pH= 6.302±0.057) ($p=0.985$), despite the carbonated brine being more acidic (pH= 4.75) than the conventional NaCl brine (pH= 7.2). The samples from treatment γ (pH= 6.263±0.074) were significantly different from treatment β, but the difference is likely to be significant only in the statistical

sense. Such a small increases in acidity is unlikely to have had a notable effect on bacterial growth, according to Farber (1991).

Note that the methodology for pH measurements may be considered less than ideal. Due to workload constraints, only one measurement per replicate was feasible. Ideally the measurements would be made in duplicate or triplicate.

3.1 Aerobic plate count

In part 1 of the study there was a significant effect from both heat treatment ($p<0.001$) and packaging technology ($p<0.001$) on APC (figure 3.2). A combination of SGS and 50°C heat treatment prevented all growth of above detection limit for the duration of the storage, while 50°C heat treatment with only MAP produced detectable APC within day 6. This inhibition of all growth may be attributed to the successful implementation of multitarget preservation, as described by (Leistner, 2000). In that case, the high CO_2-concentrations present during the heat treatment would have disrupted cellular homeostasis and reduced their ability to survive heat treatment. Such an effect has been shown by Loss and Hotchkiss (2002) in milk, where dissolved CO_2 significantly decreased the D-values of *Pseudomonas fluorescens* and *Bacillus cereus*. A high microbial stability, similar to the stability achieved by 60°C heat treatment combined with MAP, was achieved with a milder heat treatment that would presumably lead to a more attractive product for consumers.

The final bacterial load of both packaging technologies was not significantly different for samples heated to 40°C ($p=0.356$), reaching bacterial loads of 10^5-10^6 CFU/g. While the final loads were similar, samples subjected to SGS maintained a better microbial quality for longer, with bacterial counts at days 10, 13 and 17 being significantly lower ($p=0.017$).

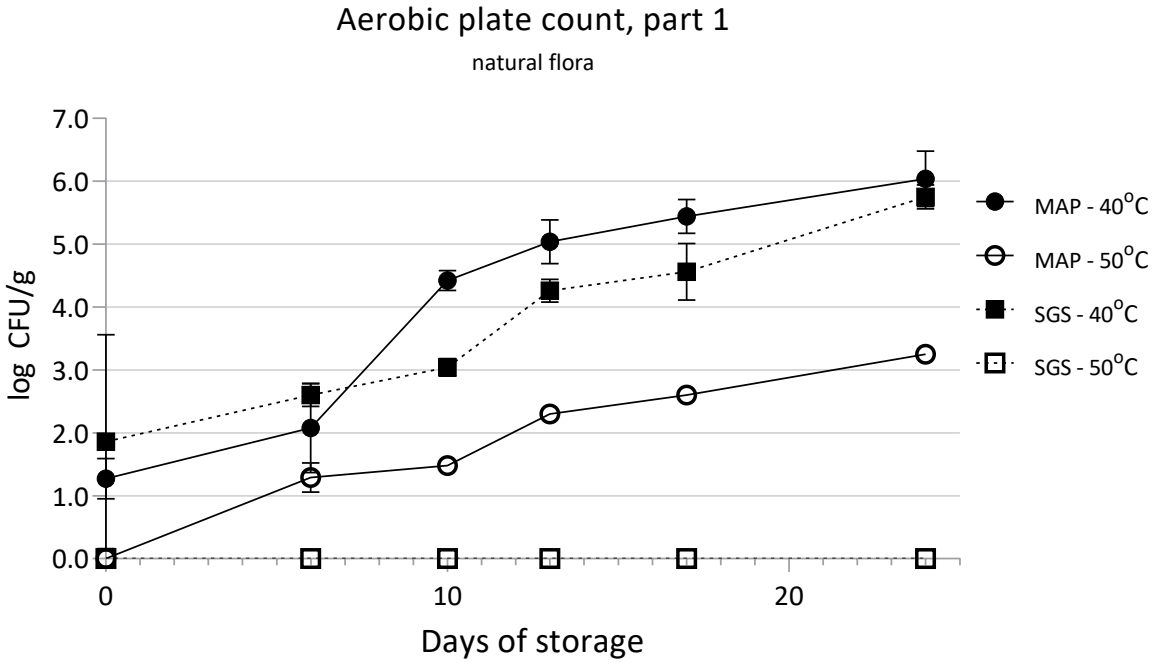

Figure 3. 2 Aerobic growth of natural flora in part 1 of the study, separated by processing temperature target and packaging technology. No growth was detected in any samples across the study at 60˚C and has thus been left out. Error bars = mean ± 1 standard deviation.

The effect of heat treatment was still significant in APC in part 2 of the study ($p<0.001$), but the final bacterial loads were higher in both the 40°C and 50°C heat treatments when compared to part 1 (figure 3.3). Final bacterial loads for the 50°C samples of all three treatments were comparable to the samples subjected to 40°C heat treatment in part 1 of the study, while 40°C samples in part 2 of the study reached final loads of 10^8 CFU/g. The International Commission on Microbiological Specifications for Foods (ICMSF, 1986) suggests a sampling plan for raw and ready-to-eat fish with a critical limit of 10^7 CFU/g, so samples from (and including) day 17 should be considered unacceptable, packaging and brine composition (at 40°C).

While this decrease in microbial stability may be correlated to differences in raw materials, it is well established that processing steps like brine injection and mechanical tenderization reduces microbial stability (Gill et al., 2005a, 2005b). The injection of needles can contaminate the otherwise sterile interior of animal flesh with microorganisms that have colonised the flesh surface, as well as microorganisms that have colonised the machinery. The heating-process in this study employs a treatment temperature of 5°C above the targeted core temperature, resulting in a heat gradient with a higher attained temperature at the surface

of the product (Baldwin, 2012). The core of the product, which was likely to have harboured microorganisms after the injection process, will have received a milder heat treatment than the bacterial communities on the surface, explaining the higher growth.

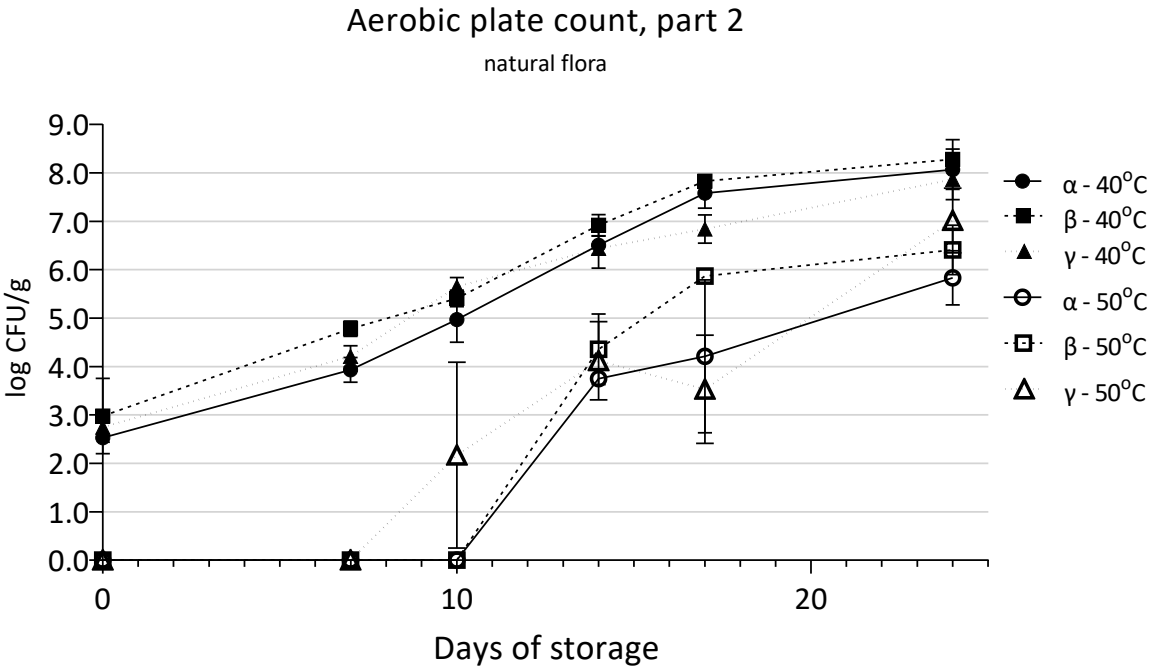

Figure 3. 3 Aerobic growth of natural flora in part 2 of the study, separated by processing temperature target and treatment. Error bars = mean ± 1 standard deviation. α= carbonated 10% NaCl (w/v) brine + MAP, β= 10% NaCl (w/v) brine + MAP, γ= 10% NaCl (w/v) brine + SGS

3.2 Lactic Acid Bacteria

The analysis of LAB in part 1 of the study showed no detectable growth for the duration of storage for the samples heated to 50°C (figure 3.4). This might have been a result of the significantly lower initial load of LAB for part 1.1 (1.04 log CFU/g) compared to part 1.2 (1.99±0.14 log CFU/g) ($p<0.001$), or may have been a result of lower heat resistance in the types of LAB present, compared to APC.

The effect of packaging on LAB growth was not statistically significant in part 1 ($p=0.324$). The author assumes that this is an issue with the statistical methodology employed, with the identical means of the 50°C samples affecting the model. As with APC, SGS treated samples have significantly better microbial quality day 0 throughout day 17 ($p<0.001$) but attains similar microbial loads by end of storage. The final LAB counts are in both cases $<10^6$

CFU/g; microbial spoilage due to LAB is associated with numbers in the 10^7-10^9 CFU/g interval (Gram and Huss, 1996).

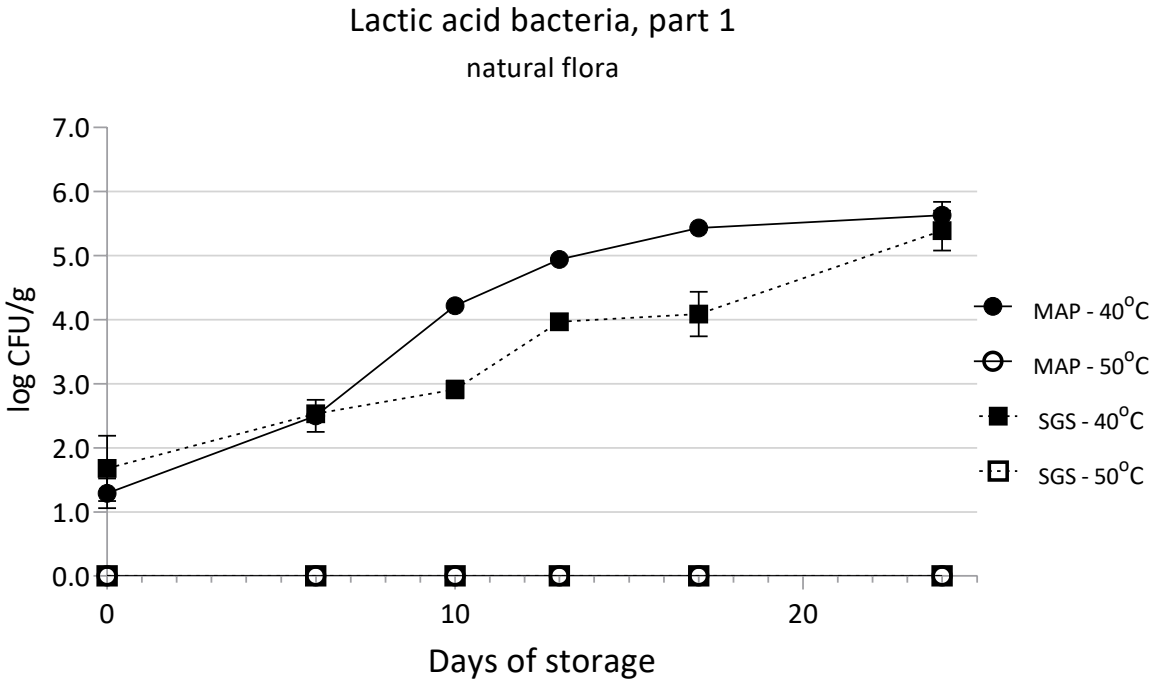

Figure 3. 4 Growth of lactic acid bacteria in natural flora samples in part 1 of the study, separated by processing temperature target and packaging technology. No growth was detected in any samples across the study at 60°C and has thus been left out. Error bars = mean ± 1 standard deviation.

As with APC, the growth of LAB in part 2 was higher than in part 1 (figure 3.5). There was no significant effect of treatment (p=0.177), and once again there was significant growth at the samples that were heated to a core temperature of 50°C. Growth of LAB in the SGS treated 50°C samples were detectable already at day 7. This may be attributed to the ballooning effect of CO_2-dissapation during heat treatment. Lactic acid bacteria reached concentrations of 10^7-10^8 CFU/g in 40°C samples by the end of storage, which are bacterial loads which has been associated with LAB spoilage in cold-smoked salmon (Gram and Huss, 1996).

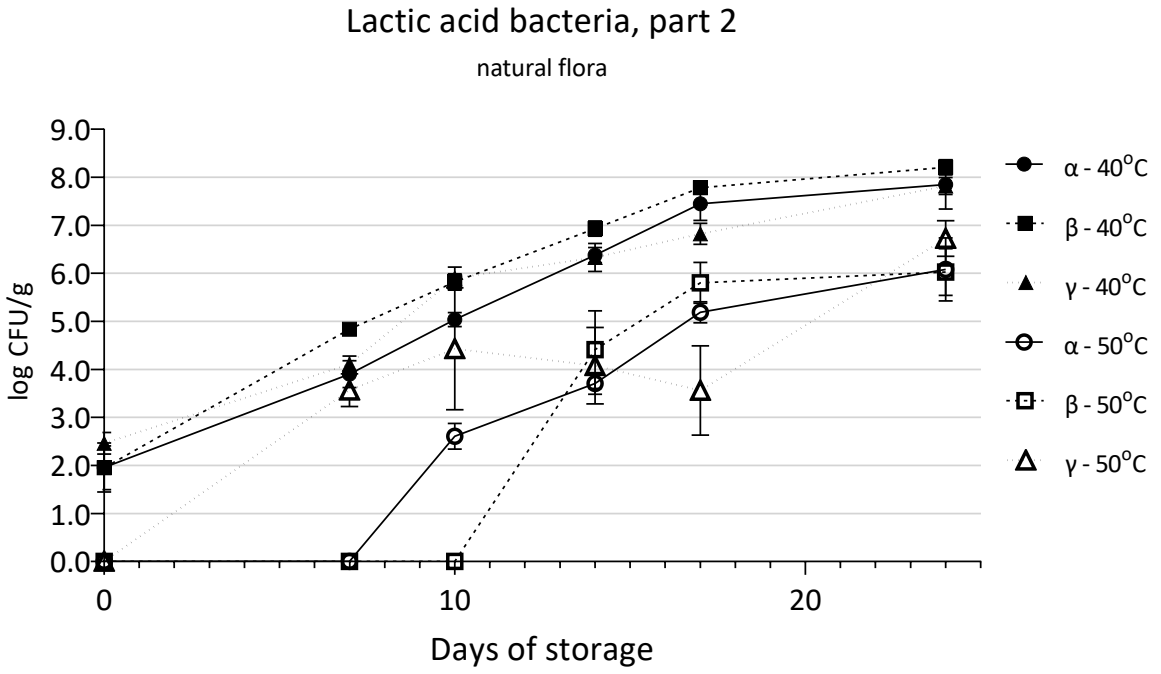

Figure 3. 5 Growth of lactic acid bacteria in natural flora samples in part 2 of the study, separated by processing temperature target and treatment. Error bars = mean ± 1 standard deviation. α= carbonated 10% NaCl (w/v) brine + MAP, β= 10% NaCl (w/v) brine + MAP, γ= 10% NaCl (w/v) brine + SGS

3.3 Hydrogen sulphide producing bacteria

No growth of HSPB was detected in any sample of part 1 which had received heat treatment, despite being shown to be present in the raw materials (part 1.2), or in negative samples (part 1.1). This suggests a higher heat sensitivity in these bacteria compared to those enumerated as APC or LAB. Absence of this class of bacteria in the spoilage microbiota are considered as beneficial because of the particularly foul brand of spoilage they may induce (Boziaris and Parlapani, 2017; Dalgaard, 1995).

As a result of the milder heat treatment of the flesh interior, HSPB was able to survive in the 40°C samples of part 2, proliferating to final concentrations >10^7 CFU/g in treatments α and β by the end of storage (figure 3.6). H_2S-producing bacteria in chilled storage fish are commonly found to be *S. putrefaciens*, a notable producer of TMA and volatile sulphur compounds (Dalgaard, 1995). *Shewanella putrefaciens* is found in concentrations of 10^8-10^9 CFU/g when spoilage occurs. The organism is not commonly found as an SSO in products packed in high CO_2-atmosphere, and the utilisation of SGS had a significant inhibitive effect on its growth (p>0,001). The HSPB were the only analysis in part 2 to be below threshold

level for the duration of storage after receiving 50°C heat treatment, and suffered a higher degree of inactivation at 40°C relative to APC and LAB, as can be seen in figure 3.7.

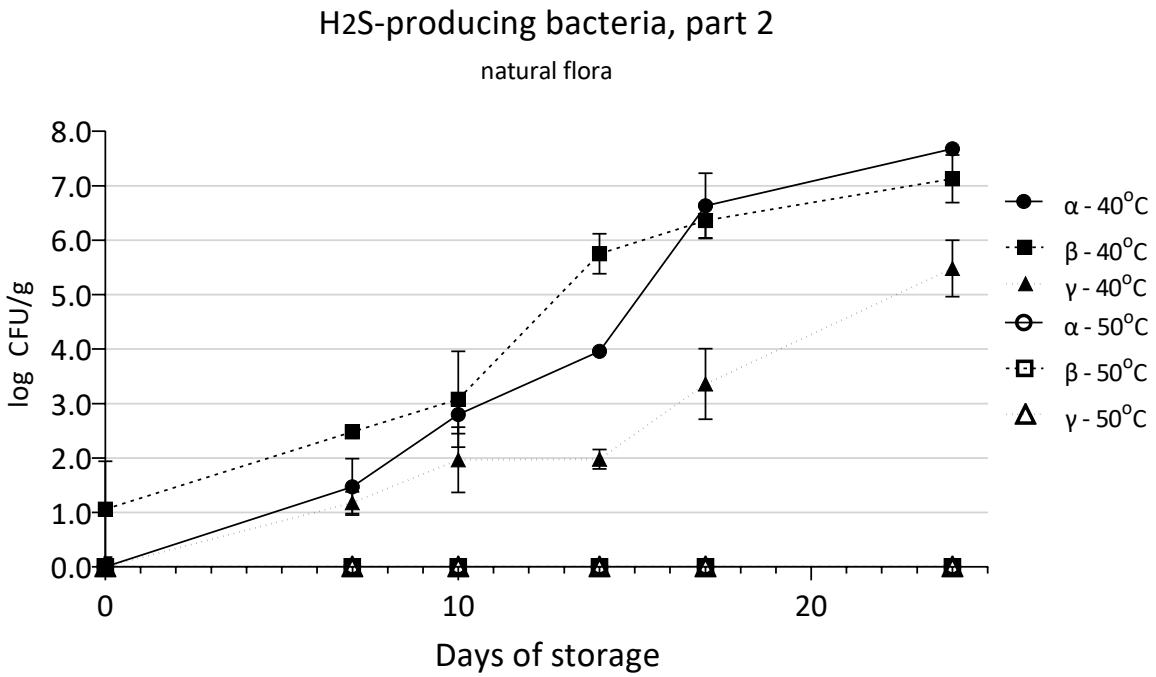

Figure 3. 6 Growth of hydrogen sulphide producing bacteria in natural flora samples in part 2 of the study, separated by processing temperature target and treatment. Error bars = mean ± 1 standard deviation. α= carbonated 10% NaCl (w/v) brine + MAP, β= 10% NaCl (w/v) brine + MAP, γ= 10% NaCl (w/v) brine + SGS.

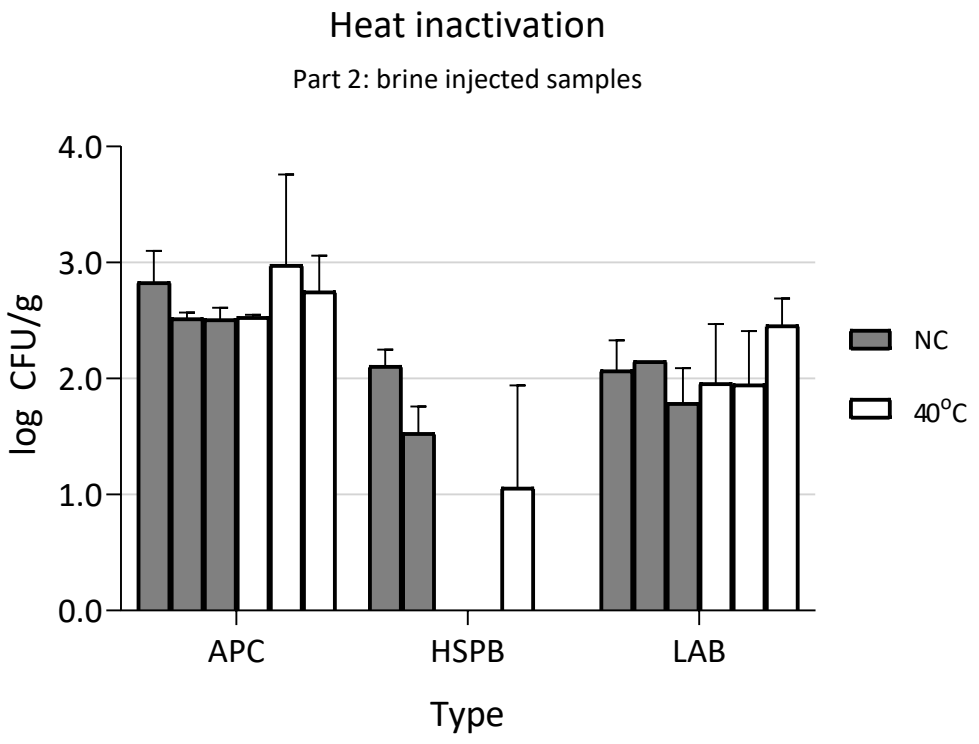

Figure 3. 7 Bacterial load of day 0 samples from part 2 to illustrate effect of heat treatment to 40˚C core temperature, separated by negative control samples and processing temperature target. Error bars = mean + 1 standard deviation. APC = aerobic plate count, HSPB = hydrogen sulphide producing bacteria, LAB = lactic acid bacteria, NC= negative control.

3.4 *Listeria*

The application of SGS resulted in a significant bacteriostatic effect throughout the storage period in *L. innocua* inoculated samples, when compared to MAP ($p<0.001$) (figure 3.8). There is however no discernible synergistic effect of SGS upon heat inactivation, as was observed in APC for part 1, but rather the opposite if one is to view the results in a vacuum. This is attributed to a particularly notable expression of the previously discussed ballooning of the vacuum pouches. While the insulation-effect of gas diffusion into the vacuum pouch led to a milder heat process and lower heat-inactivation, it did appear to cause sufficient stress in combination with SGS to elongate the lag phase of *L. innocua* until after the end of storage. The retarding effect of SGS on growth is also evident in the 40°C samples, attaining a final bacterial load similar to the 50°C samples stored in MAP at approximately 10^4 CFU/g.

The observed bacteriostatic effect of SGS combined with other preservative hurdles on *L. innocua* correlates with findings in other studies (Abel et al., 2019a; Rode et al., 2015). If the

assumption that *L. innocua* served as a conservative analogue for *L. monocytogenes* in this study was correct, SGS combined with the 50°C heat treatment may produce a microbially safe ready-to-eat product with a long shelf life. This would first require optimisation of the heating-process so that it accounts for the ballooning effect caused by CO_2-dissapation. Other methods for adding salt should also be explored. *Listeria* spp. are not noted to be less heat resistant than NBF-organisms (Abel et al., 2019a; Lorentzen et al., 2010; Ryder, 2014b), but rather the opposite. The identified trend of significantly higher bacterial loads and lower effect of heat treatment in part 2 would likely be observed in *L. innocua* too, if inoculated samples were included, potentially resulting in an unsafe product. Salt plays an important role in the flavour profile of seafoods, and consumers are more likely to find product bland, if salt is absent (Lynum, 2011; Pedro and Nunes, 2007). Less invasive salting methods than brine injection may be used, such as addition of salt to the vacuum pouch during cooking or brine margination (Lynum, 2011). The option of making a low-salt product may also be explored, as there is a demand for such products by the increasingly health-aware consumers (Kearney, 2010; Pedro and Nunes, 2007)

Variability between samples in part 1.1 and part 1.2 was assumed to be low, as there were no significant differences between the inoculums ($p=0.656$) and start-samples ($p=0.989$). Still, interaction-effects and competition with other bacteria cannot be ruled out to have an effect, as the compositions of raw materials differed (figure 3.1)

Natural samples were also tested for *Listeria* spp. at day 0 and day 24, but no growth was detected in any NBF-samples.

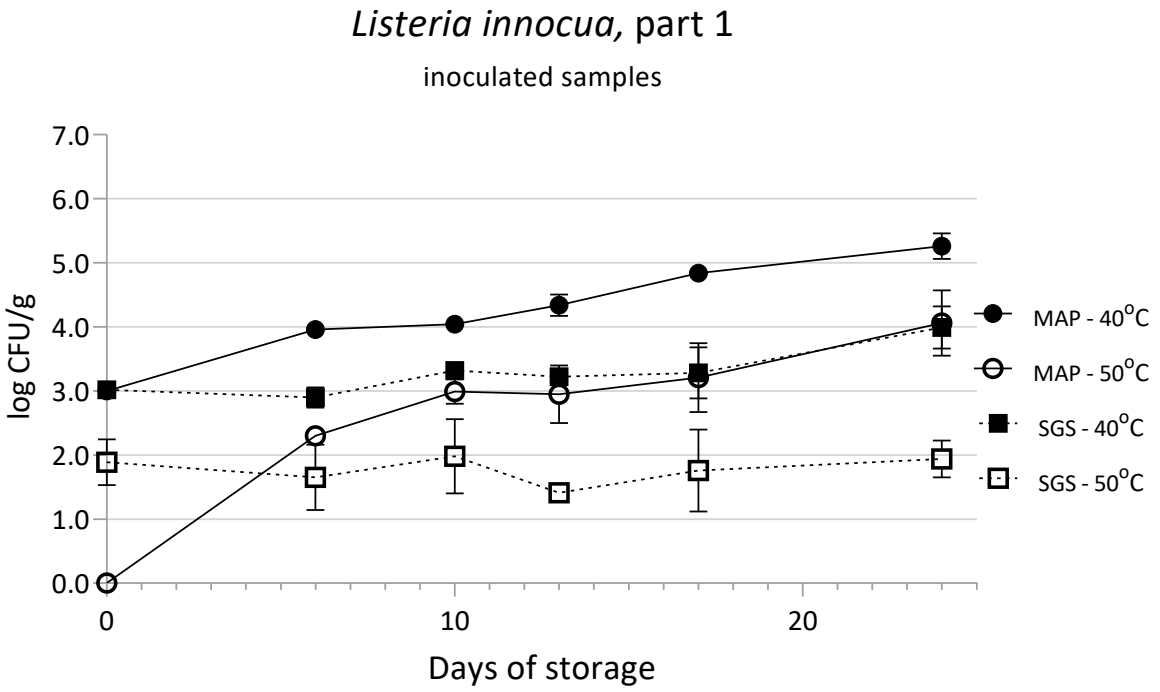

Figure 3. 8 Growth of *Listeria innocua* on inoculated samples in part 1 of the study, separated by processing temperature target and packaging technology. No growth was detected in any samples across the study at 60˚C and has thus been left out. Error bars = mean ± 1 standard deviation.

3.5 *Clostridium* species

No growth of hydrogen sulphide reducing *Clostridium* spp. spores were detected at day 0 and day 24 in any samples. As the pathogenic *C. perfringens* is unable to grow notably under chilled storage, it would have to proliferate to minimal infective doses during heat treatment. *Clostridium perfringens* have indeed been shown to exhibit fast growth, with generation times <10 min (Labbé et al., 2014), but it is unlikely to reach virulent concentrations in the short time-span of a reasonable sous-vide heating-process for fish products.

Non-proteolytic *C. botulinum* is a notable pathogen which were not enumerated in this study. While the severity of the disease and its ability to survive preservative hurdles makes it a threat in MAP products (Ryder, 2014c), it has been shown that it does not produce significant amounts of toxins in similar products within the storage period of this study (Huss and Gram, 2003). As Leistner (2000) says that toxin formation in bacteria may be initiated earlier than expected due to stressors such as temperature abuse, more focus on *C. botulinum* in further studies may be prudent.

3.6 Further development

As mentioned in section 3.4, the 50°C heating-process should be subject to further optimisation to counteract the effects of CO_2-dissapation. In combination with SGS, heating to 50°C core temperature is likely to produce a highly microbially stable product. By performing a storage challenge study with strains of *L. monocytogenes*, a conclusive determination upon the safety of the product could be made. Favourable results may allow the product to be subject to less stringent criteria if the experimental design is thorough, and may make the product suitable for special medical purposes (European Commission, 2005). As the product is subjected to temperature abuse that is insufficient to inactivate *Clostridium* spp. spores (Huss and Gram, 2003), inoculation with non-proteolytic *C. botulinum* types should be included in such a storage challenge study.

A determination of how to include salt in the product can be made by sensory analysis of a product subjected to various salting methods (brine margination, dry salting, inclusion in vacuum pouch, massaging with salt (Lynum, 2011)), as there is a need to replace brine injection to improve microbial stability.

3.8 Concluding remarks

The present study showed that SGS could be successfully implemented in a simple multivariate preservative system, providing a high microbial stability despite a lack of optimisation in the heat-processing step. Importantly, the growth of *L. innocua* was fully inhibited by a mild heat treatment when combined with SGS. A collaborating researcher showed that this increase in microbial stability came at no cost to chemical quality parameters (WHC, drip loss, surface colour, and texture). To approximate a product that consumers find attractive, salt was added by brine injection. The well-known contamination spreading effect of such a processing step produced a product of far lesser microbial quality. With further refinement of the heating-process and salt inclusion, a convenient and safe ready-to-eat Atlantic salmon product may be produced with target core temperatures as low as 40-50°C. The year-round availability of Atlantic salmon and its image as a healthy food by consumers should make this an attractive prospect for food producers and consumers alike.

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Appendices

Appendix A: pre-trial to determine heating-process

Appendix A contains the description of the pre-trials that were carried out to determine the heating-process that was used in the study. The document is translated as is from Norwegian.

Three pre-trials were performed in advance of the associated study:

1. Establishing times for heating-process
2. The effect of heat treatment on microbial growth
3. Establishment between OD_{600} (optic density at wavelength = 600 nm) and microbial growth

1. Establishing times for heating-process

Samples of 80-85g (equivalent to 1/3 DF) was prepared to be approximately uniform and packed in vacuum pouches. Core- and water bath -temperature was logged with a temperature-logger throughout the trial. The three different water bath temperatures was 45, 55, and 65°C. The time it took the samples to reach core temperatures of respectively 40, 50, and 60°C was measured in consecutive rounds.

The results were as follows:

Sample #	Heating-process time		Mean time
1	15:28	40°C	15:10
2	14:43		
3	15:20		
5	16:18	50°C	17:00
6	16:12		
7	19:0		
Extra	18:23	50°C extra	17:43
Extra	18:43		
Extra	17:34		
Extra	17:52		
8	20:34	60°C	21:01
9	21:20		
10	21:10		

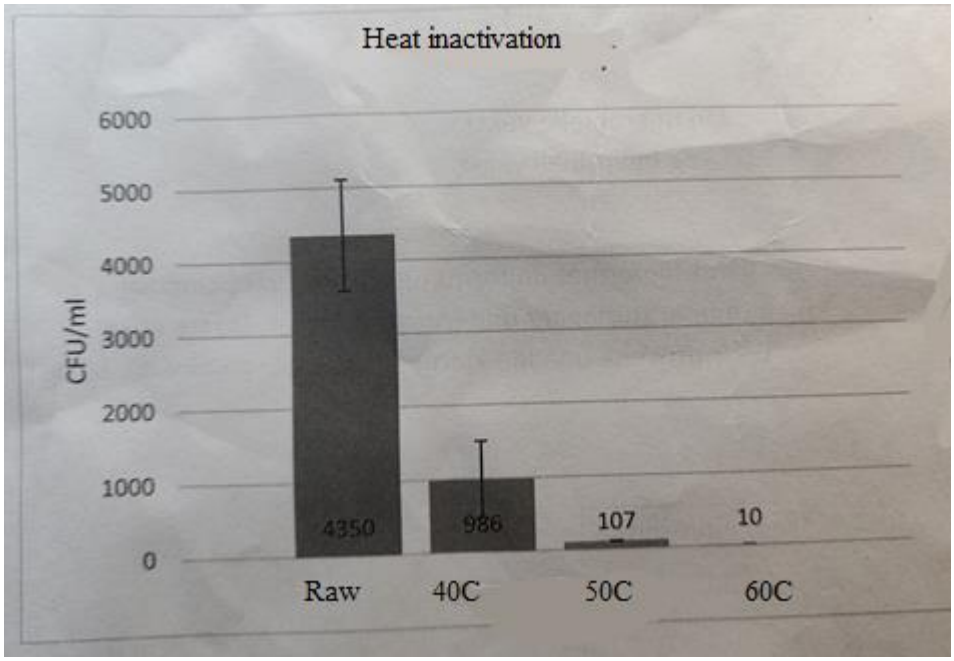
Sample #4 was removed due to error during heating-processing

Samples #5-7 showed great variations, and new samples were made and tested at 50°C. Based on the results, the following treatment times were determined

Core temperature	Heat treatment time
40°C	15
50°C	18
60°C	21

2 Heat inactivation

Samples of raw fish as well as heat treated salmon was analysed for aerobic plate count. The results are as follows



3 OD to CFU

Omitted

Appendix B: Input for statistical calculation

Appendix B contains the means, standard deviations (SD), and number of samples used (N) for statistical calculations throughout part 1 and part 2 of the study. All microbial analysis (sections B.1, B.2, B.3) values are given as log-transformed CFU/g.

MAP = modified atmosphere packaging

SGS = soluble gas stabilisation

APC = aerobic plate count

LAB = lactic acid bacteria

HSPB = hydrogen sulphide producing bacteria

α = carbonated 10% NaCl (w/v) brine + MAP,

β = 10% NaCl (w/v) brine + MAP,

γ = 10% NaCl (w/v) brine + SGS

B.1 Microbiological analysis, part 1

Table B 1 Shows values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		APC part 1		
Day	ID	mean (log)	SD (log)	N
0	MAP40	1.27	0.32	2
0	MAP50	0.00	0.00	3
0	SGS40	1.86	1.70	4
0	SGS50	0.00	0.00	4
6	MAP40	2.08	0.71	4
6	MAP50	1.29	0.23	3
6	SGS40	2.60	0.18	4
6	SGS50	0.00	0.00	4
10	MAP40	4.42	0.16	4
10	MAP50	1.48	0.12	4
10	SGS40	3.04	0.14	4
10	SGS50	0.00	0.00	4
13	MAP40	5.04	0.35	4
13	MAP50	2.30	0.05	4
13	SGS40	4.26	0.18	4
13	SGS50	0.00	0.00	4
17	MAP40	5.44	0.27	4
17	MAP50	2.60	0.09	4
17	SGS40	4.56	0.45	4
17	SGS50	0.00	0.00	2
24	MAP40	6.04	0.44	3
24	MAP50	3.25	0.08	4
24	SGS40	5.75	0.19	3
24	SGS50	0.00	0.00	1

Table B 2 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		APC negative samples part 1		
Day	ID	mean (log)	SD (log)	N
0	MAP NC	2.55	0.04	3
0	SGS NC	2.96	0.35	4
6	MAP NC	2.71	0.18	2
6	SGS NC	3.50	0.20	3
10	MAP NC	3.71	0.29	4
10	SGS NC	4.18	0.26	3
13	MAP NC	4.48	0.29	4
13	SGS NC	4.29	0.14	3
17	MAP NC	4.90	0.16	4
17	SGS NC	6.03	0.29	3
24	MAP NC	5.82	0.58	4
24	SGS NC	7.11	0.31	3

Table B 3 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		LAB part 1		
Day	ID	mean (log)	SD (log)	N
0	MAP40	1.29	0.23	3
0	MAP50	0.00	0.00	4
0	SGS40	1.68	0.51	2
0	SGS50	0.00	0.00	4
6	MAP40	2.50	0.25	3
6	MAP50	0.00	0.00	4
6	SGS40	2.53	0.04	2
6	SGS50	0.00	0.00	4
10	MAP40	4.22	0.07	3
10	MAP50	0.00	0.00	4
10	SGS40	2.91	0.14	4
10	SGS50	0.00	0.00	4
13	MAP40	4.94	0.05	3
13	MAP50	0.00	0.00	4
13	SGS40	3.97	0.07	4
13	SGS50	0.00	0.00	3
17	MAP40	5.43	0.03	3
17	MAP50	0.00	0.00	4
17	SGS40	4.09	0.35	4
17	SGS50	0.00	0.00	4
24	MAP40	5.63	0.21	3
24	MAP50	0.00	0.00	4
24	SGS40	5.39	0.31	3
24	SGS50	0.00	0.00	2

Table B 4 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		LAB negative samples part 1		
Day	ID	mean (log)	SD (log)	N
0	MAP NC	1.04	0.00	2
0	SGS NC	2.11	0.14	4
6	MAP NC	2.55	0.33	3
6	SGS NC	3.15	0.09	2
10	MAP NC	3.73	0.34	4
10	SGS NC	4.00	0.62	3
13	MAP NC	4.20	0.04	4
13	SGS NC	3.80	0.43	3
17	MAP NC	4.57	0.28	4
17	SGS NC	5.06	0.22	2
24	MAP NC	5.92	0.64	4
24	SGS NC	6.10	0.40	3

Table B 5 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		Listeria spp.part 1, *L. innocua* inoculated samples		
Day	ID	mean (log)	SD (log)	N
0	MAP40	3.00	0.00	2
0	MAP50	0.00	0.00	4
0	SGS40	3.02	0.07	4
0	SGS50	1.89	0.36	4
6	MAP40	3.96	0.04	4
6	MAP50	2.30	0.00	2
6	SGS40	2.90	0.16	3
6	SGS50	1.65	0.51	4
10	MAP40	4.04	0.08	4
10	MAP50	2.99	0.19	4
10	SGS40	3.32	0.12	4
10	SGS50	1.98	0.58	4
13	MAP40	4.34	0.17	3
13	MAP50	2.95	0.45	4
13	SGS40	3.22	0.06	2
13	SGS50	1.41	0.10	4
17	MAP40	4.84	0.06	4
17	MAP50	3.21	0.54	4
17	SGS40	3.28	0.40	4
17	SGS50	1.76	0.64	3
24	MAP40	5.26	0.20	4
24	MAP50	4.06	0.51	3
24	SGS40	3.99	0.33	4
24	SGS50	1.94	0.29	4

Table B 6 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		HSPB negative samples part 1		
Day	ID	mean (log)	SD (log)	N
0	MAP NC	0.00	0.00	4
0	SGS NC	0.00	0.00	3
6	MAP NC	1.32	0.00	2
6	SGS NC	1.04	0.00	2
10	MAP NC	1.69	0.69	3
10	SGS NC	1.83	0.18	2
13	MAP NC	2.26	0.12	2
13	SGS NC	1.75	0.35	3
17	MAP NC	2.68	0.04	2
17	SGS NC	2.95	0.20	2
24	MAP NC	2.64	0.29	2
24	SGS NC	4.20	0.87	2

B.2 Microbiological analysis, part 2

Table B 7 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		APC part 2		
Day	ID	mean (log)	SD (log)	N
0	α40	2.53	0.02	2
0	β40	2.98	0.78	2
0	γ40	2.75	0.31	4
0	α50	0.00	0.00	4
0	β50	0.00	0.00	4
0	γ50	0.00	0.00	4
7	α40	3.93	0.26	4
7	β40	4.78	0.16	4
7	γ40	4.21	0.22	4
7	α50	0.00	0.00	4
7	β50	0.00	0.00	4
7	γ50	0.00	0.00	4
10	α40	4.97	0.47	4
10	β40	5.41	0.17	4
10	γ40	5.65	0.19	3
10	α50	0.00	0.00	4
10	β50	0.00	0.00	4
10	γ50	2.17	1.92	3
14	α40	6.51	0.19	4
14	β40	6.92	0.22	4
14	γ40	6.44	0.41	4
14	α50	3.75	0.10	2
14	β50	4.36	0.73	3
14	γ50	4.12	0.81	4
17	α40	7.58	0.31	3
17	β40	7.83	0.10	4
17	γ40	6.84	0.29	4
17	α50	4.21	1.58	3
17	β50	5.87	0.01	3
17	γ50	3.53	1.12	3
24	α40	8.07	0.62	4
24	β40	8.28	0.21	4
24	γ40	7.87	0.21	4
24	α50	5.83	0.56	3
24	β50	6.41	0.51	2
24	γ50	7.019	0.667	4

Table B 8 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		APC negative samples part 2		
Day	ID	mean (log)	SD (log)	N
0	αNC	2.83	0.27	3
0	βNC	2.52	0.05	3
0	γNC	2.51	0.10	2

Table B 9 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		HSPB part 2		
Day	ID	mean (log)	SD (log)	N
0	α40	0.00	0.00	2
0	β40	1.06	0.88	4
0	γ40	0.00	0.00	3
0	α50	0.00	0.00	4
0	β50	0.00	0.00	4
0	γ50	0.00	0.00	4
7	α40	1.47	0.52	3
7	β40	2.48	0.09	2
7	γ40	1.18	0.20	2
7	α50	0.00	0.00	4
7	β50	0.00	0.00	4
7	γ50	0.00	0.00	4
10	α40	2.80	0.35	2
10	β40	3.08	0.88	4
10	γ40	1.97	0.60	4
10	α50	0.00	0.00	4
10	β50	0.00	0.00	4
10	γ50	0.00	0.00	3
14	α40	3.96	0.09	3
14	β40	5.75	0.37	4
14	γ40	1.98	0.18	3
14	α50	0.00	0.00	3
14	β50	0.00	0.00	4
14	γ50	0.00	0.00	4
17	α40	6.63	0.60	3
17	β40	6.36	0.32	4
17	γ40	3.36	0.65	4
17	α50	0.00	0.00	3
17	β50	0.00	0.00	4
17	γ50	0.00	0.00	4
24	α40	7.68	0.10	3
24	β40	7.13	0.44	4
24	γ40	5.48	0.52	4
24	α50	0.00	0.00	4
24	β50	0.00	0.00	4
24	γ50	0.00	0.00	4

Table B 10 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		HSPB negative samples part 2		
Day	ID	mean (log)	SD (log)	N
0	αNC	2.11	0.14	2
0	βNC	1.53	0.23	3
0	γNC	0.00	0.00	2

Table B 11 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		LAB part 2		
Day	ID	mean (log)	SD (log)	N
0	α40	1.96	0.51	3
0	β40	1.95	0.46	4
0	γ40	2.46	0.23	2
0	α50	0.00	0.00	4
0	β50	0.00	0.00	4
0	γ50	0.00	0.00	3
7	α40	3.90	0.28	4
7	β40	4.84	0.14	4
7	γ40	4.11	0.17	4
7	α50	0.00	0.00	3
7	β50	0.00	0.00	4
7	γ50	3.57	0.34	2
10	α40	5.04	0.15	3
10	β40	5.82	0.17	4
10	γ40	5.90	0.23	4
10	α50	2.61	0.27	2
10	β50	0.00	0.00	3
10	γ50	4.43	1.27	2
14	α40	6.38	0.16	4
14	β40	6.93	0.16	4
14	γ40	6.33	0.29	4
14	α50	3.71	0.23	2
14	β50	4.41	0.81	3
14	γ50	4.08	0.80	4
17	α40	7.45	0.35	3
17	β40	7.78	0.12	4
17	γ40	6.82	0.22	4
17	α50	5.19	0.22	2
17	β50	5.80	0.43	4
17	γ50	3.56	0.93	3
24	α40	7.85	0.51	4
24	β40	8.21	0.16	4
24	γ40	7.82	0.18	4
24	α50	6.08	0.66	4
24	β50	6.03	0.49	2
24	γ50	6.725	0.373	3

Table B 12 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		LAB negative samples part 2		
Day	ID	mean (log)	SD (log)	N
0	αNC	2.07	0.26	3
0	βNC	2.15	0.00	2
0	γNC	1.79	0.30	3

B.3 Microbiological analysis of raw materials, inoculum and start samples, part 1 and 2

Table B 13 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		raw part 1 and 2		
analysis	ID	mean (log)	SD (log)	N
APC	MAP	2.55	0.04	3
HSPB	MAP	0.00	0.00	4
LAB	MAP	1.04	0.00	2
APC	SGS	3.23	0.24	4
HSPB	SGS	1.04	0.00	3
LAB	SGS	1.99	0.14	4
APC	brine	3.31	0.06	4
HSPB	brine	1.72	0.18	4
LAB	brine	2.44	0.14	4

Table B 14 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		start part 1		
Media	ID	mean (log)	SD (log)	N
APC	MAP	3.69	0.27	4
APC	SGS	3.69	0.12	2
Listeria spp.	MAP	-	-	-
Listeria spp.	SGS	3.47	0.21	3

Table B 15 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

		inoculum part 1		
Media	ID	mean (log)	SD (log)	N
APC	MAP	5.34	0.03	4
APC	SGS	5.45	0.83	4
Listeria spp.	MAP	5.42	0.13	4
Listeria spp.	SGS	5.46	0.08	4

B.4 CO_2 concentration in package headspace, part 1 and part 2

All values in section B.4 is given as CO_2 % of total headspace gas composition.

Table B 16 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

Brine	Mean	SD	N
brine	47.283	6.851	78
c brine	41.555	1.212	40
no brine	49.596	6.109	179

Table B 17 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

Core temp	Mean	SD	N
40	48.088	6.47	131
50	47.324	6.277	136
NC	49.75	7.32	30

Table B 18 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

Packaging	Mean	SD	N
MAP	42.832	3.096	171
SGS	54.792	1.922	126

B.5 pH measurements of samples, part 2

All values in section B.5 are given as pH.

Table B 19 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

Treatment	Mean	SD	N
α	6.3009	0.065	45
β	6.3017	0.0565	46
γ	6.2626	0.074	46

Table B 20 Shows the values used for statistical calculations to determine significant differences. SD= standard deviation, N= number of samples

Core temp	Mean	SD	N
40	6.2651	0.0663	69
50	6.3119	0.0608	68