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THE EFFECTS OF STORAGE TEMPERATURE ON BIO-PRESERVATION OF LAB ON ATLANTIC SALMON

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Abstract

Fish is a nutritious, protein-rich food which is highly perishable and has a short shelf-life unless preservation methods are used. The common spoilage bacterial in fish are *Pseudomonas putida, P. fluorescens P. perolens, P. fragi*, *Shewanella putrefaciens, Brochothrix thermosphacta*, *Alteromonas nigrifaciens, Aeromonas salmonicida, A. hydrophila* and *Photobacterium* phosphorous. The most common pathogens associate to fish are *Listeria monocytogenes, Aeromonas spp., Salmonella spp., Staphylococcus aureus, Cl. perfringens, Escherichia coli, V. cholera* and *Vibrio parahaemolyticus.* In addition to microorganisms, enzyme activities in the food are also responsible for fish muscle softening. It is very important to develop methods that can help ensure safety and extend the shelf life of seafood. Bio-preservation by using lactic acid bacteria is a natural and powerful method used to extend the shelf life and improve the safety of foods. The efficiency using LAB bio-preservation is affected by physicochemical factors such as temperature, pH. The objective of this study is to investigate the effect of temperature on Bio-preservation of LAB on Atlantic salmon by including both effect on microbial growth and effect on enzymatic activity.

To conduct this study, *Carnobacterium ssp* (SF1994) Lactic Acid Bacterial strains were used. The salmon fillets were dipped into the LAB strain solutions and the control was dipped in sterile distilled water. These samples were packed in plastic bags and stored at three different storage temperatures (0 °C, 4 °C, 8 °C). For analysis of effect on microbial growth, the growth rate of spoilage microorganisms and the LAB growth rates were measured for the three different storage temperatures during 7 storage days. Furthermore, for enzyme activity analysis, the activity of cathepsin B was measured during the storage days for the three temperatures.

The result indicated that storage temperature significantly affected the efficiency of LAB to inhibit spoilage microorganisms. The LAB strain showed the highest inhibition efficiency at lowest storage temperature (0 °C) and the lowest inhibition efficiency at highest storage temperature (8 °C). The lower storage temperature increased the susceptibility of spoilage microbes to *carnobacterium* bacteriocin. Furthermore, the enzyme activity study revealed 8°C storage temperature measured the highest cathepsin B activity, the highest protein degradation, high amount of free amino acid and acid soluble peptides.

Key words: Bio-preservation, LAB, *carnobacterium,* temperature, spoilage microorganisms, cathepsin B activities, Atlantic salmon.

Table of Contents

Abstract i
List of tables iv
List of Figure v
List of Appendix vi
List of Abbreviations vii
1. Introduction 1
1.1 Bio-preservation with Lactic acid bacteria (LAB) 1
1.2 Factors that affect the effectiveness of Bio-preservation 3
1.3 Study questions 4
2. Literature review 5
2.1 Food spoilage 5
2.1.1 Bio-preservation of food with Lactic acid bacteria (LAB) 6
2.1.2 Carnobacterium 8
2.1.3 The effects of temperature on bio-preservation of LAB 9
2.2. Study on enzyme activities 10
2.2.1 Protein in fish 10
2.2.2 Proteolytic enzyme 11
2.2.3 Cathepsin 12
3. Material and methods 13
3.1 Lactic acid bacterial strains and culture condition 13
3.2 The effect of Temperature on the growth of Lactic acid bacterial 14
3.3 Microbial analysis 14
3.4 Sensory analysis 15
3.5 Enzyme analysis 15
3.5.1 Extraction of proteolytic enzymes 15
3.5.2 Protein content of the extract 15
3.5.3 Activity of specific proteolytic enzyme 15
3.5.4 Amount of acid soluble peptides 16
3.5.5 Determination of amount of free amino acids 16
4. Result & discussion 17

4.1 Lactic acid bacterial strains and culture condition 17
4.2 The effect of Temperature on the growth of *Carnobacterium* 18
4.3 Microbial analysis 19
4.3.1 Growth of LAB on fish determined on NAP agar 19
4.3.2 The effect of LAB on the growth of spoilage microorganisms 21
4.3.3 The effect of LAB on the growth of sulphide reducing microorganisms 24
4.4 Sensory analysis 27
4.5 Enzyme analysis 29
4.5.1 Protein content of the extract 29
4.5.2 Acid soluble peptides 31
4.5.3 Determination of amount of free amino acids. 33
4.5.4 Proteolytic activity 35
5. Limitation of the study 39
6. Relevance of the study 39
7. Conclusion 40
8. Recommendation 41
9. Acknowledgement 42
10. Reference 43
11. Appendix 51

List of tables

Table 1 Study questions, hypothesis and mechanisms. 4
Table 2 Characterized of common bacteriocins from lactic acid bacteria 7
Table 3 Sensory evaluation performed by the author during each experimental day. 28

List of Figure

Figure 1 Storage treatments that used during the experiment... 14
Figure 2 Turbidity of LAB culture (SF1994) as a function of time.. 17
Figure 3 Turbidity of LAB culture (SF1994) as a function of CFU/mL 17
Figure 4 The effect of temperature on the growth of LAB... 18
Figure 5 Growth of LAB as a function of storage time... 20
Figure 6 Growth of LAB from different storage temperatures as a function of storage time… .. 21
Figure 7 Growth of spoilage bacteria as a function of storage time. 22
Figure 8 Growth of spoilage bacteria from different storage temperature as a function of storage time.. 24
Figure 9 Growth of spoilage bacteria after 5 days of storage from different storage temperature 24
Figure 10 Total count of sulphide reducing bacteria after 7 days of storage from different storage temperature.. 25
Figure 11 Growth of sulphide reducing bacteria after 7 days of storage from different storage temperature. 26
Figure 12 Amount of water soluble protein from different storage temperature as a function of storage time. 30
Figure 13 Change in water soluble proteins for the LAB treatments 31
Figure 14 Amount of acid soluble peptides as a function of storage time.................................. 32
Figure 15 Change in amount of acid soluble peptides for the LAB treatments........................... 33
Figure 16 Amount of free amino acids as a function of storage time... 34
Figure 17 Change in amount of free amino acids for the LAB treatments.................................. 35
Figure 18 Cathepsin B+ activity as a function of storage time... 37
Figure 19 Cathepsin B+ activity of for the LAB treatments from different storage temperature as a function of storage time. 38

List of Appendix

Appendix 1 Composition and preparation of different growth media. 51
Appendix 2 Standard Curve for triplet of the samples. Procedure followed by Lowry method with BSA as stock protein to build standard curve. 52
Appendix 3 Statistical analysis output for acid soluble peptides between SF1994 8°C and SF1994 0°C 53
Appendix 4 Statistical analysis output for acid soluble peptides between SF1994 8°C and SF1994 4°C 53
Appendix 5 Statistical analysis output for cathepsin B+ activity between SF1994 8°C and SF1994 0°C 54
Appendix 6 Statistical analysis output for cathepsin B+ activity between SF1994 0°C and SF1994 8°C 55

List of Abbreviations

- AD = after dipping
- ANOVA= analysis of variance
- BD = before dipping
- CFU = colony forming unit
- FAA = free amino acid
- LAB= lactic acid bacteria
- SSOs = specific spoilag**e** organisms
- TCA = Trichloroacetic acid
- BSA= bovine serum albumin
- HPLC = high pressure liquid chromatography

1. Introduction

Consumption of fatty fish including salmonid species has many health benefits. Uauy et al., (2001) indicated that due to high content of EPA and DHA fatty acid in fatty fish, they play an important role in controlling brain development, prevent against cardiovascular diseases, inflammatory conditions, mental disorders, arthritis and asthma.

However, due to its high moisture content, high nutritional values, high proportions of free amino acids, low in less digestible connective tissue, and neutral pH, seafood is easily perishable and easily contaminated with spoilage bacteria and food-borne pathogens. This can cause disease outbreaks that can have a potential negative impact on human health (Cruz et al., 2015; Mizan et al., 2015). Pilet and Leroi, (2011) reported that 10–20% of food-borne diseases are due to the consumption of fish. Moreover, Ghanbari et al., (2013) indicated that the common bacterial hazards in fish and seafood products are *Listeria monocytogenes, Aeromonas spp., Salmonella spp., Staphylococcus aureus, Cl. perfringens, Escherichia coli, V. cholera* and *Vibrio parahaemolyticus.* Furthermore, spoilage microorganisms have a negative effect on the shelf life of food and are responsible for the loss of 25% of post-harvested food (Gram and Dalgaard, 2002). In addition to microorganisms, enzyme activities in the food are also responsible for fish muscle softening. Therefore, it is very important to develop methods that can help ensure safety and extend the shelf life of seafood (Fall et al., 2012; Ghanbari et al., 2013). In recent years, consumers have shown increasingly interest for food with reduced amount of chemical preservatives (Daeschel, 1993). Consequently, biopreservation as an alternative to chemical preservatives has been increasingly investigated (Ghanbari et al., 2013).

1.1 Bio-preservation with Lactic acid bacteria (LAB)

Biopreservation of food involves the use of natural microbiota and their antimicrobial products to increase food shelf life and improve safety. This can be done by controlling the growth of spoilage microorganisms and by slowing down natural breakdown processes of the food by own enzyme activities.

Bio-preservation of food by controlling the growth of spoilage microorganisms is a natural and powerful tool used to reduce microbial risks, extend the shelf life and improve the safety of

foods by inhibiting the growth of spoilage and pathogenic microorganisms without negative effect on the quality of the product (Hwanhlem & Aran, 2015). This method is conducted by inoculation of food products with certain bacterial strains or their antimicrobial compounds to prevent the growth of unwanted microorganisms and to increase shelf life. Lactic acid bacteria are the main preferred candidates for this method (Sidira et al., 2014).

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming coccobacilli, cocci or rods shaped bacteria. They generally grow anaerobically but can also grow in the presence of oxygen as “aerotolerant anaerobes”. Most species of LAB lack catalase (Makarova et al., 2006), they are powerful competitors to pathogenic and spoilage microorganisms. Lactic acid bacteria produce different types of antimicrobial metabolites that have different mode of antagonistic action on pathogens such as diacetyl (interacts with arginine-binding), hydrogen peroxide (oxidizes basic proteins), carbon dioxide (reduces membrane permeability and inhibits decarboxylation), organic acids (lactic acid lowers the intracellular pH, penetrates membranes, interferes with metabolic processes such as oxidative phosphorylation), acetoin, reuterin, reutericyclin, antifungal peptides, and bacteriocins (affect membranes, protein synthesis and DNA-synthesis) (Ghanbari et al., 2013; Mandal et al., 2011). The common genera of LAB that is used for bio-preservation are *Carnobacterium, Lactobacillus*, *Lactococcus, Leuconostoc, Enterococcus, Vagococcus* (Gancel et al., 1997; Wiernasz, et al., 2017). Other genera are: *Leuconostoc*, *Lactosphaera*, *Oenococcus*, *Pediococcus*, *Bifidobacterium*, *Melissococcus*, *Weissella,* *Aerococcus*, *Streptococcus*, *Tetragenococcus, Propionibacterium, Microbacterium* (Carr et al., 2002).

In addition to the effect microbial spoilage, the low pH will also influence the enzyme in the fish muscle.Lactic acid bacteria produce lactic acid which leads to the reduction of the pH of a food (Helander et al., 1997). Low pH has a great effect on biochemical spoilage processes that occur by enzyme activities in the food. Enzyme have maximum activity at their optimum pH. The activity is slow below or above the optimum pH. Enzymes have active sites where the substrates bind. Changing the pH can change the shape of the active sites and the substrates no longer able to fit the active site, which prevent a reaction to occur. LAB can lower the pH and slow down the enzyme activities that are responsible for fish muscle softening during storage. Fish muscle softening or mushiness is considered as muscle protein degradation result of the proteolytic activity. The presence of active proteases in muscle makes it susceptible to degrade. According

to Yamashita et al., (1992) cathepsin B had been studied as a major cause of post-mortem muscle softening proteases. Furthermore, it has been indicated that post-mortem muscle softening caused by proteolytic degradation of muscle structural proteins is due to the hydrolysis of the major components of connective tissue and myofibril, such as myosin, connectin, α-actinin, collagen and troponins.

1.2 Factors that affect the effectiveness of Bio-preservation

The efficiency of bio-preservation is often affected by physicochemical factors such as temperature, pH, CO_2, aw, O_2, time of incubation, redox potential as well as food-related factors such as the food composition and structure, processing conditions: freezing and thawing, thermal treatments. (Gálvez et al., 2007). According to Delgado et al., (2005) bacteriocin production of LAB greatly depend on temperature, the author indicated a suboptimal temperature (22 °C) stimulated bacteriocin production.

Temperature is the most important factor that affects the efficiency of LAB bio-preservation ability. Even though there are many studies about bio-preservation in the previous time, there are limited publications on factors that affect bio-preservation ability of LAB. The general objective of this research is to see the effect of storage temperature on bio-preservation of LAB. Furthermore, the specific objectives are to investigate the effect of storage temperature on bio-preservation capability of LAB (*Carnobacterium ssp)* both by controlling growth of spoilage microorganisms and studying the effect of LAB on enzyme (Cathepsin) activities in Atlantic salmon muscle.

1.3 Study questions

Table 1. Study questions, hypothesis and mechanisms.

Study question 1	Hypothesis (H1)	Mechanism 1	Reference
How does Temperature affect the growth of LAB.	Increasing temperature up to a certain point increases the growth of LAB	LAB maximum specific growth rate increase with increasing temperature up to the certain temperature	(Pikuta et al., 2005; Qin, et al., 2012)
Study question 2	Hypothesis (H2)	Mechanism 2	Reference
How does Temperature affects bio-preservation ability of LAB.	Low temperature and LAB will decrease the growth of marine spoilage bacteria in salmon.	LAB can grow in various temperature range; they produce a wide range of antimicrobial metabolites and they are powerful competitors to spoilage microorganisms.	Ghanbari et al., 2013
Study question 3	Hypothesis (H3)	Mechanism 3	Reference
How do storage Temperature and LAB affect activity of enzyme in salmon filet	Low temperature and LAB slow down enzyme activity	Most biochemical processes are slowed down at a lower temperature	Ashie et al. 1996).

2. Literature review

2.1 Food spoilage

Food spoilage is a complex process that causes food to be unacceptable or undesirable for consumption due to alterations in sensory characteristics (visual, flavor, olfactory or tactile). Spoilage in fish can occur at any stage along the food chain. It may arise from microbial infections, physical damage or by indigenous enzyme activity in the muscle. The main cause of food spoilage is invasion by microorganisms such as bacteria, yeast and moulds. In addition, Enzymes can cause degradation of polymers in the foods. These facts make the fish to be easily perishable and have a short life (Rawat, 2015).

Different chemical reactions that cause sensory changes in fish are a result of microorganisms that use food as energy and carbon source. Some microorganisms present in many types of spoiled food while others are found in specific food types. Raw fish are initially contaminated with different type of microbes; however, only specific contaminants can successfully colonize the food, show higher growth and cause food spoilage (Gram & Huss, 1996). The common seafood spoilage bacteria include *Pseudomonas putida, P. fluorescens P. perolens, P. fragi*, *Shewanella putrefaciens, Brochothrix thermosphacta*, *Alteromonas nigrifaciens, Aeromonas salmonicida, A. hydrophila* and *Photobacterium* phosphorous. The most common foodborne pathogens are *Salmonella* spp, *Listeria monocytogenes, Vibrio cholera, V. vulnificus and V. parahaemolyticus (*Nychas & Drosinos, 2009). Most bacteria that are distinguished as specific spoilage bacteria produce volatile sulphides. Some *Vibrionoceae* and *Shewanella putrefaciens* produce hydrogen sulphide from the sulphur containing amino-acid L-cysteine (Gram et al 1987). The main conditions that help food spoilage due to microorganisms to take place are the presence of spoilage microbes, available nutrients for microbial growth, favorable conditions for growth such as temperature, pH, water activity, redox potential, presence or absence of oxygen. Food preservation is the process of treating food in order to control, stop, or slow down spoilage to increase the shelf life of food and reduce the possibility of foodborne illness (Lück, E., & Jager, M. 1997). There are several methods that can be used to preserve fish and seafood such as chilling, freezing, drying, heating, salting and use of preservatives. However, consumers want more fresh seafood. Among natural type of food

preservation methods bio-preservation is the common method to control spoilage by using microbiota or their antimicrobial products.

2.1.1 Bio-preservation of food with Lactic acid bacteria (LAB)

Lactic Acid Bacteria possess certain characteristics such as repulsion to dehydrate, protease action, coliphage, frostiness, sticking and populating capacity, polysaccharide and antimicrobial material manufacturing. LAB has good reputation of application, they are under GRAS (generally recognized as safe) tag. In the process of its use as biopreservative, it leads to the following products: organic acids, bacteriocins, carbon dioxide and hydrogen peroxides.

Bacteriocins: According to the definition of Klaenhammer, (1988), Bacteriocins are proteins or protein complexes that has bactericidal activity against other microorganisms. Bacteriocins produced by LAB are classified in four main groups: Group I lantibiotics (e.g. nisin), Group II the small heat-stable proteins, Group III Larger heat labile proteins and group IV Bacteriocins with carbohydrate or lipid moieties (Bruno & Montville, 1993). There are several beneficial effect of bacteriocins in food preservation: (1) bacteriocins can be used to reduce the probability of food poisoning incidents and minimizing the risk for transmission of foodborne pathogen occurrence in the entire food chain; (2) applying bacteriocins increases the shelf life of food products; (3) they are natural substitutes and alternative techniques to chemical preservatives which are used to decrease the amount of added chemical in the food; (4) they reduce economic losses that can happen due to food spoilage; and (5) increase consumer satisfaction due to lightly preserved foods, fresh tasting, and ready to eat (Thomas et al., 2000; Gálvez et al., 2007).

Majority of the bacteriocins penetrate the target bacteria through cytoplasmic membrane. There are two models that explains how the bacteriocins interact with lipid bilayer of the cytoplasm. According to carpet model, there is an interaction between positive region of the bacteriocin and negative region of the lipid bilayer of cytoplasm. The cytoplasmic membrane breaks into patches when there is an elevated level of bacteriocin molecule around the cytoplasmic membrane. The other model called Stave – Barrel model interprets that the bacteriocins incorporated in the cytoplasm will form pores or channels through which the cellular components leak out. The two model concludes that the interaction results in depletion of membrane potential, ATP generation capacity which finally lead to cell death. This is the primary effect of the interaction between

bacteriocin and the cytoplasm of the target bacteria. Followed by the primary effect is a secondary effect, which lead to complete lysis of the bacterial cell (Gomez-Lopez, 2012).

Table 2 Characterized of common bacteriocins from lactic acid bacteria. Table from (Soomro et al., 2002)

Bacteriocin	Producer organism	Properties
Nisin	*Lactococcus lactis* subsp. lactis ATCC 11454	Lantibiotic, broad spectrum, chromosome/plasmid mediated, bactericidal, produced late in the growth cycle
Pediocin A	*Pediococcus pentosaceus* FBB61 and L-7230	Broad spectrum, plasmid mediated
Pediocin AcH	*Pediococcus acidilactici* H	Broad spectrum, plasmid mediated
Leucocin	*Leuconostoc gelidum* UAL 187	Broad spectrum, plasmid mediated, bacteriostatic, produced early in the growth cycle
Helveticin J	*L.helveticus* 481	Narrow spectrum, chromosomally mediated, bactericidal
Carnobacteriocin	*Carnobacterium piscicola* LV17	Narrow spectrum, plasmid mediated, produced early in the growth cycle

Lactic Acid: The elementary antimicrobial impact deployed by Lactic Acid Bacteria is the manufacturing of lactic acid. The antimicrobial effect of lactic acids is depicted in its obtrusion of the sustenance of a cell membrane potential, hindering active transport, decreasing intracellular pH and impeding an assortment of glycolysis (Rattanachaikunsopon & Phumkhachorn, 2010). Strain, bacterial population and environment determine the reduction of

pH and lactic acid production (Olaoye & Onilude, 2011). Lactic acid possess a wide modus operandi and hamper both gram-negative and gram-positive bacteria, moulds and saccharomyces cerevisiae (Rattanachaikunsopon & Phumkhachorn, 2010). Lactic acid is in the undissociated state and pernicious to quite a number of bacteria, fungi and yeasts at low levels of pH. Even so, various microorganisms differ to a good extent in their responsiveness to lactic acid.

Hydrogen peroxide: H_2O_2 is normally utilized in the categories of textiles, environmental preservation, food, medicinal and dental products. (Abbas et al., 2010). Hydrogen peroxide can have antimicrobial activity and enzymatic effect by oxidized sulfhydryl groups. This may from peroxidation of lipid membrane leading to membrane permeability extension and can as well be a predecessor for the formation of bactericidal free radicals (Ammor et al., 2006). Hydrogen peroxide has a powerful oxidizing impact on membrane lipids and cellular proteins. An example of such is the NADH oxidase, flavoprotein oxidoreductases, and α-glycerophosphate oxidase, NADH peroxidase (Rattanachaikunsopon & Phumkhachorn, 2010). The amalgamated hydrogen peroxide can hinder the development of psychotropic and pathogenic microorganisms (Zalan et. al., 2005).

The categorization of LAB into various genera is widely based on morphology, development under diverse temperatures, mode of glucose fermentation, conformation of processed lactic acid, capacity to develop under heightened salt content, and under varying pH levels. Generally, *Lactobacillus* and *Carnobacterium* are applied as the initial culture in vegetables, fish, meat and milk fermentation (Rattanachaikunsopon & Phumkhachorn, 2010).

2.1.2 Carnobacterium

The genus *Carnobacterium* is now categorized into ten strains; two of which are usually isolated from natural surroundings or foods. *Carnobacterium* strains are presently the topic of research engagement with a specific goal to investigate preventive cultures to hamper pathogenic and spoilage microorganisms in foods. They have the capacity to mature under low temperatures, anaerobically and with increased in carbon dioxide concentrations. They are tolerant to high pressure, freezing/thawing and able to grow at low temperatures and gain their energy by fermentation. The resulting cells are normally short to slender rods, really curved small. They come in singular or in pair form, and often as short chains and could be mobile. (Leisner et al., 2005).

Culture characteristics. Cultures on agar media are usually creamy to white or shiny and convex. In the event that they are cultured on an intricate agar, *Carnobacterium pleistocenium* make typical colonies with conical shape cultures, thicker internal uniformity with a dark exterior. The exterior is coarse having narrow, asymmetric, granulated and rough with thinner torn edges (Pikuta et al., 2005). When using an ideal agar, the culture radius ranges between 0.25 and 1 mm.

Antagonistic potential. Robertson et al., (2000) observed the effect of *Carnobacteria as* probiotics against spoilage microorganisms in fish.*Carnobacteria* strains isolated from Atlantic salmon showed in vitro action to numerous Gram- negative pathogens. The culture applied to small Atlantic salmon and rainbow trout and significantly improved the survival of the fish that exposed to pathogens. Atlantic salmon was exposed to Yersinia ruckerii, Aeromonas salmonicida, Vibrio anguillarum, and V. ordalii. The studied performed with the aim to protect food from growth of pathogens, especially *Listeria monocytogenes* (Brillet et al., 2004). As *carnobacteria* do not acidify strongly, the strains commonly used bacteriocins for microbial activity. The major type of *carnobacteria* bacteriocin are lantibiotics (group I bacteriocins) and group II compounds (Vos, et al.,2011; Klaenhammer, 1993; Tahiri et al., 2004).

2.1.3 The effects of temperature on bio-preservation of LAB

Bio-preservation by using LAB is hampered by various factors. Previous studies showed that the antimicrobial activity of LAB is affected by several factors including temperature, pH, composition, structure, and natural microbiota of food (Zhou et al., 2014). Bacterial growth is strongly influenced by temperature. Most *carnobacteria* species are psychrophilic and psychrotolerant, meaning they are able to grow and reproduce at temperatures between -10 to 20 °C. According to Qin, et al., (2012) LAB maximum specific growth rate increase with increasing temperature up to a certain temperature then decrease with further increasing temperature. Temperatures affects antimicrobial production of *carnobacterium*. According to research of Sumathi & Reetha, (2012) research on the influence of storage period and storage temperature on the inhibitory activity of bacteriocin of lactic acid bacteria they concluded that bacteriocin activity decreased with increasing storage period. Moreover, Ohenhen, (2015) indicated that bacteriocin extract exhibited maximal antibacterial activity against E. coli when stored at -20ºC for 7 days, whilst at ambient temperature (28±2ºC) the extract exhibited minimal antibacterial

activity. The author indicated that cold storage temperature may be the most appropriate preservation method for the extract against E. coli. In addition to this, Brillet-Viel, (2016) described that bacteriocin activity was mainly dependent on pH and temperature, maximum bacteriocin activity was obtained at low pH and low temperature. Similarly, Buchanan and Klawitter, (1991) observed that increased effectiveness of bacteriocin-producer at refrigeration temperatures and decrease in bacteriocin production at higher temperatures. Moreover, Ananthanarayanan, (2013) indicated that a storage temperature of 0 to 4°C is satisfactory for the preservation of most of fresh food for short term storage.

Temperature can affect lactic acid production of LAB. Taleghani et al., (2016) studied the effect of temperature at 32, 37, 42 and 47°C. Results showed that the concentration of cell dry weight increased with increased temperature from 32 to 42°C. The maximum cell and lactic acid concentration was obtained at 42°C for *Lactobacillus* species. Slightly acid pH can increase cathepsin activity. According to Taylor et al., (2002) the progress of proteolysis varies depending on the processing conditions, the type of muscle and the amount of endogenous proteolytic enzymes. For instance, the increase in temperature favors the enzymatic action and a slightly acidic pH would enhance the activity of lysosomal cathepsins. In addition to this increase in temperature can speed up movement of molecules and microbial activities reproduction. Consequently, fish muscle degrades fast in higher storage temperature. Freezing keeps food safe by slowing the movement of molecules, causing bacteria to enter a dormant stage.

2.2. Study on enzyme activities

2.2.1 Protein in fish

As a result of the rapidly increasing human population, there is a need to enrich food with nutrients such as proteins for a balanced diet. A main protein supply is from fishing. On estimation, a typical fish comprises large percentage of 80% (lipids and water) then about 20% proteins. Protein content in fish are classified into three categories i.e.: myofibrillar ranging between 70% and 80%, sarcoplasmic ranging between 25% and 30% and finally stroma for 3% (Careche et al., 1999). Proteins make build up of 20 amino acids as the basic units. They are classified into primary, secondary, tertiary, quaternary structure. They are also categorized as simple, conjugated and derived proteins. Amino acid makes two polymers which are peptides and proteins. Simply put, proteins are polymers comprised of linear amino acid peptide bond

chain. Proteins need specific conditions for their activity, stability and conformation. Protein solubility, or extractability, is one of the functional properties of protein. It indicates the amount of nitrogen in the protein that is in the soluble state. Extractability of muscle proteins influences emulsion capacity and stability, water binding, gelation, textural characteristics and adhesion of muscle pieces. Based on solubility property sarcoplasmic proteins grouped under water soluble protein, myofibrillar proteins categorizes under salt soluble protein (Zayas, 1997).

2.2.2 Proteolytic enzyme

Postmortem change of a fish depends on many factors temperature, humidity, pH, microorganisms. Postmortem interval divided into 3 categories immediately after death. These are pre- rigor mortis, rigor mortis and post rigor mortis. The 3 categories are related to muscle degradation by proteolytic enzymes. Immediately after death the muscle is soft and flexible this is known as pre- rigor mortis conditions, after some time or days the muscle becomes hard and stiffen this stage is rigor mortis condition. The muscles become soft again during post rigor mortis. different biochemical reactions are responsible for the cause of rigor mortis (Nonthaput, et al., 2017). Deteriorations of proteins is speeded up by protease which acts as a streptodornase. Rates of reactions in living cells are catalyzed by enzymes. These enzymes are proteins and formed in low quantities in living cells of an organism. Their function is to reduce the amount of energy needed to start a chemical reaction. Enzymes are specific and at times require coenzymes to assist in their functioning.

Proteins are broken down into amino acid molecules and smaller peptides in a process known as proteolysis. Proteolysis is a biochemical phenomenon consisting of the degradation of proteins and the generation of small peptides and free amino acids. Muscle proteases, mainly cathepsins B and L, which are active at slight acid conditions, and calpains, which are active at neutral pH, are able to act at the pH found in post-mortem fish and break down the structural myofibrillar proteins generating large peptides and protein fragments. This breakdown has a softening effect on the texture of the fish. Further problems ensue if these large peptides become hydrolyzed by muscle peptidases which generate small peptides and free amino acids. These can be used as substrates by microorganisms for growing and/or transforming amino acids into other compounds like biogenic amines or off-flavors like ammonia (Kilcast, & Subramaniam, 2011). Normally the breakdown of proteins occurs in 3 different stages. In stage one, calpains and

cathepsins work on main myofibrillar proteins creating protein fragments and intermediate polypeptides. The resulting polypeptides are further broken down in the presence of water to small molecule peptides by di- and tri-peptidylpeptidases in the second stage. In the third stage, dipeptidases, aminopeptidases and carboxypeptidases work on the small peptides resulting in free amino acids (Bendifallah et al., 2006). These different stages of the process are determined by conditions (temperature and pH), kind of muscle and the quantity of enzyme available. For example, a higher temperature favors enzymatic action as compared to a lower temperature. In addition, a lightly acidic environment improves the function of lysosomal cathepsins. Proteases as well break down the boundary separating myofibrils from connective tissue in the presence of water. Also, proteases can breakdown collagen fibres in a fish and have an impact on the texture of the fish (Taylor et al., 2002).

Enzymes that can break down peptide bonds are classified by the location in substrate of the chemical reaction as exopeptidases and endopeptidases. Exopeptidases cleave peptide bonds by the amino or carboxyl ends of the polypeptide link, while endopeptidases cleave inside peptide bonds (Sternlicht and Werb, 2001). Classification based on pH results into the following categories: neutral, alkaline and acid proteases. Based on their substrate specificity there are four groups: cysteine, serine, metallo and aspartic proteases (Simpson, 2000). Irrespective of their origin, proteases can be categorized as per their uniformity to typical proteases, for instance cathepsin-like, trypsin-like, chymosin-like, chymotrypsin-like, and chymosin-like (Klomklao, 2008).

2.2.3 Cathepsin

Cathepsins are acidic proteases found in lysosomes. Generally they are passive in a living organism but become released at sites of injury or upon freezing and thawing of postmortem muscle. They are differentiated by their active site (aspartic, cysteine, serine proteases) as well as by their inhibitor sensitivity and substrate specificity. Normally, lysosomes contain about thirteen cathepsins (Kolodziejska and Sikorski, 1995). As part of these, cathepsins B, D, L, L-like are refined from aquatic fish. Cathepsins B, D, L, and H form the main cathepsins in a fish's muscle lysosomes (Aoki et al., 2000). Cathepsin B constitutes the initial defined member of lysosomal cysteine peptidases. Cathepsin B has been identified to constitute the main lysosomal proteases of fish (Yamashita & Konagaya, 1990).

3. Material and methods

3.1 Lactic acid bacterial strains and culture condition

To investigate the effect of LAB on the growth of spoilage bacteria, *Carnobacterium ssp* (SF1994) of Lactic Acid Bacterial strains was used. The culture was received from Ifremer (France, September 2016) frozen at -20°C. Cultivated culture was obtained from Nofima (Stavanger, January 2018) and kept in NTNU Food Chemistry Lab in the freezer at -20°C. On the first day of the experiment a small amount of the four LAB cultures were scraped from the agar plates by using a sterile toothpick and transferred to10 ml Elliker broth in a conical flask and the culture was incubated in an orbital shaker incubator at 22°C for 24 hours. Afterwards aliquots of the culture was frozen at -80ºC.

After 24hrs of incubation 1 mL of the culture was transferred to 100 mL Elliker Broth, which was further incubated at 22°C for 20hrs. Samples were taken after 4hrs and 20hrs to determine turbidity and the CFU/M of the culture. The turbidity was measured at 660nm using Thermo Scientific, Genesys 10s UV-VIS Spectrophotometer. CFU/ml of the cultures were determined by plating 100 mL of the cultures on NAP Agar and incubated under anaerobic conditions at 22°C for 3-5 days. The anaerobic incubation for NAP agar was performed by placing the petridishes in closed containers called anaerocult along with anaerobic indicator (Thermo scientific BR0055B). The indicator turns from pink to white inside the container for a positive result of anaerobic conditions. Anaerobic atmosphere generation bags (Thermo scientific AnaeroGen 2.5L or Microbiology Anaerocult) was placed inside the container within a minute.

Concentrations of 10^9 CFU/ml of LAB were required to use the cultures for bio-conservation of salmon. The culture was estimated to have a sufficient growth after 20hrs of incubation.

When concentrations of 10^9 CFU/ml of LAB was achieved, the culture was used for bio-conservation of salmon. The dipping solution was prepared by diluted lab cultures 1:100 in sterile distilled water. To determine the CFU/ml in the dipping solution, serial dilution in peptone water was made, plated on NAP Agar and incubated at 22°C for 72 hours. Afterwards, the number of colonies were counted.

3.2 The effect of Temperature on the growth of Lactic acid bacterial

To investigate the effect of temperature on the growth of Lactic acid bacteria, the LAB culture was incubated for 24hrs. After incubation 1 mL of the culture was transferred to each of nine sterilized flasks that contain 100 mL Elliker Broth, which were further incubated separately at 0°C, 4°C, 8°C, 10°C, 15°C, 22°C, 25°C, 30°C and 37°C for 20hrs. Samples were taken after 20hrs to determine turbidity and CFU/ml of the culture.

3.3 Microbial analysis

To study the effect of temperature and LAB on the growth of spoilage bacteria, salmon fillets without skin was bought from Ravnkloa Fisk & skalldry AS in Trondheim. The salmon fillets were cut into pieces of about 100 g and prepared with a total of 42 samples (7 parallels for each of the 6 treatments) (figure 1). Microbial analysis (NAP and Long & Hammer) were conducted on the samples prior to dipping, immediately after dipping, day one storage, day two storage, day three storage, day five storage and day seven storage time. Moreover, analyzing of growth of sulphide reducing microorganisms performed on the last day (day seven) of storage time. The standard Total Plate Count (TPC) were used to perform microbial analysis. This method is the most common and widely used to measure the growth of microbes in the food (Wheaton and Lawson, 1985; AOAC, 2000).

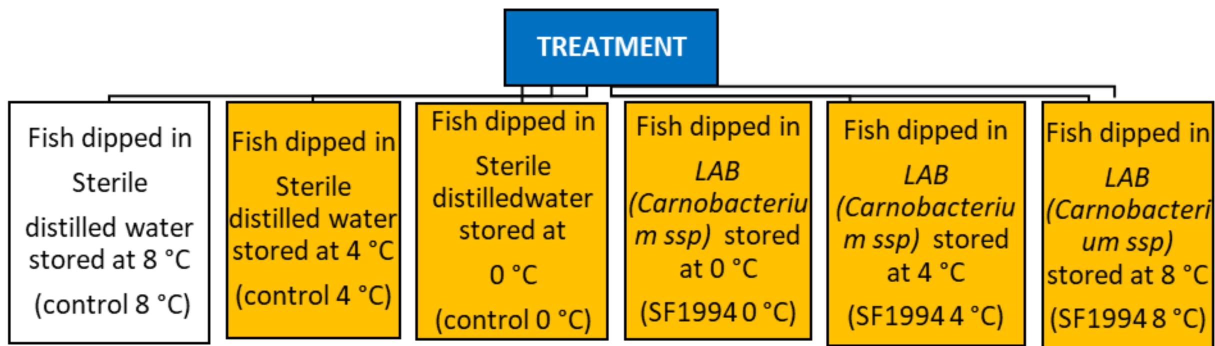

Figure 1 Storage treatments that used during the experiment.

3.4 Sensory analysis

Sensory evaluation was performed by the author by describing the appearance (visual inspection) and the smell of the samples and compare the differences of the attributes between each sampling day of the experiment (York & Sereda, 1994).

3.5 Enzyme analysis

3.5.1 Extraction of proteolytic enzymes

The extraction of enzymes was performed as described by Hultmann, & Rustad, (2004) with minor modifications in a cold room (+ 4°C). Briefly, 10 g of salmon fillet was homogenized with 20 mL cold distilled water using an Ultra Turrax homogenizer. The homogenates were kept on ice for 30 min before centrifugation at 14,600 × *g* for 20 min at 4 °C. After centrifugation the supernatants were filtered through glass wool and kept at −80 °C until analyzed

3.5.2 Protein content of the extract

To determine protein content of the extract the frozen enzyme extracts were properly thawed and centrifuged at 7840 × *g* for 10 min at 4 °C. The protein content in the extracts were determined by Lowry protein assay, using bovine serum albumin as a standard (Lowry et al., 1951). Samples were diluted with distilled water with a dilution factor of 1:150 and the analyses were run in triplicate (Hultmann, & Rustad, 2004).

3.5.3 Activity of specific proteolytic enzyme

The activity of cathepsin B+ like enzymes was measured against a synthetic fluorogenic substrate, Carbobenzoxy-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Sigma Chemical Co., St. Louis, MO, USA) (Barrett & Kirschke, 1981). The enzyme extract was diluted suitably a factor of 1:150. Diluted extract (0.1 ml) was prepared in three parallel and Incubate for 15 min to reach the desired temperature (4 °C). To start the reaction 0.1 ml substrate (150 mM bis-Tris, 30 mM EDTA, 6 mM DTT at pH 7.0) was added to the diluted enzyme extract and further incubated for 30 min at 4 °C. The reaction was stopped by adding 3 ml 1% SDS in 50 mM bis–Tris, pH 7.0. A blank was prepared by adding distilled water instead of enzyme extract to the reaction mixture. Fluorescence values were measured with excitation at 360 nm and emission at 460 nm (Hultmann, & Rustad, 2004).

3.5.4 Amount of acid soluble peptides

To determine the amount of acid soluble peptides TCA (trichloroacetic acid) was used to precipitate proteins. The experiment performed by mixing 2.0 ml water soluble extract and 2.0 ml 20 % TCA. The mixture was left for 30 min at room temperature. The prepared sample filtered and diluted with a dilution factor of 1:10 by mixing with distilled water. After that the Lowry method was used to determine the amount of acid soluble peptides by using bovine serum albumin as a standard. (Lowry et al., 1951; Hoyle & Merrltt, 1994).

3.5.5 Determination of amount of free amino acids

To determine the amount of free amino acids in enzyme extracts, Osnes and Mohr, (1985) protocol was used. 1 ml water-soluble protein extract was mixed with 0.25 ml of 10 % sulphosalicylic acid in an eppendorf tube. The mixture was kept in a cold room for 30 min and centrifuged for 10 min at 10 000 rpm. The solution was mixed again with sulphosalicylic acid until all the protein precipitated. After confirmation of no observed precipitation, the supernatant diluted with a factor of 1:50 and filtered with 0.22 µm filter for running the sample on the HPLC.0.205 ml of the diluted sample was used to measure the amino acid content in HPLC (Hultmann, & Rustad, 2004).

3.6 Statistical Analysis

The data were statistically analyzed by using Analysis of Variance (ANOVA) to determine the effect of storage time and temperatures on the treatments. Mean comparison were conducted using regression model in microsoft excel version 2016.

4. Result & discussion

4.1 Lactic acid bacterial strains and culture condition

From figure 2 and 3 the absorbance and CFU/ml for the LAB strain was increased when the incubation time increased from 16hrs to 20hrs. The recorded absorbance was 0.647 after 16hrs incubation time and 0.958 after 20hrs incubation time. Similarly, the CFU/ml of the LAB culture was increased from 8.3 to 9.9 after 16hrs and 20hrs incubation.

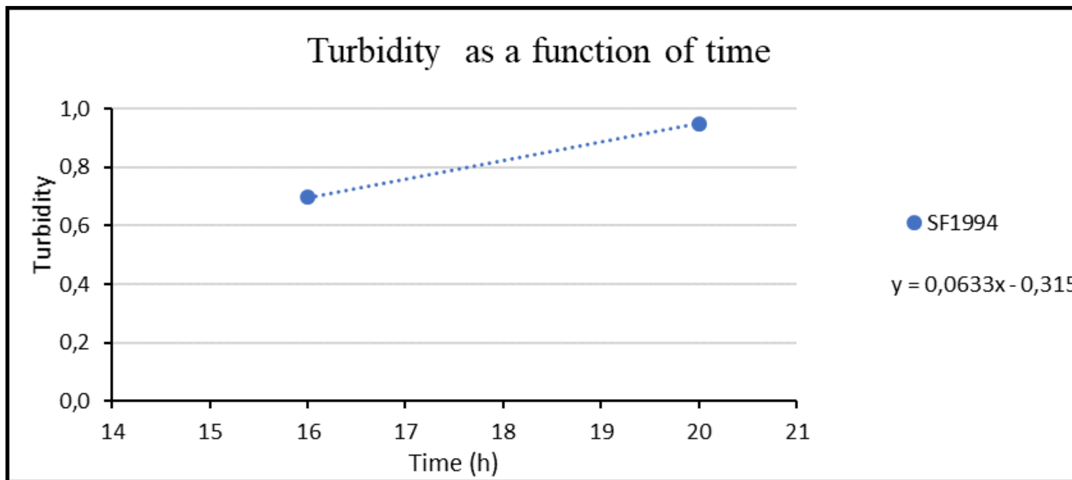

Figure 2 Turbidity of LAB culture (SF1994) as a function of time

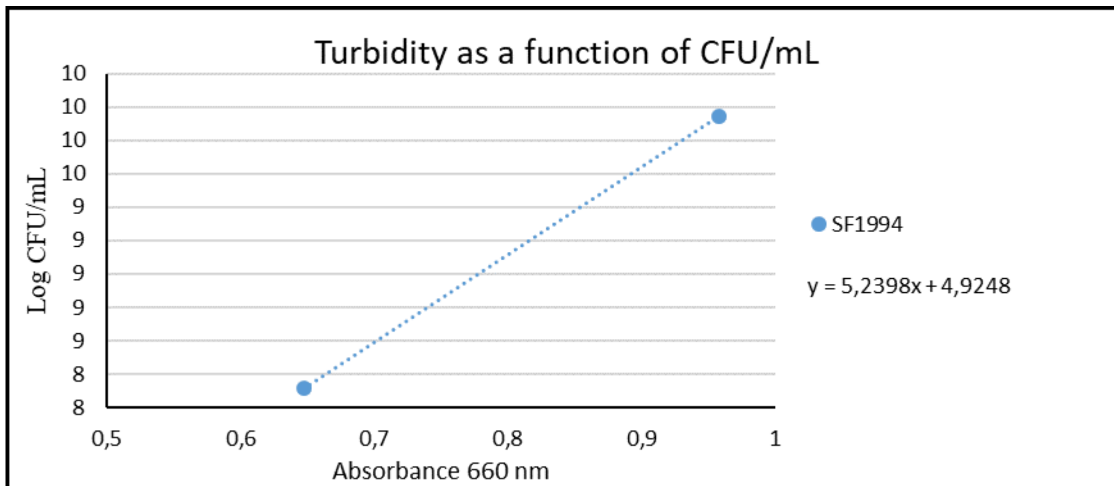

Figure 3 Turbidity of LAB culture (SF1994) as a function of CFU/mL.

The turbidity and the CFU/ml result indicated after 20hrs of incubation, the LAB strain was sufficient to perform bio-preservation of salmon at different storage temperatures. Reactivation of *carnobacterium* from the frozen culture showed they have an ability to tolerate cold temperatures. Qin, et al., (2012) described *carnobacterium* are psychrotolerant and able to grow at low temperature.

4.2 The effect of Temperature on the growth of *Carnobacterium*

Prior to perform bio-preservation of salmon, the activated LAB strain was cultured at different temperatures (0 to 37°C) to investigate the effect of temperature on the strain prior to dipping with the fish. The obtained results in this work indicated that the LAB strain was able to growth at a temperature range of 0 to 37°C. As shown on figure 4, the recorded Log (CFU/g) were 7.98 at 0°C, 8.19 at 4°C, 8.41 at 10°C, 8.93 at 15°C, 11.26 at 22°C, 11.18 at 26°C, 10.99 at 30°C and 9.51 at 37°C.

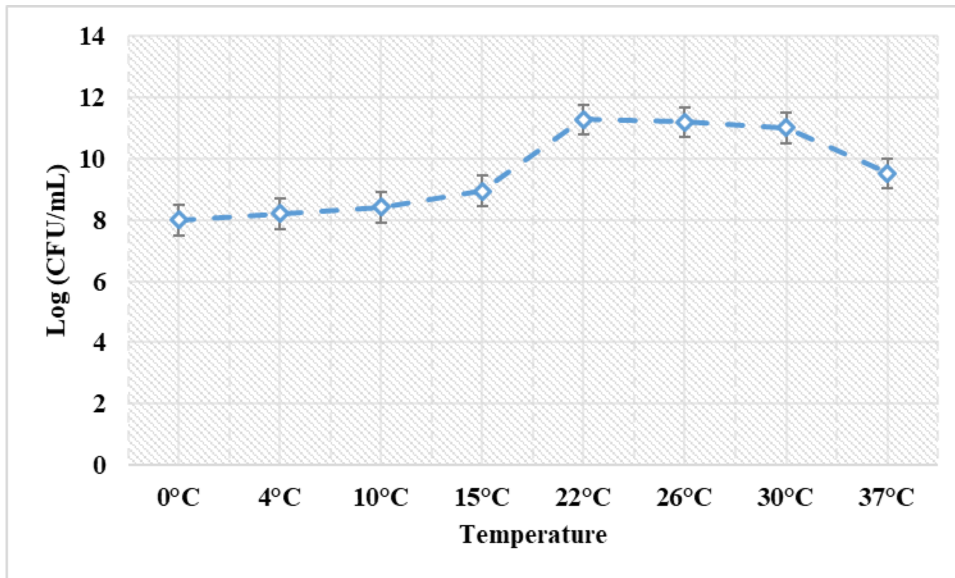

Figure 4 The effect of temperature on the growth of LAB. Standard error of the mean is shown as y-error bars. N=2.

Even though, we confirmed the ability of *carnobacterium* to grow with temperature range of 0-37°C and recorded optimal growth at 22°C, we finally selected 0°C, 4°C and 8°C storage temperature to investigate the effect of these storage temperature on bio-preservation. The main

reason for selection of these storage temperatures is fresh salmon is easily perishable at high temperature. Zhou et al., (2014) indicated selection of storage temperature is one of the main factors that can affect the bio-preservation process.

4.3 Microbial analysis

4.3.1 Growth of LAB on fish determined on NAP agar

From figure 5, the growth of LAB was changed with storage time and storage temperature. According to the result, no LAB was found on the fish before dipping. However, after dipping the fish in culture, the LAB treatments recorded a higher Log (CFU/g) of LAB than the control treatments for all storage temperature. The LAB treatments (SF1994 at 0°C, SF1994 4°C, SF1994 8°C) recorded Log (CFU/g) of 4.3, 4.8 and 5.1 at the first storage day and 6.2, 7.0 and 7.2 at the last storage day respectively. On the other hand, the control treatments (control 0°C, control 4°C, control 8°C) recorded Log (CFU/g) of 2.7, 2.9, 3.1 at the first storage day and 3.5, 4.9 and 6.1 at the end of the experiment, respectively.

The LAB treatments which were dipped the fish in the LAB culture recorded higher Log (CFU/g) of LAB than the control treatments (dipped the fish in sterile water) for all the three storage temperature. Based on the result, the control and SF1994 treatment showed a significant difference with ($p= 0.027$) at 0°C, ($p= 0.009$) at 4°C and ($p= 0.009$) at 8°C storage temperature. This conforms *carnobacterium* can managed to grow on the fish after dipping.

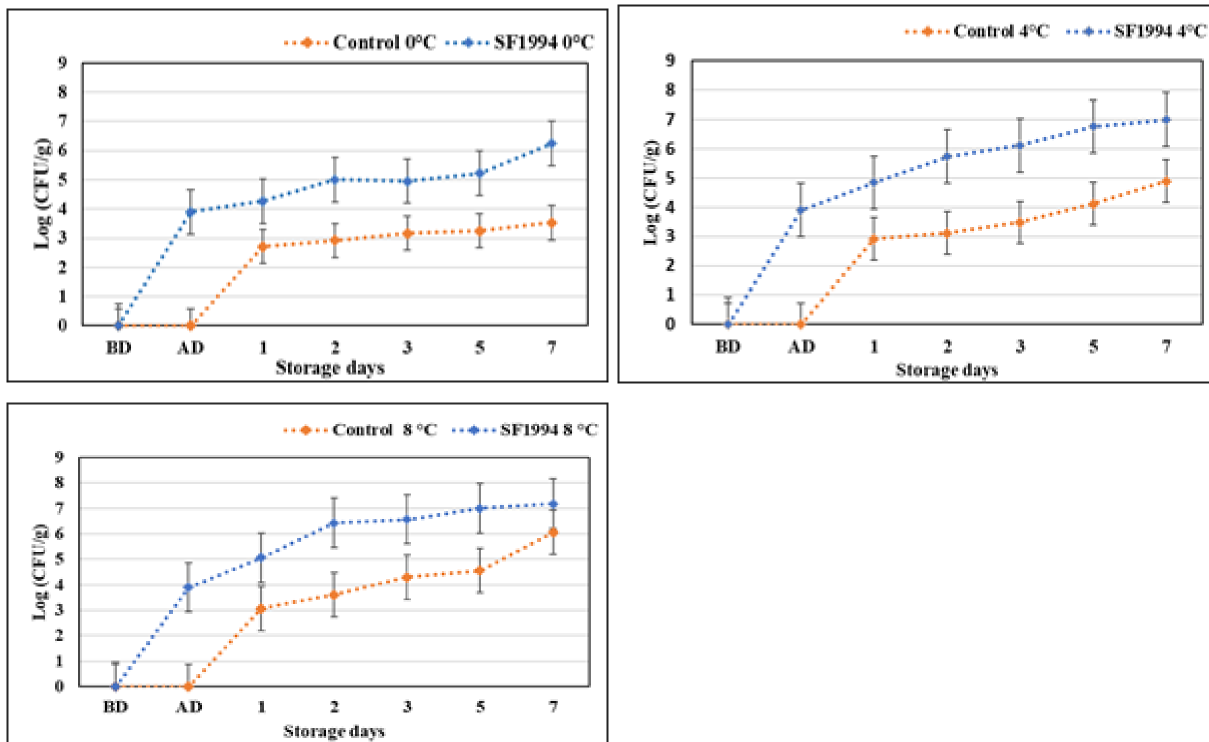

Figure 5 Growth of LAB as a function of storage time. BD indicates the number of LAB on the raw material or before dipping, AD indicates the number of LAB after dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=2

Based on figure 6, the storage temperature affects the growth of LAB. The recorded (mean±SE) for SF1994 0°C, SF1994 4°C SF1994 8°C were 4.2± 0.75, 4.9± 0.91, and 5.16 ± 0.96 Log (CFU/g) respectively. Hence, SF1994 8°C treatment showed significantly higher growth of LAB compare to SF1994 0°C and SF1994 4°C with p-value of 0.0001 and 0.00001 respectively. Moreover, the LAB strain showed better growth at the highest storage temperature (8°C) followed by 4 °C and 0°C.

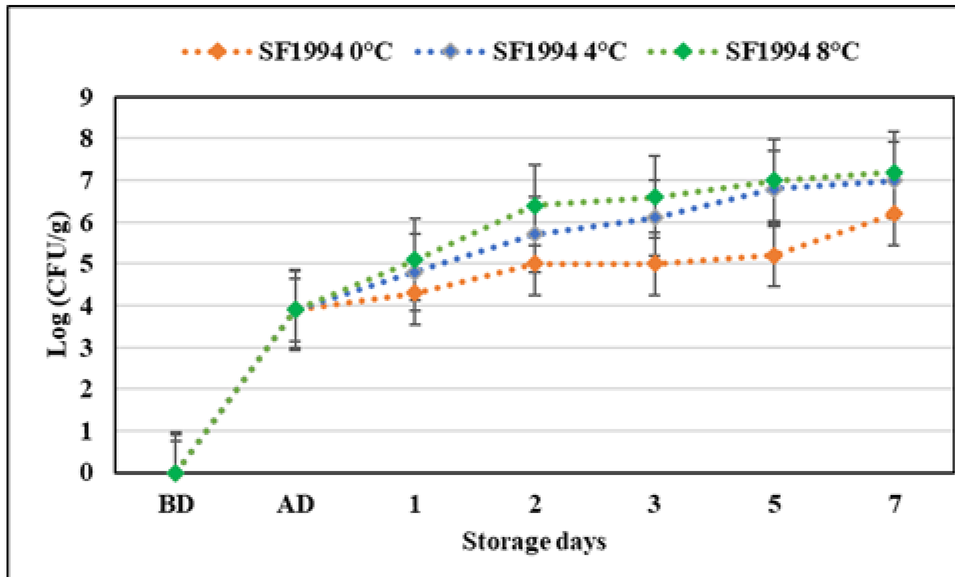

Figure 6 Growth of LAB from different storage temperatures as a function of storage time. BD indicates the number of LAB on the raw material or before dipping, AD indicates the number of LAB after dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=2.

4.3.2 The effect of LAB on the growth of spoilage microorganisms

The result shown in Figure 7 revealed that the control treatments gave higher Log (CFU/g) of spoilage microorganisms than the LAB treatments. The LAB treatments (SF1994 0°C, SF1994 4°C, SF1994 8°C) recorded Log (CFU/g) of 3.4, 3.6 and 4.0 at the first storage day and 4.2, 6.1 and 6.9 at the last storage day respectively. On the other hand, the control treatments (control 0°C, control 4°C, control 8°C) recorded Log (CFU/g) of 3.9, 4.4, 5.1 at the first storage day and 5.9, 7.8 and 8.4 at the end of the experiment, respectively.

From Fig 7 Dipping in LAB significantly decreased the growth of spoilage microorganisms in treatments SF1994 0°C, SF1994 4°C, SF1994 8°C compared to the control treatments (control 0°C, control 4°C, control 8°C). The recorded p-value between SF1994 0°C and control 0°C were p= 0.009, between SF1994 4°C and control 4°C were p= 0.001 and between SF1994 8°C and control 8°C were p= 0.0002.

The reduction of growth of spoilage microorganisms in the presence of LAB indicates that *carnobacterium* are a good candidate to preserve food by natural method. Ghanbar et al., (2013) described that bio-preservation through LAB reduce the growth of spoilage microorganisms and increase shelf life. Different mechanisms have been suggested for LAB biocontrol activity, these

include competition of nutrients to spoilage and pathogenic microorganisms, secretion of different types of antimicrobial metabolites including alcohols, acids, hydrogen peroxide, diacetyl, carbon dioxide, and other metabolites (Helander et al., 1997).

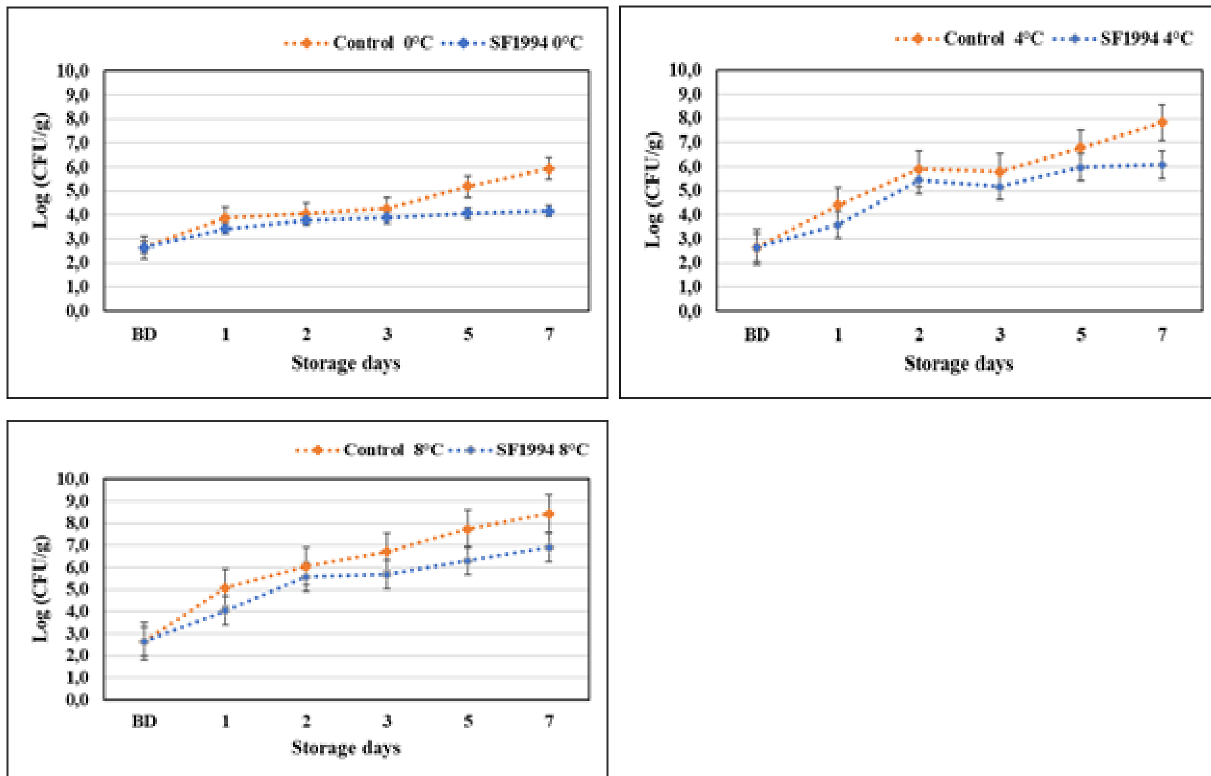

Figure 7 Growth of spoilage bacteria as a function of storage time. BD indicates the number of LAB on the raw material or before dipping, AD indicates the number of LAB after dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=2.

Figure 8&9 revealed that, among the LAB treatments SF1994 0°C 3.7± 0.21 Log (CFU/g) (mean±SE) showed significantly lower growth of spoilage microorganisms than SF1994 4°C 4.8±0.52 (mean±SE) and SF1994 8°C 5.2±0.59 (mean±SE). The recorded p-value between SF1994 0°C and SF1994 4°C were p=0.001, and between SF1994 0°C and SF1994 8°C were p=0.0003.

The above result showed bio-preservation ability of LAB was varied at different storage temperature. Even though we found the highest growth of LAB at (8°C) than (0°C) storage

temperature, the lowest storage temperature (0°C) showed lowest growth of spoilage microorganisms than (8°C) storage temperature. Ratkowsky et al., (1982) indicated that temperature is the most important physical factor for regulating the growth of spoilage organisms. For most marine spoilage microorganisms, the optimum temperature for the growth is l0-20°C. Furthermore, food storage at low temperature is an ancient food preservation technique used to slow down growth of spoilage microorganisms. Likewise Duun & Rustad, (2007) mentioned growth and toxin production of spoilage bacteria highly reduced during low storage temperature. Similarly, Sivertsvik et al., (2002) described that cold storage improve the shelf life of fish by increasing the spoilage microorganisms lag phase. The lag phase is a maturation time of the bacteria for replication. Yassoralipour et al., (2013) reported that fish stored at 8 °C showed higher plate count of spoilage bacteria than fish stored at 0 °C during 20 days storage time. This indicates growth of microorganisms influenced by many different factors, combination of different factors can achieve a better result. Nath, (2014) explained bio-preservation can be more effective by combination with other preservative factors (known hurdles) to inhibit the growth of spoilage microbes and achieve food safety. The combination of LAB and low storage temperature showed significantly lower growth of spoilage microorganisms than the combination of LAB and highest storage temperature. Our results are in accordance with those obtained by Buchanan and Klawitter, (1991) who examined the effectiveness of *carnobacterium* bacteriocin against *Listeria monocytogenes* with incubation temperature of 5, 12, 19, 28, and 37°C. The observed result was, the effectiveness of inhibition was inversely related to the incubation temperature. It was recorded highest effectiveness of *carnobacterium* bacteriocin that produced at refrigeration temperatures than at the higher temperature. This was due to the increment in susceptibility of *Listeria monocytogenes* to the bacteriocin at lower temperatures and by a decrease in bacteriocin production at higher temperatures. Moreover, the author indicated that bacteriocin production was almost equivalent at 5, 12, 19 °C and decreased at 28 °C incubation temperature. Likewise, Schillinger et al., (1993) indicated that *carnobacterium piscicola* can grow and produce bacteriocin at temperatures ranges from 1 to 30°C. Production of bacteriocin stopped for temperature above 30°C. The highest bacteriocin activity was recorded at 25°C. Almost the same bacteriocin activity was achieved for lower temperatures however; it might require increase incubation time due to lower growth rate of the strain.

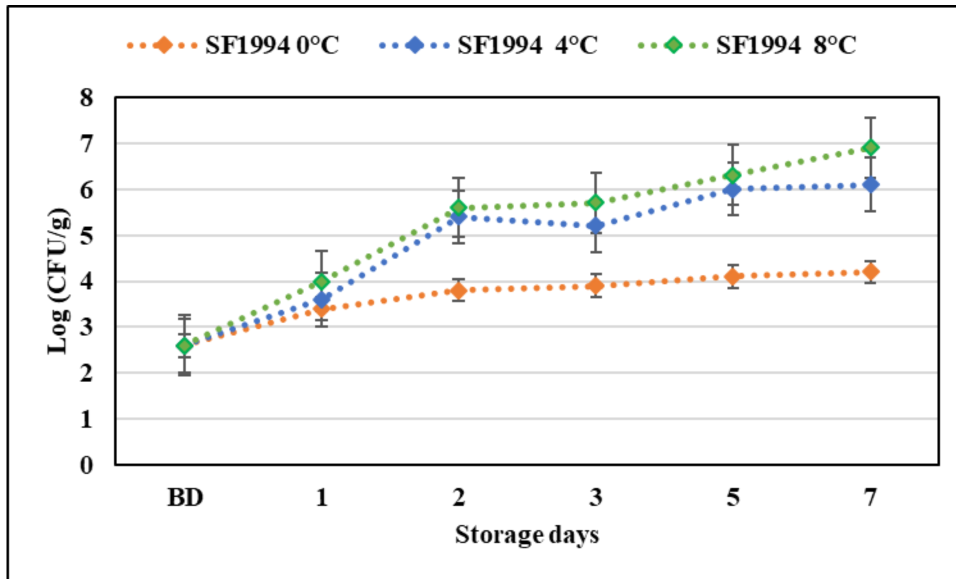

Figure 8 Growth of spoilage bacteria from different storage temperature as a function of storage time. BD indicates the number of spoilage bacteria on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=2.

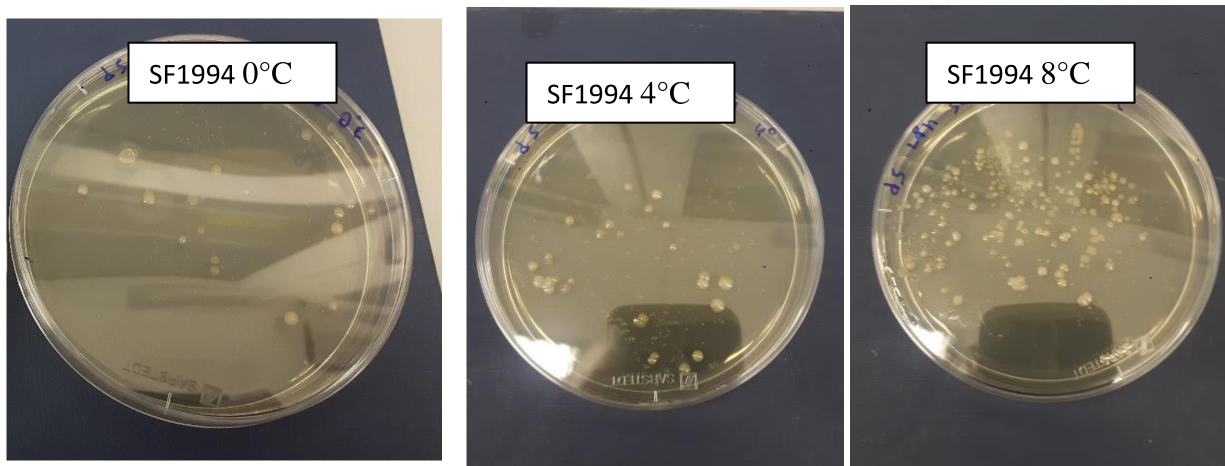

Figure 9 Growth of spoilage bacteria after 5 days of storage from different storage temperature. Plates shown are with a dilution factor of 10^{-3} for SF1994 0°C, 10^{-5} for SF1994 4°C and SF1994 8°C

4.3.3 The effect of LAB on the growth of sulphide reducing microorganisms

According to figure 10, there was a variation of growth in specific spoilage microorganisms between the control and SF1994 treatments. The control treatments recorded Log (CFU/g) of 4.9, 6.1 and 6.4 for 0°C, 4°C and 8°C storage temperature and SF1994 treatments recorded 3.9, 5.1, 5.7 for 0°C, 4°C and 8°C storage temperature, respectively.

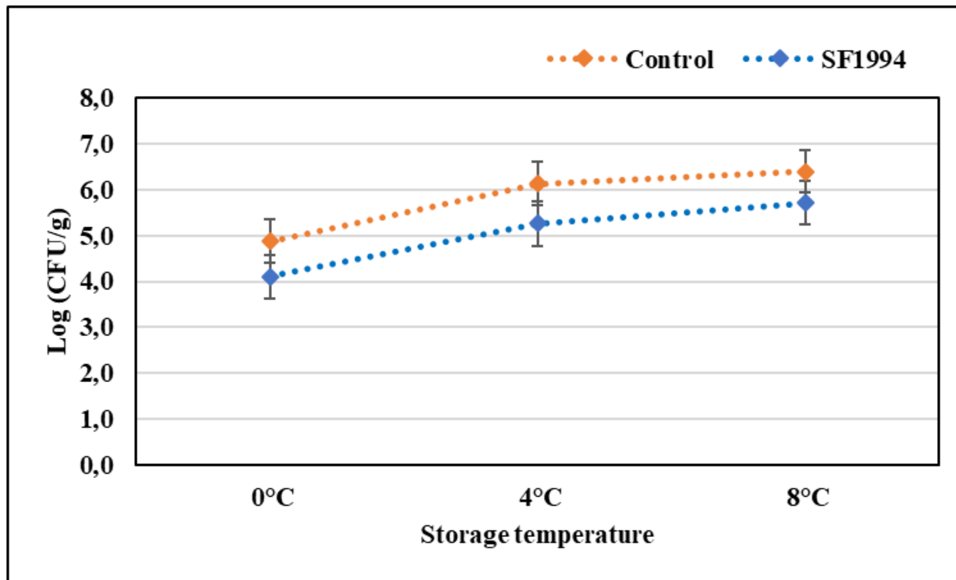

Figure 10 Total count of sulphide reducing bacteria after 7 days of storage from different storage temperature. Standard error of the mean is shown as y-error bars. N=2.

Specific spoilage organisms (SSOs) managed to grow in both treatments during the experiment. Higher growth was observed on the control treatments compared to the LAB treatments. Furthermore, the growth of SSOs showed significant correlation with storage temperature in both treatments. Supporting this finding Serio et al., (2014) who evaluated the effect of temperature on the growth of sulphide reducing microorganisms with a function of different storage temperatures (4, 8, 11, 20, 25, 30, 37 and 42°C) described that the growth of sulphide reducing microorganisms were increased with increasing temperature. Almost all the tested isolates showed growth within two days at 4 °C and most of them showed growth in one day at 8 °C. Likewise, our results revealed that, there were a significant variation between the LAB treatments. Hence, SF1994 8°C recorded the highest growth of SSOs followed by SF1994 4°C and SF1994 0°C. The low temperature improved the effectiveness of *cyanobacterium* to inhibit SSOs by increasing the vulnerability of SSOs.

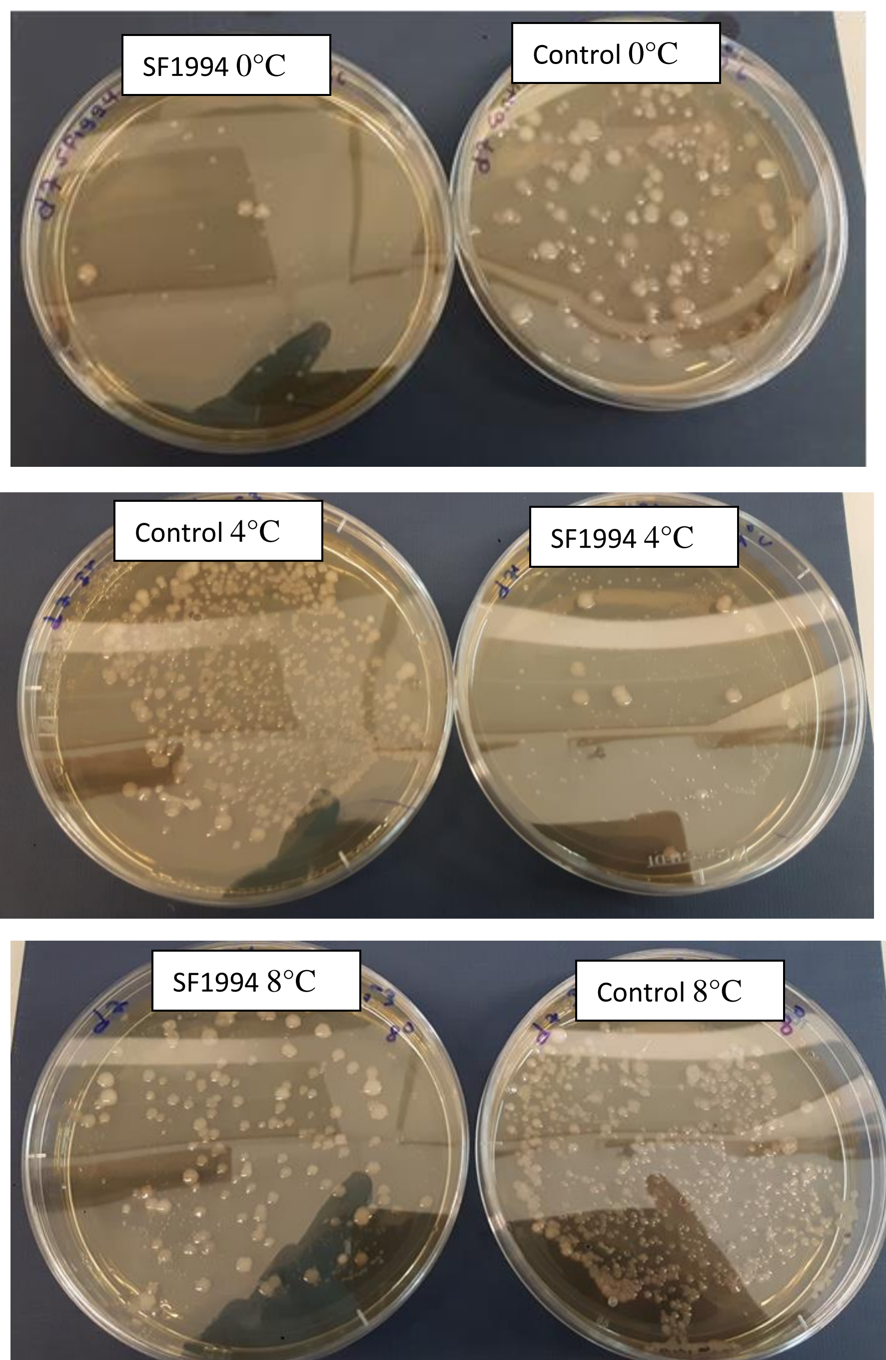

Figure 11 Growth of sulphide reducing bacteria after 7 days of storage from different storage temperature. Plates shown are with a 10-3 dilution factor

4.4 Sensory analysis

The sensory evaluation indicated that the sensory quality was decreased with storage time. The LAB treatments showed better condition than the control treatments. As it described In table 3, the sensory qualities of the samples that stored at 0°C were in good condition until the 7th day of the storage. Samples that were stored at 4°C showed good sensory qualities until the 5th day of the storage. Moreover, samples at 8°C storage temperature produce sourly smell and less transparent appearance and less firm on the 3rd day of the storage. Similarly, Jorgensen et al., (1988) indicated that growth of microorganisms increases with increasing of storage time and this reduce the sensory quality. Moreover, Wiernasz, et al., (2017) describe that fishery products are nutrient rich and have a short shelf life. Thus, this composition of the product creates favorable condition for spoilage microorganisms that affect the sensory quality. Furthermore, Fraser & Sumar, (1998) reported that the breakdown of macro and the micro components generally leads to unfavourable changes in the sensory characteristics of the fish muscle that related in most cases with its physical deterioration. It is these compounds formed in fish that are responsible for the changes in odour, flavour and texture of deteriorating fish.

The following table gives a short overview of the deterioration process that have undergone during the 7 days of storage at 0°C., 4°C, and 8°C storage temperature.

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Table 3 Sensory evaluation performed by the author during each experimental day.

Day of storage	characteristics	0°C	4°C	8°C
1	Appearance	Good	Good	Good
	Smell	Salmon smell	Salmon smell	Salmon smell
	Slim	Transparent	Transparent	Transparent
	Texture	Firm	Firm	Firm
2	Appe arance	Good	Good	Good
	Smell	Salmon smell	Salmon smell	Salmon smell
	Slim	Transparent	Transparent	Transparent
	Texture	Firm	Firm	Firm
3	Appearance	Good	Good	No freshness
	Smell	Salmon smell	Salmon smell	fermented smell
	Slim	Transparent	Transparent	Slightly turning to milky
	Texture	Firm	Firm	Less frim
5	Appearance	Good	No freshness	No freshness
	Smell	Salmon smell	fermented smell	Soury, fermented smell
	Slim	Transparent	Less transparent	Glassy and milky
	Texture	Firm	Less firm	Soft and bends
7	Appearance	Good	No freshness	Bad
	Smell	Salmon smell	fermented smell	Soury, fermented smell
	Slim	Less transparent	Slightly turning to milky	Glassy and milky
	Texture	Less firm	Soft and Bends	Soft and Bends

4.5 Enzyme analysis

Fish degradation is caused by enzymatic activities. The extracted enzyme were used to determine the amount of protein content, amino acid content, acid soluble peptides and proteolytic activities.

4.5.1 Protein content of the extract

After the enzyme extraction method performed, 30ml of enzyme extract was extracted from both the control and the treatment samples. The enzyme used to analysis the protein content, amino acid content, amount of acid soluble peptide and proteolytic activities. The lowry assay was used to determine the protein content of in the solution. This biochemical assay works by changing the color of the sample proportional to the protein concentration. It is based on the principle by the reaction of peptides with Cu+2 in the presence of alkaline condition and reduction of folin Ciocalteay reagent (Lowry et al., 1951). According to Figure 12 the protein content in the control and the LAB treatments changed during the storage time. Both treatments measured comparable amount of soluble proteins. The obtained (mean±SE) for SF1994 0°C, SF1994 4°C SF1994 8°C were 7.55± 0.21, 8,16.24 ± 0.46, and 8,67 ± 0.49 respectively. On the other hand the recorded (mean±SD) for the control treatments (control 0°C, control 4°C, control 8°C) were 7.63± 0.24, 8.22± 0.49, 9.97± 0.51 respectively.

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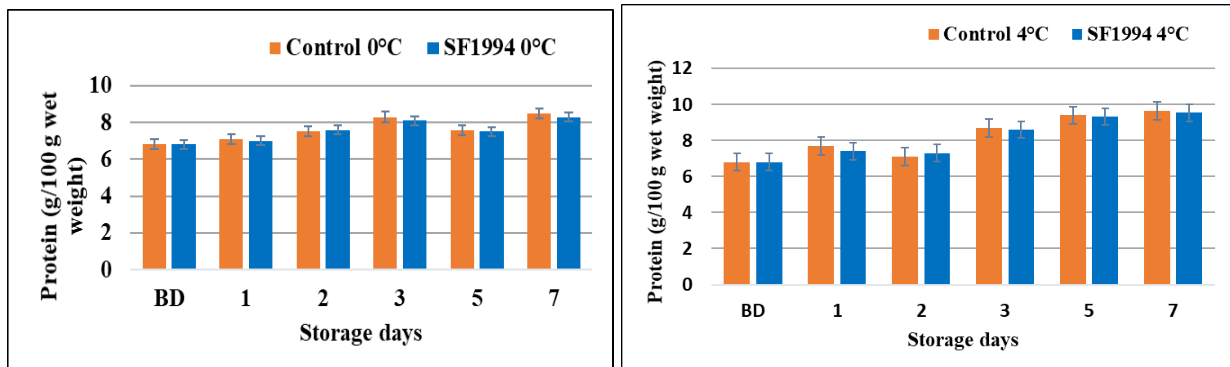


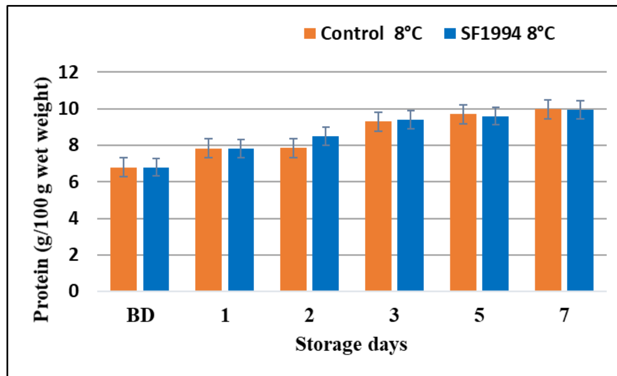


Figure 12 Amount of water soluble protein from different storage temperature as a function of storage time. BD indicates the amount of water soluble protein on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=3.

Figure 13 shows storage temperature has a significant effect on water soluble protein content of the LAB treatments. SF1994 0°C recorded the lowest total soluble protein content 7.55± 0.21 than SF1994 4°C (8,16.24 ± 0.46), SF1994 8°C (8,67 ± 0.49) (mean±SE) during the storage period. This result indicated proteins were stable at the lower temperature. Water soluble proteins are build up of amino acid that has hydrophilic (water soluble) side chain. Proteolytic activity can enhance the extractability of water soluble proteins and result to formation of small peptides. Stabilization of myofibril proteins associated with good fish quality (Rodriguez et al., 2006). Furthermore, (Deutscher, 1990) explain that Unfavorable conditions leads to protein denaturation, degradation or precipitation. Various factors have enormous impact on the stability of protein; change in temperature, hydrophobicity, pH, mechanical forces and enzymatic activity are the main once. Likewise, Dutson, (1983) reported that increased postmortem temperature rises the activity of enzymes that cause water soluble protein increament

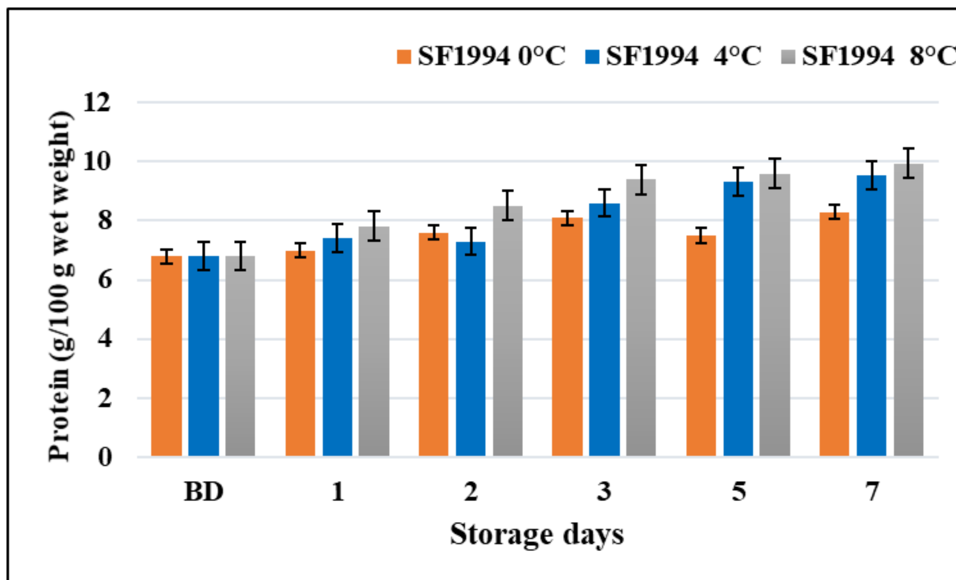


Figure 13 Change in water soluble proteins for the LAB treatments. BD indicates the amount of water soluble protein on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=3.

4.5.2 Acid soluble peptides

The precipitated protein by using TCA was used to determine the amount of acid soluble peptides. BSA was used as a standard compound and used to plot the standard curve. The absorbance result of the sample used to calculate the amount of acid soluble peptides. The result showed the amount of acid soluble peptides varies between LAB and control treatments. As expected for both treatments the amount of acid soluble peptides increased from the first storage day to the last storage day (figure 14). The reason for this could be the increment of degradation of protein through autolysis during the storage experiment. Fraser & Sumar, (1998) explained that autolysis of fish muscle proteins results formation of peptides and free amino acids. According to Lougovois & Kyrana, (2005) autolysis results various structural changes in the tissues. Thes structural changes in protein, lipids, nucleotides, carbohydrates and lipids derived compounds decrease the desirable flavors and odors related with fresh fish. Moreover, this creates favorable conditions for microbial growth and production of biogenic amines that affect the safety of the fish. This result matches with the microbial and sensory analysis findings.The highest microbial growth and low sensory quality was observed parallel with the increasing of storage days for both treatments.

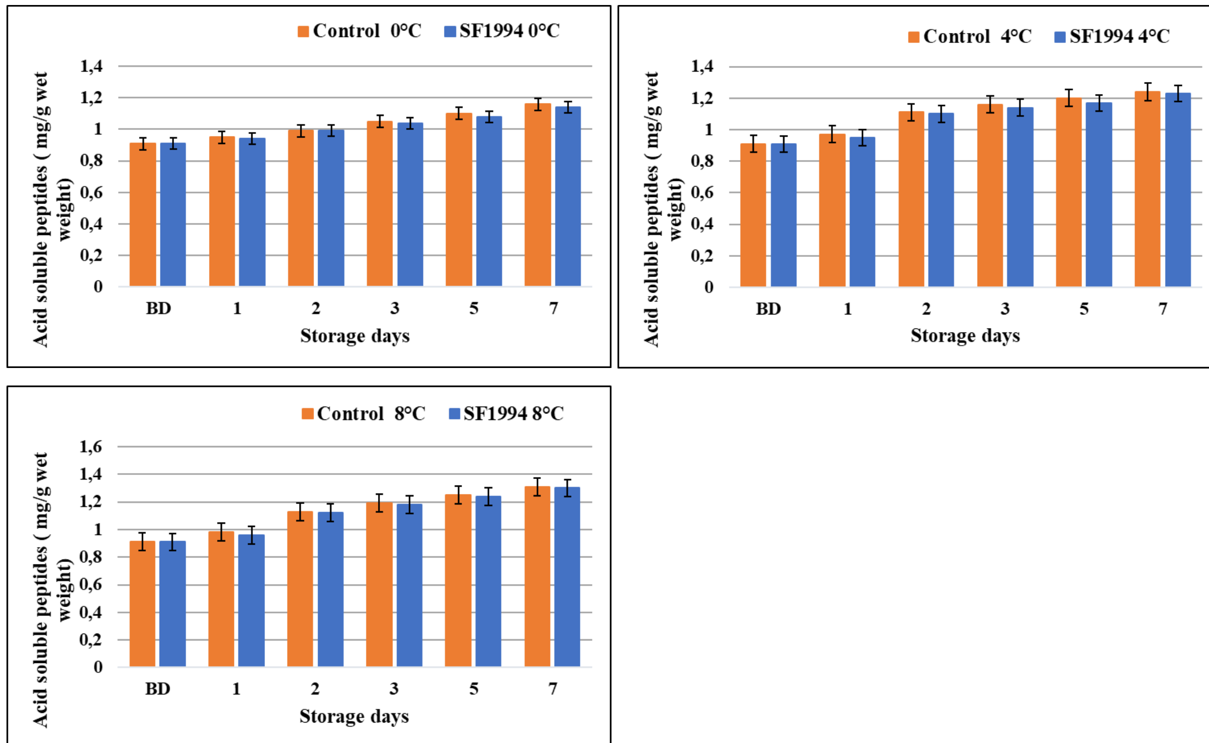


Figure 14 Amount of acid soluble peptides as a function of storage time. BD indicates the amount of acid soluble peptides on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=3.

In addition to this as it is indicated on figure 15 the amount of acid soluble peptides increased when storage temperature increased for the LAB treatments. Hence, SF1994 8°C recorded the highest amount of acid soluble peptides 1.11± 0.06 (mean±SE) than SF1994 0°C 1.01± 0.03 (mean±SE) and SF1994 4°C 1.08± 0.05 (mean±SE). The difference was statistically significant, the p value between SF1994 8°C and SF1994 0°C was 0.0006 and the p value between SF1994 8°C and SF1994 4°C was 0.001. The reason why the higher storage temperature (SF1994 8°C) recorded the highest amount of acid soluble peptides could be due to the protein content was most degraded at this temperature than SF1994 4°C and SF1994 0°C.

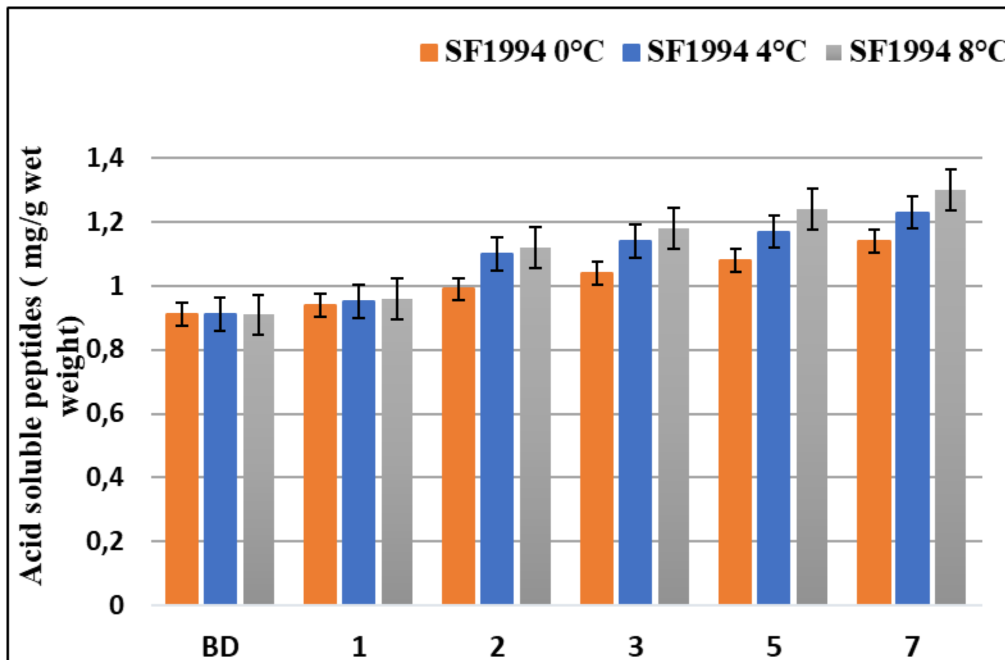


Figure 15 Change in amount of acid soluble peptides for the LAB treatments. BD indicates the amount of acid soluble peptides on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=3.

4.5.3 Determination of amount of free amino acids.

Samples were analysis by using high pressure liquid chromatography (HPLC). Different types of amino acids and their concentration was measured by this method. The result presented on figure 16 showed that the total free amino acid content of the extract was increased when storage time increased (from the first storage day to the last storage day) for both treatments. The reason for this result could be the increment of degradation of protein through autolysis during the storage experiment. Liston, (1990) explained that protein is one of the main food components of fish; its basic units are amino acids which are formed when protein is hydrolyzed by enzymes, alkalis or acids. Likewise, Duun & Rustad (2007) described the change in the amount of free amino acid is a good indication of the effect of proteolytic activity. Moreover, Hultmann, & Rustad, (2004) reported prolonged storage of salmon fillet increase the free amino acid content in the muscle. The most common FAA that are related to fish spoilage during storage are histidine, lysine, glutamine, phenylalanine, arginine and tyrosine. These FAA produced biogenic amine by decarboxylation (histamine, cadaverine, putrescine, phenylethylamine, agmatine and tyramine respectively). These product contribute the change in quality and toxicity (Onal, 2007; Yassoralipour, et al., 2013).

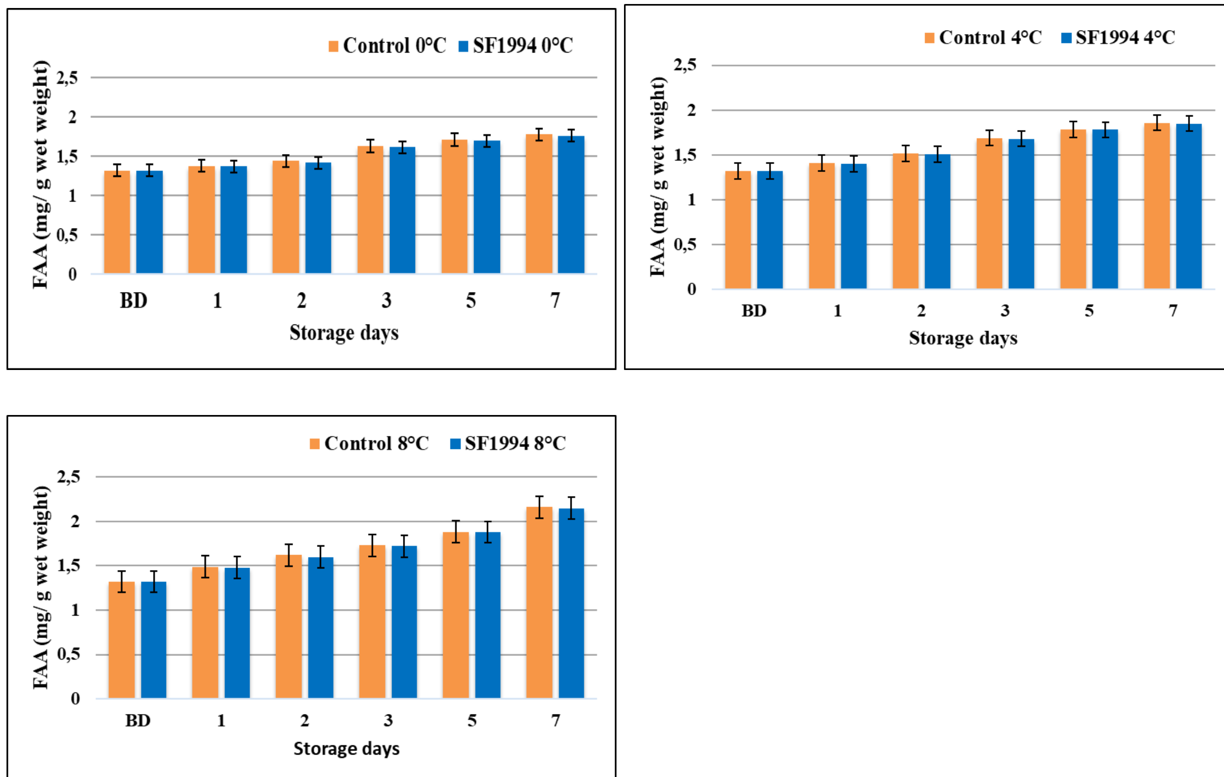


Figure 16 Amount of free amino acids as a function of storage time.BD indicates the amount of free amino acid on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=2.

Among the treatments of LAB SF1994 0°C showed low amount of total free amino acid 1.53± 0.07 (mean±SE) than SF1994 4°C 1.59±0.08 (mean±SE) and SF1994 8°C 2.15±0.1 (mean±SE). The difference was statistically significant. The p value between SF1994 0°C and SF1994 4°C was 0.12 and the p value between SF1994 0°C and SF1994 8°C was 0.03.

Moreover, the LAB treatments recorded variation in free amino acid content; SF1994 0°C recorded lower free amino acid in the samples compared to SF1994 4°C, SF1994 8°C. This result matches with the above protein content outcome, the low storage temperature showed significantly lowest degradation of protein than the higher storage temperatures.

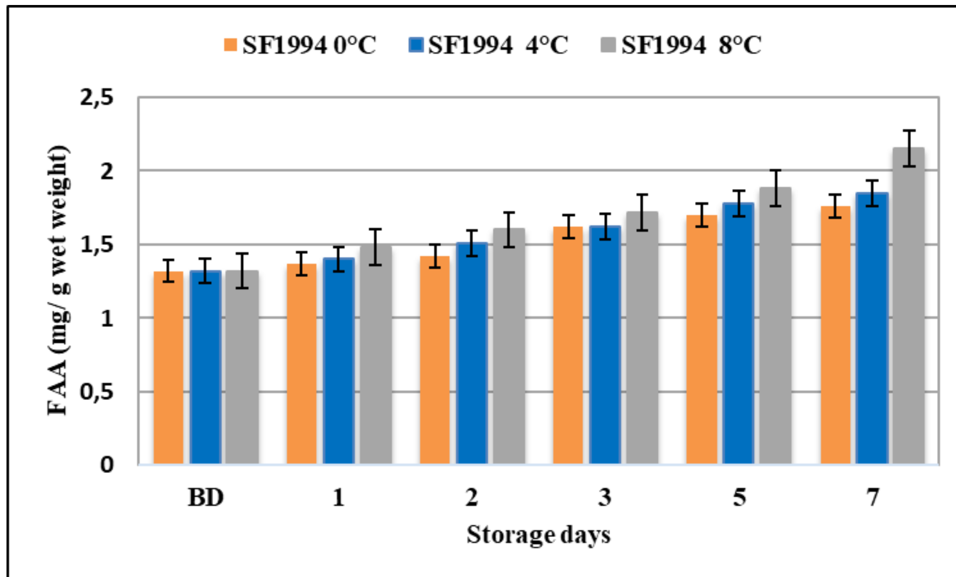


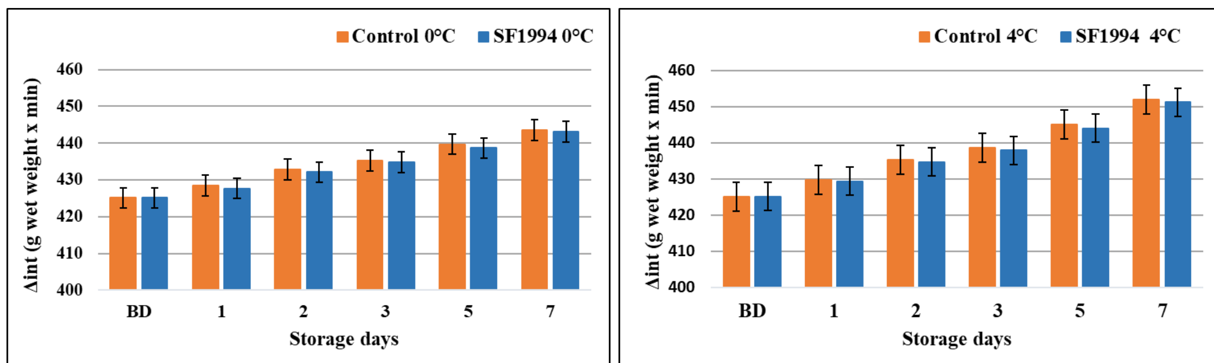
Figure 17 Change in amount of free amino acids for the LAB treatments. BD indicates the amount of free amino acid on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Standard error of the mean is shown as y-error bars. N=3.

4.5.4 Proteolytic activity

Muscle protein degradation increase when storage time increases. Lin and Park, (1996) discovered that 23.5 percent of protein degradation occurred with prolonged storage. The main reason for the degradation could be fish are highly susceptible to autolysis. According to Toldra & Flores, (2000) the term autolysis is a self-degradation process that involves postmortem chemical changes in animal tissues with the action of the enzymes (amylolytic, lipolytic and proteolytic) which are responsible for degradation of carbohydrates, fats and proteins into simpler form resulting in discoloration and muscle softening. Storage day and autolysis are directly proportional.

Figure 18 showed that the proteolytic activity (activity of cathepsins B+) was increased when storage time increased for the control and the LAB treatments. The increase in product (FAA and small peptides) formation in our previous result related with the increase in enzyme activity.Similarly, Gaarder, (2012) finding indicated the activity of cathepsins in postmortem muscle increases significantly with storage time. Furthemore, Bahuaud et al., (2008) discovered a significant correlation between enzymatic activity of cathepsins B and muscle softening in Atlantic salmon. These results are in agreement with Yamashita & Konagaya, (1991) that indicated high activity of cathepsins B in postmortem salmon muscles increased myofibrillar

proteolysis. In this study, the control treatment showed slightly higher enzymatic activity of cathepsins B compare to the LAB treatments. The sensory analysis confirmed the LAB treatments (especially samples from low storage temperature) recorded good sensory qualities (good transparent appearance and had less sour smell) than the control treatments. Moreover, the microbial analysis indicated the LAB treatments had lower growth spoilage microorganisms. Ertbjerg, et al., (1999). investigated the effect of low pH on the activity of lysosomal enzymes by injected lactic acid in the meat muscle. The researcher obtained change in pH during storage and the cathepsins B activity was influenced by the change. The enzyme was more active with the acidic pH range of 3-6 and high storage temperature. However, in our result we observe slightly lower cathepsins B+ activity in the LAB treatments than the control treatments. Most LAB strain capable to produce lactic acid. This could be due to lactic acid production ability of *carnobacteria* species. According to Vos, et al., (2011) some *carnobacteria* species are not strong acidifier, the primary antimicrobial product is their bacteriocins. The change in pH was not measured in this study. Based on our cathepsin B activity result and Vos, et al., (2011) findings the *carnobacteria* species might not lower the pH in the LAB treatments. The author suggest to include pH change for further studies.



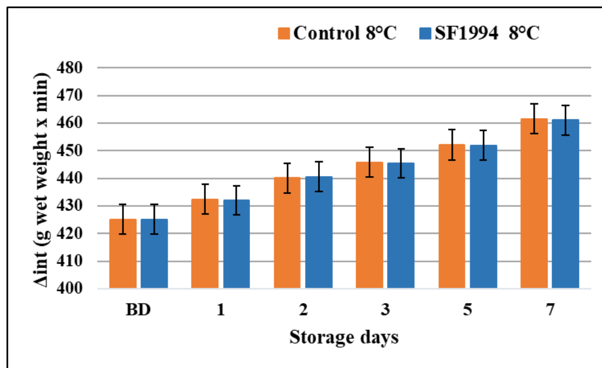


Figure 18 Cathepsin B+ activity as a function of storage time. BD indicates the activity of cathepsin B+ on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Values are given as increase in fluorescence intensity/(g wet weight x min). Standard error of the mean is shown as y-error bars. N=3.

Furthermore, Cathepsins B+ activities was significantly changed in the LAB treatments from the first day to the last storage day. The storage temperatures showed direct proportion with the enzyme activity. SF1994 8°C recorded highest 442.65± 4.87 (mean±SE) Cathepsin *B* activity than SF1994 4°C 43.03± 6.1 (mean±SE) with p= 0.001 and SF1994 0°C 433.58± 2.51 (mean±SE) with p= 0.0006. More information included in appendix (5&6).

Ertbjerg, et al., (1999) reported high temperature conditioning increased free lysosomal enzyme activity. Singh, & Benjakul, (2018) indicated that proteases in muscle tissue, such as cathepsins and calpains, hydrolyze myofibrillar proteins, mainly myosin heavy chain (MHC). Benjakul, et al., (1997) observed a marked degradation of MHC in Pacific whiting during iced storage for 8 days. Moreover, Ashie et al., (1996) reported most biochemical processes are slow down at a lower temperature. Our sensory evaluation confirmed the lower storage temperature showed better sensory quality.

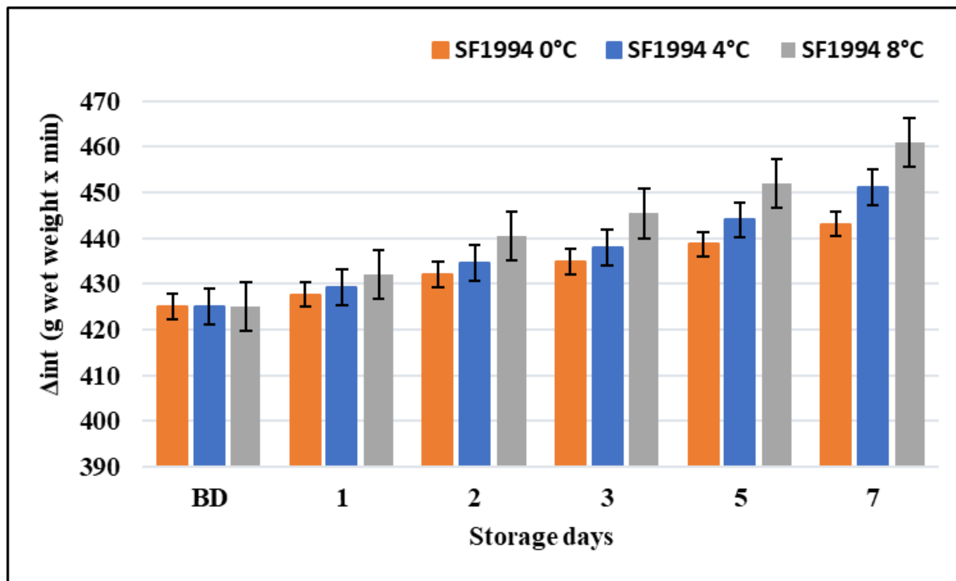


Figure 19 Cathepsin B+ activity of for the LAB treatments from different storage temperature as a function of storage time. BD indicates the activity of cathepsin B + on the raw material or before dipping, the numbers from 1-7 on the x-axis indicates the storage day of the sample. Values are given as increase in fluorescence intensity/(g wet weight x min). Standard error of the mean is shown as y-error bars. N=3.

5. Limitation of the study

The aim of this study is to investigate the effect temperature on bio-preservation of LAB. The study confirms low storage temperature increases the efficiency of LAB to control growth of spoilage microorganisms. Even though *Carnobacteria* showed a promising result at low temperature, deeper investigation is needed to ensure that *Carnobacterium* species do not themselves cause unforeseen problems. According to food safety regulation, LAB should not produce toxins compounds such as biogenic amines (histamine, tyramine) and should not have enterotoxic activity (EFSA, 2007). However, Leisner et al., (2007) report that *Carnobacterium maltaromaticum* produce tyramine. When the concentration of tyramine become higher than 100–800 mg/kg, it can cause headaches and hypertensive (Ten et al., 1990; Wiernasz, et al., 2017). It is important to identify which species of *Carnobacteria* are associated with the production of tyramine. Furthermore, Wessels et al., (2004) reported that no adverse health problem reported so far due to consumption of LAB added into food products.

6. Relevance of the study

In Earlier studies the importance of LAB to increase the shelf life of food have been researched in detail. Hwanhlem & Aran, (2015); Sidira et al., (2014) reported bo-preservation with LAB is a natural and powerful tool used to extend the shelf life and improve the safety of foods. This study confirms LAB significantly prolong the shelf life of Atlantic salmon by decreasing the growth of spoilage microbes and slow down enzyme activities. Furthermore, in previous studies very few papers have been published on the factors that can affect the bio-preservation ability of LAB. This paper gives information how temperature can affect LAB bio-preservation capacity. The findings of this result benefits to improve the efficient of bio-preservation method during food preservation.

7. Conclusion

Growth of *Carnobacterium* showed variation with different incubation temperature. The growth was increased up to the optimal temperature. The microbial study confirmed that the LAB strain reduced the growth of spoilage microorganisms. Moreover, Storage temperature showed a significant effect on microbial inhibition of *Carnobacterium*. Lower storage temperature measured higher microbial inhibition of *Carnobacterium* and lower growth of spoilage microorganisms and SSOs.

The enzyme activity study confirmed that the activity of cathepsin B was significantly affected by storage temperature. The highest storage temperature (8°C) recorded the highest enzyme activity. Similarly, highest soluble protein, high amount of free amino acid and acid soluble peptides obtained at this storage temperature. On the other hand 0°C recorded the lowest cathepsin B activity, soluble protein, free amino acid and acid soluble peptides.

The above results supported by the sensory analysis. The evaluation revealed samples at the highest temperature (8°C) showed low sensory quality (sour smell, less firm and Slightly turning to milky) during the last experimental day. This low sensory quality is expected with highest growth of spoilage microbes and highest enzyme activity at this storage temperature.

8. Recommendation

Suggested recommendations for future study based on the above conclusion are:

- ✓ This study was investigated growth of general spoilage microbes, therefore, further studies recommended on identification of the microorganisms and the effect of LAB on the identified species as a function of different storage temperature.
- ✓ The enzyme activity study was performed based on cathepsin B activities. Further investigations recommended on the activity of other proteolysis enzymes.

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10. Reference

Abbas, M. E., Luo, W., Zhu, L., Zou, J., & Tang, H. (2010). Fluorometric determination of hydrogen peroxide in milk by using a Fenton reaction system. *Food chemistry*, *120*(1), 327-331.

Ashie, I. N. A., Simpson, B. K., & Smith, J. P. (1996). Mechanisms for controlling enzymatic reactions in foods. *Critical Reviews in Food Science & Nutrition*, *36*(1-2), 1-30.

Ananthanarayanan, P. N. (2013). *Basic refrigeration and air conditioning*. Tata McGraw-Hill Education.

AOAC, 2000. Official Methods of Analysis. 17th Edn., Association of Official Analytical Chemists. Washington D.C

Aoki, T., & Ueno, R. (1997). Involvement of cathepsins B and L in the post-mortem autolysis of mackerel muscle. *Food Research International*, *30*(8), 585-591.

Ashie, I. N. A., Smith, J. P., Simpson, B. K., & Haard, N. F. (1996). Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science & Nutrition*, *36*(1-2), 87-121.

Ammor, S., Tauveron, G., Dufour, E., & Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1—Screening and characterization of the antibacterial compounds. *Food control*, *17*(6), 454-461.

Bahuaud, D., Mørkøre, T., Langsrud, Ø., Sinnes, K., Veiseth, E., Ofstad, R., & Thomassen, M. S. (2008). Effects of− 1.5 C super-chilling on quality of Atlantic salmon (Salmo salar) pre-rigor fillets: Cathepsin activity, muscle histology, texture and liquid leakage. *Food chemistry*, *111*(2), 329-339.

Barrett, A. J., & Kirschke, H. (1981). [41] Cathepsin B, cathepsin H, and cathepsin L. In *Methods in enzymology* (Vol. 80, pp. 535-561). Academic Press.

Benjakul, et al., (1997) recorded a marked degradation of MHC in Pacific whiting during iced storage for 8 days.

Bendifallah, N., Rasmussen, F. W., Zachar, V., Ebbesen, P., Nielsen, P. E., & Koppelhus, U. (2006). Evaluation of cell-penetrating peptides (CPPs) as vehicles for intracellular delivery of antisense peptide nucleic acid (PNA). *Bioconjugate chemistry*, *17*(3), 750-758.

Brillet, A., Pilet, M. F., Prevost, H., Bouttefroy, A., & Leroi, F. (2004). Biodiversity of Listeria monocytogenes sensitivity to bacteriocin-producing Carnobacterium strains and application in sterile cold-smoked salmon. *Journal of applied microbiology*, *97*(5), 1029-1037.

Brillet-Viel, A., Pilet, M. F., Courcoux, P., Prévost, H., & Leroi, F. (2016). Optimization of Growth and Bacteriocin Activity of the Food Bioprotective Carnobacterium divergens V41 in an Animal Origin Protein Free Medium. *Frontiers in Marine Science*, *3*, 128.

Buchanan, r. L., & klawitter, l. A. (1991). Characterization of a lactic acid bacterium, carnobacterium piscicola lk5, with activity against listeria monocytogenes at refrigeration temperatures 1. *Journal of food safety*, *12*(3), 199-217.

Bruno, M. E. C. & Montville, T. J. (1993). Common mechanism of bacteriocins from lactic acid bacteria. Appl. Environ. Microbial., 59, 3003-10.

Benjakul, S., Seymour, T. A., Morrissey, M. T., & AN, H. (1997). Physicochemical changes in Pacific whiting muscle proteins during iced storage. *Journal of Food science*, *62*(4), 729-733.

Carr, F. J., Chill, D., & Maida, N. (2002). The lactic acid bacteria: a literature survey. *Critical reviews in microbiology*, *28*(4), 281-370.

Cruz R, Cunha SC, Casal S (2015) Brominated flame retardants and seafood safety: a review. Environ Int 77:116–131. doi:10.1016/j.envint.2015.01.001

Daeschel, M. A. (1993). Applications and interactions of bacteriocins from lactic acid bacteria in foods and beverages. In Bacteriocin of Lactic Acid Bacteria, ed., D. G. Hoover & L. R. Steenson. Academic Press, Inc., New York, U.S.A. Chapter 4. p. 63- 92.

Delgado, A., Brito, D., Peres, C., Noe-Arroyo, F., & Garrido-Fernández, A. (2005). Bacteriocin production by Lactobacillus pentosus B96 can be expressed as a function of temperature and NaCl concentration. *Food Microbiology*, *22*(6), 521-528.

Deutscher, M. P. (1990). [8] Maintaining protein stability. In *Methods in enzymology* (Vol. 182, pp. 83-89). Academic Press.

Dutson, t. R. (1983). Relationship of pH and temperature to disruption of specific muscle proteins and activity of lysosomal proteases. *Journal of Food Biochemistry*, *7*(4), 223-245.

Duun, A. S., & Rustad, T. (2007). Quality changes during superchilled storage of cod (Gadus morhua) fillets. *Food Chemistry*, *105*(3), 1067-1075.

Ertbjerg, P., Larsen, L. M., & Moøller, A. J. (1999). Effect of prerigor lactic acid treatment on lysosomal enzyme release in bovine muscle. *Journal of the Science of Food and Agriculture*, *79*(1), 95-100.

Fraser, O., & Sumar, S. (1998). Compositional changes and spoilage in fish-an introduction. *Nutrition & Food Science*, *98*(5), 275-279.

Gaarder, M. O., Bahuaud, D., Veiseth-Kent, E., Morkore, T., & Thomassen, M. S. (2012). Relevance of calpain and calpastatin activity for texture in super-chilled and ice-stored Atlantic salmon (Salmo salar L.) fillets. *Food Chemistry*, *132*(1), 9-17.

Ga lvez A, Abriouel H, Lopez RL, Ben Omar N (2007) Bacteriocin-based strategies for food biopreservation. Int J Food Microbiol 120:51–70. doi:10.1016/j.ijfoodmicro.2007.06.001

Gancel, F., Dzierszinski, F., & Tailliez, R. (1997). Identification and characterization of Lactobacillus species isolated from fillets of vacuum-packed smoked and salted herring (Clupea harengus). *Journal of applied microbiology*, *82*(6), 722-728.

Ghanbari M, Jami M, Domig KJ, Kneifel W (2013) Seafood biopreservation by lactic acid bacteria—a review. LWT Food Sci Technol 54:315–324. doi:10.1016/j.lwt.2013.05.039

Gram, L., Trolle, G., & Huss, H. H. (1987). Detection of specific spoilage bacteria from fish stored at low (0 C) and high (20 C) temperatures. *International journal of food microbiology*, *4*(1), 65-72.

Gram, L., & Huss, H. H. (1996). Microbiological spoilage of fish and fish products. *International journal of food microbiology*, *33*(1), 121-137.

Gram, L., & Dalgaard, P. (2002). Fish spoilage bacteria–problems and solutions. *Current opinion in biotechnology*, *13*(3), 262-266.

Gomez-Lopez, V. M. (Ed.). (2012). *Decontamination of fresh and minimally processed produce*. John Wiley & Sons.

Helander, I., Von Wright, A., & Mattila-Sandholm, T. M. (1997). Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. *Trends in Food Science & Technology*, *8*(5), 146-150.

Hoyle, N. T., & Merrltt, J. H. (1994). Quality of fish protein hydrolysates from herring (Clupea harengus). *Journal of food Science*, *59*(1), 76-79.

Hultmann, L., & Rustad, T. (2004). Iced storage of Atlantic salmon (Salmo salar)–effects on endogenous enzymes and their impact on muscle proteins and texture. *Food Chemistry*, *87*(1),31-41.

Hwanhlem, N., & Aran, H. (2015). Biopreservation of Seafood by Using Bacteriocins and Bacteriocinogenic Lactic Acid Bacteria as Potential Bio-control Agents. In *Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas* (pp. 183-213). Springer, Cham.

Jorgensen, B. R., Gibson, D. M., & Huss, H. H. (1988). Microbiological quality and shelf life prediction of chilled fish. *International Journal of Food Microbiology*, *6*(4), 295-307.

Kilcast, D., & Subramaniam, P. (Eds.). (2011). *Food and beverage stability and shelf life*. Elsevier.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. Biochimie., 70, 33749.

Klomklao, S. (2008). Digestive proteinases from marine organisms and their applications. *Songklanakarin Journal of Science & Technology*, *30*(1).

Leisner, J. J., Laursen, B. G., Prévost, H., Drider, D., & Dalgaard, P. (2007). Carnobacterium: positive and negative effects in the environment and in foods. *FEMS microbiology reviews*, *31*(5), 592-613.

Liston, J. (1990). Microbial hazards of seafood consumption. *Food Technology, Chicago*.

Lougovois, V. P., & Kyrana, V. R. (2005). Freshness quality and spoilage of chill-stored fish. *Food policy, control and research*, *1*, 35-86.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*, 265-275.

Luck, E., & Jager, M. (1997). Antimicrobial action of preservatives. In *Antimicrobial Food Additives* (pp. 36-57). Springer, Berlin, Heidelberg.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., ... & Shakhova, V. (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences*, *103*(42), 15611-15616.

Mandal V, Sen SK, Mandal NC (2011) Isolation and characterization of pediocin NV 5 producing Pediococcus acidilactici LAB 5 from vacuum-packed fermented meat product. Indian J Microbiol 51:22–29. doi:10.1007/s12088-011-0070-0

Mizan MFR, Jahid IK, Ha S-D (2015) Microbial biofilms in seafood: a food-hygiene challenge. Food Microbiol 49:41–55. doi:10.1016/j.fm.2015.01.009

Nonthaput, T., Hahor, W., Thongprajukaew, K., Yoonram, K., & Rodjaroen, S. (2017). Cathepsin activities and thermal properties of Nile tilapia (Oreochromis niloticus) meat during ambient storage. *Agriculture and Natural Resources*, *51*(3), 206-211.

Nychas, G. J., & Drosinos, E. H. (2009). Detection of fish spoilage. *Handbook of seafood and seafood products analysis*, 537-555.

Olaoye, O. A., & Onilude, A. A. (2011). Quantitative estimation of antimicrobials produced by Lactic Acid Bacteria isolated from Nigerian beef. *International Food Research Journal*, *18*(3).

Ohenhen, R. E., Isibor, J. O., Emonfonmwan, G., & Enabulele, S. A. (2015). Effects of PH and Storage Temperatures on Antibacterial Activity of Bacteriocin Produced by Lactic Acid Bacteria Isolated from OGI. *British Microbiology Research Journal*, *9*(3), 1-9.

Onal, A. (2007). A review: Current analytical methods for the determination of biogenic amines in foods. *Food chemistry*, *103*(4), 1475-1486.

Osnes, K. K., & Mohr, V. (1985). Peptide hydrolases of Antartic krill, Euphausia superba. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *82*(4), 599-606.

Ouwehand, A.C., 1998. Antimicrobial components from lactic acid bacteria. In: Salminen, S. & von Wright, A. (eds.) Lactic Acid Bacteria: Microbiology and Functional Aspects 2nd Edition. New York: Marcel Dekker Inc., 139-160

Pikuta, E. V., Marsic, D., Bej, A., Tang, J., Krader, P., & Hoover, R. B. (2005). Carnobacterium pleistocenium sp. nov., a novel psychrotolerant, facultative anaerobe isolated from permafrost of the Fox Tunnel in Alaska. *International journal of systematic and evolutionary microbiology*, *55*(1), 473-478.

Pilet, M. F., & Leroi, F. (2011). Applications of protective cultures, bacteriocins and bacteriophages in fresh seafood and seafood products. In *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation* (pp. 324-347).

Qin, H., Gong, S. S., Ge, X. Y., & Zhang, W. G. (2012). The effect of temperature on L-lactic acid production and metabolite distribution of Lactobacillus casei. *Preparative Biochemistry and Biotechnology*, *42*(6), 564-573.

Ratkowsky, D. A., Olley, J., McMeekin, T. A., & Ball, A. (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology*, *149*(1), 1-5.

Rawat, S. (2015). Food Spoilage: Microorganisms and their prevention. *Asian Journal of Plant Science and Research*, *5*(4), 47-56.

Rattanachaikunsopon, P., & Phumkhachorn, P. (2010). Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Ann Biol Res*, *1*(4), 218-228.

Robertson, P. A. W., O'Dowd, C., Burrells, C., Williams, P., & Austin, B. (2000). Use of Carnobacterium sp. as a probiotic for Atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss, Walbaum). *Aquaculture*, *185*(3-4), 235-243.

Rodriguez, O, Barros-Velázquez, J., Piñeiro, C., Gallardo, J. M., & Aubourg, S. P. (2006). Effects of storage in slurry ice on the microbial, chemical and sensory quality and on the shelf life of farmed turbot (Psetta maxima). Food Chemistry, 95(2), 270-278.

Sanlıbaba, P., & Güçer, Y. (2015). Antimicrobial activity of lactic acid bacteria. *J. Int. Sci. Publ*, *3*, 451-457.

Schillinger, U., Stiles, M. E., & Holzapfel, W. H. (1993). Bacteriocin production by Carnobacterium piscicola LV 61. *International journal of food microbiology*, *20*(3), 131-147.

Serio, A., Fusella, G. C., López, C. C., Sacchetti, G., & Paparella, A. (2014). A survey on bacteria isolated as hydrogen sulfide-producers from marine fish. *Food Control*, *39*, 111-118.

Sidira M, Galanis A, Nikolaou A, Kanellaki M, Kourkoutas Y (2014) Evaluation of Lactobacillus casei ATCC 393 protective effect against spoilage of probiotic dry-

Sikorski, Z. E., & Kołakowski, E. (2000). Endogenous Enzyme Activity and Seafood Quality: Influence of Chilling, Freezing, and Other. *Seafood enzymes: Utilization and influence on postharvest seafood quality*, 451.

Sivertsvik, M., Rosnes, J. T., & Kleiberg, G. H. (2003). Effect of modified atmosphere packaging and superchilled storage on the microbial and sensory quality of Atlantic salmon (Salmo salar) fillets. *Journal of food science*, *68*(4), 1467-1472.

Sternlicht, M. D., & Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annual review of cell and developmental biology*, *17*(1), 463-516.

Sumathi, V., & Reetha, D. (2012). Effect of storage time and temperature for maximum bacteriocin production by lactic acid bacteria. *IJPBA*, *3*, 831-834.

Tahiri, I., Desbiens, M., Benech, R., Kheadr, E., Lacroix, C., Thibault, S., ... & Fliss, I. (2004). Purification, characterization and amino acid sequencing of divergicin M35: a novel class IIa bacteriocin produced by Carnobacterium divergens M35. *International journal of food microbiology*, *97*(2), 123-136.

Taleghani, H. G., Najafpour, G. D., & Ghoreyshi, A. A. (2016). A study on the effect of parameters on lactic acid production from whey. *Polish Journal of Chemical Technology*, *18*(1), 58-63.

Taylor, R. G., Fjaera, S. O., & Skjervold, P. O. (2002). Salmon fillet texture is determined by myofiber-myofiber and myofiber-myocommata attachment. *Journal of Food Science*, *67*(6), 2067-2071.

Ten Brink, B., Damink, C., Joosten, H. M. L. J., & In't Veld, J. H. (1990). Occurrence and formation of biologically active amines in foods. *International journal of food microbiology*, *11*(1), 73-84.

Thomas, L.V., Clarkson, M.R, Delves-Broughton, J., 2000. Nisin. In: Naidu, A.S. (Ed.), Natural food antimicrobial systems. CRC Press, Boca-Raton, FL, pp. 463–524.

fermented sausages. Food Control 42:315–320. doi:10.1016/j.foodcont.2014.02.024.

Uauy, R., Hoffman, D.R., Peirano, P., Birch, D.G., Birch, E.E., 2001. Essential fatty acids in visual and brain development. Lipids 36, 885– 895.

Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., ... & Whitman, W. B. (Eds.). (2011). *Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes* (Vol. 3). Springer Science & Business Media.

Wheaton, F. W., & Lawson, T. B. (1985). *Processing aquatic food products*. Wiley.

Wiernasz, N., Cornet, J., Cardinal, M., Pilet, M. F., Passerini, D., & Leroi, F. (2017). Lactic acid bacteria selection for biopreservation as a part of hurdle technology approach applied on seafood. *Frontiers in Marine Science*, *4*, 119.

Yamashita M., Konagaya S. Hydrolytic action of salmon cathepsin B and L to muscle structural proteins in respect of muscle softening. Nippon Suisan Gakkaishi. 1991;57:1917–1922. doi: 10.2331/suisan.57.1917.

Yassoralipour, A., Bakar, J., Rahman, R. A., Bakar, F. A., & Golkhandan, E. (2013). Effect of different temperatures on the free amino acids, physico-chemical and microbial changes during storage of Barramundi (Lates calcarifer) fillets. *Adv. J. Food Sci. Technol*, *5*, 822-828.

York, R. K., & Sereda, L. M. (1994). Sensory assessment of quality in fish and seafoods. In *Seafoods: Chemistry, Processing Technology and Quality* (pp. 233-262). Springer, Boston, MA.

Zalán, Z., Németh, E., Baráth, Á., & Halász, A. (2005). Influence of growth medium on hydrogen peroxide and bacteriocin production of Lactobacillus strains. *Food Technology and Biotechnology*, *43*(3), 219-225.

Zayas, J. F. (1997). Solubility of proteins. In *Functionality of proteins in food* (pp. 6-75). Springer, Berlin, Heidelberg.

11. Appendix

Appendix 1. Composition and preparation of different growth media.

NAP Agar

Pepton ……………………………………………………………………………………...10 g

Peptonized milk…………………………………………………………...…...……….. ..10g g

Yeast Extract ………………………………………………………………..…..……..... 10.00 g

Glucose ………………………………..…………………………………………..…....7.5 g

Beef Extract………………………………………………..…………………….…..….. ..2.5g

Yeast Extract ……………………………………………..………………..……….... 10.00 g

Magnesium sulphate (Mgso$_4$ 7H2O) …………………………………..…………..….. .0.575 g

Manganese sulphate (Mnso$_4$ 4H2O) …………………….………………..………....….. .0.05 g

Tween 80 ……………………………….…………………………………….………..….... 1.0g

Agar – agar ……………………………………..………………………….…………………15.0g

Distilled water (to make up)……………………….……………………………….………… 970ml

All the above ingredients dissolved in 9700 ml of distilled water. Adjusted to pH= 6.1 mixed. The solution was sterilized at, 121 ^{o}C for 15 min. After cooling the media the following antibiotics were added: 6% sodium nitrite (1ml), 0.1% Cycloheximide (1ml). 0.03% Polymyxin (1ml).

Long & Hammer Agar

Proteose- Peptone ……………………………………………………………………....10.00 g

Sodium chloride (NaCL)) ……………………………………..………...................... 10.00 g

Gelatin) ………………………..…………………………………………………..….. .10.00 g

Potassium hydrogen phosphate (K$_2$2HPO$_4$)……………………….……………………….. ..0.5 g

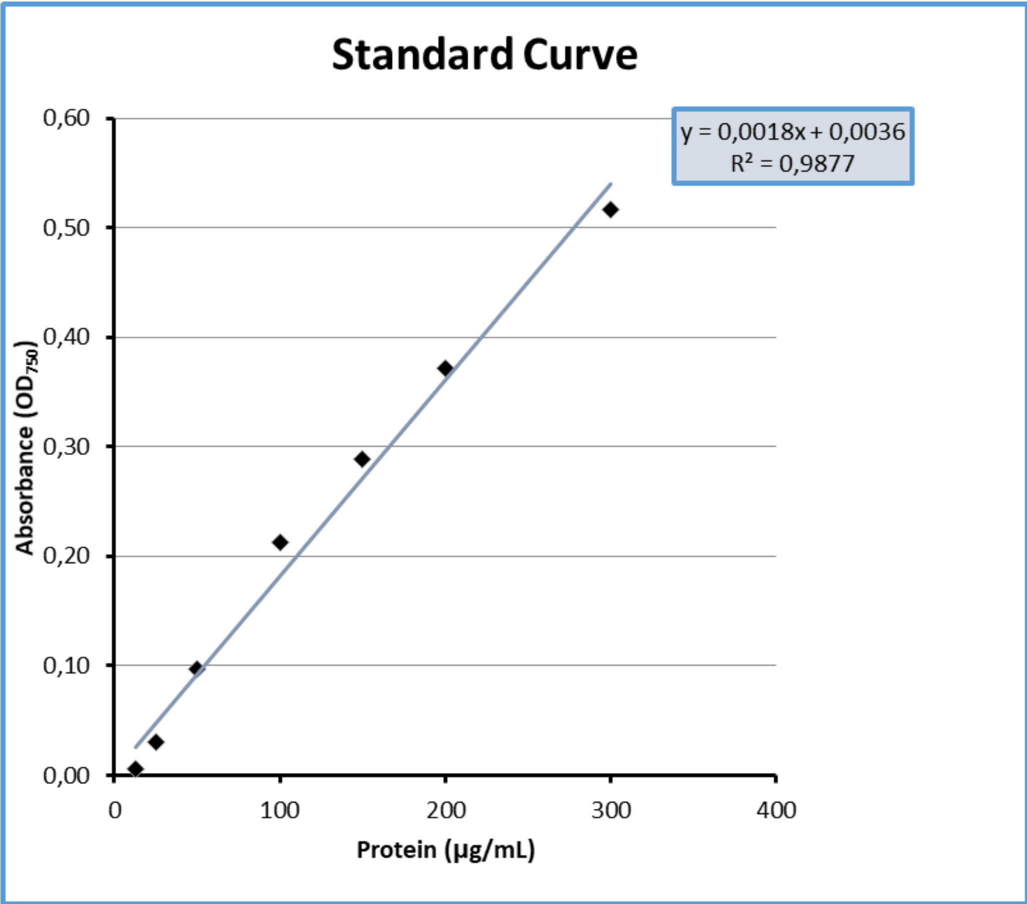
Ammonium Ferric Citrate (NH4)$_5$, (Fe(C$_6$H$_4$O$_7$) $_2$)) …………….…………………..…..... 0.25g

Agar – agar ……………………………………………………….…………………….……15.0g

Distilled water (to make up)…………………………………………………………………. 1000ml

All the above ingredients dissolved in 1000 ml of distilled water. The solution were sterilized at, 121 ^{o}C for 15 min.

Appendix 2 Standard Curve for triplet of the samples. Procedure followed by Lowry method with BSA as stock protein to build standard curve.



Appendix 3 Statistical analysis output for acid soluble peptides between SF1994 8°C and SF1994 0°C

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,978502
R Square	0,957466
Adjusted R Square	0,946833
Standard Error	0,035734
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0,114976	0,114976	90,04292	0,000688
Residual	4	0,005108	0,001277		
Total	5	0,120083			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0,65634	0,187591	-3,4988	0,024923	-1,17718	-0,13551	-1,17718	-0,13551
SF1994 0°C	1,745583	0,183957	9,489095	0,000688	1,234837	2,256329	1,234837	2,256329

Appendix 4 Statistical analysis output for acid soluble peptides between SF1994 8°C and SF1994 4°C

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,997106
R Square	0,99422
Adjusted R Square	0,992775
Standard Error	0,013173
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*

Regression	1	0,119389	0,119389	688,0568	1,26E-05
Residual	4	0,000694	0,000174		
Total	5	0,120083			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0,20234	0,050635	-3,99616	0,016182	-0,34293	-0,06176	-0,34293	-0,06176
SF1994 4°C	1,219087	0,046475	26,23084	1,26E-05	1,090051	1,348123	1,090051	1,348123

Appendix 5 Statistical analysis output for cathepsin B+ activity between SF1994 8°C and SF1994 0°C

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998289
R Square	0,99658
Adjusted R Square	0,995725
Standard Error	0,855451
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	852,9678	852,9678	1165,58	4,39E-06
Residual	4	2,927189	0,731797		
Total	5	855,895			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-397,812	24,62015	-16,158	8,58E-05	-466,168	-329,455	-466,168	-329,455
SF1994 0°C	1,938409	0,056777	34,14058	4,39E-06	1,78077	2,096048	1,78077	2,096048

Appendix 6 Statistical analysis output for cathepsin B+ activity between SF1994 0°C and SF1994 8°C

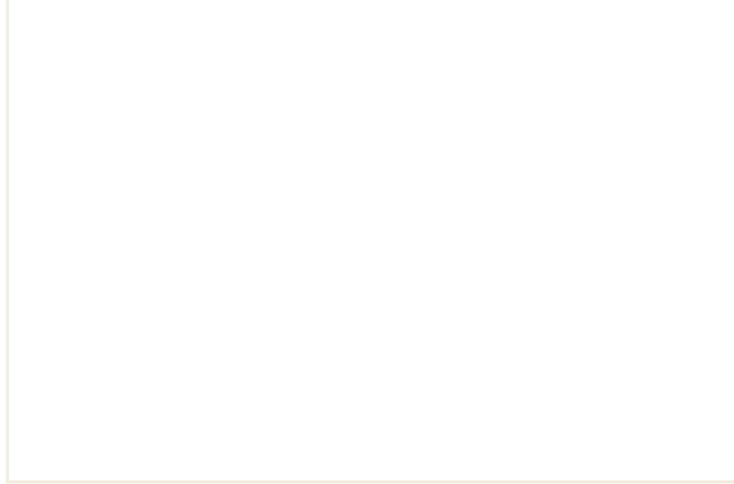
SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,996392
R Square	0,992796
Adjusted R Square	0,990995
Standard Error	1,241533
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	849,7294	849,7294	551,2693	1,95E-05
Residual	4	6,165621	1,541405		
Total	5	855,895			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-152,87	25,36885	-6,02588	0,003822	-223,305	-82,4344	-223,305	-82,4344
SF1994 4°C	1,362641	0,058036	23,47912	1,95E-05	1,201507	1,523776	1,201507	1,523776



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