Master's thesis

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The effect of natural antioxidants in combination of modified atmospheric packaging to prolong shelf life and enhance quality of Atlantic salmon

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Abstract

A growing market for fresh aquaculture fish is emerging since fish can provide several important nutrients to the human diet, including omega-3 fatty acids. Despite its high susceptibility to biochemical and microbial spoilage, the use of this product is limited. In earlier studies, modified atmosphere packaging (MAP), antioxidant addition and temperature control have been shown to extend fresh fish's shelf life. This study was aimed at investigating the combination of natural antioxidants, MAP and temperature control to prolong the shelf life of Atlantic salmon.

Antioxidant solution obtained from natural sources (Holy basil, *Ocimum sanctum*) were prepared to use them in packaging methods for salmon. The study was conducted in two storage experiment setups lasting for 16 days. The first storage experiment was combined with MAP packaging (CO_2:N_2 60:40) in which one sample was treated with antioxidant and other without antioxidant. Several quality changes during storage have been analyzed, including drip loss, microbial growth, and measurement of primary and secondary oxidation products by PV. The second experiment setup for experiment was performed using vacuum packaging method while treating one sample with antioxidant and other without it. Same analyzing methods were used to determine the quality changes during storage.

Samples treated with Holy basil (*Ocimum sanctum*) in combination with MAP packaging showed lower microbial growth activity as compared to the other methods used. On the other hand, the sample treated with holy basil in combination of vacuum packaging was the second significant method which helped to slow down microbial growth after MAP with antioxidant packaging. All other samples which were not treated with any antioxidant showed less resistance towards increase of shelf life despite using the MAP and vacuum packaging. There was a difference between the results obtained from MAP packaging and vacuum packaging as MAP showed little bit better results towards slowing down the microbial growth.

In conclusion, the results of this study demonstrate that temperature affects microbiota growth on Atlantic salmon, and that MAP keeps the quality. Neither lipid oxidation nor drip loss was affected by antioxidant treatment.

Sammendrag

En økende etterspørsel for fersk oppdrettsfisk vokser frem siden fisk kan gi flere viktige næringsstoffer til menneskets kosthold, inkludert omega-3 fettsyrer. Til tross for dets høye følsomhet for biokjemisk og mikrobiell ødeleggelse, er bruken av dette produktet begrenset. I tidligere studier har modifisert atmosfære-pakning (MAP), antioksidanttilsetning og temperaturkontroll vist seg å forlenge fersk fisks holdbarhet. Denne studien var rettet mot å undersøke kombinasjonen av naturlige antioksidanter, MAP og temperaturkontroll for å forlenge holdbarheten til atlantisk laks.

Antioksidantløsning hentet fra naturlige kilder (hellig basilikum, *Ocimum sanctum*) ble tilberedt for å bruke dem i pakkingsmetoder for laks. Studien ble utført i to lagringseksperimentoppsett som varte i 16 dager. Det første lagringsforsøket ble kombinert med MAP-emballasje (CO_2:N_2 60:40) hvor en prøve ble behandlet med antioksidant og en annen uten antioksidant. Flere kvalitetsendringer under lagring er analysert, inkludert drypptap, mikrobiell vekst og måling av primære og sekundære oksidasjonsprodukter med PV. Det andre eksperimentoppsettet av eksperimentet ble utført ved bruk av vakuumpakkingsmetode mens en prøve ble behandlet med antioksidant og en annen uten. Samme analysemetoder ble brukt for å bestemme kvalitetsendringene under lagring.

Prøver behandlet med hellig basilikum (*Ocimum sanctum*) i kombinasjon med MAP-emballasje viste signifikant økt holdbarhet sammenlignet med de øvrige metodene som ble brukt. Derimot var prøven behandlet med hellig basilikum i kombinasjon med vakuumemballasje som en annen signifikant metode der økt holdbarhet ble bevist etter MAP med antioksidantemballasje. Alle andre prøver som ikke ble behandlet med noen antioksidant viste mindre motstand mot økt holdbarhet til tross for bruk av MAP og vakuumemballasje. Det var en forskjell mellom resultatene oppnådd fra MAP-emballasje og vakuumpakning, da MAP viste noe bedre resultater mot forlengelsen av holdbarheten.

Avslutningsvis viser resultatene fra denne studien at temperaturen påvirker mikrobiotaveksten på atlantisk laks, og at MAP holder kvaliteten. Verken lipidoksidasjon eller drypptap ble påvirket av antioksidantbehandling.

Abbreviations

MAP	Modified atmosphere packaging
PV	Peroxide value
TBARS	Thiobarbituric acid reactive substances
SSO	Specific spoilage organism
FRS	Free radical scavenger
TMAO	Trimethylamine oxide
TMA	Trimethylamine
CFU	Colony forming bacteria
PUFA	Polyunsaturated fatty acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
CFU	Colony forming units
AO	Antioxidant

Contents

1. Introduction

1.1. Introduction and Review of Tulsi as a Medicinal Plant

Botany

Holy basil (*Ocimum sanctum*) is included in the family Lamiaceae, along with several other medicinal herbs such as salvia and mint and over 65 species of basil that are used in the food, pharmacology, and perfume industry. The species is closely related to sweet basil (*Ocimum basilicum*), a commonly used herb in Europe and North America. Holy basil has also been described as *Ocimum tenuiflorum* (basil with small flowers) or *Ocimum gratissimum* (very grateful basil). Of these, the species name *O. sanctum* is favored due to the wide range of its uses in religious and cultural traditions (1, 2).

Holy Basil: the elixir of life

Traditional uses of holy basil as medicine usually include but are not limited to the treatment of abdominal issues, oral infections, cough, colds, tumours and cancer, digestive tract problems, respiratory inflammation, arthritis, asthma, ulcers, wounds, hypoglycaemia, bronchitis, urinary issues, cardiovascular problems, and many others. Although the whole plant is medicinally important, the leaves are generally the most common form of treatment and are either used raw or steeped in hot water. The medicinal properties of holy basil leaves include antioxidant, antimicrobial, anticancer, antistress, adaptogenic, stimulant, expectorant, nervine, antipyretic and antiperiodic. These beneficial properties have led to holy basil being named “Queen of Herbs”, “Incomparable One” and “The Mother Medicine of Nature” and one of the most valued medicinal and religious herbs in India. The knowledge of this traditional medicinal herb is expanding to other cultures due to its unique therapeutic properties (1).

Production of holy basil

Holy basil is an important part of the agricultural system in large part of the world and is among one of the 178 highly cultivated plant species (3). The consumption of holy basil is approximately 3500MT annually, with trade requirements between 2000 – 5000 MT, sourced through traditional cultivation practices (4).

Basil plants have small seeds that require friable, well tilled soil and which are planted in late spring (April – May). Germination of seeds, planted in rows 10-15cm apart and with plants 2cm apart, occurs after 4 - 14 days. It is common to see variation in the production and appearance of the plants based on the environmental alterations. Using a fertilizer with a 1:1:1 ratio of P, K, N is desirable, and the species is not water tolerant, so a consistent, slow application of water is ideal. The plant grows well under long days and high temperatures, preferably in full sun, but can tolerate partial shade (5). Removal of the inflorescence enhances vegetative growth and results in a flush of new leaves throughout the growing season. In tropical climate, the first holy basil crop can be harvested in about three months and under optimized growth conditions, as many as three to four harvests are easily possible. The method of harvest affects overall yield of essential oil content as well as individual components of the oil. For example, secondary branches were found to have decreased biomass but increased essential oil content with high levels of eugenol, and low levels of methyl-eugenol – two main chemical components of holy basil. The biomass harvested from above 30cm from ground had decreased essential oil yield (6).

Medicinal properties of holy basil

Medicinal effect of holy basil is believed to be due to the complexity of constituents in the plant, leading to many positive influences in the human body. Innumerable medicinal benefits of holy basil have been recorded in many different regions and local languages around world. Recently, scientific evidence of therapeutic effect of holy basil has started to emerge in mainstream medical journals mostly from studies using in vitro bioassays and small clinical trials. A few recent examples of such studies on holy basil include: antimetastatic activity against Lewis Lung carcinoma cells, cardioprotective inhibition of lipid peroxidation in rats induced with myocardial infarction, and high cholesterol or high cadmium diets, neuroprotection and normalization of brain function through modulation of neurotransmitters (7) and prevention of radiation mediated cell death in mice. Other examples of the medicinal properties of holy basil are antimicrobial benefits as a mouth rinse, inhibition of bacterial gonorrheal, anthelmintic activity, wound healing and reduced levels of plasma glucose, triglyceride and cholesterol. Holy basil can also reduce diabetic symptoms and blood pressure and stimulate insulin production in the pancreas as well as, subdue skin, breast, and gastric cancer because of the antioxidative properties (8).

Although the most widespread use of holy basil remains to be medicinal, recent studies have shown

that this plant is effective against a variety of biological pollutants present in water such as virus and bacteria that are responsible for several diseases. Holy basil essential oils have antifungal and anti-aflatoxigenic effects which can be used for storage and improvement of shelf life of food products such as 'Tofu'. There is evidence that holy basil extracts can improve the shelf life of bananas and may also be a viable alternative to chemical fungicides for the management of crown rot disease. Strong antimicrobial effects of the essential oil constituents against pathogenic fungi and both gram-positive and gram-negative bacteria have also been demonstrated (9).

1.2. Chemistry

A wide range of technologies have been used for the quantification of the chemical composition as well as antioxidant potential of holy basil including gas chromatography (GC), high performance thin layer chromatography (HPTLC), GC-mass spectrometry (GC-MS), 1D and 2D NMR spectroscopic analysis, solid phase microextraction (SPME), flame ionization detection (FID), and olfactoric evaluation (10) Antioxidant potentials have been measured separately by assays such as DPPH (1-diphenyl-2-picrylhydrazyl), or have been combined with HPLC (high performance liquid chromatography) (11). Techniques such as x-ray fluorescence analysis and Fourier transform infrared spectroscopy coupled with principal component analysis have been used to evaluate the uniformity and identity of herbal plants for quality control purposes (12). This study will follow up some of these methods and more will come about later in this study.

The chemical composition of holy basil analyzed by multiple techniques described above revealed a highly complex mixture of many nutrients, essential oils and other biologically active compounds. The plant contains a diverse range of compounds including alkaloids, carbohydrates, fats, flavonoids, glycosides, phenols, proteins, saponins, tannins and terpines. Generally, the main components of the leaf tissue are eugenol and methyl-eugenol, but their levels vary in different plants. These constituents along with the numerous other compounds found in holy basil at lower levels, give rise to the variety in medicinal properties (11).

Spice	Bioactive compound	Chemical structure
Holy basil 	Eugenol	

Figure 1.1. Chemical structure of holy basil (13)

Major constituents	% in Fresh leaves oil	% in Dried leaves oil
Eugenol	57.94	6.34
Beta-Caryophyllene	15.32	18.20
Beta-elemene	7.57	11.38
GermacreneD	9.10	3.24
Caryophyllene oxide	3.20	29.36

Figure 1.2. Chemical structure of holy basil (14)

1. Antioxidants in plants

Antioxidants scavenge free radicals within the body and externally. Free radicals are unstable and react with cell membranes, proteins, DNA, and lipids causing structural and functional damage. This is undesirable as it can lead to premature aging of the cells, causing degenerative diseases, cancer and cardiovascular problems and other health issues. Antioxidants provide protection by offering a free proton and are oxidized themselves, thereby stopping the oxidation chain reaction (15).

1.1 Free radicals

The body is under constant attack from oxidative stress. Oxygen in the body splits into single atoms with unpaired electrons. Electrons like to be in pairs, so these atoms, called free radicals,

scavenge the body to seek out other electrons so they can become a pair. This causes damage to cells, proteins, and DNA. Free radicals are associated with human disease, including cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease, and many others. They also may have a link to aging, which has been defined as a gradual accumulation of free-radical damage, according to Christopher Wanjek (16), the Bad Medicine columnist for Live Science. Substances that generate free radicals can be found in the food we eat, the medicines we take, the air we breathe and the water we drink, according to the Huntington's Outreach Project for Education at Stanford University (17). These substances include fried foods, alcohol, tobacco smoke, pesticides, and air pollutants.

Free radicals are the natural byproducts of chemical processes, such as metabolism. Dr. Lauri Wright, a registered dietitian, and an assistant professor of nutrition at the University of South Florida, said, "Basically, I think of free radicals as waste products from various chemical reactions in the cell that when built up, harm the cells of the body." Yet, free radicals are essential to life, The body's ability to turn air and food into chemical energy depends on a chain reaction of free radicals. Free radicals are also a crucial part of the immune system, floating through the veins and attacking foreign invaders (16).

1.2 The danger of free radicals

According to Halsted et al (18), once free radicals are formed, a chain reaction can occur. The first free radical pulls an electron from a molecule, which destabilizes the molecule and turns it into a free radical. That molecule then takes an electron from another molecule, destabilizing it and turning it into a free radical. This domino effect can eventually disrupt and damage the whole cell. The free radical chain reaction may lead to broken cell membranes, which can alter what enters and exits the cell. The chain reaction may change the structure of a lipid, making it more likely to become trapped in an artery. The damaged molecules may mutate and grow tumors. Or the cascading damage may change DNA code.

Oxidative stress occurs when there are too many free radicals and too much cellular damage. Oxidative stress is associated with damage of proteins, lipids and nucleic acids, according to an article in the Pharmacognosy Review. Several studies throughout the last few decades have suggested that oxidative stress plays a role in the development of many conditions, including macular degeneration, cardiovascular disease, certain cancers, emphysema, alcoholism,

Alzheimer's disease, Parkinson's disease, ulcers, and all inflammatory diseases, such as arthritis and lupus. Free radicals are also associated with aging. "The free radical theory of aging states that we age because of free radical damage over time," said Wright. Free radicals can damage DNA's instructional code, causing our new cells to grow incorrectly, leading to aging.

1.3. Symptoms of oxidative stress

According to a Elleuche et al (19), there are no officially recognized symptoms of oxidative stress. According to naturopathic doctor Donielle Wilson's website (20), however, symptoms include fatigue, headaches, noise sensitivity, memory loss and brain fog, muscle and joint pain, wrinkles and gray hair, vision trouble and decreased immunity.

Testing for free radicals

It is not possible to directly measure the number of free radicals in the body, according to Rice University. According to Halsted et al (18), there are indirect methods of measuring oxidative stress, usually involving analysis of the byproducts of lipid peroxidation. The article warns that all methods should "should be used with caution because of the lack of accuracy, validity or both." It also states that kits for testing oxidative stress are increasingly available, though their accuracy and validity are still under scrutiny.

Antioxidants and free radicals

Antioxidants keep free radicals in check. Antioxidants are molecules in cells that prevent free radicals from taking electrons and causing damage. Antioxidants can give an electron to a free radical without becoming destabilized themselves, thus stopping the free radical chain reaction. "Antioxidants are natural substances whose job is to clean up free radicals. Just like fiber cleans up waste products in the intestines, antioxidants clean up the free radical waste in the cells," said Wright (21). Well-known antioxidants include beta-carotene and other carotenoids, lutein, resveratrol, vitamin C, vitamin E, lycopene, and other phytonutrients.

Our body produces some antioxidants on its own, but an insufficient amount. Oxidative stress occurs when there is an imbalance of free radicals and antioxidants (too many free radicals and too few antioxidants), according to the Pharmacognosy Review. Antioxidants can be acquired through diet. "Antioxidants are plentiful in fruits and vegetables, especially colorful fruits and vegetables," said Wright. "Some examples include berries, tomatoes, broccoli, spinach, nuts and

green tea."

H_2O_2 free radical

Hydrogen peroxide (H_2O_2) is a strong oxidizer. It is also known as a wound disinfectant or as a bleaching agent for hair and teeth, but it is also created naturally in our bodies, as part of our cellular oxidation.

H_2O_2 belongs to a group of natural chemicals called reactive oxygen species (ROS) and when the process gets out of hand, too much oxidation can have a damaging effect on cells and their components. Unchecked free radicals, the most well-known ROS, are believed to play a role in carcinogenesis, degenerative diseases, and even aging. To prevent that, our cells also contain antioxidant enzymes known as peroxiredoxins that degrade H_2O_2 molecules. Under most conditions, H_2O_2 is not an undesired side product but rather an essential chemical messenger that plays an important role in regulating the way in which body cells respond to signals from outside such as hormones and growth factors," says Dr. Tobias Dick of the German Cancer Research Center (Deutsches Krebsforschungszentrum, DKFZ) (22). "We know today that the body's own H_2O_2 is vital for signal processing in a healthy organism."

H_2O_2 transmits signals by oxidizing specific proteins at sites, thereby alternatively turning them on or off. Dick and colleagues have shown the molecular mechanisms behind this signaling through specific oxidation in human cells, a mechanism that has long been enigmatic for scientists. A signaling molecule needs to act specifically. How can a tiny molecule like H_2O_2, which is hardly any larger than a water molecule (H_2O), specifically oxidize proteins while leaving others completely unaffected, and why is it that the relatively small amounts of H_2O_2 that are produced for signaling are not immediately captured by peroxiredoxins before H_2O_2 can even react with target proteins "Tumor cells produce larger quantities of H_2O_2 and use oxidative signals at higher levels than normal cells in order to drive their own growth," says Mirko Sobotta (23), first author of the publication. "Now that we have identified the peroxiredoxins as key players in specific oxidation, we can target them in order to interfere with cancer-relevant oxidative signals." (German Cancer Research Center (Deutsches Krebsforschungszentrum, DKFZ 2014)

1.4. Atlantic salmon

Atlantic salmon (Salmo Salar) are predominantly anadromous species that are found primarily in the northern Atlantic Ocean and in rivers that flow into it. Reproduction and nursery phases of the

species occur in freshwater before migrating to marine environments where they undergo rapid growth before reaching sexual maturity. While salmonids from the Pacific usually die after reproduction, Atlantic salmon, typically found in Russian and Norwegian rivers, does not die after reproduction, and can therefore grow to substantial sizes and weights (24).

The production of farmed Atlantic salmon has been steadily growing since the 1970s, and Norway is one of the largest exporters of farmed salmon in the world. Due to a long coastline, deep fjords, a favorable ocean current, oxygen-rich water, and moderate temperatures, Norway has a unique advantage in aquaculture. Large scale commercial operations replaced wild salmon fisheries in few decades through salmon farming. Nowadays, farmed salmon production far exceeds that of wild salmon (25). Since this is exported to many parts of the world from Norway and is a fatty fish, there is a need for good preservation methods in order to enhance the quality and integrity of export.

1.5. Aim of thesis

The aim of this study has been centered on the effect of antioxidants extracted from Tulsi to extend the shelf life of salmon using two different packaging methods, MAP packaging and vacuum packaging. The main goal of the thesis was to find out which one of the above-mentioned packaging methods is more effective in combination of antioxidant. To address this, the treatment was compared with two control groups, MAP and vacuum packaging which were further divided into subgroups of MAP with antioxidant, MAP without antioxidant, vacuum with antioxidant and vacuum without antioxidant.

Materials and Methods

All the chemicals and equipment used in this experiment is mentioned below in appendix.

2.1. Preparation of plant extract

About 11 grams of Tulsi plant was obtained after drying the sample plant for five days and then grounded the powder from that dried plant residues to make antioxidant. 11g the powder was soaked in 300 ml of methanol (70%) by continuous shaking at room temperature. The mixture was then filtered with the help of Whatman filter paper after 168 hours (7 days). When filtration was done then with the help rotary organic solvents was removed. The extract was then placed into water bath to evaporate remaining methanol at 40°C. For in-vitro studies of antioxidant activity methanolic extract of Tulsi was kept in the refrigerator at 4°C in dark room for 1 day.

Then stock and sub stock solution were prepared by mixing 0.006g of extract in 6ml of methanol, stock solution was prepared. Sub solution was prepared in tubes as 200µg/ml, 400µg/ml, 600µg/ml and 800µg/ml.

2.2. Salmon packaging and storage

Salmon fillets with skin were delivered at the NTNU lab in Kalvskinnet one day before experiments. The fillets were first deskinned and then cut those fillets into fine and equally divided portions with the sample weight of around 80-90 grams each. The part used for the sample preparation was the middle part of the fish hence cutting off the tail and belly region to avoid the fat content difference in those areas.

After cutting into samples, the samples for MAP packaging and Vacuum packaging. The samples were divided into two different groups for MAP packaging and vacuum packaging. In total 16 samples were prepared on the day one of experiment.

For the MAP sample packaging, tray sealing machine Webomatic TL250 was used with the combination of Carbon dioxide and Nitrogen gas as in ratio of 60:40. To analyze the gas composition a headspace gas analyzer PBI Dansensor was used in one empty pack in each sample packaging batch. The average composition that was obtained after analyzing was 60.25:39.75. The antioxidant was directly sprayed on the surface of the fish after placing it into the plastic container that was going inside the MAP and vacuum packaging machine. Two spray per sample was done on the surface of the filets. One sample was without spray so that we can differentiate the effects later in comparison with the sample with antioxidants.

For the vacuum packaging, a vacuum chamber machine was used, and machine was Webomatic Supermax. The packaging criteria was like MAP packaging as one sample was packed with antioxidants while other was without it.

After packaging of all the 16 samples for different analysis days, it was transferred into a container with ice to keep the fish cold. Then these samples were transferred to cold room at the lab in Gloshaugen NTNU. The temperature for the cold room was around 4°C. The samples were stored there for two weeks.

2.3. Microbial growth

The microbial growth during storage of samples was continuously monitored to check the microbial activity in the samples. The procedure was followed as per defined by Dunn and Rustad (26). All the equipment was sterilized while using autoclave machine.

A 10g of the sample was weighed accurately using a digital analytical balance. After weighing, the samples were transferred into stomacher bag and then homogenized with the help of stomacher homogenizer while keeping rpm 230 for 30sec. 100ml of saline peptone water (0.1% w/v peptone, 0.85% w/v NaCl) was added into sample to make dilution properly. The medium was then used to make a dilution series of four with an expanded level of dilution make up for the expanded microbial development during storage. Then 1ml of the diluted samples were transferred into plates using pipette. These plates were then transferred into an incubator while keeping the temperature at 38°C. After 72 hours, visible microbial colonies growth was observed and counted manually.

2.4. Lipid Extraction

For sampling, the samples were analyzed every fourth day while checking the lipid oxidation occurring in them during the whole experiment. For the process of lipid extraction, “Bligh, EG & Dyer, W.J 1959 (27). A rapid method of total lipid extraction and purification. Canadian J. Biochem. 37:911-917.” was used (27).

Following this method, ice cold chloroform was used to avoid any problems during pipetting because ice cold chloroform is easier to handle while pipetting. Also, it was kept on ice to avoid the evaporation.

For all the samples, the 10g of the fish was weighed accurately using a digital scale and then it was

transferred to homogenizer containers which are chloroform resistant. After transferring, the fish samples were homogenized for two minutes then 20ml of chloroform was added and homogenized for 30 seconds. Then 20ml of distilled water was added and homogenized for 30 more seconds. Now the samples are ready for centrifuge machine. Samples were placed in centrifuge machine for 10 minutes on 9000 rpm.

The chloroform phase after centrifugation were transferred into glass tubes using a pipette with accurate amount of 1ml from each sample. Glass tubes were weighed before poring the samples into them so that it becomes easy to observe the oxidation happening in the samples. After pouring, the glass tubes were placed into the evaporation unit (60°C) under the stream of N2. The evaporation process was continued until the chloroform was evaporated completely. The chloroform evaporation was evaluated by smelling the sample after different time intervals. When chloroform was fully evaporated, the samples were weighed again, and the final weight of the tubes were calculated for the results purposes. The method used to calculate is stated below:

$$g\ lipid/100\ g\ sample\ =\ Lipid\ in\ the\ tube\ *\ (volume\ chloroform\ in\ total)\ *\ 100$$

$$\frac{}{Amount\ of\ sample\ weighed\ in\ (g)\ *\ (ml\ chloroform\ evaporated)}$$

2.5. Total lipid content

The kimax tubes were pre weighed and the lipid containing chloroform extracts were added about 1ml. Using evaporating unit (Pierce Reacti-Vap), the chloroform was evaporated by using a steam of N_2 within that evaporating unit while heating the moulde (Reacti-Therm, 60°C) for 20 ± 5 min. The tubes were weighed and stored in desiccator for 12 hours.

2.6. PV

Peroxide values (PV) were determined using iodometric titration of chloroform extracts according to AOCS official methods (28). PV was measured three times on day 1 and day 16 of each treatment, with three parallel measurements each. If one of the parallels was an outlier, it would be rejected, and the two remaining values would be used to determine the average. In cases where none of the values matched, a parallel treatment group would be employed. Titration was

performed with an automatic titrator (TitroLine 7000) and a platinum electrode. A fresh potassium iodide solution (KI) was prepared for each titration day and stored in a refrigerator (4°C) when not in use. After the titration, a record was made of how much titrant had been consumed for the blank. The same procedure was followed when titrating the samples, except that chloroform was substituted with lipid chloroform extracts. PV was calculated using equation 2 and expressed as meq peroxide/kg lipids:

$$PV(\frac{m_{eq}\ peroxide}{kg\ lipid}) = \frac{(V - B) * T * 1000}{w}$$

V is the volume of titrant consumed (mL), B is the volume of titrant consumed for blank (mL), T is titrant (0,001 M), w is weight of lipids in sample.

3. Results and Discussion

In this section, results obtained from the experimental procedure conducted at NTNU Gløshaugen lab is presented. Different effects from MAP and Vacuum packaging are discussed and explained with certain limitations. According to experimental data the shelf life of salmon fish treatment with MAP and VAC with antioxidant treatment and without antioxidant is given below in the tables and data will elaborate the difference. Also, the microbial growth is summarized in this section.

3.1. Microbial Growth

Microbial activity during the storage period is visualized in a graph below that shows the microbial activity during different days of experiments. During the initial stages, the average bacterial count was found around 2.53CFU/g (29) (30) (31). This value is normal within the marine fish, and it indicates the well hygiene practices while slaughtering or harvesting the fish.

The microbial growth of different days is shown in the figure (figure numbers are yet to decide, will be updated afterwards) below where we can see the different microbial activity in several days of experiment.

Fish arrived one day before the experiment and all the samples were stored 4°C in cold room during the whole experiment and first sample was analyzed right after the next day of storage. As we can see in the figure, there is no significant microbial activity on the first days of analysis in both vacuum and MAP samples.

Previous studies have determined an enormous change in microbial growth increase among MAP and vacuum packaging which may follow the small change observed among vacuum-packed and MAP samples saved at 0°C in first batch of the samples but this change is not visible as seen in the graph, the activity is negligible (32) (33).

On the fourth days of experiment the number of bacterial colonies start spreading and multiple colonies were counted on the plate. After counting colonies of bacteria on all samples, a prominent difference of microbial activity was monitored in MAP a vacuum sample where the MAP samples and vacuum samples with the antioxidant present in them were less effected with bacterial growth as compared to non-antioxidant vacuum and antioxidant MAP package samples. There was a clear difference in microbial growth between samples with and without antioxidants. The microbial count on samples with antioxidants was low in both the MAP and vacuum packaging. The CFU/g value for both samples with antioxidants with MAP and vacuum packaging was around 2.5CFU/g.

While on the other hand, there is the difference of CFU/g values in non-antioxidant samples of both packaging methods. The CFU/g value of vacuum sample was slightly higher than MAP sample. For vacuum package this value is around 4.0 CFU/g while for the MAP its 3.7CFU/g. These values show that on the fourth day of experiment, the MAP packaging is performing slightly better than the vacuum packaging.

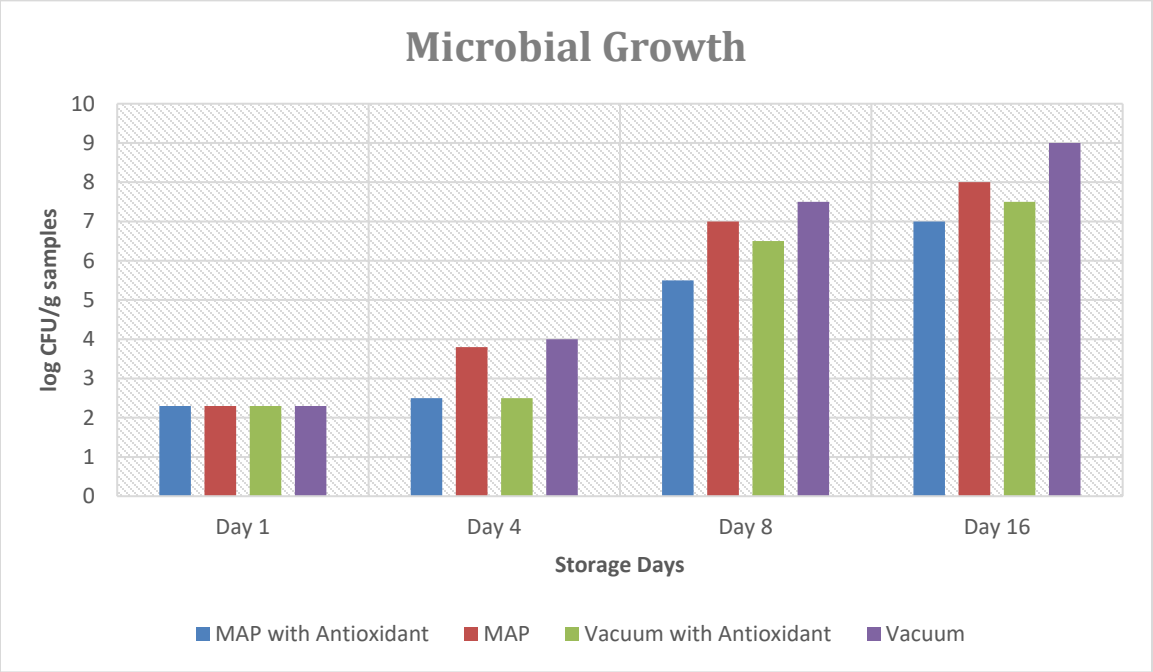

Figure 3.1. CFU per gram salmon during storage for 1-16 days for two different packaging methods with and without antioxidant usage. MAP with AO (Blue), MAP without AO (Orange), Vacuum with AO (Grey), Vacuum without AO (Yellow).

Day 8th of the experiment shows a gradual change in microbial growth in all the samples and there were more microbial colonies were visible on all the plates. Below in the line graph we can see that change clearly where the number of bacterial counts is increasing. This growth was steeper after the 5th day, and it kept increasing until the last day of experiment.

According to the International Commission on Microbiological Specifications for Food (ICMSF), the acceptable limit of CFU/g in fish for consumption of human is 10^6 (34).

The MAP packaging sample with antioxidant remained below the 10^6 CFU while rest of all the samples surpassed this level with more microbial growth. This means, sample with MAP

packaging is still on a safe side for consumption without any problems. On the other hand, Vacuum packaging with antioxidant is also little bit above the standard CFU/g which is $10^{6.5}$ as compared to other samples. Due to increased growth of microbes, we can suggest that the antioxidant with MAP is performing better than Vacuum or the sample without antioxidants.

On the last day of sampling, as shown in figure (..) all the samples are crossing the CFU/g standard value of 10^6 CFU and hence they are not good for consumption. However, the MAP with antioxidant sample is still at lower CFU/g value than the other samples. The CFU/g value of MAP with antioxidant is 10^7 which shows that this method performed better than all the others that were used in this experiment.

One of the previous research projects has also found that the extracts from Tulsi showed antimicrobial activities against the *S. aureus* (including MRSA) and *E. coli* (35). This research can explain why microbial activity was slowed down by antioxidants used in this study.

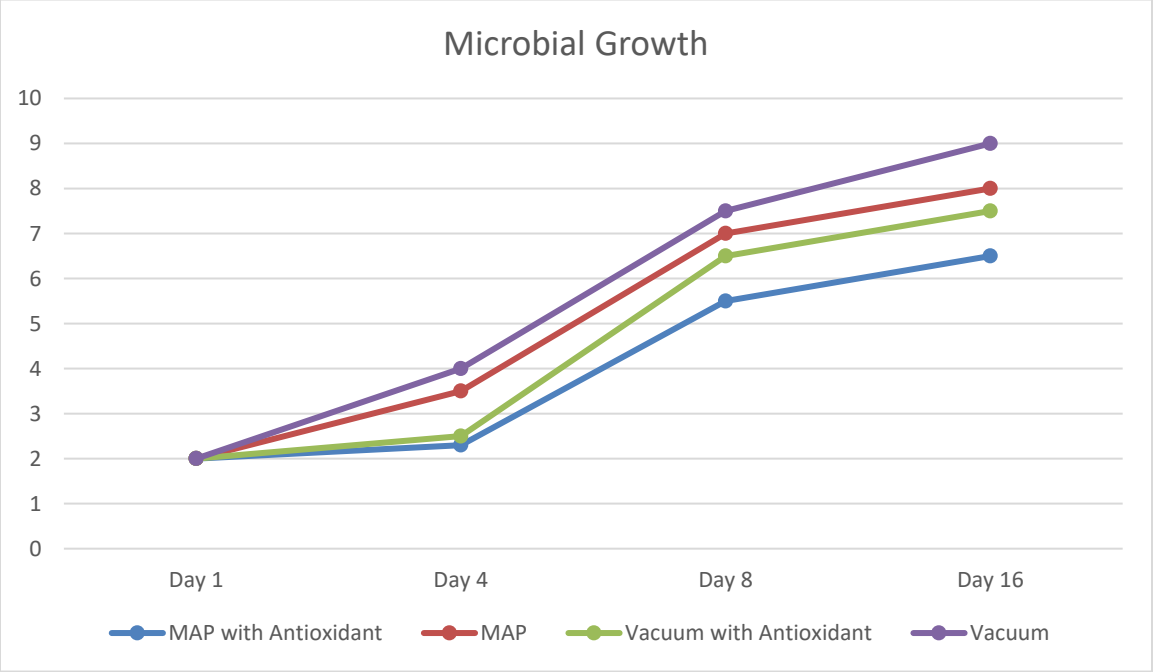

Figure 3.2. CFU per gram salmon during storage for 1-16 days for two different packaging methods with and without antioxidant usage. MAP with AO (Blue), MAP without AO (Orange), Vacuum with AO (Grey), Vacuum without AO (Yellow).

Following the data shown in figures, there is a significant difference between the treatments used for the experiment. From the previous studies, it has been found that the microflora of salmon can

be dominated by the *psychrotrophic aerobic* or *facultative anaerobic* Gram-negative bacteria. The bacteria that is responsible for the spoilage of salmon is *Photobacterium phosphoreum.* These bacteria produce TMAO and TMA hence making them resistance to CO_2 (36).
Sajjanshetty Mallikarjun and Ashwini Rao have investigated the effect of Tulsi to halt the microbial activity. They have suggested that the Tulsi is effective against some pathogens which are most associated with food (37).

Previous research has also shown that absence of divergency in CFU/g samples within antioxidant samples. Scientists investigated contrasts between fundamental oil in lean and greasy fish where they recommended that the dynamic parts of the cell reinforcement broke up in the lipid period of salmon making them less accessible to follow up on microorganisms in the aquas stage (32, 38, 39).

Based on all the results data, it can be finally concluded that the MAP packaging in combination of antioxidant extracted from *Ocimum tenuiflorum* performed better than all the other methods used in the experiment.

3.2. Drip Loss:

Drip loss of the MAP samples remained low as compared to the vacuum samples, while an increase in drip loss was observed on the 8th day of experiment. On average only 1% of the drip loss was found in MAP samples which is not that high as previous studies showed(31, 40). The drip loss is demonstrated in the graph below. Previous studies have also shown that the drip loss increases with the storage time, meanwhile this change was not that significant for the salmon samples packed with MAP packaging. Vacuum packaging gives higher drip loss if we compare it with MAP packaging (41).

Microbial activity can be one of the many reasons responsible for the drip loss and it can be corelated with it. Drip loss can provide the medium for the growth of the bacteria hence initiating or triggering the growth of microbes present in the fillets (26). Drip loss can be increased with the higher CFU/g samples. However, no correlation was found between CFU/g and drip loss.

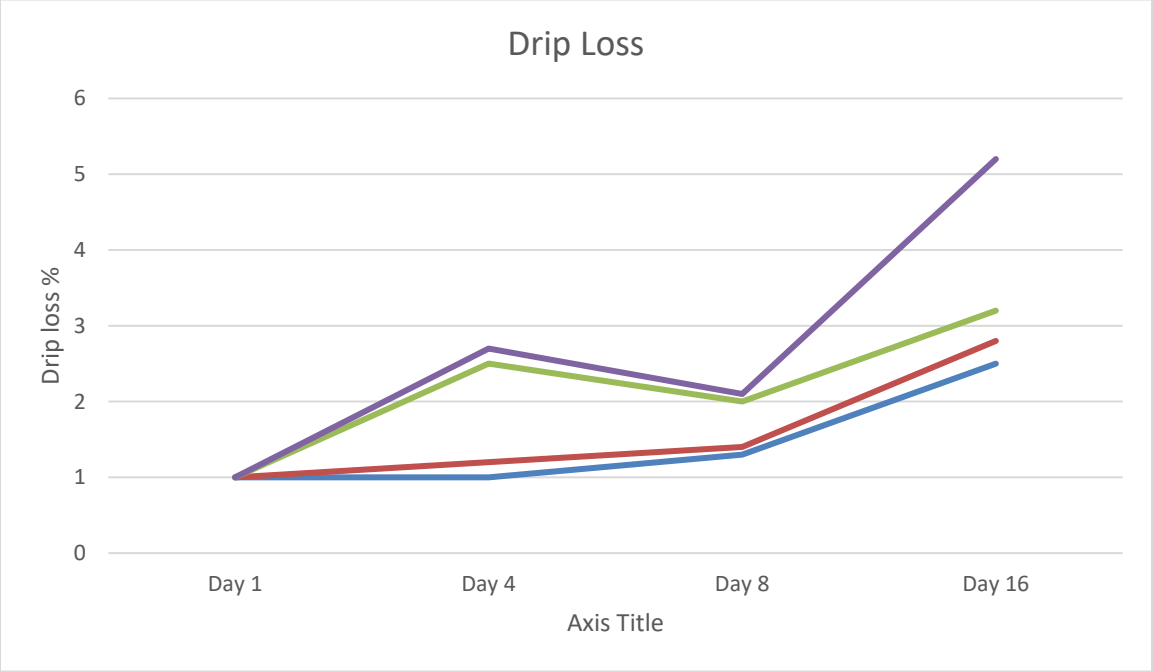

Figure 3.3. Drip loss (%) during 1-16 days of storage for MAP with Antioxidant (Blue), MAP (Orange), Vacuum with Antioxidant (grey), Vacuum (Yellow).

If we look at the values of the graph closely, there is not very big difference between values of the drip loss in MAP with Antioxidant sample and MAP without antioxidants. Same case with the samples of vacuum. While if we compare both methods combined, then there is a significant

difference between the values as the vacuum sample without antioxidant tend to have more drip loss. These results were also achieved by one of the senior students at NTNU where she found quite similar behavior of drip loss process. She found out that there is no major change found using antioxidant to prevent the drip loss (42).

During all these studies, no color analysis or sensory analysis were performed. While some of the visible changes were observed on the vacuum samples but not on the MAP. The reason that the vacuum packaging showed high values after 8^{th} day, can be the mechanical force applied while packaging. Some of the previous studies have also observed the higher drip loss in vacuum packaging as in contrast to MAP packaging, especially in Atlantic salmon and beef (32) (43).

3.3. Total lipid content

Lipid content was determined to see the difference between the amount of lipid present in fillets from day 1 to day 16 of the experiment. The average lipid content for the samples was around 12.90%, with the highest value of 24.20, 11.54% as middle value and 2.96% as lowest value. These values are the expected values for the fat content in Atlantic salmon, specifically if fish is grown inside a farm (43) (44) (45) (46). After going through all the data, there was no significant difference between the lipid content of the samples packed with MAP packaging and vacuum packaging. Also, there was not a huge difference between the values for the samples that were treated with the antioxidant within both packaging methods. The figure (..) shows the change in lipid content throughout the experiment within those 16 days.

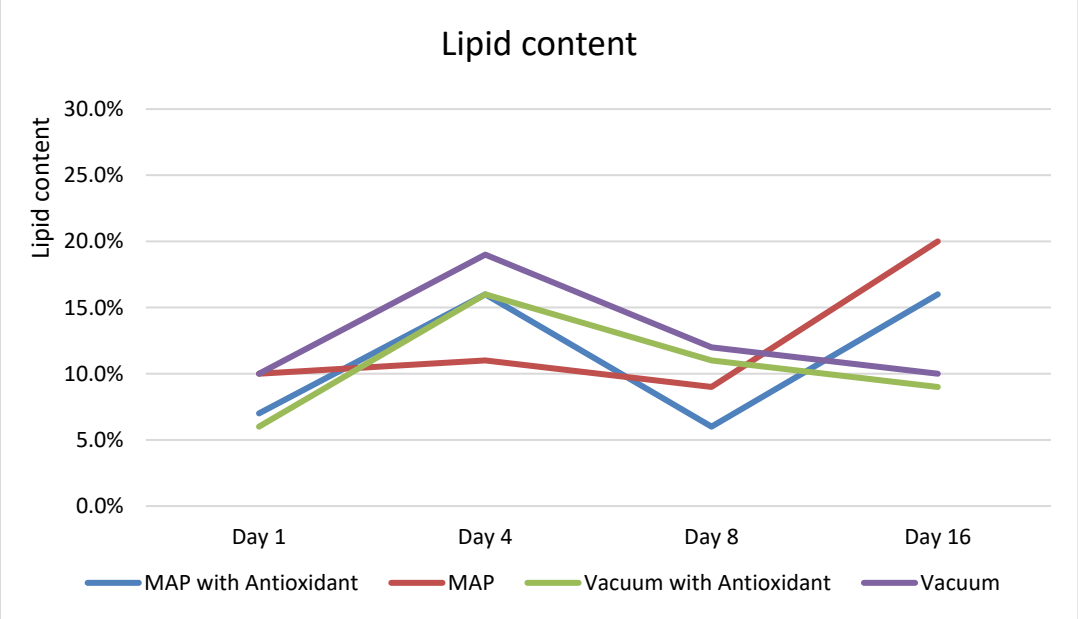

Figure 3.4. Lipid content (%) day 1-16 of storage for MAP with AO (Blue), MAP without AO (Orange), Vacuum with AO (Grey), Vacuum without AO (Yellow).

This variation in lipid content is probably because of the amount of different lipid content present in different parts of the fillets of the salmon. Another reason behind this change could be also the amount of lipid content present in individual salmon. For this experiment, the excess amount of fat was cut and removed prior to packaging however explanation for the difference between highest and lowest lipid content values can be suggested as the samples came from different parts of the fillets and different regions have different fat content. The noticeable thing over here is that all the samples even with lowest and highest values falls within lipid content changes that can occur in same fillet as explained by Katikou and fellow writers in their article (45).

3.4. Lipid oxidation (Peroxide values PV)

Graph shown below in figure (..) shows the results of PV analysis. Initial PV was found to be 1.99 meq peroxide /kg lipids. PV values increased overall by the end of the 16th day of experiment and significant difference was observed between both packaging methods and different days as the experiment continued. Day 0 and day 1 samples showed different values, but these values were significantly lower. While these values kept changing during other days and there was not much difference between them and they look stable except for the 16th day, as the values were higher. On average, the samples treated with antioxidant in combination of MAP packaging showed the greatest resistance as regards to lipid oxidation by showing the lowest average PV values during the whole time period of storage. On the other hand, sample treated with antioxidant in combination with vacuum packaging showed the second lowest values towards the lipid oxidation. While rest of the samples without antioxidants added went towards higher values by the last day of the experiment with the minimum difference between the values in them.

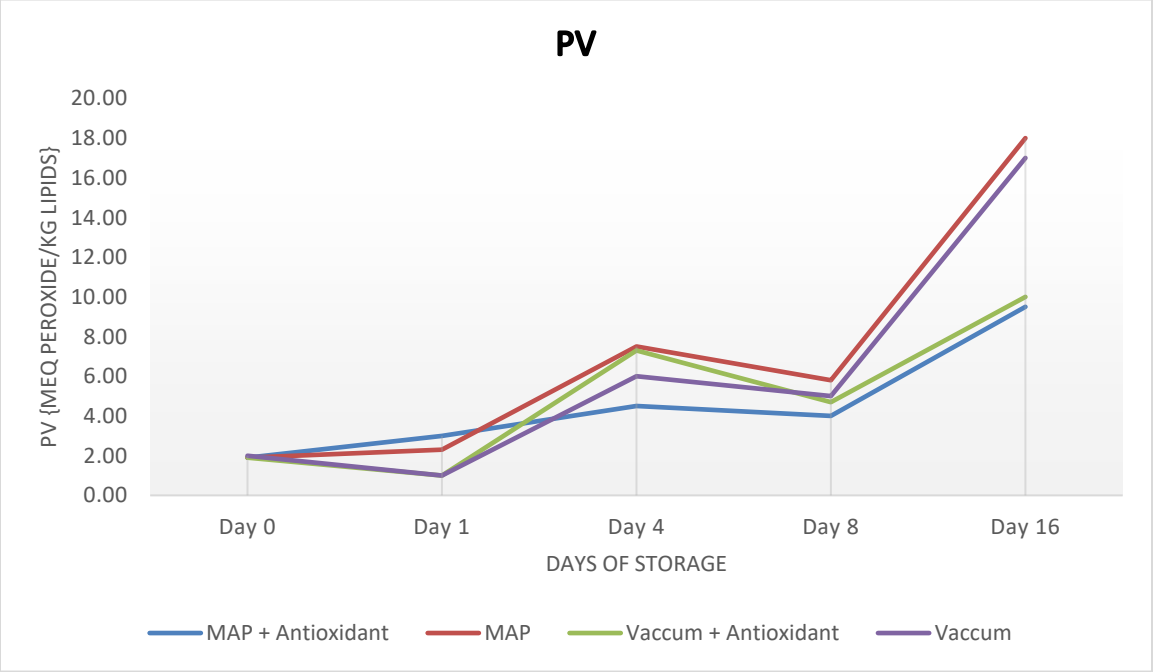

Figure 3.5. Peroxide values (meq peroxide/kg lipids) for samples after 0-16 days of storage, MAP with AO (Blue), MAP without AO (Orange), Vacuum with AO (Grey), Vacuum without AO (Yellow).

Change in values of PV among day 1 to day 16 is significant ($P<0.05$) which shows us clearly that lipid oxidation is occurring. But this does not clearly cover all the things as PV only shows the primary oxidation products and those products do not take part in rancidity (47) (48). However,

we can conclude anything from current data about the effect of antioxidant on lipid oxidation. The difference between PV values ($P<0.05$) was already expected as PV values usually increases early during the time of sample storage.

Considering the quality, maximum values for PV are set to PV<5 meq/kg for the consumption of the humans. This value was set by the European Pharmacopeia (Ph.Eur) and GOED (Global Organization for EPA and DHA). (ref). In this experiment, the limit for PV had already crossed PV <5 on the 4^{th} day of examination of the samples but during the next few days, it showed some variation between 4-6 meq/kg. While on the last day of the experiment, the PV already crossed the strict limit of PV<10 meq/kg which makes it extremely unhealthy for the consumption of humans (49) (47) (50) (51).

3.5. Limits and challenges of the study and future work

The main challenge for this study was limit to use of only one antioxidant. Also, the number of samples used in this study was suffered because of the low number of available samples. Most of the data was collected by using the parallel samples. Also in early stages, someone transferred the samples from cold room to freezer hence causing the experimental data delay and all the samples were prepared again for the experiment from the scratch.

Several challenges were also faced in the evaluation of the microbial growth phase. Many of the plates had low plate count and that leads to unreliable CFU/g values. Reason behind this could be the use of low concentration suspensions while homogenizing the samples in stomacher. For the future work, proper concentrations for the homogenizing should be used to avoid the low plate count in samples.

While determining the oxidation products, large fluctuation was found within the samples. While keeping PV in mind, it is hard to extract more and more lipids than what was done in this study in order to get more well-grounded results. Also, for the PV, there are other various methods available that can be used to get the desired results while requiring the less amount of lipid extracts, like spectrophotometric ferric thiocyanate method (52).

Another restriction for the current study was time. Fish analyzing and getting desirable is a sensitive method which can be affected with the time as it requires consistency while keeping many events in mind. The number of samples chosen were not that high and that's why parallel samples were used for different methods. This is because of the working alone in lab and it was not easy to manage all the stuff by one individual. Also, the sample analysis was done every 4^{th} day of experiment instead of each day, because data collection and having track of data was bit hectic as other things were also going parallel with experiment. Future suggestion for this challenge is to split the work among at least two people who can be responsible for different parts of experiment.

4. Conclusion

Samples treated with Holy basil (*Ocimum sanctum)* in combination with MAP packaging showed significantly increased shelf life as compared to the other methods used. On the other hand, the sample treated with holy basil in combination of vacuum packaging was the second significant method which showed the increased shelf life after MAP with antioxidant packaging. All other samples which were not treated with any antioxidant showed less resistance towards increase of shelf life despite using the MAP and vacuum packaging. There was a difference between the results obtained from MAP packaging and vacuum packaging as MAP showed little bit better results towards the shelf-life extension.

In terms of drip loss, it simultaneously increased during the storage days regardless of the packaging methods used. There is no significant difference between values of the drip loss in MAP with antioxidant sample and MAP without antioxidants as well as samples of vacuum packaging. If we compare both methods, then there is a significant difference between the values as the vacuum sample without antioxidant has more drip loss. The consumption limit already exceeded after 8^{th} day.

There was no significant change among the lipid content of the samples packed with MAP packaging and vacuum packaging. Also, there was not a considerable difference between the values for samples that were treated with the antioxidant within both packaging methods.

The limit for PV exceeded on the 4^{th} day of examination of the samples but during the following few days, it showed some variation. While on the last day of the experiment, the PV exceeded the strict limit of PV<10 meq/kg which makes it extremely unhealthy for the consumption of humans.

To sum up the whole study, *Ocimum sanctum* is not suggested to use as an antioxidant for now because it does not show too much influence in increasing the shelf life of salmon, but it can be suggested for the future research in combination of other antioxidants.

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atmosphere packaging. 2020.
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Appendix 1. Materials and chemicals

Chemicals:

- Methanol
- Ascorbic Acid
- Distilled Water
- Ethanol
- Carbon dioxide gas
- Nitrogen gas
- Chloroform

Raw materials:

- Fresh Tulsi from the REMA1000 near Moholt Studentby, Trondheim.
- Salmon supplied by VikenkoAS.

Instruments:

- Beakers
- Funnel
- Micro Pipette
- Micro centrifuge tubes
- Mortar
- Digital scale (.00001 g accuracy range)
- Filter Paper (CompactDry TC)
- Cold Room (4°C)
- Spray Bottles
- Tray sealing machine (Webomatic TL250)
- Vacuum chamber machine (Webomatic supermax)
- Homogenizer (Ultra Turrax T25)
- Stomacher homogenizer
- Stomacher bags (Grade)
- Incubator (Termaks)
- Vortex (MS2 Minishaker IKA)

- Filter papers (CompactDry TC)
- Gas analyzer (PBI Dansensor CheckMate 9900)
- Centrifuge (Sorvall RC-5B Plus)
- Desiccator
- Kimax glass tubes
- Evaporating unit with N2-gas (Pierce Reacti-Vap)

A.1 Microbial growth

Table A.1 shows the weight of the samples, and it shows the CFU during the 1-16 days of storage.

Table A.1: CFU counted during 1-16 days of storage for samples treated with antioxidant in combination of MAP and vacuum packaging

	Treatment, parallel	Weight(g)	Dilutions	Number of colonies
Day 1	Vacuum with AO	10.23	1	3
	Vacuum without AO	10.05	1	9
	MAP with AO	10.30	1	2
	MAP without AO	9.93	1	2
Day 4	Vacuum with AO	10.47	1	14
	Vacuum without AO	10.27	1	38
	MAP with AO	9.85	1	8
	MAP without AO	10.15	1	18
Day 8	Vacuum with AO	9.28	1	37
	Vacuum without AO	10.05	1	121
	MAP with AO	10.23	1	24
	MAP without AO	10.05	1	65
Day 16	Vacuum with AO	10.19	1	76
	Vacuum without AO	9.96	1	125
	MAP with AO	10.24	1	68
	MAP without AO	9.98	1	91

A.2 Drip loss

Table A.2 shows all the raw data from drip loss measurements for each storage.

Table A.2: All the experimental data from drip loss measurements for the multiple treatment groups MAP with AO, MAP, vacuum with AO, vacuum.

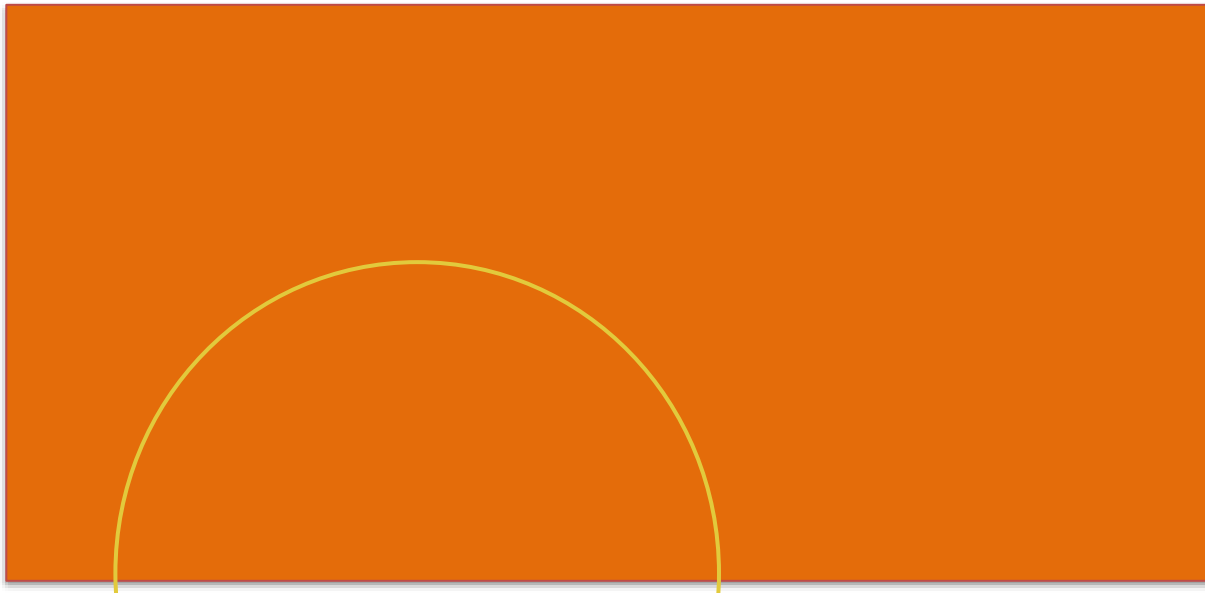
	MAP with AO	**MAP**	**Vacuum with AO**	**Vacuum**
Fish in bag	196.21	188.54	184.25	177.32
MB (2 x samples)	20.12	20.00	19.42	20.65
Fish after drying	154.63	138.32	142.21	139.38
Dried fish incl. MB	172.32	159.32	162.32	158.35
Bag after drying	20.41	12.54	20.84	12.66
Mass of drip	4.21	4.89	2.24	4.96
Drip loss (%)	**2.38**	**2.01**	**1.36**	**3.01**

A.3 Total lipid content

Table A.3 shows the raw data from lipid content measuring experiment setup.

Table A.3: Raw data for the measurement of lipid content including weight of sample (g), empty test tube weight (g), weight of test tubes with lipid content (g) and final lipid per mL (g/mL).

Treatment method	**Sample weight (g)**	**Weight of test tube w/o lipids (g)**	**Weight of test tubes w/ lipids (g)**	**Lipids/mL extract (g/mL)**
MAP with AO	09.81	11.1883	10.9854	0.0349
MAP	09.74	10.9548	11.1954	0.0153
Vacuum with AO	10.34	10.7456	11.3214	0.0182
Vacuum	10.33	10.7361	10.9524	0.0430



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