Effects of ergothioneine-rich mushroom extract supplementation on the oxidative stability of astaxanthin in salmonids

Doctoral Dissertation

EFFECTS OF ERGOTHIONEINE-RICH MUSHROOM EXTRACT SUPPLEMENTATION ON THE OXIDATIVE STABILITY OF ASTAXANTHIN IN SALMONIDS

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Abstract

Certain species in the family of Salmonidae are known for their distinct reddish-orange muscle coloration, and the same characteristic also dictates the commodity's market quality and value. This pigmentation is due to the accumulation of astaxanthin in their muscles. Fish and other animals cannot synthesize this compound but can be acquired through their diet. Salmonid meat also contains a considerably high amount of polyunsaturated lipids which make it susceptible to oxidation. This could lead to quality deterioration due to discoloration, loss of nutritional value, production of unhealthy by-products, and compromised organoleptic properties. The supplementation of naturally-derived products with high antioxidative properties is one of the strategies being used to control oxidative degradation. Ergothioneine is a potent hydrophilic antioxidant abundantly found in several edible mushroom species and has been widely used to control oxidation and quality deterioration in several post-harvest seafood commodities.

This study was conceptualized with the aim of preserving astaxanthin-rich salmonid meat from oxidation through the supplementation of ergothioneine-rich mushroom extracts (ME). Specifically, this study aimed to determine the feasibility of preserving astaxanthin through the addition of ergothioneine-rich ME in an *in vitro* cell model. With the feasibility of ergothioneine to protect astaxanthin from oxidation demonstrated in the initial study, a subsequent study was conducted to determine the antioxidative effects of ME, added to fish meat, during low temperature storage. In addition, the feasibility of ME dietary supplementation and ergothioneine uptake in fish was determined by conducting a feeding trial. Moreover, another feeding trial was conducted to evaluate the feasibility of ME dietary supplementation with other salmonid species and evaluate its antioxidative effects in fish meat during post-harvest low temperature storage. Furthermore, another study was carried out with the aim to determine the most probable gene sequences encoding for ergothioneine transporter proteins (ETTs) in certain *Oncorhynchus* spp. through bioinformatics.

Giant-sized astaxanthin-filled liposomes were used as *in vitro* cell models mimicking the astaxanthin-pigmented cells of salmon meat to elucidate the interactions of astaxanthin and lipid compounds with ergothioneine-rich mushroom extract under oxidation-induced conditions, to provide a better understanding of the agricultural and post-harvest applications of mushroom extract applications to post-harvest commodity preservation. Azocompounds 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were used as hydrophilic and lipophilic radical initiators, respectively. Liposomes were added with either AAPH or AMVN, incubated, and monitored for oxidative stability. Results of this study effectively demonstrated that the presence of ergothioneine or ME together with astaxanthin in the liposomes have additive synergistic antioxidant functions that could neutralize reactive radical species to control the progress of lipid oxidation and delay astaxanthin degradation. Crude mushroom extracts had higher antioxidative capacity than the equimolar concentrations of ergothioneine alone, which demonstrates the antioxidative properties of other compounds present in the extract such as the phenolics.

The positive outcome from the hypotheses in the *in vitro* experiment led to the application of ergothioneine-rich ME to astaxanthin-pigmented rainbow trout (*Oncorhynchus mykiss*) meat to evaluate its effects against lipid oxidation and astaxanthin degradation during low-temperature storage (-10 °C). Results showed promising effects of ME-treated meat in controlling astaxanthin degradation and lipid oxidation. Subsequently, a 10-week feeding trial on rainbow trout was done to evaluate feed acceptability and ergothioneine uptake using different concentrations of ME. In addition, this preliminary feed-supplementation trial was conducted to assess the effects of this strategy on the growth and pigmentation of the fish. Results of this feeding trial showed a positive response of fish towards the acceptability of the different concentrations of ME-supplemented feeds. Considerable ergothioneine uptake was confirmed and was correlated with the decrease in the levels of lipid hydroperoxides in blood. Moreover, no adverse effects were observed on the

growth, lipid content, and pigmentation of the fish, as a response to the dietary supplementation of the ME concentrates.

The next ME-supplemented feeding experiment was done with coho salmon (*Oncorhynchus kisutch*) to evaluate the applicability and effects of feeding supplementation strategy on other salmonid species. Different concentrations of ergothioneine-rich ME were incorporated into the astaxanthin-rich commercial diets of coho salmon and were administered for 8 weeks. Results of the supplemented feeding showed no adverse effects on the growth, pigmentation, and fat deposition in the ME-supplemented coho salmon. Moreover, a positive uptake of ergothioneine from the diet was noted in the ME-supplemented group. Ergothioneine-containing meat samples from ME-supplemented group exhibited radical scavenging activities. Moreover, meat samples were collected from the cultured fish, kept at low temperature (-2 and -18 °C), and evaluated for the effects of ME dietary supplementation on the lipid hydroperoxide formation, astaxanthin content, and changes in visual coloration during storage. Results showed mitigation of lipid oxidation and discoloration in the meat of ME-supplemented fish. The findings of this study demonstrated the feasibility of incorporating ergothioneine into the diet of fish during grow-out culture as a strategy to preserve the quality of the fish as well as to provide added value to the commodity.

To be able to further understand the effects of dietary ergothioneine supplementation on the fish, further studies were conducted to elucidate the mechanism of ergothioneine absorption, transport, and accumulation in fish. The SLC22 gene family to which ergothioneine and carnitine transporters belong is one example of a relatively large gene group that shares a considerable amount of homology among its members but also exhibit distinct and specific functions for each unique homolog. Based on the various bioinformatics analyses conducted for salmonid SLC22 homologs, a clearer picture of the most appropriate candidates for salmonid ergothioneine

transporter gene was thus obtained. The candidate genes determined through evolutionary phylogeny, sequence analysis, and topology comparison should, therefore, be the subject of future studies related to ergothioneine uptake in salmonids. This candidate gene for *O. mykiss* was used to evaluate the effects of ergothioneine-rich ME dietary supplementation on the expression of ETT in certain tissues. Results demonstrated that ETT is expressed in *O. mykiss* blood and muscle tissues and that ETT expression was actually downregulated with ME supplementation, despite increased ergothioneine accumulation in these tissues. This provides stronger evidence of the feasibility of dietary supplementation of ergothioneine-rich mushroom extracts in maintaining salmonid flesh quality against oxidative damage and degradation.

In summary, the crude hydrophilic extracts from edible mushroom species are efficient sources of ergothioneine as well as other potent antioxidants, which has demonstrated synergistic effects with astaxanthin, as tested in both *in vitro* and *in vivo* applications. The utilization of underutilized commercial food processing waste such as mushroom cuttings or spent culture media is a rich and economical source of ergothioneine and crude mushroom extracts that can be used as feed additives and dietary supplements in the grow-out culture of certain salmonid species, which have considerable effects in the mitigation of lipid oxidation during post-harvest storage. The results obtained in this study could also provide insights into the possible antioxidative properties of the other bioactive components present in edible mushrooms. Furthermore, this study could expand the understanding and application of naturally-derived antioxidants from edible mushrooms in the post-harvest preservation of oxidation-susceptible food commodities.

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Madamo guid nga salamat.
どうもありがとうございました。

Table of Contents

List of Figures

List of Tables

List of Appendices

Abbreviations Used

MEP	Methylerythritol 4-phosphate
IPP	Isopentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
PUFA	Polyunsaturated fatty acid
L•	Alkyl radical
LOO•	Peroxyl radical
LOOH	Lipid hydroperoxide
AH	Oxidation inhibitors or antioxidants
A•	Free antioxidant radical
OCTN1	Organic cation transporter, Novel, Type 1
SLC22	Solute Carrier Family 22
ETT	Ergothioneine transporter
DPPH	2,2-diphenyl-1-picrylhydrazyl
AAPH	2,2'-Azobis(2-methyl-propionamidine)dihydrochloride
AMVN	2,2'-Azobis(2,4-dimethylvaleronitrile)
ME	Mushroom extract
PBS	Phosphate buffered saline
AST	Astaxanthin
ESH	Ergothioneine
NBD	Nitrobenzoxadiazole
HPO	Hydroperoxide
FIA	Flow injection analysis
PC	Phosphatidylcholine
IS	Internal standard
FAME	Fatty acid methyl ester
AOCS	American Oil Chemists' Society
TBARS	Thiobarbituric acid reactive substances
MDA	Malondialdehyde
EC_{50}	Half-maximal effective concentration
ORAC	Oxygen absorbance radical capacity
PDB	Protein Data Bank
NIH	National Institutes of Health
CTT	Carnitine transporter
MSA	Multiple sequence alignment
pBLAST	Protein Basic Local Alignment Search Tool
TMD	Transmembrane domain

CHAPTER 1
General Introduction

1.1 Salmonids

Fish from the family Salmonidae include salmon, trout, and char. Although commonly caught as sports or trophy fish, several species are already being cultured as a mass-produced seafood commodity. Salmonids, as they are more commonly called, are known for their characteristic red-orange muscle pigmentation. The same characteristic can also dictate its market quality[5, 6]. A natural compound, astaxanthin, is responsible for the color imparted in the muscle of these fish species[7].

1.1.1 General characteristics

Salmonids are widely distributed throughout marine and freshwater ecosystems in the northern hemisphere, occurring mainly along the coasts of both the Pacific and Atlantic oceans. Some species though, have been introduced to parts of the southern hemisphere such as New Zealand[8] and Patagonia in Chile[9]. Most of the species within this family are anadromous, spending much of their adult life in oceanic waters and migrating up freshwater streams and rivers to spawn. Salmonids that hatch in freshwater streams or rivers spend a few months to several years in these habitats as juveniles before migrating back to sea until sexual maturation. Other species are known to be potamodromous, with reproductive migrations and all other parts of their life cycle occurring only within freshwater systems[10]. The average lifespan of salmonids ranges from three to eight years with sexual maturity usually reached within two to eight years depending on the species. The smallest species (Pink salmon - *Oncorhynchus gorbuscha*) attain average weights of 1.3 to 2.3 kg while the largest recorded species (Chinook salmon - *Oncorhynchus tshawytscha*) can reach weights exceeding 50 kg[10].

All salmonids are mainly carnivorous. In freshwater systems, salmonids mainly feed on aquatic insects such as the larvae of a variety of flies. This food type is similar for juveniles of anadromous species as well as juveniles and adults of potamodromous species. In marine waters, maturing and adult salmonids feed on a variety of small crustaceans and smaller species of fish such as herrings and capelins[11].

Although salmonids are mainly known for the red-orange coloration of their muscles only 4 genera out of a total of 10 within this family exhibit red to pinkish muscle coloration. All species falling within the salmonid genera of *Oncorhynchus*, *Salmo*, *Salvelinus,* and *Parahucho* possess this unique characteristic among all other fishes[12]. It is mainly because of this unique characteristic that species from these genera have been the main focus of fishery and aquaculture activities, including a considerable amount of research effort in understanding this particular trait.

1.1.2 Supply and demand

Salmonids have been traditionally fished in the northern areas of both the Pacific and Atlantic oceans. Production of salmonids through capture fisheries reached its peak in the mid-1990s, after which, steady declines have been reported. Salmonid aquaculture began in the 1970s and sharp increases in aquaculture production have been continuous since the 1980s. Given that the red-orange coloration of salmonid muscle is one of the main factors dictating its market price, nearly all salmonid farmers feed cultured stocks with artificial diets containing supplemental astaxanthin, the pigment that imparts the red-orange coloration to salmonid muscle. Thus, the increase in market demand for salmon farming since the 1980s has resulted in the increased market demand for astaxanthin as well[13].

1.2 Astaxanthin

Astaxanthin, a keto carotenoid compound (3,3-dihydroxy-β,β-carotene-4,4-dione)[14], is a naturally occurring pigment perceived as orange to red in color. The most commonly known source of astaxanthin is the freshwater microalgae *Haematococcus pluvialis*[15] but is also produced by other marine algae as well, and it is commonly found in seafood products such as salmonids and crustaceans. It has high antioxidative properties which makes it a valued food component, which can also be found in forms of nutritional supplements and cosmetic products. Astaxanthin is a C_{40} isoprenoid that consists of a conjugated polyene chain and two terminal ring moieties, giving it both lipophilic and hydrophilic characteristics, respectively[3, 16]. The molecular structure of astaxanthin is shown in Figure 1.1.

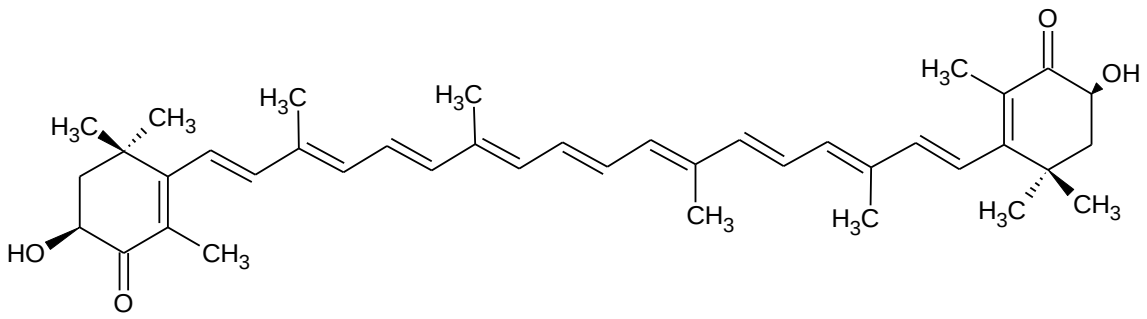


Figure 1.1 Structure of astaxanthin.

1.2.1 Biosynthesis of astaxanthin

Astaxanthin and other carotenoid compounds (classified as tetraterpenoids) are biosynthesized through the plastid-based isoprenoid methylerythritol 4-phosphate (MEP) pathway (Figure 1.2), the same pathway responsible for the synthesis of other isoprenoid compounds such as monoterpenes and diterpenes. In the strictest terms, the final products of the MEP pathway are the 5-carbon prenyl pyrophosphates isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate at an average ratio of 85:15 (IPP:DMAPP)[17]. These 5-carbon

pyrophosphates are the main base unit of all subsequent isoprenoids[1, 18]. IPP and DMAPP are then conjugated by the enzyme geranyl pyrophosphate synthase to form the 10-carbon geranyl pyrophosphate (GPP), which serves as the precursor for monoterpenes[1]. Two additional IPP molecules can also be added to GPP to form the 20-carbon geranylgeranyl pyrophosphate (GGPP), two molecules of which form the 40-carbon prenyl precursor (phytoene) of all carotenoids, including astaxanthin[19].

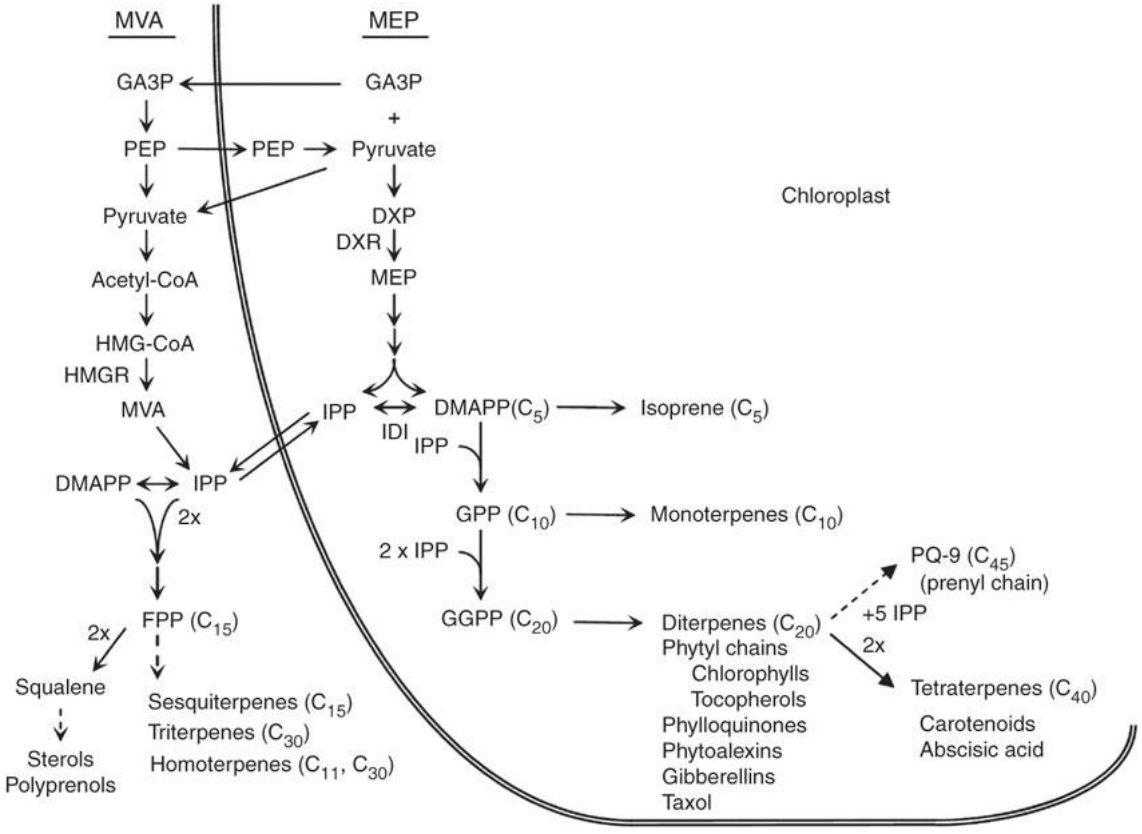


Figure 1.2 Astaxanthin biosynthesis through the plastid-localized MEP pathway in plants. Adapted from Vickers *et al.*[1].

As a commercially valuable compound, a considerable amount of research has gone into understanding the biosynthesis of astaxanthin[19-22]. Given that it is only produced through the plastid-localized MEP pathway in plants, overall production of astaxanthin is reliant on the photosynthetic activity of plastids to take up carbon dioxide that serves as a substrate for the

precursors of the MEP pathway. This aspect of astaxanthin biosynthesis thus makes its commercial production through biological platforms such as microalgae a potentially carbon neutral endeavor that could help offset the larger carbon footprints of farming systems reliant on astaxanthin such as salmonid farms. In *H. pluvialis*, the astaxanthin production is a response to certain environmental stress conditions such as high irradiation[15], nutrient deficiency[23], increase in salinity[23], or physical stress[24]. Under these conditions, a change in the biochemical components and morphological structure takes place in *H. pluvialis* from being vegetative motile green algal cells to distinctly red cysts containing astaxanthin in the cytoplasm, as shown in Figure 1.3. Understanding both biosynthetic mechanisms as well as culture conditions conducive for maximum astaxanthin accumulation has allowed for the production of astaxanthin in commercial quantities.

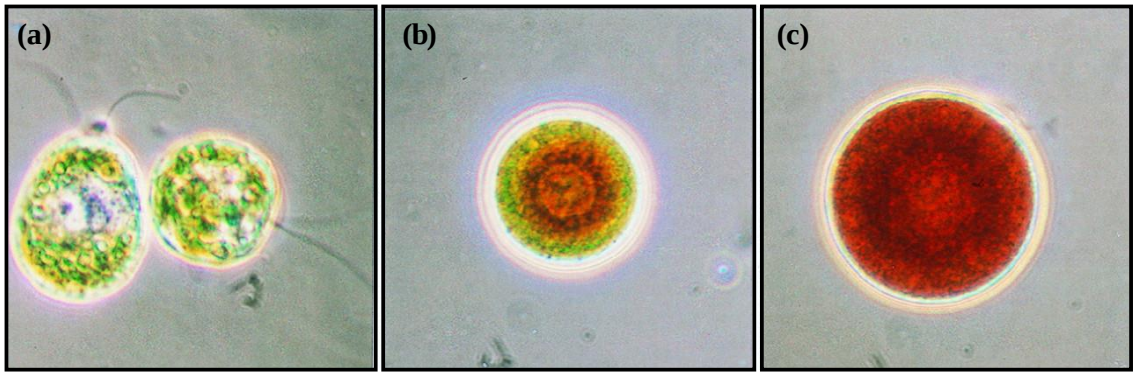


Figure 1.3. The developmental sequence of *Haematococcus pluvialis* cells from the green stage (A), to extraplastidic carotenoid accumulation and increase in cell size (B), to the enlarged red-cyst stage (C). (1,600.)[2]

1.2.2 Function as pigment

Carotenoids are widely distributed in nature as natural pigments. In terrestrial environments, most of the carotenoids are found in several species of plants, giving them distinct characteristic colorations. In the aquatic environment, the most abundant and common carotenoid is astaxanthin, which is commonly found in marine organisms such as in the muscles of wild

salmonid species and in the carapace of certain crustacean species. Some of the common examples of astaxanthin-pigmented seafood products are shown in Figure 1.4. These animals cannot synthesize carotenoids, hence the carotenoids, such as astaxanthin, are acquired through the diet.

Figure 1.4. Examples of astaxanthin-pigmented seafood products: salmon meat (a); lobster (b); and shrimp (c).

Chromophores are certain parts of a molecule that are responsible for the projection of visible colors[25]. Within the structure of astaxanthin as well as other carotenoids, the main component that acts as a chromophore is the conjugated polyene chain, which imparts the yellow to the reddish coloration of these compounds. Other carotenoid compounds with longer polyene chains have been found to exhibit darker red to purple colorations. For instance, the naturally occurring bacterioruberin, found mainly in halophilic archaea, is an acyclic C_{50} carotenoid that imparts a reddish tinge to these organisms[26]. Other researchers have also managed to engineer the bacterium *Escherichia coli* to produce non-natural C_{50}-astaxanthin which gives the *E. coli* cells a dark red coloration[27-29].

1.2.3 Function as an antioxidant

Several studies on astaxanthin have demonstrated the various functions and properties of astaxanthin as an antioxidant. It was shown to quench singlet oxygen or triple, scavenge radical species, and protect against photooxidation. Astaxanthin has strong antioxidative properties[3, 16],

and is often regarded as valuable for preventing lipid peroxidation. The antioxidative action of astaxanthin occurs via singlet oxygen quenching at the terminal rings and via radical scavenging within cell membranes by the conjugated double bonds[16, 30].

Carotenoids are known to have relatively stronger antioxidative properties than other antioxidants such as α-tocopherol, Coenzyme Q10, and α-lipoic acid. Amongst the carotenoids with antioxidative properties, astaxanthin exhibits the strongest activity for singlet oxygen quenching and lipid peroxidation suppression[16] wherein astaxanthin has been shown to be twice as effective as β-carotene in terms of inhibiting hydroperoxide production. In addition, the astaxanthin structure with conjugated polyene chains and terminal ring moieties makes it considerably efficient for scavenging lipid peroxyl radicals and reactive oxygen species, both within and at the surface of the cell membrane[3] (Figure 1.5).

Despite being a strong antioxidant, the highly unsaturated structure of astaxanthin makes it susceptible to lipid oxidation and discoloration as well, which in turn could compromise the market quality of commodities containing this compound. Previous studies have demonstrated that incorporating either hydrophilic (ascorbic acid) or hydrophobic (α-tocopherol) antioxidants in a heterogeneous system together with astaxanthin significantly improves the oxidative stability of astaxanthin[31, 32].

1.3 Lipid oxidation

Food lipid components contribute to both the functional and organoleptic properties of a food commodity. Fish lipid consists mostly of polyunsaturated fatty acids (PUFA), which have been found to have certain health benefits. However, the unsaturated structure of PUFA makes it unstable and susceptible to lipid oxidation. Lipid oxidation is the degradation process wherein free

radical species abstract electrons from a lipid molecule resulting to cell damage and food quality deterioration. The process of lipid oxidation proceeds through a chain reaction mechanism, which starts with an initiation phase, followed by a propagation phase, and ends in the terminal phase. Lipid oxidation may be initiated by several factors and conditions such as the presence of radical species, singlet oxygen, or photo sensitizers.

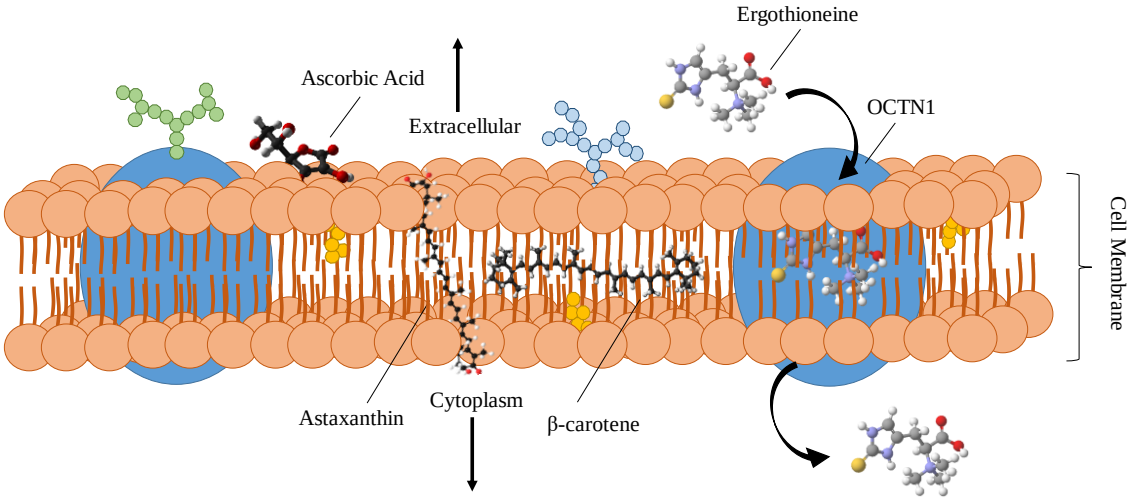


Figure 1.5. Superior position of astaxanthin in the cell membrane (Adapted and slightly modified from Yamashita et al, 2001[3])

During the initiation step, a lipid molecule exposed to a radical species can generate an alkyl radical (L•) (Eq. 1.1) with which when exposed to atmospheric oxygen can be converted to a peroxyl radical (LOO•) (Eq. 1.2). During this propagation phase, the alkyl radical can react with another lipid molecule by abstracting a hydrogen atom to form lipid hydroperoxide (LOOH) and generate another alkyl radical (Eq. 1.3). The decomposition of a lipid hydroperoxide molecule can lead to the further propagation of free-radical generation until all lipid substrates are used up (Eqs. 1.4 to 1.7). When free-radical concentration becomes too high, two free-radicals may react and combine to and terminate the chain reaction process (Eqs. 1.8 to 1.10).

Initiation phase:	$LH + X\bullet \rightarrow L\bullet + HX$	(Eq. 1.1)
Propagation phase:	$L\bullet + O_2 \rightarrow LOO\bullet$	(Eq. 1.2)
	$LOO\bullet + LH \rightarrow L\bullet + LOOH$	(Eq. 1.3)
	$LOOH \rightarrow LO\bullet + \bullet OH$	(Eq. 1.4)
	$LOOH + LO\bullet \rightarrow LOO\bullet + LOH$	(Eq. 1.5)
	$LO\bullet + LH \rightarrow L\bullet + LOH$	(Eq. 1.6)
	$\bullet OH + LH \rightarrow L\bullet + H_2O$	(Eq. 1.7)
Termination phase:	$L\bullet + L\bullet \rightarrow L\text{-}L$	(Eq. 1.8)
	$L\bullet + LOO\bullet \rightarrow LOOL$	(Eq. 1.9)
	$LOO\bullet + LOO\bullet \rightarrow LOOL + O_2$	(Eq. 1.10)

However, the oxidation chain reaction can be terminated earlier or can be inhibited by the presence of oxidation inhibitors or antioxidants. These compounds (AH) terminate the progress of the oxidation chain reaction by donating a hydrogen atom to a radical species, resulting in the formation of a free antioxidant radical (A•) (Eqs. 1.11 to 1.12). Subsequently, these free antioxidant radicals can react with a peroxyl radical or another free radical antioxidant to terminate the chain reaction by forming a more stable compound (Eqs. 1.13 to 1.14).

$LOO\bullet + AH \rightarrow LOOH + A\bullet$	(Eq. 1.11)
$L\bullet + AH \rightarrow LH + A\bullet$	(Eq. 1.12)
$LOO\bullet + A\bullet \rightarrow LOOA$	(Eq. 1.13)
$A\bullet + A\bullet \rightarrow A\text{-}A$	(Eq. 1.14)

1.4 Ergothioneine

Certain fungal species biosynthesize the hydrophilic antioxidant (2*S*)-3-(2-sulfanylidene-1,3-dihydroimidazol-4-yl)-2-(trimethylazaniumyl)propanoate (ergothioneine)[33,34]. It is also reportedly produced by certain cyanobacteria as well[35]. Despite its ubiquitous presence in the tissues of higher organisms such as vertebrates, these organisms cannot synthesize ergothioneine and thus obtain this compound from their diets.

Ergothioneine is a betaine derivative of 2-thiolhistidine characterized by the presence of a sulfur atom bonded to the carbon at position 2 of its imidazole ring (Figure 1.6)[36]. Depending on the pH of the environment wherein ergothioneine is located, it can exist as either a thiol or a thione. In aqueous solutions such as the environment within cells where ergothioneine is mainly detected, it predominantly exists as a thione[36].

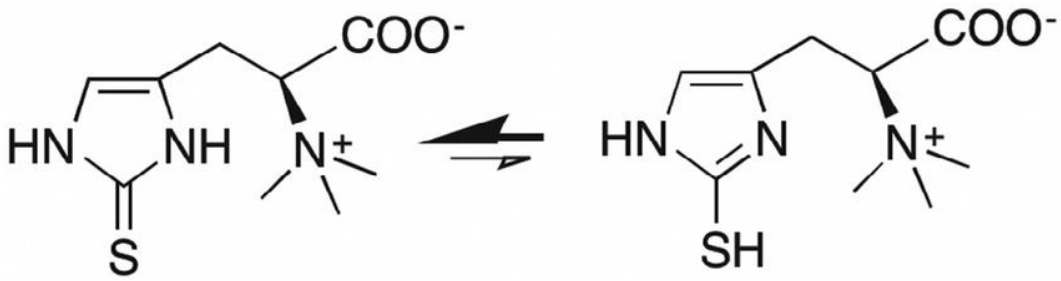


Figure 1.6. Chemical forms of ergothioneine as thione (left) and thiol (right).

1.4.1 Function as an antioxidant

Since the discovery of ergothioneine in 1909, the specific function of this compound in the human body has remained unclear, however, several studies consider it as an intracellular antioxidant[34]. As a hydrophilic antioxidant, ergothioneine has been shown to act as an intracellular singlet oxygen quencher and a non-radical species scavenger that prevents photooxidation and allows DNA repair of UV-damaged cells[37, 38], protects against lipid peroxidation, conserves endogenous antioxidants glutathione and α-tocopherol[39], acts as a hydroxyl radical scavenger and transition metal (ferrous or copper(II)) ion-dependent oxidation inhibitor, and slows reaction rates with superoxide or hydrogen peroxide[34]. Moreover, ergothioneine exists in a mostly concentrated state in the mitochondria suggesting that it functions as a cytoprotectant for specific mitochondrial materials such as DNA[40].

1.4.2 Ergothioneine transporter protein

Given that the antioxidative properties of ergothioneine have been associated with important physiological functions, understanding the mechanisms involved in its uptake in non-producing organisms has been the focus of considerable research and debate[41]. An organic cation transporter (OCTN1) has been identified to be the main protein involved in ergothioneine transport across cellular membranes[42]. This protein is a member of a larger transporter family known as the solute carrier family (SLC22). Although several studies have shown the potential of OCTN1 to transport other compounds aside from ergothioneine[43, 44], the most recent and detailed studies on its function has pointed to its main function *in vivo* as a specific transporter for ergothioneine[41]. Because of this, a more specific nomenclature of OCTN1 as the ergothioneine transporter (ETT) has increasingly been used[41, 45].

1.5 Edible mushroom extracts

Several species of mushrooms are widely consumed as food, and the increasing demand for this food commodity has led to the rise of the mushroom cultivation industry. However, with the increase in the supply of cultivated mushroom, follows an increase in processing waste products. In this study, trimming wastes and spent culture media have been used as feed supplements for salmonid fish to provide ergothioneine through the diet.

1.5.1 Ergothioneine content in mushrooms

Several studies have been conducted to determine and evaluate the ergothioneine content of certain edible mushroom species. Bao *et al*. (2010)[4] quantified the ergothioneine content, total phenolic compounds, and DPPH radical scavenging activity of certain edible mushroom species, and the results are presented in Table 1.1. As reported in this comprehensive study, specific mushrooms appear to have higher concentrations of ergothioneine compared to others.

Interestingly, for the mushroom *F. velutipes*, ergothioneine concentrations are markedly higher in parts considered as waste as compared to its fruiting body. This, therefore, provides a crucial factor in ergothioneine production and utilization: the non-competition of ergothioneine sources for human consumption and nutrition (i.e. fruiting bodies) and ergothioneine sources for other uses such as post-harvest applications (i.e. from mushroom processing waste).

Table 1.1. Concentration of antioxidative compounds and antioxidant activity in various species of mushroom. (Adapted from Bao et al. (2010)[4].)

Mushroom species	Ergothioneine content, mg/10g[a]	Total phenolics, Equiv mg/mL gallic acid	DPPH radical scavenging EC_{50}[b], mg[a]
Fruiting body			
Flamullina populicola	0.48±0.01	2.30±0.07	0.98±0.04
Flamullina velutipes	2.98±0.02	2.91±0.04	0.81±0.03
Grifola frondosa	0.30±0.01	4.26±0.20	1.41±0.01
Hypsizygus tessellates	0.06±0.01	6.06±0.28	1.65±0.02
Lentinula edodes	2.84±0.04	6.72±0.25	0.56±0.04
Pholiota nameko	0.02±0.01	1.21±0.06	1.48±0.03
Pleurotus cornucopiae	20.82±0.12	6.72±0.19	0.29±0.01
Pleurotus eryngii	3.17±0.11	2.95±0.09	0.81±0.02
Waste			
Flamullina velutipes	5.08±0.08	6.16±0.07	0.71±0.01

[a]mushroom wet weight
[b]$EC_{50,}$ Half maximal effective concentration

1.5.2 Post-harvest Applications

Ergothioneine-rich mushroom extracts have been applied in several post-harvest techniques to preserve the quality of certain meat and products such as cattle meat, chicken, and seafood products like salmon, yellowtail, shrimp, and crab[46-51]. Given that mushroom processing wastes have been found to be a rich source of ergothioneine for such applications, it presents itself as a cheap and cost-effective supplement to aid in mitigating quality deterioration in a wide range of food

commodities. Although a considerable amount of previous studies has simply focused on the direct application of ergothioneine-rich mushroom extracts to processed food products, another strategy that has shown increasing feasibility is the incorporation of ergothioneine-rich mushroom extracts to the feeding regime of food commodities (i.e. cattle, swine, chicken, and fish) during rearing. The ubiquitous presence of the ergothioneine transporter protein across nearly all taxa of vertebrates does not only give credence to this strategy but also serves as an impetus for further research to optimize aspects such as supplementation doses and strategies.

1.6 The Current Study

Given the economic importance of salmonids to the fisheries industry, not only in Japan but in other countries across the northern Pacific and Atlantic oceans as well, research on both the culture and post-harvest strategies for this commodity will have resounding effects in terms of improving its overall contribution. Moreover, with the orange to red coloration of salmonid muscle due to astaxanthin being one of the main determinants of fish quality and value, ensuring the preservation of this characteristic through cost effective means such as the use of processing wastes with antioxidative properties from other industries will be an intuitive approach to improve its economic value.

In general, although hydrophilic antioxidants tend to be less effective in heterogeneous systems such as in oil-in-water emulsions[52], ergothioneine has been shown to have exceptional antioxidative properties in various model and food systems[53]. Based on these previous works, it was hypothesized ergothioneine could also improve the stability of astaxanthin when incorporated together in food products such as salmonid muscle. As such, the current study was conducted with the general aim of improving the stability of astaxanthin present in the muscle of salmonids through the application of ergothioneine-rich mushroom extracts as an antioxidative agent.

To provide a better understanding of the potential of ergothioneine present in mushroom extracts in the mitigation of oxidative degradation of astaxanthin, Chapter 2 of the current study focuses on their more detailed interactions in an *in vitro* liposome model. Although previous studies have already demonstrated that the presence of other antioxidants together with astaxanthin helps prevent the oxidation of the latter, these have been mainly conducted in simplified model systems that do not necessarily simulate the actual positioning of astaxanthin within a physiological context. By utilizing liposomes as a model system to reflect the actual occurrence of astaxanthin in cellular membranes, the results from this chapter provide a resounding rationale as to why ergothioneine-rich mushroom extracts would be capable of improving the stability of astaxanthin found in salmonid muscles.

Equipped with the basis for utilizing ergothioneine-rich mushroom extracts as a protective agent for membrane-bound astaxanthin, Chapter 3 focuses on demonstrating the effectiveness of the *in vitro* application of ergothioneine-rich mushroom extracts in stabilizing astaxanthin in salmonid (*Oncorhynchus mykiss*) muscles. Additionally, the feasibility of incorporating ergothioneine-rich mushroom extracts into the feeds of salmonids (*O. mykiss*) as a strategy to accumulate ergothioneine within the fish prior to harvest was also investigated. The latter experiment was conducted in light of previous studies that reported the conserved occurrence of the mechanism for ergothioneine uptake (i.e. ergothioneine specific transporter proteins) across several taxa of vertebrates. Results from this experiment showed a considerable accumulation of ergothioneine into the blood and meat of *O. mykiss* suggesting the presence of uptake mechanisms. Furthermore, supplementation of mushroom extracts to feeds given to experimental fish did not adversely affect growth rates and other characteristics, pointing to the viability of this strategy for salmonid culture.

To test whether ergothioneine supplementation through mushroom extracts during culture translated into a harvested product with improved astaxanthin stability, a feeding experiment was conducted in another salmonid species, *Oncorhyncus kisutch* after which, harvested muscles were monitored for astaxanthin stability during low temperature storage. These experiments comprise Chapter 4 of this study. Results from this feeding experiment confirmed that the ability to take in ergothioneine through their diet appears to be conserved among salmonids. Furthermore, the storage studies not only demonstrated the ability of ergothioneine to preserve astaxanthin in salmonid muscle but also effectively reduced lipid oxidation, which also affects the overall quality of salmonid products.

In order to further support the observed uptake of ergothioneine in salmonids, Chapter 5 of this study focuses on the identification of genes encoding for the ergothioneine transporter (ETT) in some of the most commercially important salmonid species. Given the vast amount of genetic data made available for the salmonids *O. mikyss*, *O. kisutch*, and *Salmo salar*, and previous work on the sequences of ETTs from other organisms, a bioinformatics approach to mine salmonid genomes for their corresponding ETTs resulted in specific genes for each species that exhibit the highest probability for ergothioneine transport. The information provided in this chapter should, therefore, be used as a rational basis for more resource intensive molecular biology work to understand the various aspects of ergothioneine uptake in salmonids.

The final chapter of this thesis summarizes all these results and provides possible directions for future work on the application of ergothioneine-rich mushroom extracts in salmonid culture and post-harvest.

CHAPTER 2

Effects of the ergothioneine-rich mushroom extract on the oxidative stability of liposomal astaxanthin under radical oxidation-induced conditions

2.1 Introduction

Astaxanthin is a naturally occurring pigmenting compound that has potent antioxidative properties. It is commonly found in aquatic organisms which is synthesized by certain algae such as the *Haematococcus pluvialis*[15, 24], which are consumed by crustaceans and can be deposited into the carapace. These crustaceans are later eaten by larger fish like salmon, and astaxanthin is metabolized and deposited into the flesh and results to a bright reddish orange muscle pigmentation. Astaxanthin and other carotenoid compounds are known to have relatively stronger antioxidative properties than other antioxidants such as α-tocopherol, Coenzyme Q10, and α-lipoic acid[3, 30]. Amongst the carotenoids with antioxidative properties, astaxanthin exhibits the strongest activity for singlet oxygen quenching and lipid peroxidation suppression[16] wherein astaxanthin has been shown to be twice as effective as β-carotene in terms of inhibiting hydroperoxide production. In addition, the astaxanthin structure with conjugated polyene chains and terminal ring moieties makes it considerably efficient for scavenging lipid peroxyl radicals and reactive oxygen species, both within and at the surface of the cell membrane[3].

However, the highly unsaturated structure of this pigment makes it susceptible to lipid oxidation and discoloration, which in turn could compromise the market quality of commodities containing this compound. Previous studies have demonstrated that incorporating other antioxidants, such as ascorbic acid and α-tocopherol, in a system together with astaxanthin significantly improves the oxidative stability of astaxanthin[31, 32]. Based on these findings, it was hypothesized that other antioxidants such as (2*S*)-3-(2-sulfanylidene-1,3-dihydroimidazol-4-yl)-2-(trimethylazaniumyl)propanoate (ergothioneine) could also improve the stability of astaxanthin

when incorporated together in food products such as salmonid muscle. Lipid oxidation is a process of fatty acid degradation wherein free radical species abstract electrons from a lipid molecule resulting in the alteration of the structure and function of certain fatty acids. As previously discussed (Chapter 1), lipid oxidation process proceeds in a chain reaction mechanism, which starts with an initiation phase, followed by the propagation phase, and ends in the terminal phase. The presence of a free radical species coupled with certain extrinsic factors can initiate the oxidation process, which could then result in the entire oxidation process.

Ergothioneine is a hydrophilic antioxidant compound that is mostly synthesized by certain fungal species, and several edible mushroom species were found to contain a relatively high concentration of ergothioneine with corresponding antioxidative properties[54]. In some previous studies, the addition of ergothioneine-rich mushroom extracts (both externally onto the surface and through incorporation into the diet) into certain meat products showed significantly positive effects in controlling lipid oxidation and discoloration during post-harvest storage[48, 55, 56]. These previous studies were tested on crustaceans, cattle meat, and high myoglobin-containing fish meat. However, no studies have been conducted regarding the antioxidative and other effects of ergothioneine in astaxanthin-rich muscle tissues of salmonid fish. With this, an *in vitro* study was conceptualized to assess the feasibility of the antioxidative protection of ergothioneine in preventing the oxidative degradation of astaxanthin in a cellular model using liposomes.

Liposomes are self-enclosed circular vesicles made up of phospholipid bilayer enclosing in a compartment of an aqueous medium from a bulk aqueous medium it is suspended from[57]. The membrane is made up of amphiphiles such as phospholipids[58] that orients together in an orderly manner according to their polarity. These vesicles can be classified based on their size and lamellarity. A giant vesicle membrane resembles that of a biological cell[57] and is the reason why

liposomes are widely used in research as to mimic or as a cell model. Other applications of liposomes have also been done in drug delivery system and in molecular biology studies.

This study aimed to elucidate the effects of the ergothioneine-rich mushroom extract on the oxidative stability of astaxanthin and lipid components in a liposome under oxidation-induced conditions. Specifically, this study aimed to evaluate the effects of astaxanthin and ergothioneine-rich mushroom extract on lipid hydroperoxide formation and fatty acid stability in a liposome. In addition, this study aimed to determine the effects of different ergothioneine and astaxanthin concentrations on the stability of liposomal astaxanthin and lipid components in the presence of radical initiators. Furthermore, this study aimed to determine and compare the antioxidative properties of the pure authentic standard of ergothioneine with crude hydrophilic extracts of mushroom.

2.2 Materials and methods

2.2.1 Materials and chemicals.

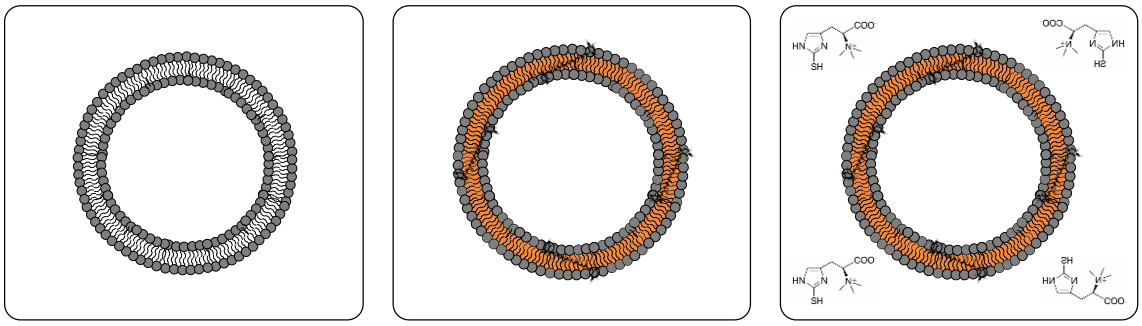
Egg yolk lecithin (95.0% purity) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Authentic astaxanthin (97.0% purity) and L-ergothioneine (98.0% purity) were purchased from Abcam, Inc. (Cambridge, UK), and Focus Biomolecules (Plymouth Meeting, PA), respectively. Radical initiators 2,2'-azobis(2-methyl-propionamidine)dihydrochloride (AAPH, 97.0% purity) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN, 98.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively.

2.2.2 Mushroom extract (ME) preparation

A 10 g portion of lyophilized *Pleurotus cornucopiae* was added to 200 mL 70% (v/v) ethanol, sonicated for 5 min, and filtered. The recovered residue was extracted with 100 mL 70% (v/v) ethanol, and this extraction was repeated 3 times. All filtrates were combined and evaporated to dryness *in vacuo* using a rotary evaporator. A 100 mL aliquot of PBS pH 7.4, was added to the flask to obtain the crude ME, with total dissolved solids of 2.80 ± .010 °Bx. The ergothioneine content of the ME amounted to 44.82 ± 0.12 mg ergothioneine per mL. The extract was further diluted to a final ergothioneine concentration of 0.25 mg/mL, which was used in the subsequent experiments.

2.2.3 Liposome preparation

Liposomes were prepared according to the method of Moscho et al. (1996)[59] with a slight modification. For the preparation of liposomes without astaxanthin or ME, a 0.4 mL portion of 0.1 M lecithin in chloroform was added to a 1000 mL round-bottom flask containing 4 mL chloroform and 0.8 mL methanol, followed by the careful addition of 17 mL 10 mM PBS along the interior walls of the flask. Subsequently, organic solvents were evaporated using a rotary evaporator under reduced pressure at 40°C. The resultant opalescent liquid containing the liposomes was diluted to a final volume of 17 mL. For the preparation of liposomes containing astaxanthin, 4 mL of astaxanthin solution in chloroform (100 µg/mL) was used instead of chloroform. For liposome preparation with ergothioneine, 6.8 mL of ME containing 0.25 mg/mL ergothioneine was added after the evaporation step and subsequently diluted to a 17-mL final volume. The liposome solutions were aliquoted into 1.5 mL polypropylene snap-cap microcentrifuge tubes. Liposome formation was confirmed by bright field and fluorescence microscopy. A graphical illustration of the different liposome treatments is presented in Figure 2.1.



no AST, no ME/ESH | with AST, no ME/ESH | with AST, with ME/ESH

Figure 2.1. Different liposome preparations

AST: astaxanthin
ME: crude mushroom extract
ESH: L-(+)-ergothioneine *standard*

2.2.4 Microscopy evaluation

Liposomes containing astaxanthin were observed under the bright-field setting of an Olympus CKX41 Microscope (Tokyo, Japan) equipped with a WRAYCAM G130 Wraymer microscope camera (Osaka, Japan) to evaluate the aggregation of astaxanthin pigments. 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-glycero-3-phospho-choline (NBD-labeled PC, 99.0% purity, Avanti Polar Lipids, Inc., Alabaster, AL) was mixed with lecithin and chloroform prior to liposome preparation, and fluorescence in the liposomes was observed at an excitation wavelength of 463 nm and an emission wavelength of 536 nm to determine the structure of the formed vesicles.

2.2.5 Oxidative stability tests

The first oxidative stability test evaluated the effects of incubation temperature and the addition of radical initiators on the progress of oxidation in liposomes. The 3 liposome preparations described in Figure 1 were subjected to oxidative conditions induced by the addition of either hydrophilic AAPH or lipophilic AMVN radicals. The molar ratio of lecithin to radical initiators added was 2:1. The liposome preparations were then incubated at either 25 or 37 °C, and

the progress of oxidation and stability of lipid, astaxanthin, and ergothioneine were monitored quantitatively.

The second oxidative stability test evaluated the effects of varying ME concentration on the progress of oxidation in liposomes. Six different liposomes were prepared: liposomes without astaxanthin or ME, liposomes with astaxanthin without ME, liposomes with astaxanthin and ME containing 0.15 mM ergothioneine, liposomes with astaxanthin and ME containing 0.30 mM ergothioneine, liposomes with astaxanthin and ME containing 0.45 mM ergothioneine, and liposomes with astaxanthin and ME containing 0.70 mM ergothioneine. These different liposomes were separately added with either AAPH or AMVN, and incubated at 37 °C for 24 h, and evaluated accordingly.

The third oxidative stability test evaluated the effects of varying astaxanthin concentration on the progress of oxidation in liposomes. Four different liposomes with ME were prepared with varying astaxanthin concentration: liposomes without astaxanthin, liposomes with 15 µM astaxanthin, liposomes with 30 µM astaxanthin, and liposomes with 60 µM astaxanthin. These different liposomes were separately added with either AAPH or AMVN, and incubated at 37 °C for 24 h, and evaluated accordingly.

2.2.6 Crude mushroom extract and authentic ergothioneine comparison

The fourth oxidative stability test evaluated and compared the effects of ME and authentic ergothioneine on the progress of oxidation in liposomes. The different liposome treatments prepared were: liposomes without astaxanthin or ME/ergothioneine, liposomes with astaxanthin but no ME/ergothioneine, liposomes with astaxanthin and ME containing 100 µg/mL ergothioneine, and liposomes with astaxanthin and 100 µg/mL ergothioneine. These different

liposomes were separately added with either AAPH or AMVN, and incubated at 37 °C for 24 h, and evaluated accordingly.

Comparison of the radical scavenging activities of ME and authentic ergothioneine was done using aqueous solutions, not liposomes, and these were compared with other hydrophilic antioxidants such as glutathione and ascorbic acid.

2.2.7 Quantitative evaluation

The effects of the addition of radical initiators to the oxidative stability of liposomal components were quantitatively evaluated for total HPO formation, astaxanthin, ergothioneine, and fatty acid stability. Methods used for each quantification procedure are described below.

Total lipid HPO quantification. Total lipid HPO was quantitatively measured using the flow injection analysis (FIA) system, according to the method of Sohn et al. (2005)[60]. Briefly, the lipid components of liposomes were extracted by the Bligh and Dyer method[61] with NBD-labeled PC used as an internal standard (IS). The chloroform layer was collected and subjected to analysis. A calibration curve was obtained using different concentrations of authentic cumene hydroperoxide (80.0 % purity, Sigma-Aldrich). All data are expressed as equivalent nmol cumene hydroperoxide per mL liposome.

Fatty acid quantification. Total lipids in liposomes were extracted using the Bligh and Dyer method[61]. Fatty acid methyl esters (FAMEs) were derivatized according to the Official Methods and Recommended Practices of the AOCS, using methyl tricosanoate (99.0% purity, Nu-check Prep, Inc., Waterville, MN) as an IS. FAMEs were separated and quantified using a Shimadzu gas chromatograph model GC-2010 (Kyoto, Japan) equipped with a Supelcowax™ 10 fused silica

capillary column (60 m × 0.32 mm i.d. × 0.25 µm film thickness), and a flame ionization detector. A calibration curve was obtained using different concentrations of FAME (99.6% purity, Nu-check Prep, Inc.). All data are expressed as mM FAME.

Determination of thiobarbituric acid reactive substances (TBARS). TBARS present in liposomes were quantified as secondary products of lipid oxidation, adapting the methods of Schmedes and Hølmer (1989)[62] and Uchiyama and Mihara (1978)[63], with slight modifications. A calibration curve was obtained using varying concentrations of authentic 1,1,3,3'-tetraethoxypropane. All data are expressed as µM malondialdehyde (MDA) equivalents.

Total astaxanthin quantification. Total astaxanthin content was quantified using the method previously described[64]. Briefly, collected liposomes were extracted using the Bligh and Dyer method[7] with the addition of *trans*-β-apo-8'-carotenal (96.0% purity, Sigma-Aldrich) as an IS. The chloroform layer was collected and subjected to HPLC analysis. A calibration curve was obtained using different concentrations of authentic astaxanthin. All data are expressed as µg astaxanthin per mL liposome.

Ergothioneine quantification. Ergothioneine content was determined following the methods described by Nguyen et al. (2012)[65]. Liposomes were subjected to Bligh and Dyer extraction[61] with the addition of 3-methyl-1H-imidazole-2-thione (99.0% purity, Sigma-Aldrich) as an IS. The water-methanolic layer was collected, evaporated to dryness *in vacuo* using a rotary evaporator, and dissolved in 3 mL distilled water. A calibration curve was obtained using different concentrations of authentic ergothioneine. All data are expressed as µg ergothioneine per mL liposome.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity. The radical scavenging activity of ergothioneine in crude ME was determined according to the method of Giri, et al. (2011)[66] with a slight modification. The samples used for ergothioneine analysis were subjected to HPLC analysis, and bleaching of 0.1 mM methanolic 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95.0% purity, Sigma-Aldrich) solution in the post-column reaction coil was monitored at 517 nm with an SPD-M10AVP. Varying concentrations of authentic ergothioneine were used to obtain a calibration curve, with all data expressed as equivalent mmol ergothioneine per mL sample.

Determination of DPPH radical scavenging EC_{50}. The concentration of a sample that possessed a half-maximal DPPH radical scavenging activity was determined following the methods of Chen and Ho (1995)[67] and Bao et al. (2010)[68], with slight modifications. The aqueous sample was added to a 0.1 mM methanolic DPPH solution and measured at 517 nm using a Shimadzu UV-1600-PC spectrophotometer. All data are expressed as DPPH radical scavenging EC_{50}.

Oxygen absorbance radical capacity (ORAC) assay. The assay used to determine the ORAC of the samples was adapted and slightly modified from the method of Ou et al. (2001)[69], using 0.08 M fluorescein as a probe in the oxidation-induced condition in the presence of 0.15 mM AAPH. Fluorescence signals were monitored at 1 min intervals for 90 min using a TECAN SPECTRAFluor Plus microplate reader (Männedorf, Switzerland) set at 37 °C. Varying concentrations of authentic Trolox were used to obtain a calibration curve. All data are expressed as µmol Trolox equivalent per g sample.

Determination of total phenolic compounds. The total amount of phenolic compounds present in the ME samples was measured using the method described by Bao et al. (2010)[68]. Varying

concentrations of authentic gallic acid were used to obtain a calibration curve, with all data expressed as µg gallic acid per mL sample.

Statistical analysis. Microsoft Excel 2016 was used to analyze the means and standard deviations of the collected data and to generate graphs. IBM SPSS Statistics 20 was used to determine significant differences among values at a 5% level of significance.

2.3 Results

2.3.1 Liposome formation

Representative photos of the liposomes are shown in Figure 2.2. The bright field micrograph (Figure 2.2(a)) shows the aggregation of astaxanthin in liposomes seen as orange-colored spherical vesicles floating in the aqueous phase, and the phase-contrast micrograph (Figure 2.2(b)) shows the distinct circular outline of the liposomes. The fluorescence micrograph (Figure 2.2(c)) shows that NBD-labeled PC mixed with lecithin formed distinct circular outlines corresponding to a phospholipid bilayer emitting bright fluorescence, which distinctly separated the intracellular and the extracellular aqueous phases. Average sizes of the liposomes formed were 10–25 µm in diameter; thus, they can be classified as giant-sized vesicles. These observations confirm the physical features of the liposomes.

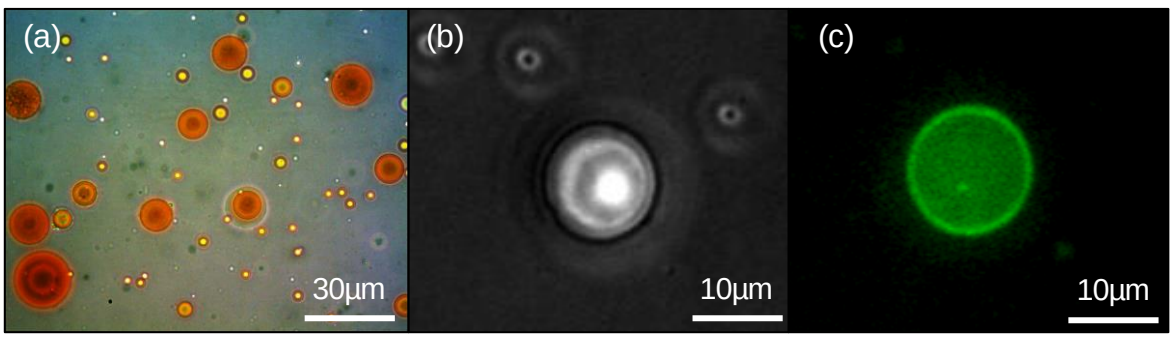


Figure 2.2. Micrograph of liposomes formed as viewed by bright-field (a), phase-contrast (b), and fluorescence (c) microscopy.

2.3.2 Effects of the ergothioneine-rich mushroom extract on the stability of liposomal lipid and astaxanthin under oxidation-induced conditions.

The first hypothesis that astaxanthin and ergothioneine-rich crude mushroom extracts in the liposomal solution can inhibit the progress of oxidation was tested under oxidation induced conditions in the presence of radical initiators AAPH and AMVN in different temperatures (25 °C and 37 °C). Three different liposomes were used—liposome A contained no astaxanthin or ME, liposome B contained astaxanthin but no ME, and liposome C contained both astaxanthin and ME—and evaluated in a time course experiment.

Effects of the oxidation-induced conditions on the liposomal lipid components. Changes in the total lipid HPO content of the different liposome treatments in varying incubation conditions are shown in Figure 2.3. Liposomes without radical initiators did not show any significant increase in lipid HPO ($p > 0.05$) after 17 days of incubation at 25 °C (Figure 2.3(a)). Addition of AAPH at 25 °C significantly increased ($p < 0.05$) the HPO content of liposomes without ME, while liposomes with ME did not exhibit any significant increase ($p > 0.05$) during the 8-day incubation period (Figure 2.3(b)). The increase of the incubation temperature to 37 °C resulted in a further increase ($p < 0.05$) in lipid HPO content of liposomes without ME, while liposomes with ME continued to have the lowest content, with no significant increase during the 24-h incubation period (Figure 2.3(c)). The addition of AMVN resulted in a higher magnitude of lipid HPO increase in all the liposome treatments during the 5-day incubation at 25 °C (Figure 2.3(d)). Liposomes without astaxanthin or ME had the highest level of lipid HPO formed, followed by liposomes with astaxanthin and without ME, while liposomes with both astaxanthin and ME had the lowest levels. The increase of the incubation temperature to 37 °C with AMVN further increased lipid HPO formation in all liposomes, exhibiting no significant difference ($p > 0.05$) (Figure 2.3(e)). It was confirmed that the addition of both hydrophilic and lipophilic radical

initiators and elevation of incubation temperature remarkably increased the amount of lipid HPO formed.

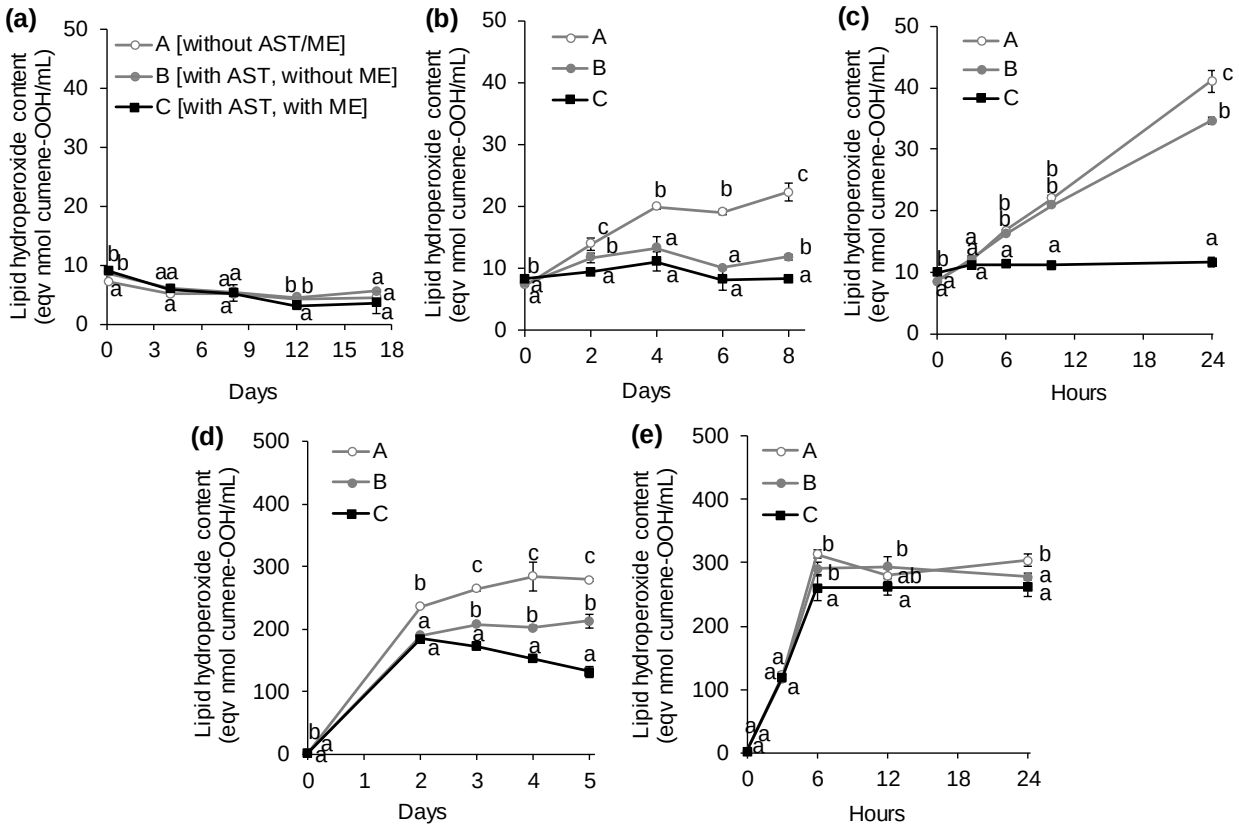


Figure 2.3. Changes in total lipid HPO content of liposomes at 25 °C without radical initiator (a), 25 °C with AAPH (b), 37 °C with AAPH (c), 25 °C with AMVN (d), and 37 °C with AMVN (e). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters indicate significant differences among treatment groups at each time point ($p < 0.05$).

Another parameter used to evaluate the effect of lipid oxidation in the liposomes was the quantitative determination of the fatty acids. Dominant constituent fatty acids of the liposomes were identified as C14:0, C16:0, C16:1n-7, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, and C20:4n-6 (Figure 2.4). After 24 h incubation with AAPH at 37 °C, no observable decrease ($p > 0.05$) was noted in most of the fatty acids except for PUFAs, for which concentrations in liposomes with both astaxanthin and ME were higher ($p < 0.05$) than those in all other liposomes (Figure 2.5).

Incubation with AMVN showed a decreasing trend for most constituent fatty acids, wherein the liposomes without ME had lower ($p < 0.05$) concentrations than the those of the liposomes with ME. However, no significant difference among liposomes was observed for C14:0, C18:1n-7, and C20:4n-6 (Figure 2.6).

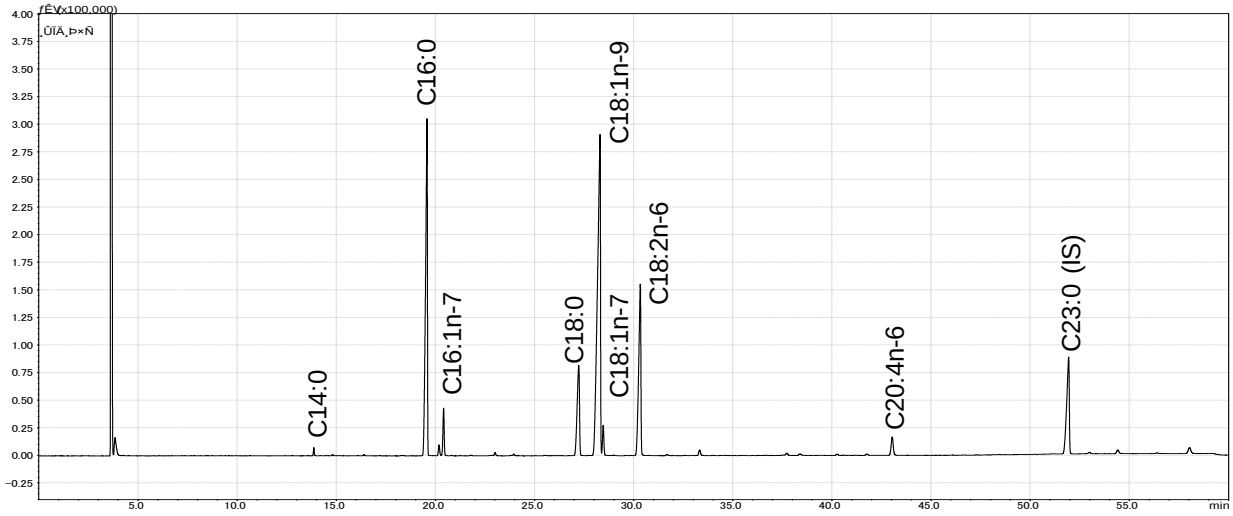


Figure 2.4. Representative chromatogram showing the dominant fatty acids present in the liposomes.

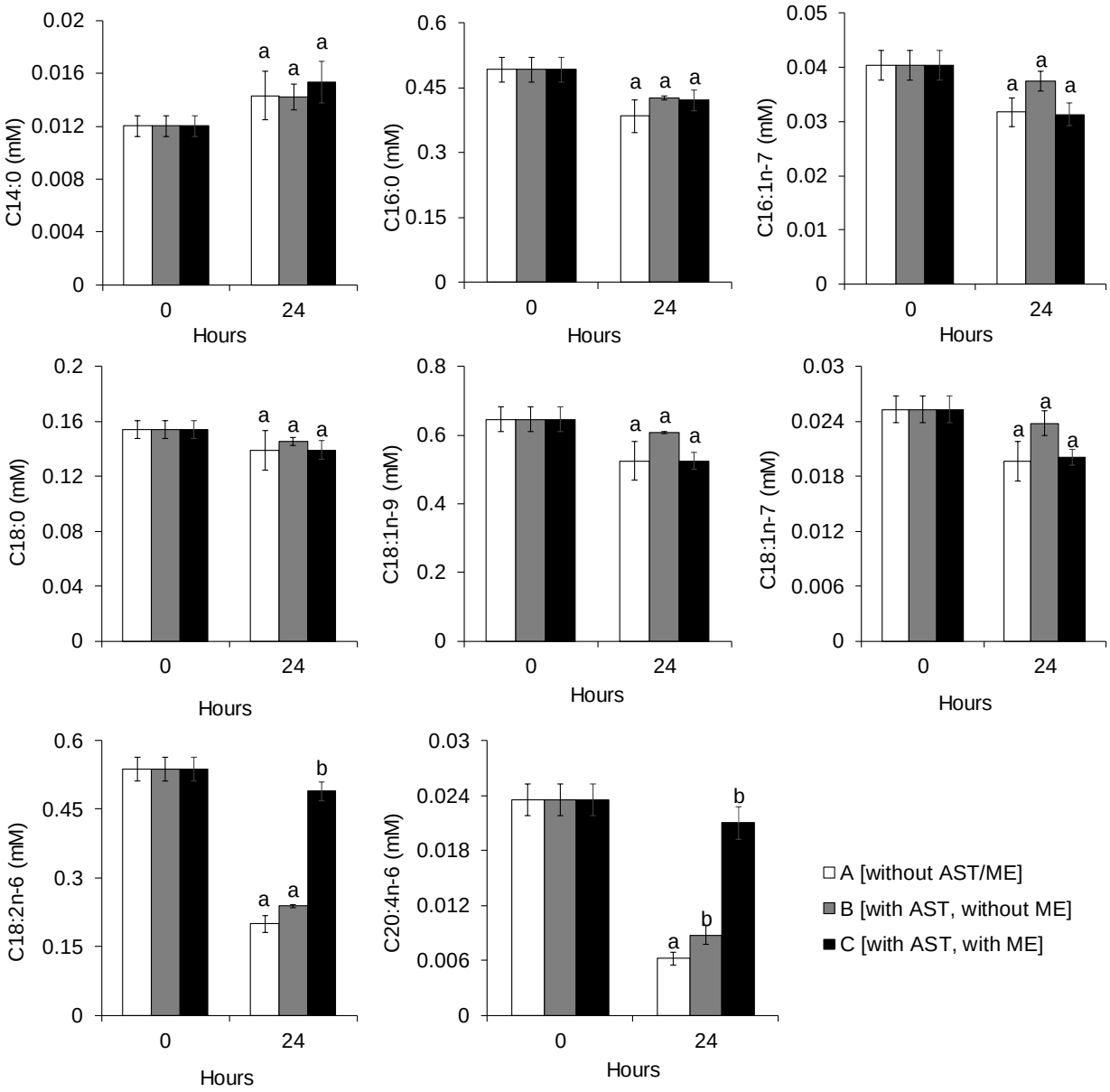


Figure 2.5. Changes in the fatty acid content of liposomes after 24 h incubation at 37 °C with AAPH. Data are presented as mean ± standard deviation (n=3). Values with different superscript letters indicate significant differences among treatment groups at each time point ($p < 0.01$).

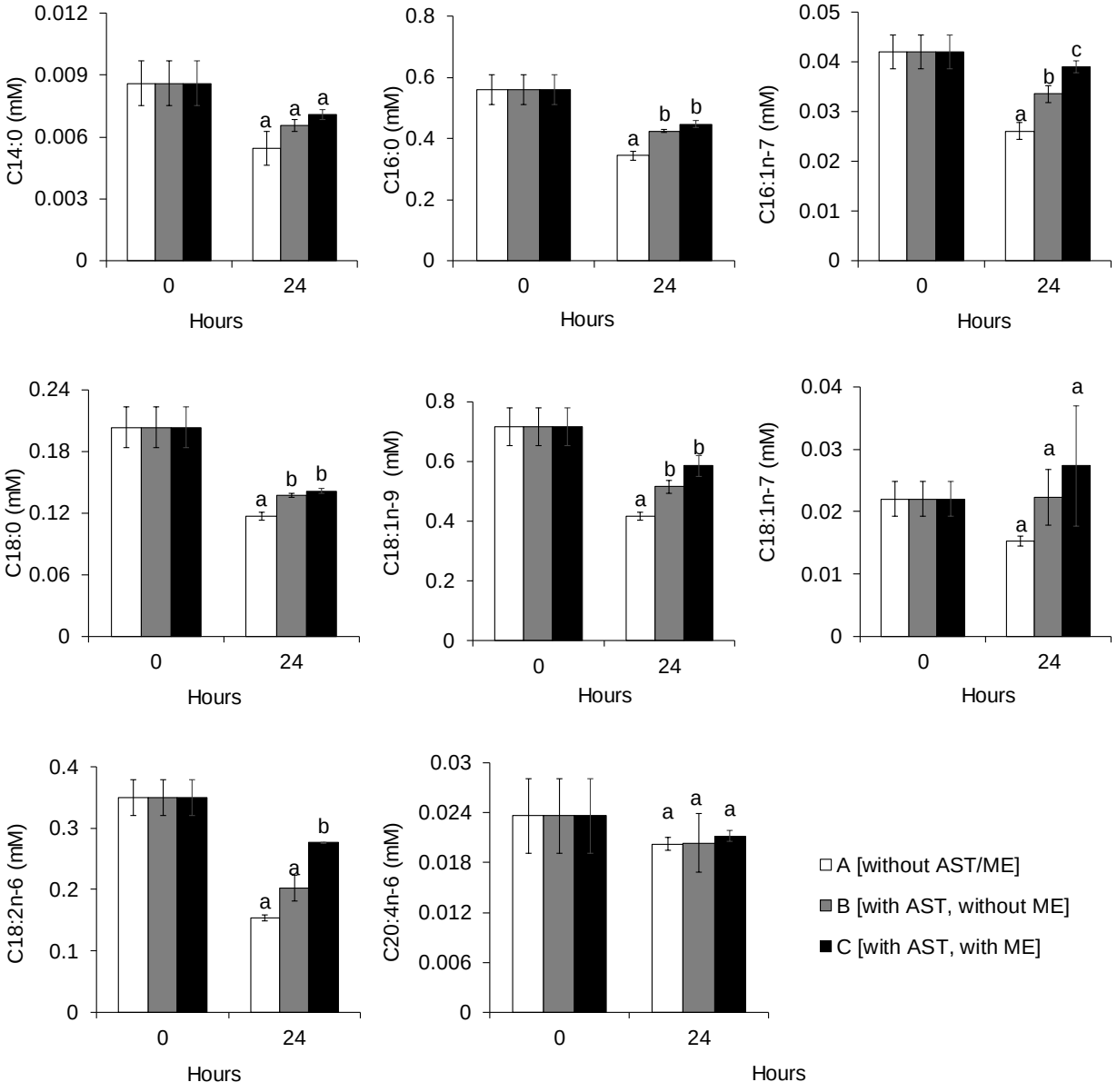


Figure 2.6. Changes in the fatty acid content of liposomes after 24 h incubation at 37 °C with AMVN. Data are presented as mean ± standard deviation (n=3). Values with different superscript letters indicate significant differences among treatment groups at each time point ($p < 0.01$).

Effects of the oxidation-induced conditions on the astaxanthin content. Changes in astaxanthin content of liposomes during incubation are shown in Figure 2.7. The decrease in astaxanthin content of liposomes was noted for all incubation conditions, and the addition of radical initiators and increase of incubation temperature further accelerated the degradation rate of astaxanthin. Despite the continuous decrease in astaxanthin content, a significant difference ($p < 0.05$) in the degradation rate was observed between liposomes with and without ME.

Liposomes incubated at 25 °C without radical initiators had the slowest rate of astaxanthin degradation among all incubation conditions tested (Figure 2.7(a)). The addition of AAPH and 8-day incubation period resulted in the decrease of astaxanthin content to 76% and 47% in the liposomes with and without ME, respectively (Figure 2.7(b)). The increase of the incubation temperature to 37 °C with AAPH further accelerated astaxanthin degradation during a 24-h incubation period to 66% and 32% for the liposomes with and without ME, respectively (Figure 2.7(c)). The degradation rate of astaxanthin in liposomes was increased when incubated with AMVN compared to the rate in liposomes incubated with AAPH, wherein after 5 days of incubation, the liposomes without ME contained 14% total residual astaxanthin while the liposomes with ME contained 27% (Figure 2.7(d)). The increase of the incubation temperature in the presence of AMVN further accelerated astaxanthin degradation, as residual astaxanthin dropped in liposomes with and without ME to 36% and 25%, respectively, after 24 h incubation (Figure 2.7(e)).

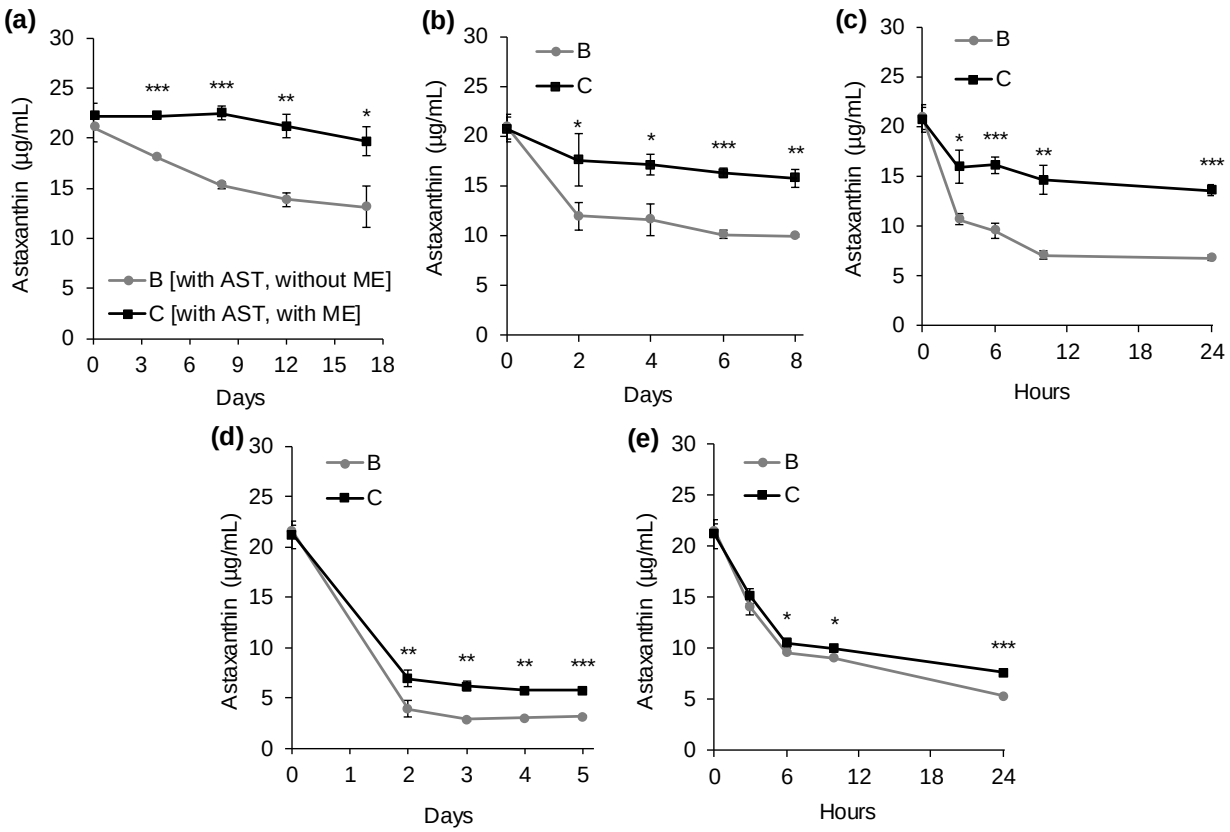


Figure 2.7. Changes in astaxanthin content of liposomes at 25 °C without radical initiator (a), 25 °C with AAPH (b), 37 °C with AAPH (c), 25 °C with AMVN (d), and 37 °C with AMVN (e). Data are presented as mean ± standard deviation (n=3). Asterisks represent significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between treatments at each time point.

Effects of the oxidation-induced conditions on the ergothioneine content. Based on the initial results, it was observed that liposome with ME exhibited controlled lipid HPO formation and decreased rate of astaxanthin degradation during incubation. This led to the monitoring of the stability of ergothioneine in this liposome. Effects of the addition of radical initiators on ergothioneine content in liposomes are shown in Figure 2.8. The total ergothioneine content was measured in liposomes with ME, and no significant decrease ($p > 0.05$) was observed in all the incubation conditions tested.

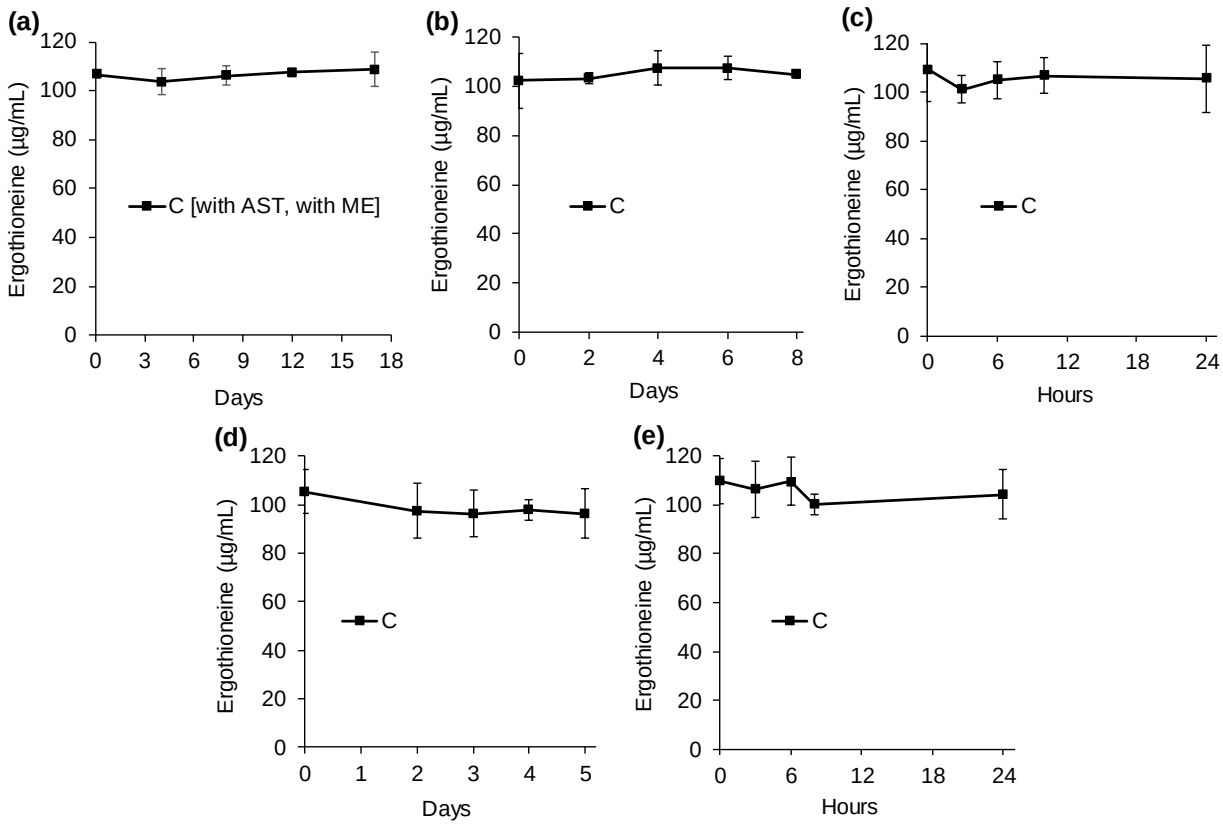


Figure 2.8. Changes in ergothioneine content of liposomes at 25 °C without radical initiator (a), 25 °C with AAPH (b), 37 °C with AAPH (c), 25 °C with Values with asterisks represent significant differences (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) compared to the initial concentration.

2.3.3 Effects of different concentrations of ergothioneine-rich ME on the stability of liposomal lipid and astaxanthin under oxidation-induced conditions.

The results of the initial hypotheses testing showed that the addition of ergothioneine-rich ME in liposomes controlled lipid oxidation and astaxanthin degradation during oxidation-induced conditions. Based on these results, the next hypothesis was tested to determine the effects of the varying concentrations of ergothioneine in the stability of lipid and astaxanthin components of liposomes in the presence of radical initiators.

Effects of different concentration of ergothioneine-rich ME on the formation of lipid HPO in the presence of radical initiators. The results of the effects of varying ME concentration on the lipid HPO formation are shown in Figure 2.9. In the incubation with AAPH, liposomes without

ME showed significant increase in HPO formation during the 24-h incubation (Figure 2.9(a)). The addition of ME mitigated the formation of HPO, wherein the level of total HPO remained almost the same with the initial value at 0 hour, and no significant difference ($p > 0.05$) was observed among the 4 liposomes with different ME concentrations. In the incubation with AMVN, all six of the liposomes increased the HPO content with no significant difference ($p > 0.05$) observed (Figure 2.9(b)).

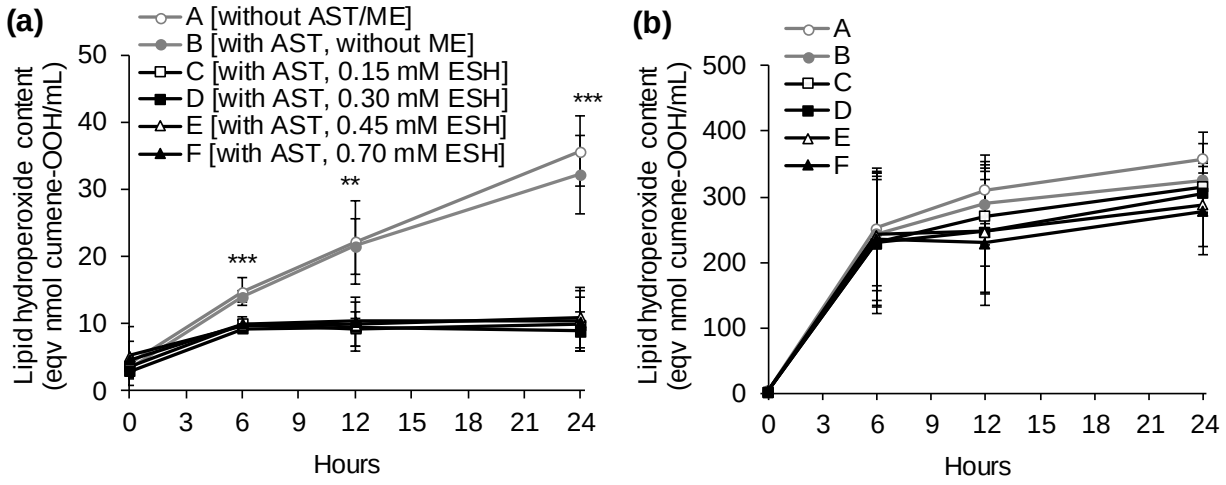


Figure 2.9. Changes in total lipid hydroperoxides content of liposomes with different ME concentration incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among liposomes (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

Effects of different concentration of ergothioneine-rich ME on the stability of liposomal astaxanthin in the presence of radical initiators. The stability of astaxanthin was monitored during the incubation with radical initiators and results are shown in Figure 2.10. In the incubation with AAPH, the liposome without ME has the significantly lowest ($p < 0.05$) astaxanthin content among all (Figure 2.10(a)). The liposomes with different ME concentrations had a minimal decrease in astaxanthin content after the 24-h incubation period, wherein no significant difference ($p > 0.05$) was observed among these liposomes. The incubation with AMVN decreased the astaxanthin content all the liposomes as most notable after 6th and 12th hour of incubation, wherein

liposome without ME was significantly lower ($p < 0.05$) compared with the other liposomes that have ME (Figure 2.10(b)). On the 24th hour of incubation, the decrease in astaxanthin content of liposomes showed no significant difference ($p > 0.05$) among the 5 liposomes.

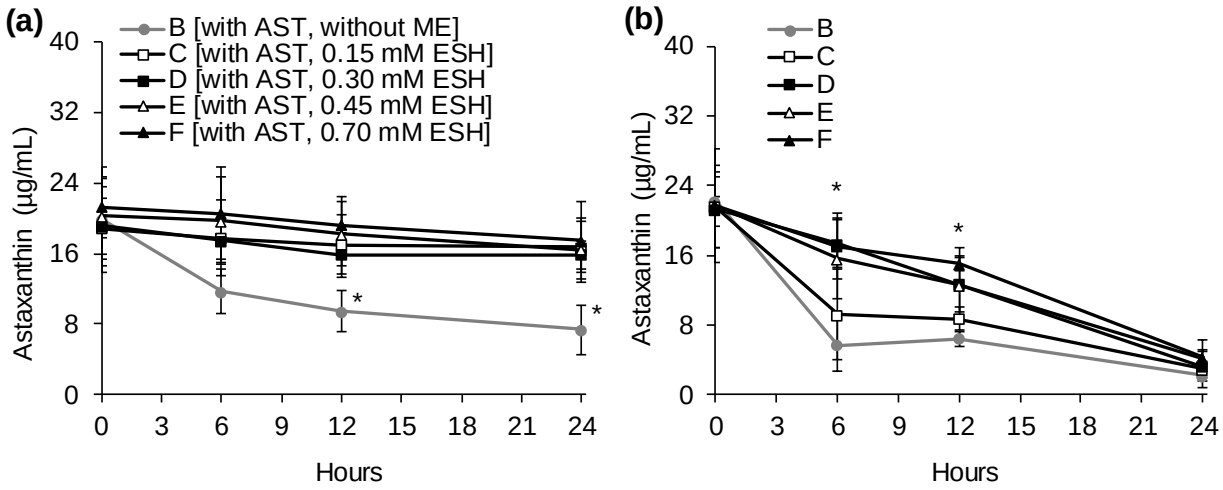


Figure 2.10. Changes in total astaxanthin content of liposomes with different ME concentration incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

Effects of incubation with hydrophilic and lipophilic radical initiators on the different concentrations of ergothioneine in crude ME. Changes in the ergothioneine content of liposomes with different ME concentration are shown in Figure 2.11. The 24-h incubation of liposomes with AAPH (Figure 2.11(a)) or AMVN (Figure 2.11(b)) did not significantly affect the stability of ergothioneine in all liposomes with ME, wherein no significant decrease ($p > 0.05$) was observed in both AAPH and AMVN incubation.

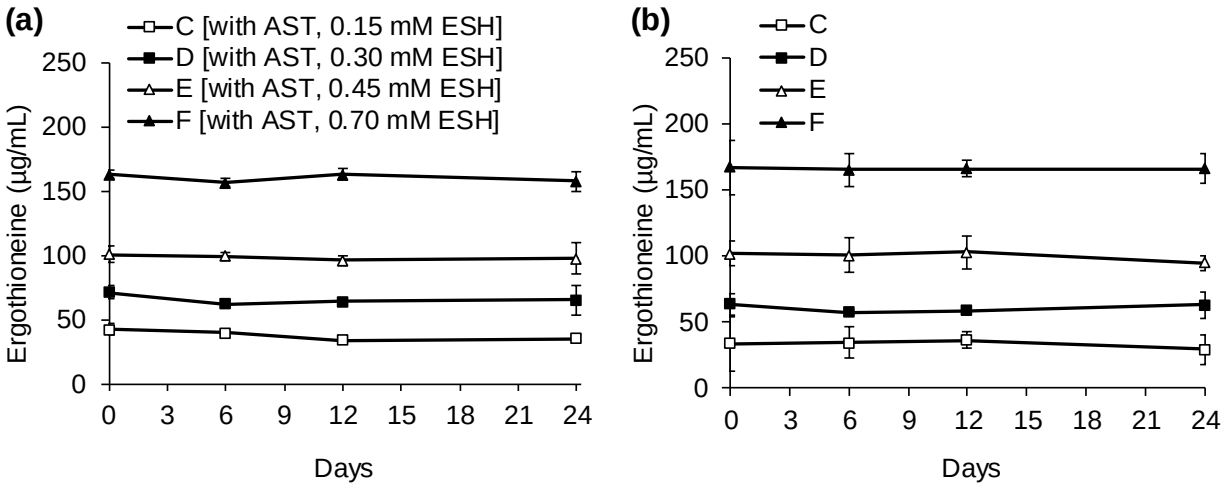


Figure 2.11. Changes in total ergothioneine of liposomes with different ME concentration incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

2.3.4 Effects of different concentrations of astaxanthin on the lipid HPO formation and astaxanthin stability

Changes in the lipid HPO content of liposomes with varying astaxanthin content during the incubation period are shown in Figure 2.12. The incubation with AAPH at 37 °C showed that HPO formation increased over time, however, the increase in liposome without astaxanthin was significantly faster ($p < 0.05$) compared to the other 3 liposomes with astaxanthin (Figure 2.12(a)). No significant difference ($p > 0.05$) was observed in the HPO content of the liposomes with astaxanthin. The all liposomes incubated with AMVN at 37 °C showed a much faster rate of HPO formation than those same liposomes incubated with AAPH (Figure 2.12(b)). Moreover, no significant difference ($p > 0.05$) was observed in the HPO content of all 4 liposomes during the incubation period.

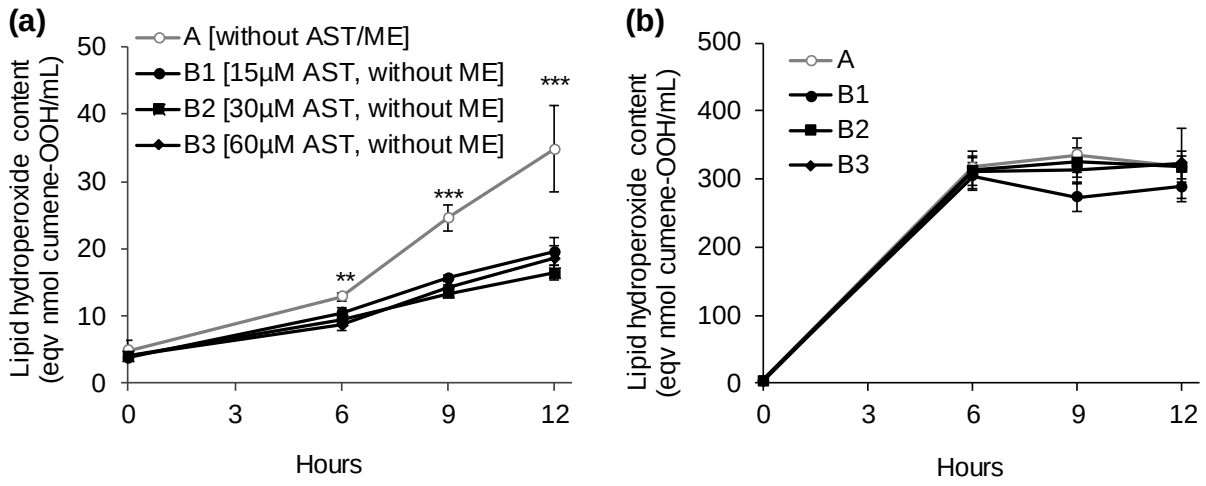


Figure 2.12. Changes in total HPO content of liposomes with different astaxanthin content incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

The stability of the different concentrations of liposomal astaxanthin was evaluated and results are shown in Figure 2.13. The incubation with both APPH (Figure 2.13(a)) and AMVN (Figure 2.13(b)) shows that astaxanthin content in all the liposomes decreased over time. The significant decrease ($p < 0.05$) in astaxanthin started 6 hours after the start of incubation, and continuously decreased until the end of the incubation period. It was also noted that the decrease of astaxanthin in liposomes with AMVN was faster than the rate of decrease than those incubated with AAPH.

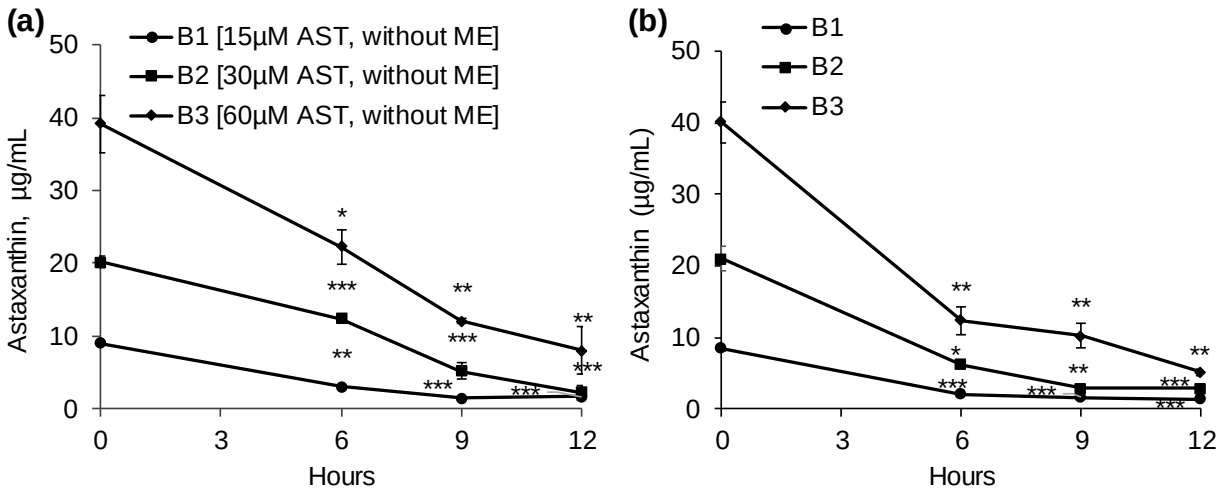


Figure 2.13. Changes in total astaxanthin content of liposomes incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

2.3.5 Evaluation of the effects of crude mushroom extract and authentic ergothioneine in the oxidative stability of liposomes

In this part of the experiment, the effects of crude ME in controlling oxidative degradation in liposomes were tested in parallel with an authentic ergothioneine, and alongside liposomes without ME or ergothioneine.

Effects on the progress of lipid oxidation. Changes in the lipid HPO content and TBARS of the liposomes are shown in Figures 2.14 and 2.15, respectively. Incubation of liposomes with AAPH at 37 °C resulted in a significant increase ($p < 0.05$) of lipid HPO in the liposomes without ME or ergothioneine, whereas the liposomes without AST or ME/ergothioneine had the highest HPO formed, while the liposomes containing either ME or ergothioneine did not exhibit any significant increase during the 24-h incubation period (Figure 2.14(a)). Incubation with AMVN resulted in a higher magnitude of increase in HPO formation in all liposomes (Figure 2.14(b)). Despite this increase, significant differences ($p < 0.05$) were observed wherein the liposomes without

astaxanthin or ME/ergothioneine had the highest HPO content, followed by liposomes with astaxanthin and without ME/ergothioneine, then by liposomes with astaxanthin and ergothioneine. Liposomes with astaxanthin and ME had the lowest HPO content among all the liposomes that were incubated with AMVN.

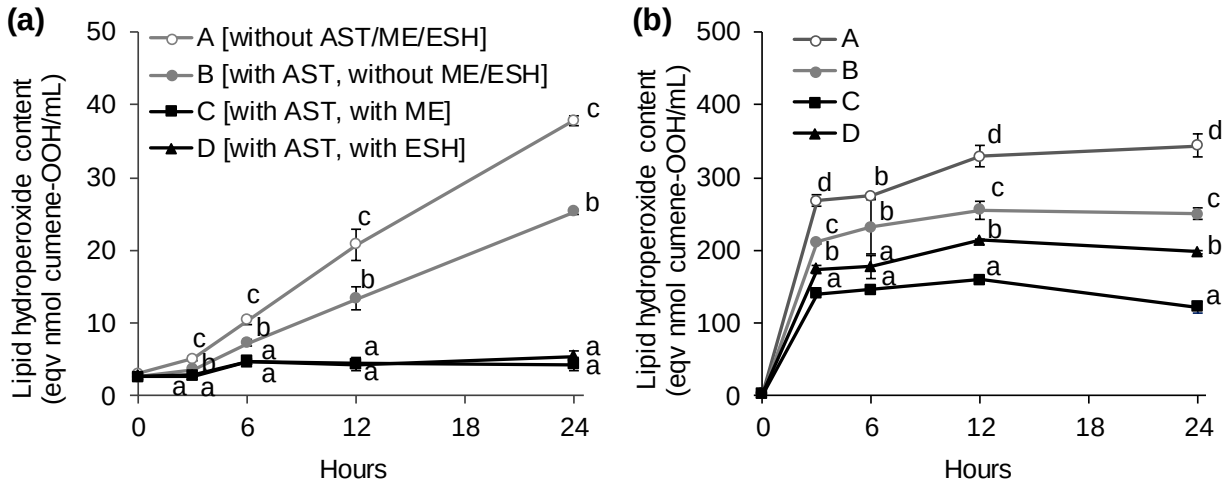


Figure 2.14. Changes in total HPO content of liposomes incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at a time point.

A similar pattern was observed for TBARS. Incubation with AAPH resulted in a significant increase ($p < 0.05$) of TBARS in liposomes without ME or ergothioneine, while liposomes with either ME or ergothioneine did not show any increase (Figure 2.15(a)). Incubation with AMVN showed an increase in TBARS in all liposomes (Figure 2.15(b)). Liposomes without astaxanthin or ME/ergothioneine had the highest levels of TBARS, followed by liposomes with astaxanthin and without ME/ergothioneine, then by liposomes with astaxanthin and ergothioneine. The liposomes containing both astaxanthin and ME had the lowest levels of TBARS at the end of the 24-h incubation period.

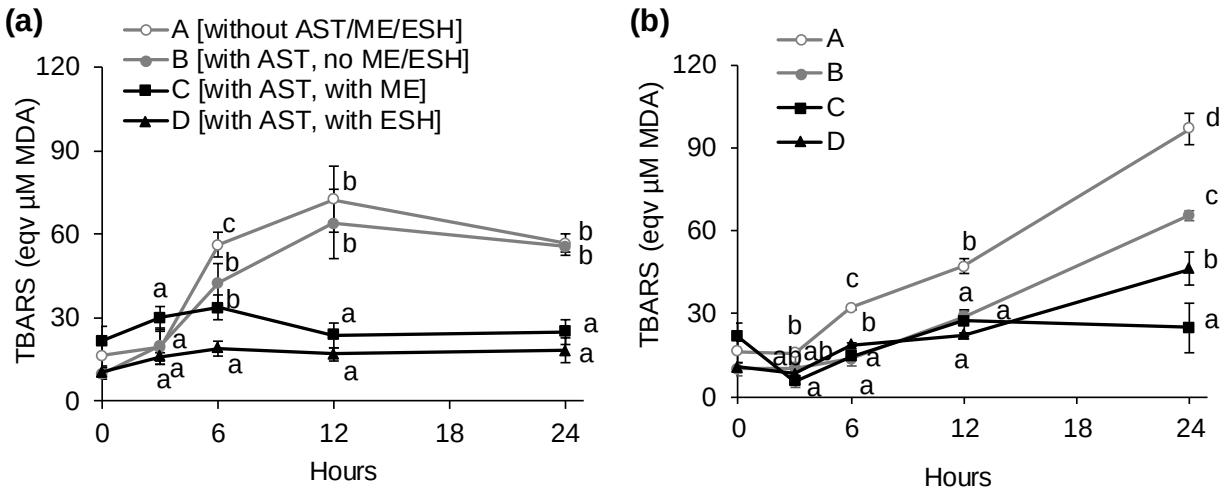


Figure 2.15. Changes in thiobarbituric acid reactive substances in liposomes incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at a time point.

Effects of crude ME and ergothioneine on the oxidative stability of astaxanthin. Changes in the astaxanthin content of liposomes as an effect of the presence or absence of ME or ergothioneine are shown in Figure 2.16. A decreasing pattern in the astaxanthin content was observed in all liposomes during incubation with either AAPH or AMVN. Incubation with AAPH showed that the liposomes without ME or ergothioneine had the lowest ($p < 0.05$) astaxanthin content and no significant difference ($p > 0.05$) was observed between the liposomes with ME or ergothioneine (Figure 2.16(a)). Incubation with AMVN demonstrated a faster rate of astaxanthin decrease wherein the liposomes without ME or ergothioneine had the lowest ($p < 0.05$) astaxanthin content, followed by liposomes with ergothioneine, and liposomes with ME had the highest residual astaxanthin content (Figure 2.16(b)).

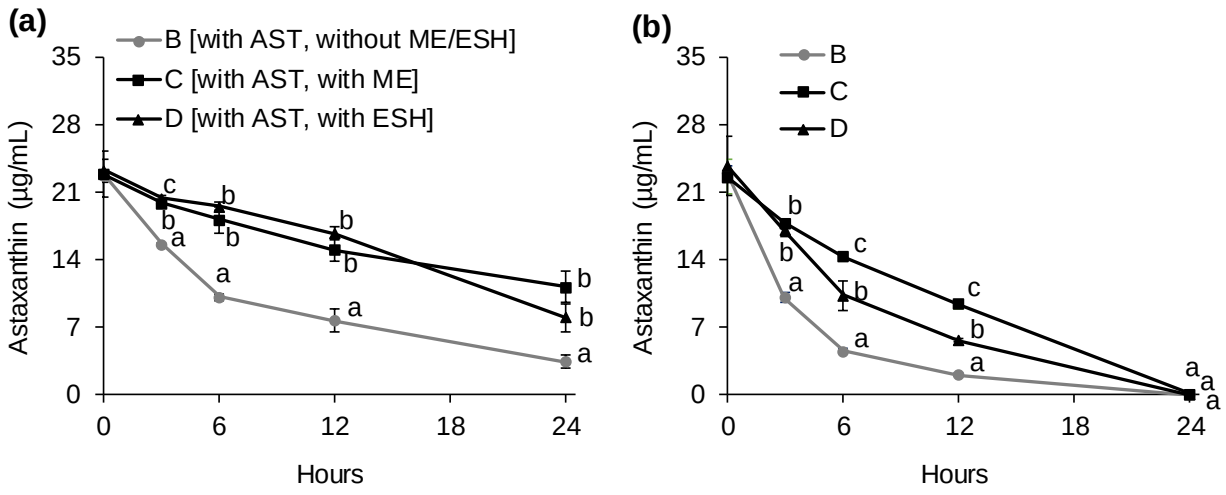


Figure 2.16. Changes in total astaxanthin content of liposomes incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

Stability of ergothioneine in the presence of radical initiators. Changes in the ergothioneine content of liposomes with ME and ergothioneine during incubation with AAPH or AMVN are shown in Figure 2.17. Ergothioneine quantification showed that after 24 h incubation with AAPH, a significant decrease ($p < 0.05$) in the ergothioneine content of liposomes with ergothioneine was observed, but not in liposomes with ME (Figure 2.17(a)). On the other hand, no significant decrease in ergothioneine content was observed in liposomes incubated with AMVN (Figure 2.17(b)).

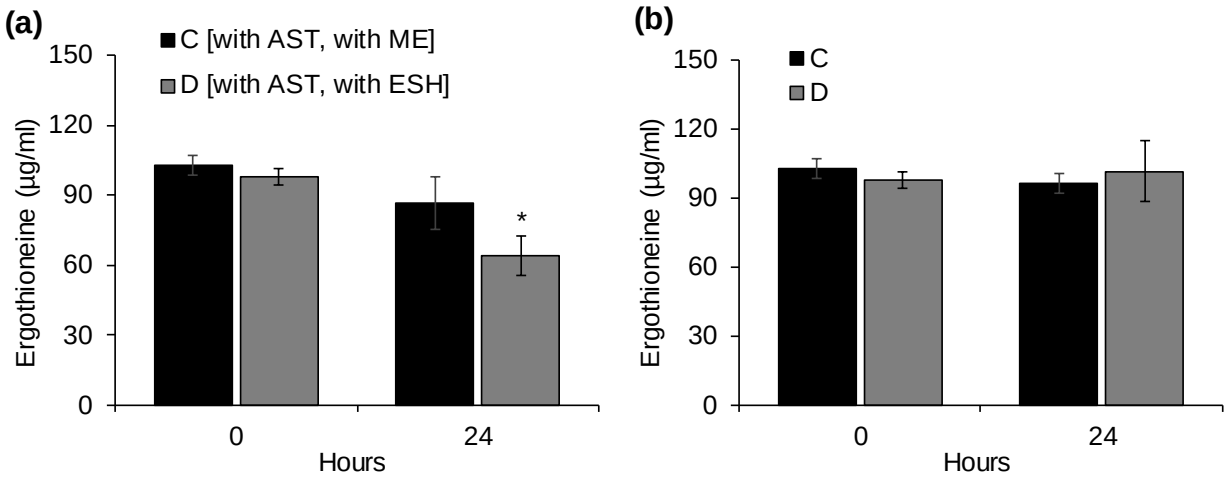


Figure 2.17. Changes in total ergothioneine content of liposomes incubated at 37°C with AAPH (a) or AMVN (b). Data are presented as mean ± standard deviation (n=3). Values with asterisk represent significant differences among treatment groups (* for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$) at each time point.

Changes in the physical appearance of liposome during oxidation-induced conditions. Representative micrographs of liposomes before and after incubation with AAPH and AMVN are shown in Figure 2.18. Liposomes without astaxanthin that were incubated with AAPH showed no observable difference in appearance after 24 h incubation (Figure 2.18(a)). Distinct changes in color from bright to pale orange were observed in the liposomes with astaxanthin, but the sizes were still within their original size range. Liposomes incubated with AMVN (Figure 2.18(b)) showed a notable decrease in size, and no discernable orange coloration was observed in the liposomes that initially contained astaxanthin. Moreover, no discernable difference was observed between the effects of ME and ergothioneine on the physical appearance of the liposomes.

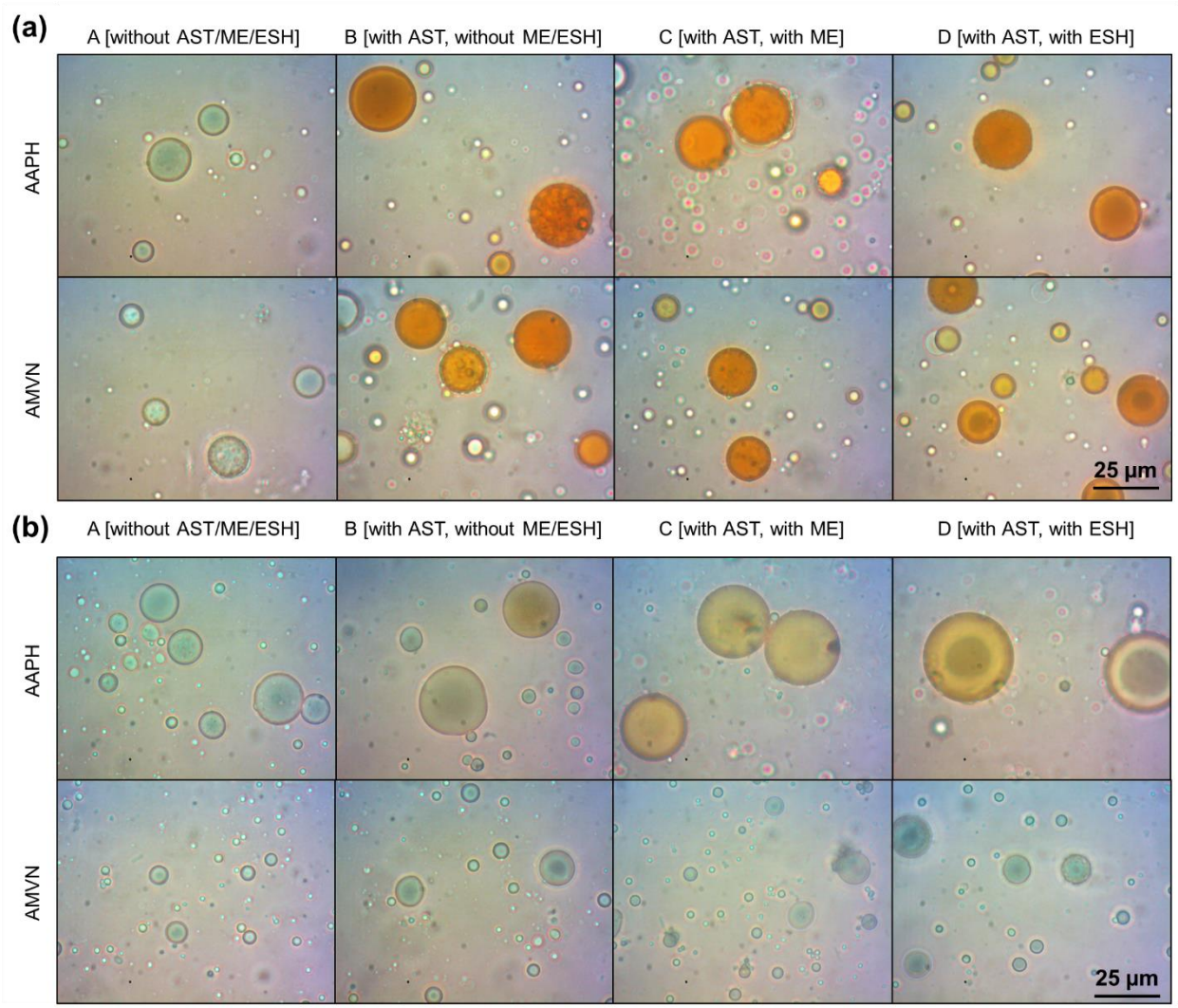


Figure 2.18. Changes in the physical appearance of liposomes observed in micrographs taken before (a) and after incubation (b) at 37 °C.

2.3.6 Comparison of the antioxidative properties of ME and ESH

Based on some of the index that was used to compare the effects of ME and ESH against lipid oxidation, certain differences were observed in their respective antioxidative effects. The next part of this experiment was the comparison of some antioxidative properties of ME and ESH, that are not incorporated in liposomes.

Correlation between ergothioneine content and DPPH radical scavenging activity. The correlation between ergothioneine content in crude ME and corresponding DPPH radical

scavenging activity is shown in Figure 2.19. Results of ME quantitative analysis showed a high correlation (R^2 = 0.9984) between ergothioneine content and DPPH radical scavenging activities. This correlation was relative to the values obtained from authentic ergothioneine equivalents, suggesting the purity of the ergothioneine peaks separated by HPLC.

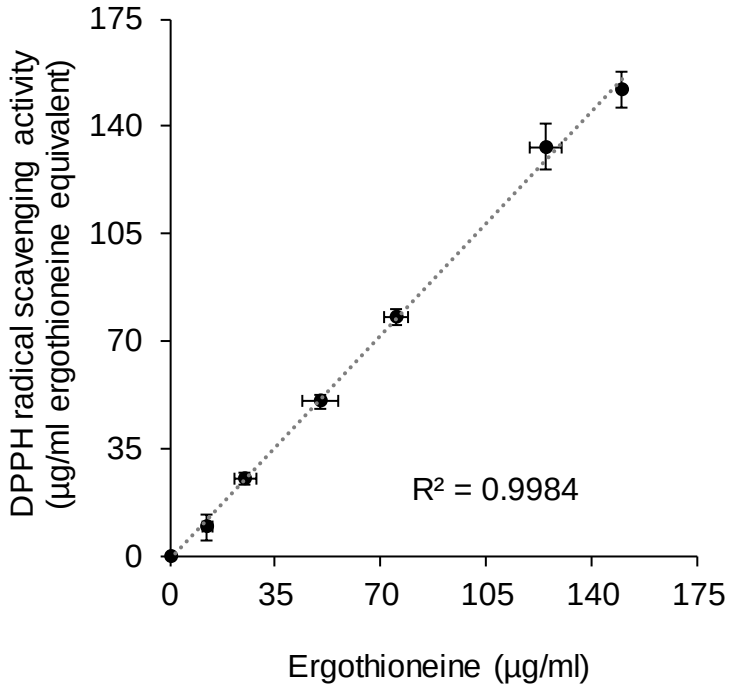


Figure 2.19. The relationship between the ergothioneine content in the crude ME and the DPPH radical scavenging activities relative to the ESH standard equivalents. Data are presented as mean ± standard deviation (n=3).

Comparison of the antioxidative properties of crude ME and ergothioneine. Some antioxidative properties of ME and ergothioneine are compared in Table 2.1. The comparison showed that 250 µg of ergothioneine per mL of crude ME had 2-fold higher levels of total phenolics, 1.6-fold more potent DPPH EC_{50}, and 2.6-fold higher ORAC values compared to the same concentration of ergothioneine.

Table 2.1. Total phenolics, DPPH EC_{50}, and oxygen radical absorbance capacity comparison of authentic ergothioneine and crude mushroom extract. Data are presented as mean ± standard deviation (n=3).

Antioxidative indices	Authentic ergothioneine	Crude mushroom extract
Total phenolics (eqv µg gallic acid)	43.2 ± 1.2[a]	89.3 ± 0.9[a]
DPPH radical scavenging EC_{50}[b] (µg)	4.6 ± 0.1	2.8 ± 0.1
ORAC[c] (µmol Trolox eqv/g)	7375.3 ± 99.7	19042.3 ± 264.1

[a]1mM ergothioneine, [b]Half maximal effective concentration. [c]ORAC, oxygen radical absorbance capacity.

Comparison of the radical scavenging activities of crude ME and ergothioneine with other hydrophilic antioxidants. Radical scavenging activities of crude ME, ergothioneine, ascorbic acid, and glutathione are shown in Figure 2.20. The DPPH EC_{50} of glutathione was the highest, and there was no significant difference ($p > 0.05$) observed in the other 3 samples (Figure 10(a)); the ORAC value of ME was the highest, followed by ergothioneine, ascorbic acid, and glutathione exhibited the lowest value.

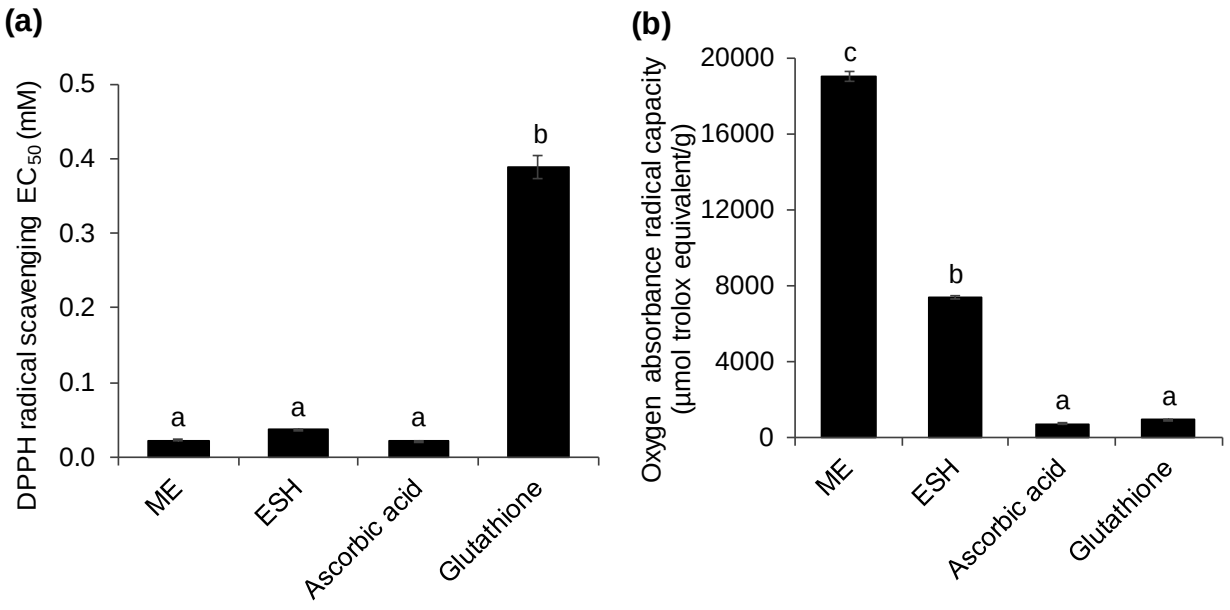


Figure 2.20. DPPH radical EC_{50} (a) and ORAC (b) of the different antioxidant samples. Data are presented as mean ± standard deviation (n=3). Values with different superscript letters indicate significant differences among samples ($p < 0.05$).

2.4 Discussion

This study used astaxanthin-filled liposomes as a model of the astaxanthin-pigmented cells of salmonid meat to investigate the effects of ergothioneine-rich ME supplementation as a strategy to protect the meat from oxidative degradation. Radical-induced oxidation conditions were employed to evaluate the individual and collective antioxidative properties of astaxanthin, ergothioneine, and ME against certain types of radicals. The results of this study demonstrate that the addition of these radical initiators to the liposomes increased the amount of lipid HPO formed and accelerated the oxidative degradation of astaxanthin. Azo compounds such AAPH and AMVN are commonly used for *in vitro* reactions as hydrophilic and lipophilic radical initiators, respectively[70, 71]. These compounds can decompose on their own to produce 2 carbon radicals and a molecular nitrogen (Eq 2.1), even without the presence of enzymes or biotransformation. These carbon radicals can recombine to form a stable molecule (Eq 2.2), but most of them diffuse apart and rapidly reacts with oxygen forming a peroxyl radical (Eq 2.3)[70]. The solubility and the rate of

decomposition are determined based on the structure of these azo compounds (Figure 2.21), wherein when a molecule of AAPH decomposes it yields 2 molecules of carbon-amide radicals (Figure 2.21(a)), which have hydrophilic properties, while the degradation of an AMVN molecule yields 2 molecules of carbon-nitrile radicals (Figure 2.21(b)), which have lipophilic properties. It was noted that AMVN resulted in a faster rate of oxidation than AAPH as demonstrated by the results of lipid HPO formation and astaxanthin degradation, due to the difference in the decomposition rates of these azo compounds as influenced by temperature[70]. At neutral pH, AAPH has a half-life of 175 h while AMVN has a half-life of 90 h[72]. The faster rate of decomposition of AMVN explains its faster rate of oxidation, which in turn produces more radicals at a faster rate.

$$R–N=N–R \rightarrow R\bullet + N=N + \bullet R \quad \text{(Eq 2.1)}$$

$$R–N=N–R \rightarrow R–R + N=N \quad \text{(Eq 2.2)}$$

$$R\bullet + O_2 \rightarrow RO_2\bullet \quad \text{(Eq 2.3)}$$

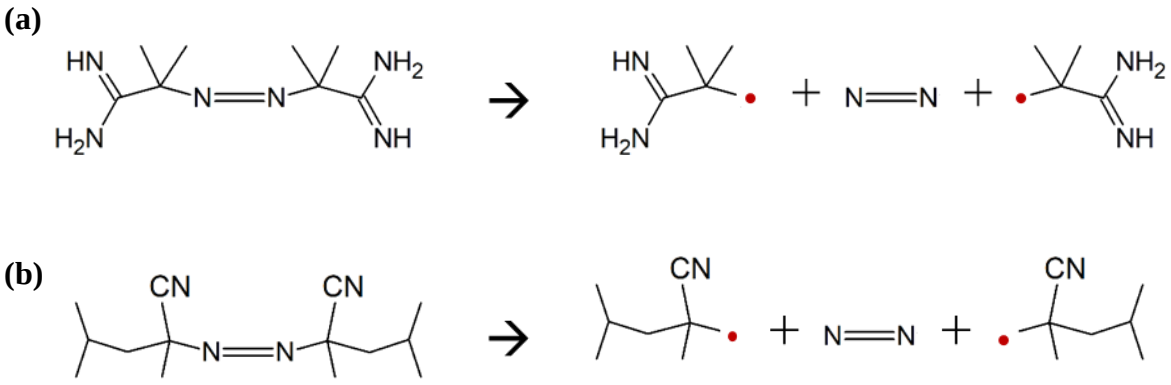


Figure 2.21. Generation of azoradicals from 2,2′-azobis(2-methyl-propionamidine) dihydro-chloride (AAPH) (a); and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) (b).

Astaxanthin in the liposomes could control HPO formation only under limited conditions at 25 °C. As incubation temperature increased, astaxanthin could not mitigate the oxidative effects of higher rates of radical formation. This can be attributed to the compromised stability of astaxanthin at higher temperatures. In a study of the stability of astaxanthin in *H. pluvialis*

powdered biomass, the low-temperature drying method resulted in a higher recovery yield of astaxanthin[73]. In addition, it was also noted in the same study that when *H. pluvialis* powder was stored at a higher temperature, less astaxanthin was retained[73]. In another study, the stability of astaxanthin in a carotenoprotein of shrimp fermentation byproduct was monitored under varying storage conditions, and the results showed that increased storage temperature compromised astaxanthin stability due to the denaturation of protein, exposing it to air and light[74]. The limited antioxidative functions of astaxanthin in liposomes can also be attributed to the amount of astaxanthin present relative to the radicals. In the present liposome system, the experimental concentration of phospholipid was approximately 3 mM, the radical initiator was 1.5 mM, and astaxanthin was 0.037 mM, which is comparable to the average astaxanthin content in the muscle of pigmented salmonids[4]. In addition, these concentrations are within the previously reported ranges for the appropriate ratio of astaxanthin to phospholipid in liposomes[75, 76]. Consequently, the amount of astaxanthin may not have been sufficient to counter the oxidative effects of radicals present in this system.

The addition of ergothioneine-rich ME to liposomes showed additive effects to the antioxidative properties of astaxanthin. It exhibited a delay in the progress of lipid oxidation and astaxanthin degradation in both the AAPH and AMVN-initiated reactions. Ergothioneine is a known antioxidant[33, 36] commonly found in certain edible mushroom species[54, 77], thus MEs have been the focus of several natural antioxidant studies. Furthermore, ergothioneine-rich MEs have been proven to have antioxidative effects when applied to certain oxidation-susceptible post-harvest commodities[78, 79]. However, few studies have been conducted regarding the antioxidative properties of crude MEs against the oxidative degradation of astaxanthin in salmon tissues.

The present study clearly shows that the antioxidative effects of ME addition to liposomes were apparent in the suppression of lipid HPO and formation of TBARS. The effects of each radical initiator on fatty acid substrates during oxidation-induced conditions provided interesting outcomes. The results suggest that AAPH was more reactive with PUFAs (C18:2n-6 and C20:4n-6), which is comparable to previous observations in which lipid stability against peroxidation decreased with increasing unsaturation[80, 81]. On the other hand, AMVN affected most of the fatty acids in the liposomes regardless of the degree of unsaturation. These results are consistent with the mode of action of AMVN as a lipophilic radical generator. Despite the decrease of certain fatty acids as an effect of lipid oxidation, liposomes with ME exhibited significant lipid stability with AAPH and AMVN when compared with liposomes without ME, suggesting the oxidative protective effects of ME against both hydrophilic and lipophilic radicals.

The different concentrations of ME that were tested for their efficacy against oxidation did not exhibit any significant difference in HPO formation and astaxanthin degradation when incubated with AAPH. However, when these liposomes were incubated with AMVN, a dose-dependent trend was observed. Furthermore, regardless of the concentration of ME and the type of radical initiator added, ergothioneine was stable throughout the incubation duration. Based on these results, even the lowest concentration of ME tested (35 µg ergothioneine/mL) exhibited a potent effect in controlling the progress of lipid HPO formation and delaying the degradation of astaxanthin.

The test on the different concentrations of astaxanthin did not have any significant dose-dependent effect in the mitigation of lipid HPO formation when incubated in AAPH but had a significant effect compared to the liposomes without astaxanthin. However, when the same liposomes were incubated with AMVN, no significant effect in the mitigation of HPO formation

was observed. These observations suggest that astaxanthin radical scavenging activity is more potent against hydrophilic radicals than lipophilic radicals. Astaxanthin as a carotenoid is not exclusively a lipophilic compound as contrary to other carotenoids such as lycopene and carotene[82]. The 2 terminal ring moieties of astaxanthin possessing hydroxyl groups make it able to bind with other hydrophilic compounds such as the phosphate groups of the phospholipids, and the conjugated polyene chain of astaxanthin is the lipophilic part of its structure[3]. In addition, these hydroxyl groups in the terminal rings of astaxanthin are also able to scavenge radicals both in the interior and at the surface of the cell membrane.

Microscopic evaluation showed that oxidation initially targeted the astaxanthin pigments present in the phospholipid membrane of the liposomes as evidenced by the distinct color change after incubation. Moreover, the effect of lipophilic radicals on decreasing the amount of several fatty acids was shown by the apparent decrease in the average size of the liposomes after 24 h incubation with AMVN. As fatty acids are oxidized, certain regions within the molecules are cleaved, compromising the integrity of the phospholipid bilayer. The collective oxidation reactions could also result in a greater effect on the overall structural integrity of the phospholipid bilayer of the entire vesicle. The damage in certain parts of the bilayer, which depends on the extent of oxidation progress, could lead to the reorientation of the bilayer position, which could then result in smaller vesicles. Mosca et al. (2001)[83] reported that azo radical-induced liposomal oxidation can lead to modifications of the z-potential of a membrane bilayer, resulting in the rearrangement of the polar phosphate head in the membrane. This phenomenon was observed in the microscopy analysis of the liposomes in this study.

The parallel tests of the crude ME and ergothioneine revealed some antioxidative properties present in ME. The effects of ME and ergothioneine against oxidation induced by

hydrophilic radicals did not show any significant difference. However, incubation with AMVN showed that crude ME exhibited more potent antioxidative properties, as shown by the significant suppression of HPO and TBARS formation as well as the delay in astaxanthin degradation. These observations led to the speculation that crude ME may contain antioxidant compounds in addition to ergothioneine that are sufficiently potent to significantly control the effects of lipophilic radicals. Moreover, the results of the ergothioneine analysis that demonstrated its stability in crude ME may be attributed to other compounds present in the extract.

In this study, the premise for the use of the ME as a source of antioxidants is based on previous findings regarding the considerable amounts of ergothioneine present in certain edible mushroom species[54, 65,] and its potential in post-harvest quality preservation[48, 64, 78, 79]. Ergothioneine together with the other components in crude ME exhibited additive and significantly higher antioxidative properties than just ergothioneine in terms of DPPH radical scavenging activity and oxygen radical absorbance capacity. Aside from ergothioneine, a wide range of compounds such as are B group vitamins, phenolic compounds, ascorbic acid, flavonoids, sterols, and carotenoids such as β-carotene, lycopene, and α-tocopherol have also been identified in a variety of edible mushroom species exhibiting certain degrees of antioxidant characteristics[84-90]. Based on the findings of this study, it can be acknowledged that other components in crude ME may have contributed to the total antioxidative property.

Knowing that ME has a superior antioxidative property than authentic ergothioneine, similar radical scavenging tests were done with other antioxidants. Glutathione was used in comparison having a similar thione constituent as ergothioneine[91], and ascorbic acid one of the most commonly known and studied hydrophilic antioxidant. Results showed that ME is almost 17.3 times more potent than glutathione, 1.6 times more potent than authentic ergothioneine, and almost has the

same potency as ascorbic acid in terms of DPPH radical scavenging activity as a reactive nitrogen species. On the other hand, the test for oxygen radical scavenging activity using the peroxyl radicals produced by AAPH showed that ME has the highest antioxidant capacity, which is 2.6 times higher than authentic ergothioneine, 20.6 times more than glutathione, and 26.4 times more than ascorbic acid.

2.5 Summary

This study was contextualized based on the premise that hydrophilic extracts of edible mushroom species contain a significant amount of the potent antioxidant ergothioneine. In addition, the use of crude extracts is an economical approach to naturally obtain ergothioneine. The findings of this study successfully demonstrated the antioxidative properties of crude *Pluerotus cornucopiae* extract in a liposomal system by delaying astaxanthin degradation and controlling the progress of lipid oxidation.

Astaxanthin alone is known to have antioxidative functions, which was also demonstrated in this liposomal system when compared to liposomes that did not contain astaxanthin. However, this antioxidative function has its limitations and the structure of the astaxanthin itself makes it susceptible to oxidation. The high radical scavenging activity of ME was shown to effectively react against radicals which controlled the formation of lipid hydroperoxides as primary products of lipid oxidation as well as the aldehydes in the form of thiobarbituric reactive substances as secondary products of lipid oxidation. Furthermore, ME also effectively demonstrated its protective function for fatty acid substrates and liposomal astaxanthin against oxidative degradation. Although the majority of the antioxidative functions of ME are more potent against hydrophilic radicals, considerable activities were still observed against a lipophilic radical initiator. Moreover, the function of ME as a protective agent against lipid oxidation is also equally valuable

in maintaining the structure of the lipid bilayer of cell membranes against oxidation, which could further translate to protective functions for other cellular organelles and genetic components. Thus, the presence of ergothioneine or ME together with astaxanthin have additive synergistic antioxidative functions that could neutralize reactive radical species and control the advancement of lipid oxidation.

In addition, even the minimum ergothioneine concentration from ME tested in this study was still able to efficiently control lipid hydroperoxide formation and astaxanthin degradation almost equally as the highest concentration that was tested even in the presence of hydrophilic radicals. On the other hand, although statistically insignificant, the effects of the different concentrations ME demonstrated a dose-dependent efficacy trend against lipophilic radical initiators. The different concentrations of astaxanthin that were tested showed no dose-dependent effects against lipid hydroperoxide formation nor astaxanthin stability in both the hydrophilic and lipophilic radical initiators.

Furthermore, the evaluation of the antioxidative properties of ESH and ME validated some of the earlier findings of their radical scavenging properties. Moreover, these tests have clearly demonstrated and provided evidence to support the hypothesis regarding the presence of other components in the crude ME that may have contributed to their synergistic antioxidative functions. In conclusion, the main hypothesis of this study wherein ESH and ME have protective functions against lipid oxidation and astaxanthin degradation was proven to be positive. Hence, these findings may be further validated through its application in preserving post-harvest commodities, such as the astaxanthin-pigmented salmonid species, against lipid oxidation and astaxanthin degradation.

CHAPTER 3

In vitro and *in vivo* supplementation of ergothioneine-rich mushroom extracts in rainbow trout (*Oncorhynchus mykiss*)

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3.1 Introduction

Salmonid species are known for their characteristic red-orange muscle pigmentation. The same characteristic can also dictate its market quality[5]. A natural compound, astaxanthin, is responsible for the color imparted in the muscle of these fish species[7]. This compound is naturally occurring and abundant in the marine environment and is mainly produced by microalgae such as *Haematococcus pluvialis*[15]. Astaxanthin is a C_{40} terpenoid that consists of a conjugated polyene chain and two terminal ring moieties, giving it both lipophilic and hydrophilic characteristics, respectively. This compound has strong antioxidative properties[3, 16], and is often regarded as valuable for preventing oxidation. The antioxidative action of astaxanthin occurs via singlet oxygen quenching at the terminal rings and via radical scavenging within cell membranes by the conjugated double bonds[16, 30].

However, astaxanthin-pigmented salmonid meat often contains a significantly high amount of fat, mostly polyunsaturated fatty acids that are highly susceptible to lipid peroxidation. With these, astaxanthin in the meat also becomes susceptible to oxidation. The oxidation of astaxanthin could lead to its degradation and loss of its antioxidative properties. In addition, oxidation in astaxanthin also affects the structure of the saturated polyene chain, resulting to the cleavage in certain parts of the unsaturated chain forming shorter chains and producing by-products such as aldehydes and ketones. Moreover, the result of this oxidation in astaxanthin is mostly evident in the significant change of coloration of salmon meat from bright reddish-orange to pale grayish-orange color.

Aqueous extracts of certain mushroom species have been analyzed to contain a significant amount of ergothioneine[65, 92-95] and have been applied in several post-harvest storage studies as pigment stabilizers which inhibited lipid oxidation and metmyoglobin formation in beef and fish meat[4, 55, 96]. This study aimed to evaluate the effects of ergothioneine-rich mushroom extract in controlling lipid oxidation and astaxanthin degradation in rainbow trout meat when added directly to meat prior storage. In addition, this study also aimed to test the feasibility of incorporating the ergothioneine-rich crude mushroom extract into the diet of rainbow trout during the grow-out period and evaluate its possible uptake.

3.2 Materials and methods

3.2.1 Materials and chemicals

Rainbow trout samples used for the *in vitro* supplementation of ME were acquired and raised in the Tokyo University of Marine Science and Technology (TUMSAT) Oizumi Research Station (Yamanashi Prefecture, Japan), and were approximately 2 years old with an average weight of 220.0±37.2 g and an average fork length of and 22.1±1.5 cm. Rainbow trout samples used for the dietary supplementation of ergothioneine-rich ME experiment were obtained and raised in Iwate Prefecture Fisheries Experimental Station (Iwate Prefecture, Japan). Fish samples were approximately 1 year old with an average weight of 109.9±13.4 g and an average fork length of and 18.2±1.3 cm. HPLC-grade acetone, methanol, chloroform, and *tert*-butyl methyl ether were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Authenticated standards of all-*trans*-astaxanthin from algae (*Haematococcus pluvialis*) of 85.0% purity, cumene hydroperoxide of 80.0% purity, and *L*-(+)-ergothioneine of 99.0 % purity were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and Bachem AG (Bubendorf, Switzerland), respectively. *trans*-β-apo-8′-carotenal of 96.0% purity and 3-methyl-1H-imidazole-2-thione (methimazole) of 99.0% purity as internal standards (IS) were purchased from Sigma-

Aldrich (St. Louis, MO). 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-glycero-3-phosphocholine (NBD-labeled PC) of 99% purity as a lipid hydroperoxide (HPO) IS was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

3.2.2 Mushroom extract preparation

***Flammulina velutipes* extract preparation.** Hydrophilic extract of *F. velutipes* used for the *in vitro* ME treatment were prepared in the laboratory. Fresh *F. velutipes* were obtained from a local supermarket. The fruiting bodies were separated, freeze-dried, and ground into a powder using a food processor (MK-K75, Matsushita Electric Corp., Osaka, Japan). A 5-gram portion of the dried powder was extracted by the addition of 40 mL of 70% acetone and subsequently homogenized using a Waring Blender (Cell Master CM-100, Iuchi Seieido Co. Ltd, Japan) at 10000 rpm for 3 min. The homogenate was vacuum filtered and the resulting filtrate was evaporated to dryness *in vacuo* using a rotary evaporator. Five mL distilled water was added into the flask to dissolve the mushroom extract which was used for the *in vitro* experiments. The ergothioneine concentration of the extract amounted to 2.7 mg/mL.

***Pleurotus cornucopiae* extract preparation.** Hydrophilic extract of *P. cornucopiae* used for the feeding experiment was prepared and donated by Three B Co., Inc. (Hokkaido, Japan). The fruiting bodies of *P. cornucopiae* (200 kg) in a stainless-steel sieve were added to water at a 1:1 proportion (w/w) and boiled for 15 min in a 400 L stainless steel vat. After boiling, the mushroom fruiting bodies were removed from the resulting aqueous extract. Using the same aqueous extract, this process was repeated for the extraction of two more batches of fresh mushroom fruiting bodies. A total of 600 kg of mushroom fruiting bodies was therefore processed which resulted in a recovered volume of 500 L aqueous extract with an approximate dissolved solid content of 2.5 °Bx. This aqueous extract was then concentrated to 25 °Bx using a thin-layer vacuum concentrator (Hisaka

REV 60/30-1-2, Osaka, Japan) resulting in 60 kg of concentrated extract. The final ME solution used for this study was prepared by diluting the concentrated extract to 10 °Bx, which had an ergothioneine concentration of 1.1 mg/mL.

3.2.3 *In vitro* supplementation of ergothioneine in salmon meat

Live rainbow trout were collected from culture tanks, immediately sacrificed, and transported within a few hours to the laboratory on ice. The fish samples were subsequently skinned and degutted. The dorsal muscles were filleted, minced using a food processor, and divided into 3 groups for the different treatments. The prepared *F. velutipes* liquid extracts were separately added into the 2 groups of minced meat at concentrations of 3 and 5% (w/w) and were further minced for a few seconds to ensure even distribution. Distilled water was added to the third group as a control group. The equivalent ergothioneine concentrations per gram of sample for each treatment group were 0, 81, and 135 µg for the control, 3, and 5% groups, respectively. Twelve 5 g portions of the samples from each treatment group were kept in polystyrene petri dishes (30 mm diameter) with lids and were stored at -10°C for 4 weeks. Treatments were prepared in 4 replicates and were evaluated every 2 weeks for quantitative analyses of lipid HPO and total astaxanthin levels, as well as visual color evaluation.

3.2.4 Dietary supplementation of ergothioneine-rich ME in rainbow trout feeds

A feeding experiment was done to evaluate the feasibility of supplementing ergothioneine-rich ME into the diet of rainbow trout during grow-out culture. The experiment was done in Iwate Prefecture Fisheries Experimental Station, in Iwate Prefecture, Japan. Three different treatment groups were assigned based on the amount of ME supplemented into the diet, which were the 0% or the control group, 3%, and 5% ME (w/w). The base diet used was an astaxanthin-rich commercial diet used for rainbow trout grow-out culture. This corresponds to an equivalent

ergothioneine content of 0, 33, and 55 µg per gram of feeds in the control, 3, and 5% groups, respectively. The daily feeding rate was based on 1% of the average fish body weight and was broadcasted twice daily over a period of 2.5 months.

Blood and meat samples were collected at 0, 1.5, and 2.5 months for quantitative analyses. blood samples were collected on site (via caudal venous puncture) and kept in vacuum-sealed tubes with sodium heparin (Terumo Co. Ltd., Tokyo, Japan). The fish were then immediately sacrificed and transported together with the blood samples within a few hours to the laboratory on ice. The fish samples were subsequently skinned and degutted. The dorsal muscles were filleted, minced using a food processor, and divided into 3 groups for the different treatments. Five-gram portions of the minced meat were used to analyze lipid and astaxanthin content.

3.2.5 Quantitative analyses

Lipid extraction and quantification. The total lipid content of the stored minced meat was extracted using the Bligh and Dyer procedure[61] and quantified gravimetrically.

Total astaxanthin quantification. Total astaxanthin in minced meat was extracted using the Bligh and Dyer procedure[61] with previous addition of *trans*-β-apo-8′-carotenal as an IS. Quantification analysis was carried out using a Shimadzu model SCL-10A HPLC system (Kyoto, Japan) equipped with two straight-lined C_{18} reverse-phase columns (Waters Symmetry Shield^{TM} RP18, 5 µm, 4.6 mm i.d. × 250 mm). A gradient elution using HPLC-grade methanol and *tert*-butyl methyl ether as the mobile phase was programmed as follows: 0-9.3 min: 100% methanol; 9.4-22.0 min: 60% methanol; and 23.0-39.0 min: 100% methanol. The sample injection volume was 20 µL and the column temperature was kept at 25°C. The eluted astaxanthin was monitored at a wavelength of 485 nm, to obtain the peak area ratio (astaxanthin/IS). A calibration curve was obtained using

varying concentrations of the all-*trans*-astaxanthin standard. All data were expressed as µg of astaxanthin per gram of meat.

Total lipid HPO quantification. Lipid oxidation was evaluated by the quantification of total lipid HPO present in the samples using a flow injection analysis (FIA) system equipped with a fluorescent detection system, as described by Sohn et al. (2005)[60]. The preference for this method was based on its specificity and high sensitivity for detecting and measuring lipid oxidation products during the early stages of lipid oxidation. Briefly, minced meat was extracted using the Bligh and Dyer procedure[61] with the previous addition of NBD-labeled PC as an IS. The fluorescence intensity of HPO reacted with diphenyl-1-pyrenylphosphine (DPPP), was monitored at the excitation wavelength (λ_{ex}) of 352 nm and at the emission wavelength (λ_{em}) of 380 nm using a Shimadzu model RF535 fluorescence detector (Kyoto, Japan). The IS was monitored at 460 nm (λ_{ex}) and 534 nm (λ_{em}) using a Shimadzu model RF-10A fluorescence detector (Kyoto, Japan). A calibration curve was obtained using different concentrations of authenticated cumene hydroperoxide. All data were expressed as equivalent nmol cumene hydroperoxide per gram of meat.

Ergothioneine quantification. Ergothioneine from blood and meat samples were quantified according to the method of Nguyen et al.[97]. Briefly, 4 mL of distilled water was added to 1 mL of whole blood and placed in a water bath at 90°C for 12 min while agitating every 3 min intervals. Samples were then cooled on ice for 3 min and 1 mL of an aqueous solution of methimazole (200 µg/mL) was subsequently added as an IS. The samples were centrifuged at 3000 x *g* for 15 min at 4°C. The resulting supernatant was collected to which 12 mL absolute ethanol was subsequently added. The solution was then agitated and kept on ice for 30 min, and centrifuged at 3000 x *g* for 15 min at 4°C. The resulting supernatant was collected and evaporated to dryness *in vacuo* using

a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water. Meat sample was extracted using the Bligh and Dyer method[61], with slight modifications. Instead of the addition of water, 0.88 % of KCl was added, and 1 mL of an aqueous solution of methimazole (200 µg/mL) was added prior to the homogenization. The water-methanolic layer was collected and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water. A 20-µL portion of the sample was subjected to analysis. Quantitative analysis was carried out by HPLC analysis following the method of Nguyen et al.[97]. Briefly, ergothioneine from the sample extract was chromatographically separated using two reversed-phase C_{18} columns (ACE C18-AR 3 µm, 150 × 4.6 mm i.d.) eluted with 10% methanol in deionized water with 0.1% acetic acid at a flow rate of 0.20 mL/min and monitored at 254 nm using a photodiode array detector (SPD-M10A Shimadzu, Kyoto, Japan). Separated ergothioneine was reacted with 0.23 M 2,2'-dipyridyl disulphide in 0.25 M HCl at a flow rate of 0.15 mL/min in a post-column reaction coil, wherein the resulting reaction product 2-thiopyridone was monitored at 343 nm (SPD-M10A). Varying concentrations of an L-(+)-ergothioneine standard were used to obtain a calibration curve. All data for this analysis are expressed as µg of ergothioneine per g or mL of sample.

DPPH radical scavenging analysis by HPLC-FIA method. The radical scavenging abilities of the meat samples were determined according to the methods of Giri, et al. (2011)[66] and Tepwong et al (2012)[53] with slight modifications. The hydrophilic extracts prepared for the ergothioneine analysis were analyzed in an HPLC system equipped with a post-column reaction system for free DPPH radical scavenging ability. Briefly, components of the injected sample were separated using a Synergi 4u Fusion - RP 80A column (4 µm, 250 mm × 4.6 mm i.d., Phenomenex Inc., Torrance, CA, USA), by 10% methanol in deionized water with 0.01% acetic acid at a flow rate of 0.2 mL/min. The eluates were monitored at 254 nm with an SPD-M10AVP detector. DPPH radical methanolic solution (150 µM) pumped at a flow rate of 0.32 mL/min was reacted with the eluted

antioxidative components in a post-column reaction coil. Negative peaks formed by DPPH radical scavenging were monitored at 517 nm with an SPD-M10AVP. Varying concentrations of L-(+)-ergothioneine standard were used to obtain a calibration curve with all data expressed as µg ergothioneine equivalent per g of sample.

Color image analysis. Visual color of the meat was evaluated quantitatively according to the method of Bao et al.[4]. Briefly, the minced meat kept in polystyrene petri dishes were positioned inside an imaging apparatus (Image Capture G3 Liponics) equipped with 2 florescent light sources (6-watt Toshiba FL6D, 210 lumens). Image data (jpeg format) was captured using a digital single-lensed CCD camera (Canon EOS Kiss X3) and were analyzed for the red (*r*), green (*g)*, and blue (*b*) values in a specified area of the sample using an image processing software (ImageJ of the National Institutes of Health, Bathesda, MD). The *R** value of the sample was calculated using the formula:

$$\mathrm{R}^{*}\ \text{value} = \frac{r}{r+g+b} \times 100$$

where *r, g,* and *b* denote the average red, green and blue values, respectively.

Statistical analysis. Microsoft Excel 2016 was used to analyze the means and standard deviations of the collected data as well as for the generation of graphs. One-way Analysis of Variance using IBM SPSS Statistics 20 was used to evaluate the data for significant differences among treatments at a 5% level of significance.

3.3 Results

3.3.1 Effects of *in vitro* addition of ergothioneine-rich ME in *Oncorhynchus mykiss* meat during storage

The effects of *in vitro* ME supplementation in the minced rainbow trout meat as presented through the changes in the visual coloration and total astaxanthin content during storage at -10 °C are shown in Figure 3.1. Digital image analysis of the red coloration of the meat revealed that the *R** value of the samples did not differ significantly ($p > 0.05$) among treatments throughout the course of storage (Figure 3.1(a)). No significant differences ($p > 0.05$) in astaxanthin content were observed among all groups on the 2nd week of storage (Figure 3.1(b)). However, significant differences ($p < 0.05$) in astaxanthin content were observed among the 3 groups on the 4th week of storage. The addition of 3% and 5% ME to the minced meat prior storage significantly retained higher ($p < 0.05$) astaxanthin content in a dose dependent manner at 18.9±0.4 µg/g and 19.9±0.2 µg/g, respectively, compared with the control group which exhibited a considerably reduced amount of astaxanthin at 18.2±0.6 µg/g.

Changes in the total lipid HPO content of the different treatments of minced rainbow trout meat during storage at -10 °C are shown in Figure 3.2. No significant differences ($p > 0.05$) in HPO content were observed among all groups on the 2nd week of storage. However, with prolonged storage to 4 weeks, the effects of 5% ME in HPO accumulation became apparent; the addition of 5% ME significantly suppressed ($p < 0.05$) the HPO accumulation on the 4th week of storage with HPO at 89.9±12.5 equivalent nmol cumene-OOH/g, compared with the control and 3% ME-treated groups that exhibited a continued increase in HPO accumulation at 180.7±19.8 and 148.2±44.9 equivalent nmol cumene-OOH/g, respectively.

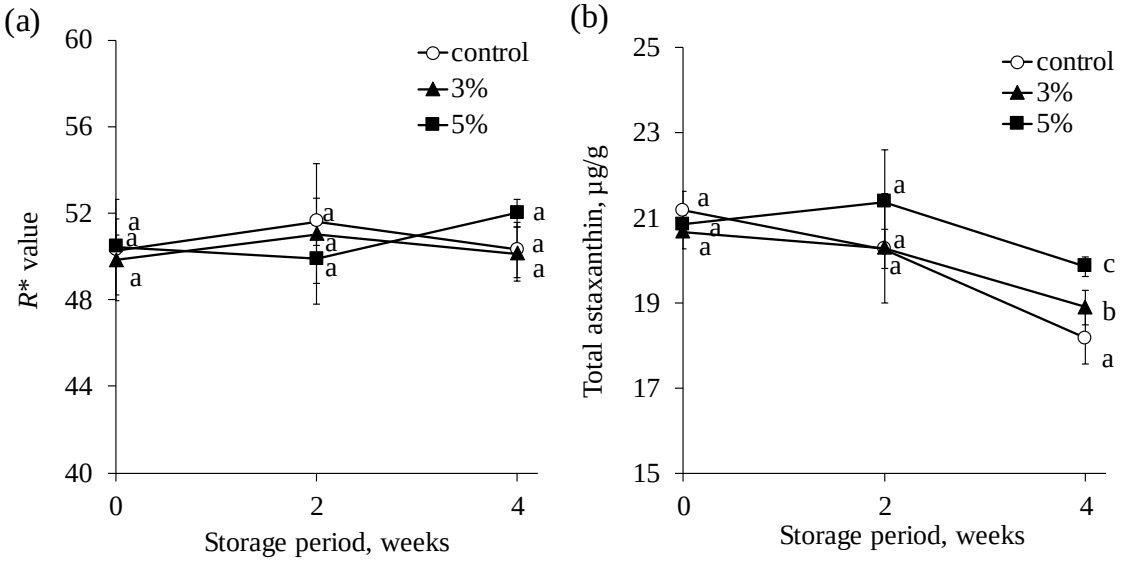

Figure 3.1. Effects of *in vitro* addition of mushroom extract in minced *O. mykiss* meat during storage at -10°C. Changes in R^* value (a); and total astaxanthin content (b) of the different treatments. Data are presented as mean ± standard deviation (n=4). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

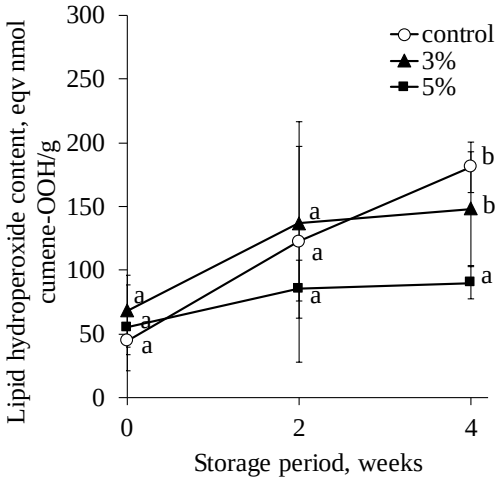

Figure 3.2. Effects of *in vitro* addition of mushroom extract in minced *O. mykiss* meat during storage at -10 °C. Changes in total lipid hydroperoxide of the different treatments. Data are presented as mean ± standard deviation (n=4). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

3.3.2 Effects of dietary supplementation of ergothioneine-rich mushroom extract in the growth and pigmentation of rainbow trout

Based on the initial results obtained from the *in vitro* addition of ergothioneine-rich mushroom extract into the meat of rainbow trout, wherein it resulted into the slower decrease rate of astaxanthin and lower lipid HPO content during storage at -10 °C, a feeding experiment was conducted to determine the feasibility of incorporating the ergothioneine-rich ME into the diet of rainbow trout.

Effects of dietary mushroom extract supplementation on the growth and lipid content of *Oncorhynchus kisutch*. The changes in the average body weight and lipid content of different fish treatments during the 2.5-month feeding duration are shown in Figure 3.3. The average total weight of all 3 feeding treatments increased during the feeding duration (Figure 3.3(a)), and no significant differences ($p > 0.05$) were observed among the different groups. The lipid content of the meat (Figure 3.3(b)) showed no significant differences ($p > 0.05$) among the different treatment groups during the feeding duration.

Effects of ME supplementation on the visual coloration and total astaxanthin content of meat. After the evaluation of the effects of the dietary ME supplementation on the growth of rainbow trout, the meat pigmentation was also evaluated to check whether the supplementation had any adverse effects on the pigment deposition in fish muscles. The changes in the meat coloration of rainbow fed for 2.5 months with varying ME concentrations are shown in Figure 3.4. Digital image analysis of the visual redness of the meat (Figure 3.4(a)) from the different dietary treatments of rainbow trout showed an increase in the R^* value after 2.5 months of feeding. It was also observed that the control group had a significantly higher ($p < 0.05$) R^* value than the 3% treatment group, while the R^* value of the 5% treatment group was in between of the 2 other groups. Total

astaxanthin content in the meat of the fish was quantitatively measured and results showed (Figure 3.4(b)) an increase in the astaxanthin content throughout the feeding duration. Moreover, contrary to the results obtained from the digital image analysis, no significant differences ($p > 0.05$) were observed among the groups fed with varying dietary ME concentration.

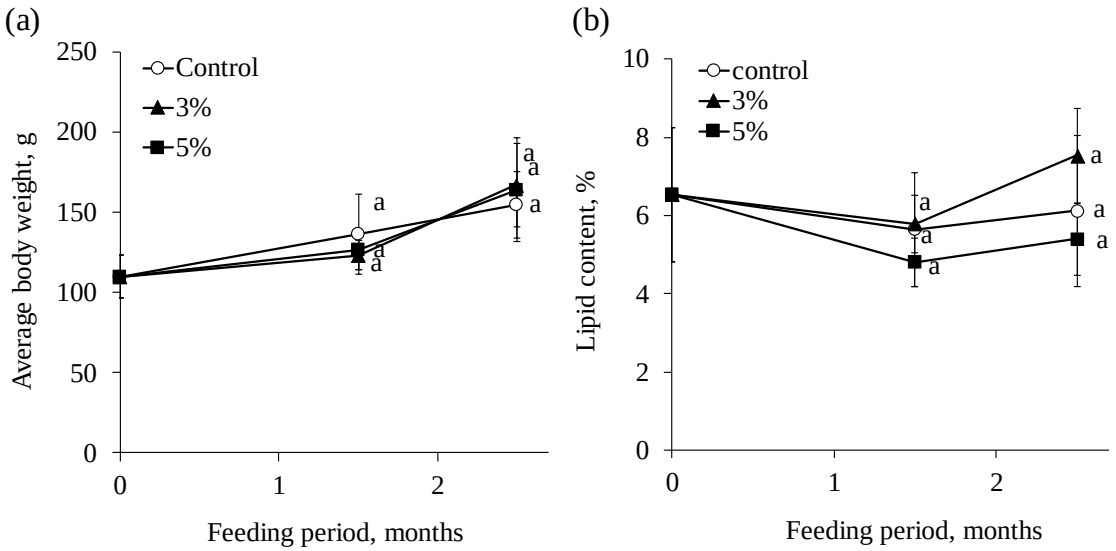

Figure 3.3. Effects of the dietary mushroom extract supplementation on the growth of *O. mykiss* during 2.5 months of feeding. Changes in the average body weight (a); and lipid content (b) of the different treatments. Data fare presented as mean ± standard deviation (n=10). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

Ergothioneine recovery in the blood of ME supplemented and non-supplemented rainbow trout. Ergothioneine content in the blood of the different fish treatments was quantitatively measured and results are shown in Figure 3.5. An increase in the ergothioneine content in the blood of the ME-supplemented groups of rainbow trout was observed in a dose-dependent manner. Wherein, 5% treatment group had a significantly higher ($p < 0.05$) ergothioneine content than the 3% treatment group throughout the feeding duration. The control group with no ME dietary supplementation remained at the same level as it was before the feeding started, wherein no ergothioneine content was detected in the samples.

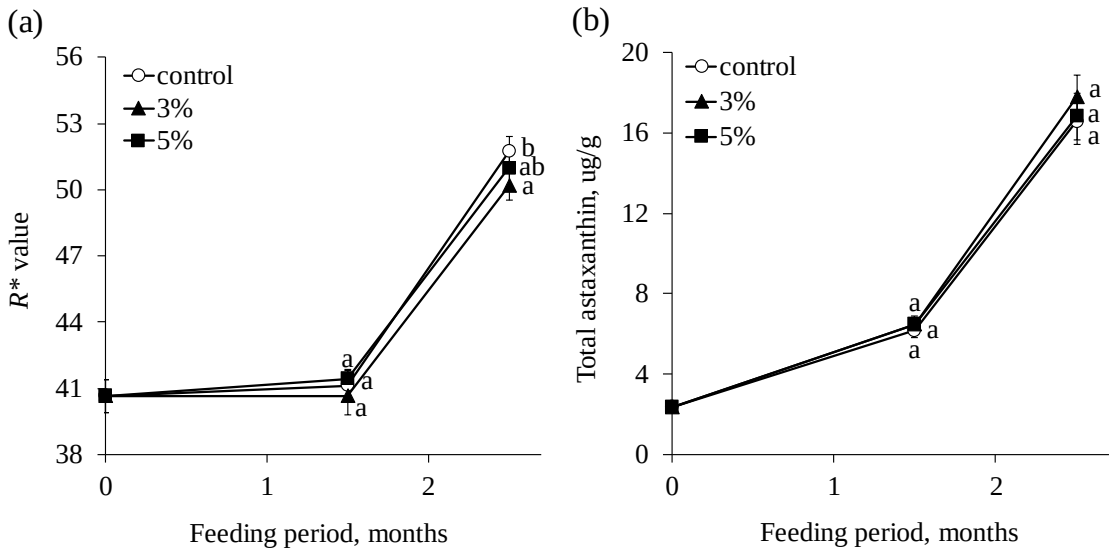

Figure 3.4. Effects of the dietary mushroom extract supplementation on the meat pigmentation of *O. mykiss* during 2.5 months of feeding. Changes in the R* value (a); and total astaxanthin content (b) of the different treatments. Data are presented as mean ± standard deviation (n=5). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

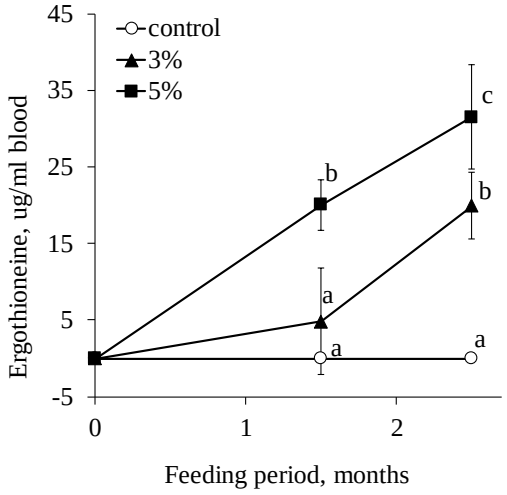

Figure 3.5. Ergothioneine concentration in blood of *O. mykiss* during the 2.5 months of feed supplementation with different concentrations of mushroom extract. Data are presented as mean ± standard deviation (n=5). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

Effects of ME supplementation on the total lipid HPO in the blood of rainbow trout. The concentration of lipid HPO in the blood of rainbow trout was quantitatively measured during the feeding duration, and the effects of the ME supplementation on the different fish treatments are shown in Figure 3.6. The control group, with no dietary ME supplementation, had the significantly highest ($p < 0.05$) lipid HPO formed during the feeding duration. The 5% treatment group had the lowest lipid HPO in blood, with very minimal changes since the start of the feeding duration. The levels of lipid HPO in the blood of the 3% treatment group is in between the control and 5% group. The effects of ME supplementation in the lipid HPO in the blood of the fish are reflected in a dose dependent manner relative to the concentration of ME supplemented into the diet.

The data obtained from the ergothioneine and lipid HPO quantification in blood after the 2.5-month feeding were correlated and results are shown in Figure 3.7. The treatment group with the highest ergothioneine content had the lowest lipid HPO formed, and with an inverse proportionality, the treatment group with the least or no ergothioneine content had the highest lipid HPO formed.

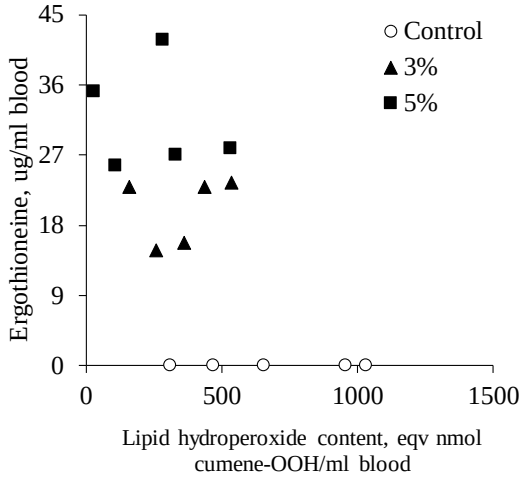

Figure 3.6. Total lipid hydroperoxide concentration in blood of *O. mykiss* during the 2.5 months of feed supplementation with different concentrations of mushroom extract. Data are presented as mean ± standard deviation (n=5). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

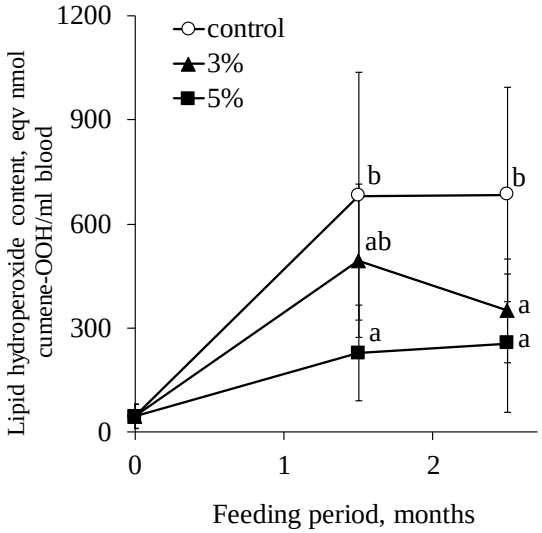

Figure 3.7. Correlation between ergothioneine content and lipid hydroperoxide concentration in blood of *O. mykiss* after 2.5 months of feed supplementation with different concentrations of mushroom extract.

Ergothioneine recovery in the meat of ME supplemented and non-supplemented rainbow trout. Significant concentrations of ergothioneine were measured in meat samples of rainbow trout that were supplemented with ergothioneine-rich ME diet. The 5% treatment group had the highest ergothioneine quantified at 18.6 ± 2.2 µg / gram (dry weight basis) of meat sample and followed by the 3% group with 7.0 ± 3.2 µg / gram. No detectable ergothioneine concentration was measured from the control group samples.

The meat samples that were analyzed for ergothioneine were also analyzed for their DPPH radical scavenging activities, and results are shown in Figure 3.9. Meat samples that had the highest ergothioneine content correspond to the highest DPPH radical scavenging activity as well, and samples with non-detectable ergothioneine concentrations did not have any antioxidative activities relative to the equivalent ergothioneine concentrations.

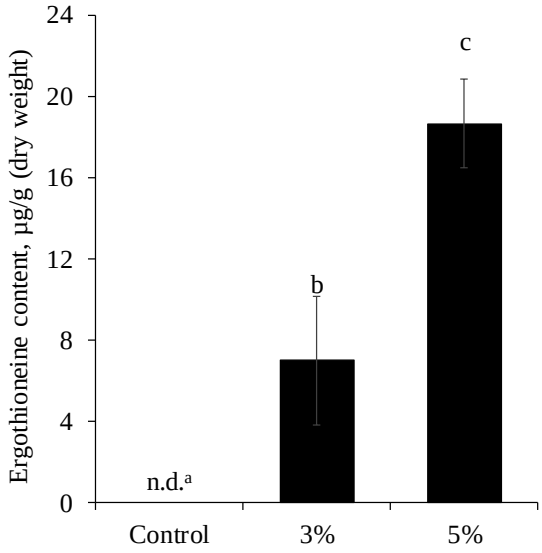

Figure 3.8. Ergothioneine content in the meat of *O. mykiss* after 2.5 months of feed supplementation with different concentrations of mushroom extract. Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$).

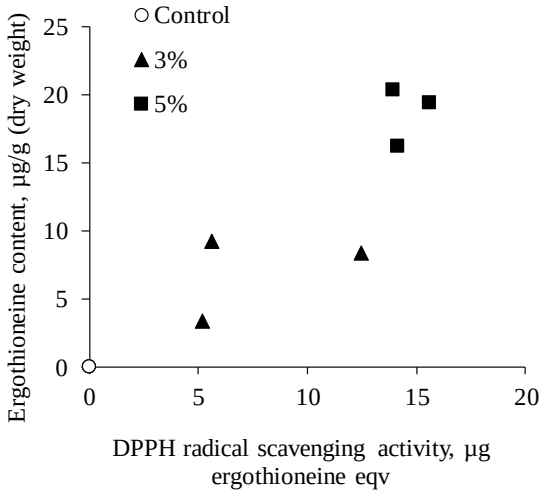

Figure 3.9. Correlation between ergothioneine content and DPPH radical scavenging activity in the meat of *O. mykiss* after 2.5 months of feed supplementation with different concentrations of mushroom extract.

3.4 Discussion

The overall results of the preliminary *in vitro* ME supplementation experiments showed that the effects of the addition of ME may not be discernable in terms of visual pigment evaluation. However, the effects of the ME treatments were significantly notable in the quantitative evaluation delaying the rate of astaxanthin degradation as well as in the control of lipid HPO formation. These results point to the potential application of ME-treatment for the control of discoloration and oxidation in fish meat during post-harvest storage. From these observations, a feeding experiment was subsequently conducted to determine whether *in vivo* supplementation of ergothioneine-rich ME in the diet of the fish would be a viable strategy without compromising the growth and pigmentation of fish during grow-out culture.

The uniform increase in body weight of the of fish under different dietary treatments showed that the ME supplementation did not have any adverse effects on their growth, contrary to the earlier hypothesis that the distinct odor imparted by the crude ME may have a negative effect on the acceptability of the feeds to the fish. However, results showed that despite the addition of ME just a few minutes prior to the feeding, the smell imparted by the feeds did not affect its acceptability to the fish. In addition, similar with the increase in body weight, the lipid content in the meat of the fish illustrated that the fat deposition in all the different treatments was not affected by the additional components added to the feeds.

The lipid content in salmonid fish is an important factor that affects the rate of absorption and deposition of the pigmenting compound astaxanthin into their muscle tissues[98]. Several studies have also reported the correlation of dietary lipid and pigment deposition in salmonid species[99-101]. The pigmentation in the muscles of rainbow trout and other salmonid fish is one essential factor to consider, as it is one of the key characteristics that influence its market quality and value.

Both digital image evaluation of the pigmentation in meat samples using software computations and the quantitative measurement of total astaxanthin content illustrated an increasing pattern wherein the no apparent effects or differences were observed in coloration despite the difference in the dietary supplementation. The visual evaluation of astaxanthin-pigmented meat may be used as a rough estimate of the astaxanthin content in meat, but may not be always precisely correlated with the actual astaxanthin content.

The presence of the ergothioneine in the blood of ME-supplemented fish demonstrates a positive effect and uptake of ergothioneine from the diet. Moreover, the accumulation of ergothioneine illustrates a dose-dependent trend, during the 2.5 months of feed supplementation period. With the increase in ergothioneine content in blood, it was noted that the lipid HPO analyzed in blood follows an inversely similar pattern. The treatment group with the 5% treatment group with the highest ergothioneine content in blood had the lowest lipid hydroperoxides in blood, and likewise, the control group with no detectable ergothioneine in blood has the highest lipid hydroperoxides quantified in blood. This may additionally support and confirm the successful uptake of ergothioneine as it was detected in blood. Ergothioneine is known for its antioxidative property[33], specifically its radical scavenging property[102]. In an aerobic biological system such as in fish and other animal metabolism, certain radical species may be present that could initiate and promote an oxidation reaction[103], that if not controlled early could have the possibility to lead to cellular damage. These results demonstrated the radical scavenging property of ergothioneine as well as other possible compounds in the mushroom extract that have antioxidative properties that may also have taken up by the fish together from the ME.

The significant quantity of ergothioneine that was measured from the meat samples of the ME-supplemented fish further confirms the positive uptake of ergothioneine in fish from the ME-supplemented diet. Furthermore, the ergothioneine that was measured from the meat samples were also analyzed for DPPH radical scavenging activity by HPLC method, and corresponding radical scavenging activities as ergothioneine equivalents were measured from samples containing ergothioneine. These results were similar to the findings of some studies[51, 95, 96], wherein the increasing concentration of ergothioneine in crude mushroom extract translated into corresponding increasing radical scavenging activities. These same extracts that were tested for ergothioneine and DPPH radical scavenging activities were also found to have antioxidative functions when applied to other fishery commodities[51, 95, 96].

3.5 Summary

The results of this study demonstrated positive effects of ME addition to salmonid meat in terms of mitigating the effects of lipid oxidation and astaxanthin degradation. It also gave positive insights into the possible protective effects of ME addition in meat during storage oxidation. These results also confirmed the findings from Chapter 1, wherein ergothioneine and ME addition successfully mitigated the progress of lipid oxidation and astaxanthin degradation in a liposome system. Moreover, the other set of results obtained from this experiment demonstrated a feasibility of incorporating ergothioneine-rich ME into the diet of the fish with a positive uptake as demonstrated in the accumulation of ergothioneine in some of the tissues tested. In addition, the positive effects of this feeding trial gave positive directions on the viability of using the strategy of ME dietary supplementation in salmonid species for post-harvest protective functions and nutritional quality preservation.

CHAPTER 4

Effects of ergothioneine-rich *Pleurotus cornucopiae* extract supplementation on the stability of lipid and astaxanthin against oxidation in *Oncorhynchus kisutch* meat during low-temperature storage

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4.1 Introduction

Salmonids are mainly known for their characteristic red-orange muscle coloration that has been one of the distinct and desirable characteristics of these group of fish. Out of the 10 genera that belong to the family of Salmonidae, only 4 genera exhibit red to pinkish muscle coloration. All species falling within the salmonid genera of *Oncorhynchus*, *Salmo*, *Salvelinus*, and *Parahucho* possess this unique characteristic among all other fishes[12]. Production of salmonids through capture fisheries reached its peak in the mid-1990s, after which, steady declines have been reported, and has led to the development of salmonid aquaculture in the 1970s wherein a sharp increase in aquaculture production has been continuous since the 1980s.

Based on the results of Chapter 3, the current study aimed to expand the application of ergothioneine-rich mushroom extracts (ME) to other salmonid species and evaluate its subsequent effects during post-harvest storage. This study was contextualized from a practical approach of using crude MEs directly as supplements to the commercial diet of coho salmon (*Oncorhynchus kisutch)* since the purification of ergothioneine from crude MEs could entail additional processing costs which could diminish the practicality of this strategy. Specifically, this study aimed to evaluate the feasibility of ergothioneine supplementation by incorporating ME in *O. kisutch* diets, with the overall objective of preserving fish meat quality by mitigating lipid oxidation and meat discoloration during post-harvest storage.

4.2 Materials and methods

4.2.1 Materials and chemicals. Coho salmon (122.1±29.3 g average body weight; 20.0±1.3 cm average fork length) used for this feeding experiment were obtained and raised in Iwahime Fish Farm (Iwate Prefecture, Japan). The base diet used for feeding consisted of an extruded pellet-type formulated feed for freshwater fish enriched with astaxanthin, which was purchased from Nosan Corporation (Kanagawa, Japan).

HPLC-grade methanol, chloroform, and *tert*-butyl methyl ether were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Authenticated standards of all-*trans*-astaxanthin from algae (*Haematococcus pluvialis*) of 85.0% purity, cumene hydroperoxide of 80.0% purity, and *L*-(+)-ergothioneine of 99.0 % purity were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and Bachem AG (Bubendorf, Switzerland), respectively. *trans*-β-apo-8′-carotenal of 96.0% purity and 3-methyl-1H-imidazole-2-thione (methimazole) of 99.0% purity as internal standards (IS) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-glycero-3-phosphocholine (NBD-labeled PC) of 99% purity as a lipid hydroperoxide (HPO) IS was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

4.2.2 Mushroom extract preparation

Hydrophilic extracts of *P. cornucopiae* used for the feeding experiment was prepared and donated by Three B Co., Inc. (Hokkaido, Japan). The fruiting bodies of *P. cornucopiae* (200 kg) in a stainless-steel sieve were added to water at a 1:1 proportion (w/w) and boiled for 15 min in a 400 L stainless steel vat. After boiling, the mushroom fruiting bodies were removed from the resulting aqueous extract. Using the same aqueous extract, this process was repeated for the extraction of two more batches of fresh mushroom fruiting bodies. A total of 600 kg of mushroom

fruiting bodies was therefore processed which resulted in a recovered volume of 500 L aqueous extract with an approximate dissolved solid content of 2.5 °Bx. This aqueous extract was then concentrated to 25 °Bx using a thin-layer vacuum concentrator (Hisaka REV 60/30-1-2, Osaka, Japan) resulting in 60 kg of concentrated extract. The final ME solution used for this study was prepared by diluting the concentrated extract to 10 °Bx, which had an ergothioneine concentration of 1.1 mg/mL.

4.2.3 Dietary supplementation of ergothioneine-rich ME in coho salmon

The feeding experiment was conducted at the Iwahime Fish Farm Co. in Iwate Prefecture, Japan. The base diet used was an astaxanthin-supplemented feed formulation, which was used for the control group. Treated groups were fed the same base diet with the additional supplementation of *P. cornucopiae* liquid extracts at concentrations of 1 and 5% (w/w). This corresponded to equivalent ergothioneine concentrations of 0, 11, and 55 µg per gram of feeds in the control, 1, and 5% groups, respectively. The daily feeding rate was based on 1% of the average fish body weight and was broadcasted twice daily over a period of 2 months. Based on this feeding scheme, it was estimated that a 200-gram fish would receive 22 or 110 µg of ergothioneine daily for the 1 or 5% groups, respectively.

After 2 months of feeding, blood samples were collected on site (via caudal venous puncture) and kept in vacuum-sealed tubes with sodium heparin (Terumo Co. Ltd., Tokyo, Japan). The fish were then immediately sacrificed and transported together with the blood samples within a few hours to the laboratory on ice. The fish samples were subsequently skinned and degutted. The dorsal muscles were filleted, minced using a food processor, and divided into 3 groups for the different treatments. Five-gram portions of the minced meat were used to analyze lipid content, HPO, and astaxanthin levels. The remaining samples were used for the storage experiment.

To determine whether the presence of ergothioneine in the blood of ME-supplemented fish translated into improved antioxidative activity in fish meat, storage studies were subsequently conducted. Five-gram portions of the samples from each treatment group were kept in polystyrene petri dishes (30 mm diameter) with lids. The total number of samples were divided into two groups and were stored separately at either -2 or -18 °C for 3 and 4 weeks, respectively. Treatments were prepared in 3 replicates and were evaluated every week for quantitative analyses of lipid HPO and total astaxanthin levels, as well as visual color evaluation.

4.2.4 Quantification analyses

Lipid extraction and quantification. The total lipid content of the stored minced meat was extracted using the Bligh and Dyer procedure[61] and quantified gravimetrically.

Total astaxanthin quantification. Total astaxanthin in minced meat was extracted using the Bligh and Dyer procedure[61] with previous addition of *trans*-β-apo-8′-carotenal as an IS. Quantification analysis was carried out using a Shimadzu model SCL-10A HPLC system (Kyoto, Japan) equipped with two straight-lined C_{18} reverse-phase columns (Waters Symmetry Shield™ RP18, 5 µm, 4.6 mm i.d. x 250 mm). A gradient elution using HPLC-grade methanol and *tert*-butyl methyl ether as the mobile phase was programmed as follows: 0-9.3 min: 100% methanol; 9.4-22.0 min: 60% methanol; and 23.0-39.0 min: 100% methanol. The sample injection volume was 20 µL and the column temperature was kept at 25 °C. The eluted astaxanthin was monitored at a wavelength of 485 nm, to obtain the peak area ratio (astaxanthin/IS). A calibration curve was obtained using different concentrations of the authentic all-*trans*-astaxanthin from algae (*Haematococcus pluvialis*). All data were expressed as µg of astaxanthin per g of meat.

Total lipid HPO quantification. Lipid oxidation was evaluated by the quantification of total lipid HPO present in the samples using a flow injection analysis (FIA) system equipped with a fluorescent detection system, as described by Sohn et al. (2005)[60]. The preference for this method was based on its specificity and high sensitivity for detecting and measuring lipid oxidation products during the early stages of lipid oxidation. Briefly, minced meat was extracted using the Bligh and Dyer procedure[61] with the previous addition of NBD-labeled PC as an IS. The fluorescence intensity of HPO reacted with diphenyl-1-pyrenylphosphine (DPPP), was monitored at the excitation wavelength (λ_{ex}) of 352 nm and at the emission wavelength (λ_{em}) of 380 nm using a Shimadzu model RF535 fluorescence detector (Kyoto, Japan). The IS was monitored at 460 nm (λ_{ex}) and 534 nm (λ_{em}) using a Shimadzu model RF-10A fluorescence detector (Kyoto, Japan). A calibration curve was obtained using different concentrations of authenticated cumene hydroperoxide. All data were expressed as equivalent nmol cumene hydroperoxide per gram of meat.

Ergothioneine quantification. Ergothioneine from blood samples were quantified according to the method of Nguyen et al.[97]. Briefly, 4 mL of distilled water was added to 1.5 mL of whole blood and placed in a water bath at 90 °C for 12 min while agitating every 3 min intervals. Samples were then cooled on ice for 3 min and 1 mL of an aqueous solution of methimazole (200 µg/mL) was subsequently added as an IS. The samples were centrifuged at 3000 x *g* for 15 min at 4 °C. The resulting supernatant was collected to which 12 mL absolute ethanol was subsequently added. The solution was then agitated and kept on ice for 30 min, and centrifuged at 3000 x *g* for 15 min at 4 °C. The resulting supernatant was collected and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water. Meat sample was extracted using the Bligh and Dyer method[61], with slight modifications. Instead of the addition of water, 0.88 % of KCl was added, and 1 mL of an aqueous solution of methimazole (200 µg/mL) was

added prior to the homogenization. The water-methanolic layer was collected and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water. A 20-µL portion of the sample was subjected to analysis. Quantitative analysis was carried out by HPLC analysis following the method of Nguyen et al.[97]. Briefly, ergothioneine from the sample extract was chromatographically separated using two reversed-phase C_{18} columns (ACE C18-AR 3 µm, 150 × 4.6 mm i.d.) eluted with 10% methanol in deionized water with 0.1% acetic acid at a flow rate of 0.20 mL/min and monitored at 254 nm using a photodiode array detector (SPD-M10A Shimadzu, Kyoto, Japan). Separated ergothioneine was reacted with 0.23 M 2,2'-dipyridyl disulphide in 0.25 M HCl at a flow rate of 0.15 mL/min in a post-column reaction coil, wherein the resulting reaction product 2-thiopyridone was monitored at 343 nm (SPD-M10A). Varying concentrations of an *L*-(+)-ergothioneine standard were used to obtain a calibration curve. All data for this analysis are expressed as µg of ergothioneine per g or mL of sample.

DPPH radical scavenging analysis by online HPLC method. The radical scavenging abilities of the meat samples were determined according to the methods of Giri, et al. (2011)[66] and Tepwong et al (2012)[53] with slight modifications. The hydrophilic extracts prepared for the ergothioneine analysis were analyzed in an HPLC system equipped with a post-column reaction system for free DPPH radical scavenging ability. Briefly, components of the injected sample were separated using a Synergi 4u Fusion - RP 80A column (4 µm, 250 mm × 4.6 mm i.d., Phenomenex Inc., Torrance, CA, USA), by 10% methanol in deionized water with 0.01% acetic acid at a flow rate of 0.2 mL/min. The eluates were monitored at 254 nm with an SPD-M10AVP detector. DPPH radical methanolic solution (150 µM) pumped at a flow rate of 0.32 mL/min was reacted with the eluted antioxidative components in a post-column reaction coil. Negative peaks formed by DPPH radical scavenging were monitored at 517 nm with an SPD-M10AVP. Varying concentrations of *L*-(+)-

ergothioneine standard were used to obtain a calibration curve with all data expressed as µg ergothioneine equivalent per g of sample.

Color image analysis. Visual color of the meat was evaluated quantitatively according to the method of Bao et al.[4]. Briefly, the minced meat kept in polystyrene petri dishes were positioned inside an imaging apparatus (Image Capture G3 Liponics) equipped with 2 florescent light sources (6-watt Toshiba FL6D, 210 lumens). Image data (jpeg format) was captured using a digital single-lensed CCD camera (Canon EOS Kiss X3) and were analyzed for the red (*r*), green (*g),* and blue (*b*) values in a specified area of the sample using an image processing software (ImageJ of the National Institutes of Health, Bathesda, MD). The *R** value of the sample was calculated using the formula:

$$R^* \text{ value} = \frac{r}{r+g+b} \times 100$$

where *r, g,* and *b* denote the average red, green and blue values, respectively.

Statistical Analysis. Microsoft Excel 2016 was used to analyze the means and standard deviations of the collected data as well as for the generation of graphs. One-way Analysis of Variance using IBM SPSS Statistics 20 was used to evaluate the data for significant differences among treatments at a 5% level of significance.

4.3 Results

4.3.1 Effects of ME dietary supplementation on the growth, lipid content, and pigmentation of coho salmon.

The effects of 2-month ME-feed supplementation on the growth and lipid content of coho salmon are shown in Figure 4.1. The total average body weight of the fish increased significantly

($p < 0.05$) in all feeding groups, particularly in the 1% and 5% ME-supplemented groups (Figure 4.1(a)). The ME supplementation showed no adverse effects on the total body weight of the fish in all the different feeding treatments. The lipid content in the meat (Figure 4.1(b)) increased in all the feeding groups after 2 months of feeding, but no significant differences ($p > 0.05$) were observed among the treatment groups.

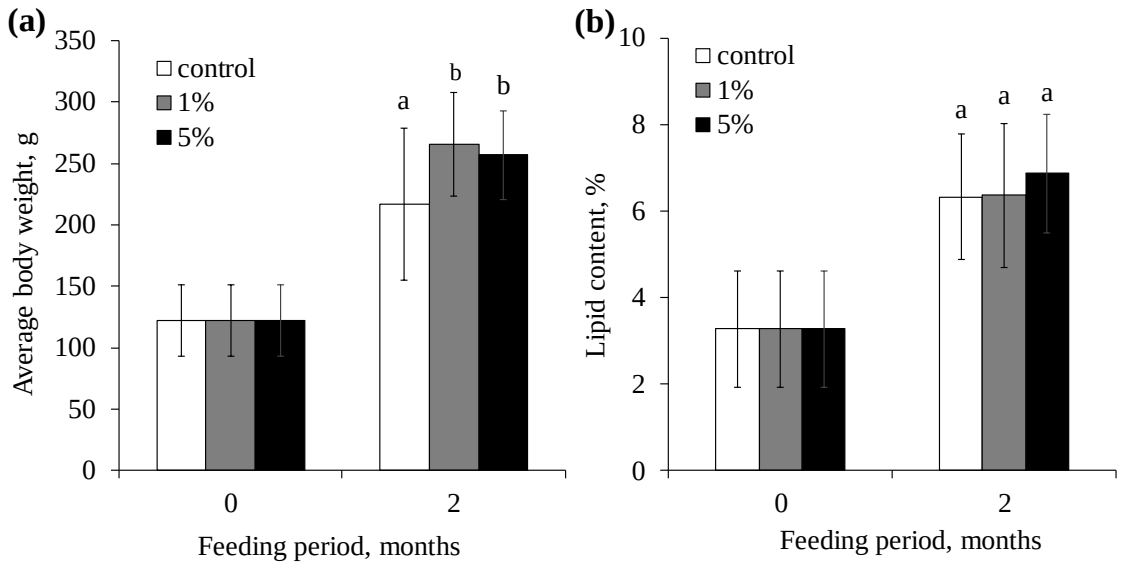

Figure 4.1. Effects of mushroom extract supplementation in *O. kisutch* growth after 2 months of feeding. Changes in average body weight (a); and lipid content (b) of the different fish treatments. Data are presented as mean ± standard deviation (n=10). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$).

The effects of 2-month ME supplementation in the meat pigmentation of coho salmon are shown in Figure 4.2. A slight increase in the R^* value was observed in all of the fish treatments (Figure 4.2(a)). However, no significant differences ($p > 0.05$) were observed among the treatment groups. The total astaxanthin content of the meat of the different fish treatment showed a remarkable increase after the 2 months of feeding (Figure 4.2(b)). However, similar to the R^* value no significant differences ($p > 0.05$) were observed among the treatment groups. These results indicated that there were no significant differences ($p > 0.05$) in astaxanthin content among the starting materials for the succeeding storage studies and that subsequent changes in the astaxanthin

contents during storage could mainly be attributed to the supplementation of ergothioneine-rich ME.

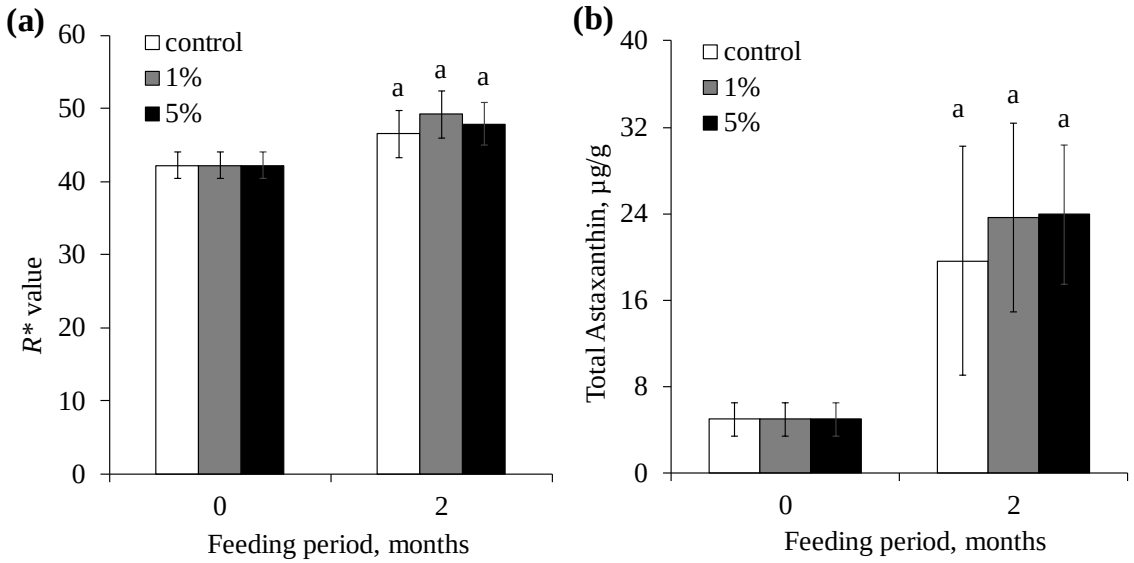

Figure 4.2. Effects of mushroom extract supplementation in *O. kisutch* meat pigmentation after 2 months of feeding. Changes in *R** value (a); and total astaxanthin content (b) of the different fish treatments. Data are presented as mean ± standard deviation (n=10). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$).

4.3.2 Effects of ME dietary supplementation on the ergothioneine recovery in blood and meat coho salmon. Ergothioneine content in the blood and meat of fish after the 2 months of feeding are shown in Figure 4.3(a) and Figure 4.3(b), respectively. The ergothioneine concentration recovered in the whole blood of fish after the feeding period showed that the 5% ME supplementation was a sufficient treatment in maintaining a significant ergothioneine concentration in the blood of fish which amounted to 44.9 µg/mL. In addition, the concentration quantified in the 5% ME-treated group was significantly higher ($p < 0.05$) than both 1% and control groups which had 5.9 µg/mL and no detectable amount of ergothioneine, respectively. Similarly, the meat samples from the 5% feeding treatment have accumulated a significant concentration of ergothioneine, which also have corresponding DPPH radical scavenging activity as shown in Figure 4.4.

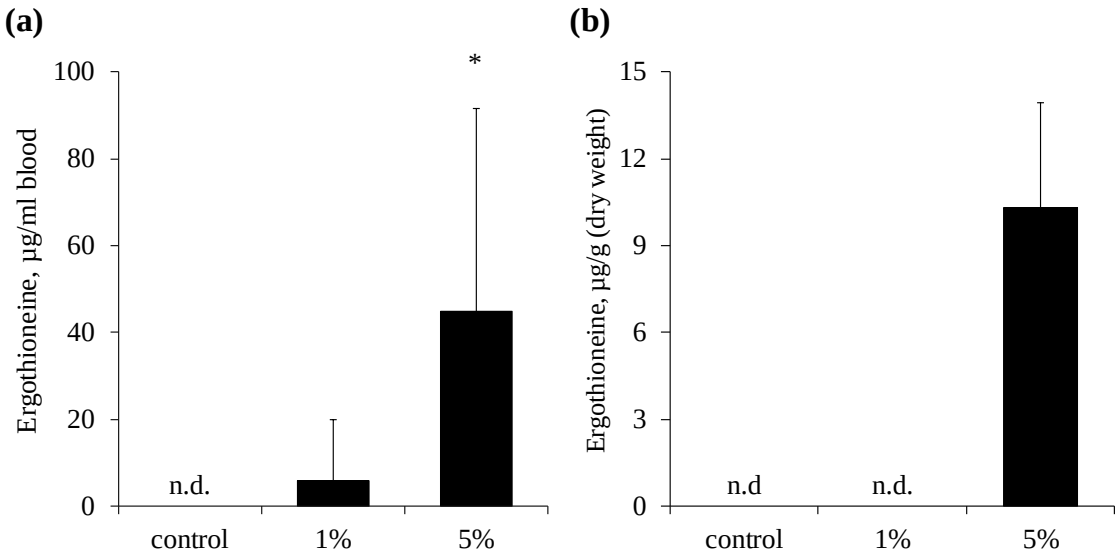

Figure 4.3. Ergothioneine content in blood (a); and meat (b) of *O. kisutch* after 2 months of feed supplementation with different concentrations of mushroom extract. Data are presented as mean + standard deviation (n=10). Significant differences among treatment groups ($p < 0.05$) are indicated by asterisks. n.d., not detected.

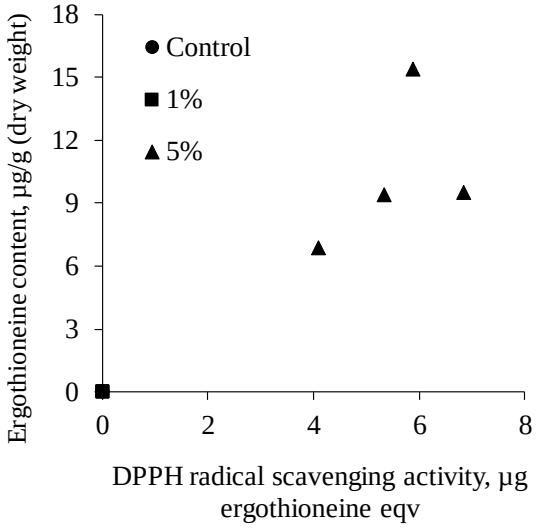

Figure 4.4. Correlation between ergothioneine content and DPPH radical scavenging activity in meat of *O. kisutch* after 2 months of feed supplementation with different concentrations of mushroom extract.

4.3.3 Effects of ME dietary supplementation on the coho salmon meat during low temperature storage

The effects of the ME supplementation in the stability of lipid and astaxanthin against oxidation in fish meat during low temperature storage were evaluated in a time course experiment.

Changes in total lipid hydroperoxide content in meat during storage. Changes in the HPO contents of the fish meat during storage are shown in Figure 4.5. ME-treated and untreated meat stored at -2 ℃ (Figure 4.5 (a)) showed no significant differences ($p > 0.05$) in HPO content among treatments throughout most of the storage duration. In all treatments at this temperature, HPO accumulation far exceeded the highest amounts observed for all treatments at -18 ℃ as early as the first week of storage. ME-treated and untreated meat stored at -18 ℃ (Figure 4.5 (b)) showed that lower temperature can suppress the accumulation of HPO during storage, wherein HPO contents recorded throughout the storage period were less than 2000 equivalent nmol cumene-OOH/g. Furthermore, the 5% ME supplementation significantly ($p < 0.05$) suppressed the accumulation of HPO in fish meat stored at -18 ℃ for 4 weeks.

Changes in Visual Coloration and Total Astaxanthin Content During Storage. Representative images of the minced meat samples are shown in Figure 4.6. Based on visual evaluation it can be noted that the discoloration during storage at -2 ℃ (Figure 4.6 (a)) was more prominent in the control group than in the ME-treated groups. Changes in the color of meat samples during storage at -18 ℃ (Figure 4.6 (b)) were not clearly discernable by visual evaluation.

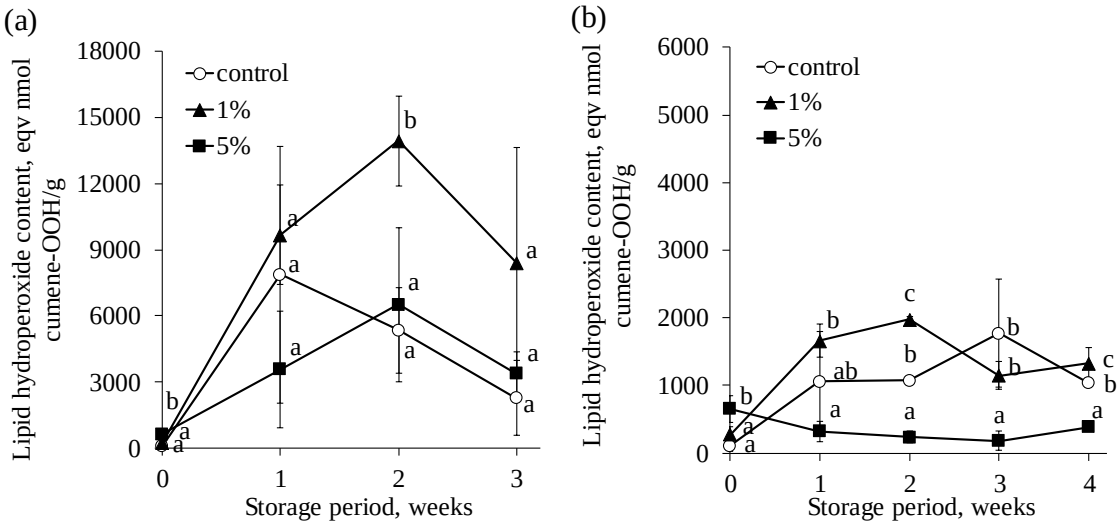

Figure 4.5. Effects of mushroom extract feed supplementation in the total lipid hydroperoxide contents of minced *O. kisutch* meat during storage. Changes during storage at -2 °C (a) and -18 °C (b). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

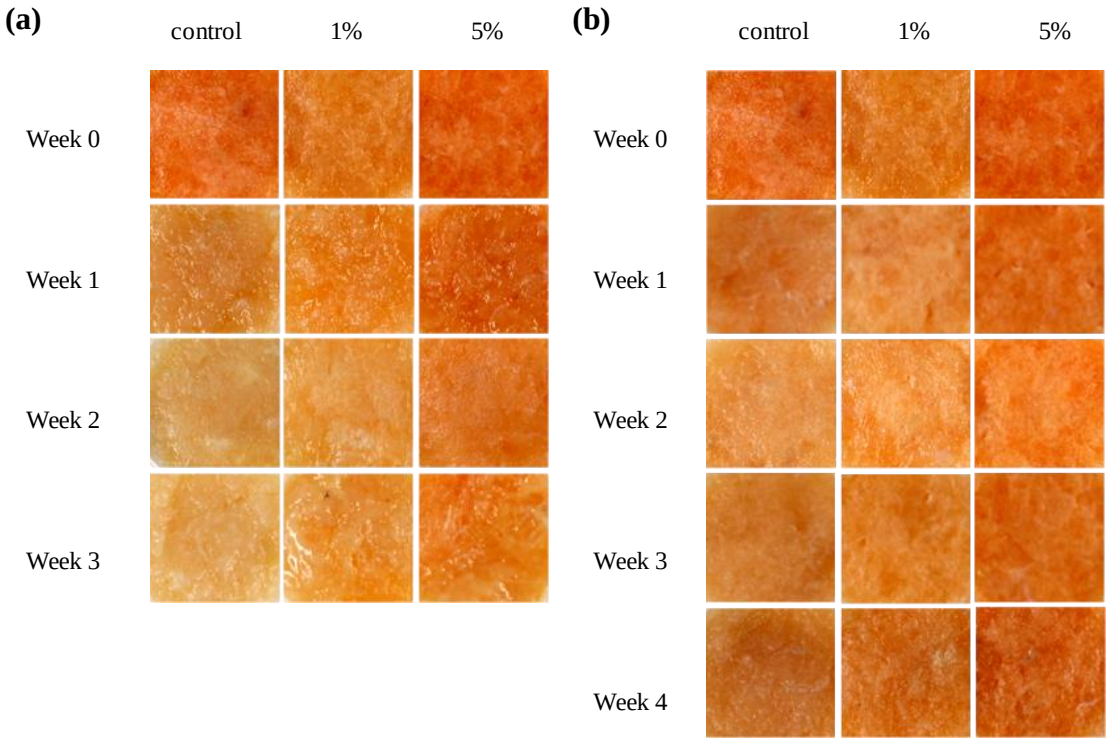

Figure 4.6. Representative photos showing the changes in visual coloration of minced meat samples of the different mushroom extract-supplemented treatments of *O. kisutch* stored at -2 °C (a) and -18 °C (b).

Changes in the R^* value of the fish meat during storage are shown in Figure 4.6. The R^* values of the control group stored at -2 °C (Figure 4.7(a)) consistently showed significantly lower ($p < 0.05$) red coloration throughout the storage duration with an R^* value of 47.3±0.6 at week 3, while the 1% and 5% ME-supplemented group had R^* values of 51.7±1.4 and 60.1±1.2, respectively. The R^* values of the 5% ME-supplemented group stored at -18 °C (Figure 4.7(b)) consistently showed a significantly higher ($p < 0.05$) red coloration throughout the storage duration with an R^* value of 64.1±0.7 at week 4, while the control and 1% ME-supplemented group had R^* values of 57.7±1.3 and 59.3±1.6, respectively.

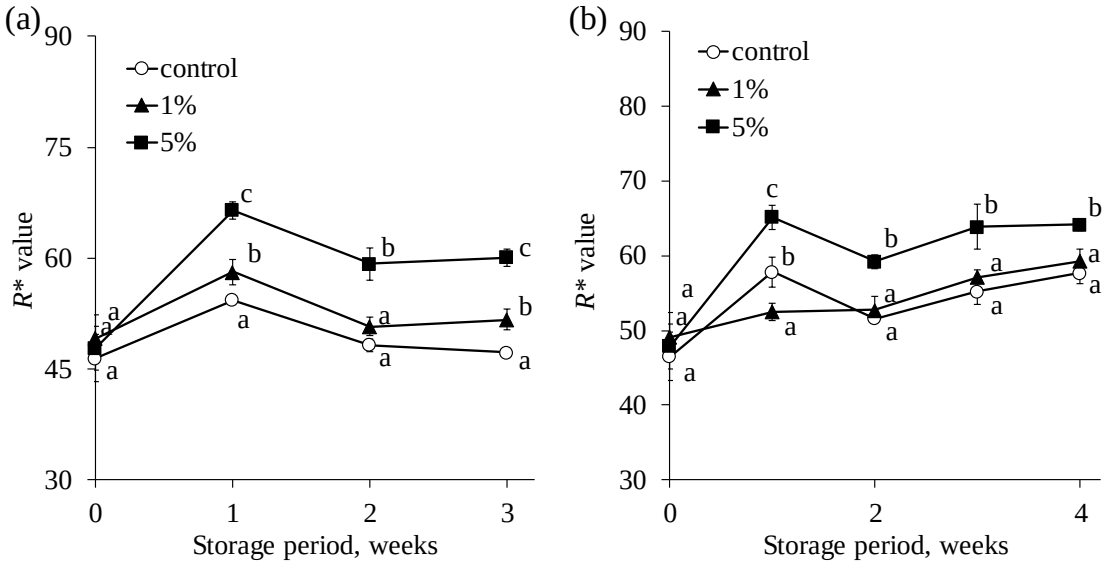

Figure 4.7. Effects of mushroom extract feed supplementation on the R^* values of minced *O. kisutch* meat during storage -2 °C (a) and -18 °C (b). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point.

Changes in astaxanthin content of the fish meat during storage are shown in Figure 4.8. The control group stored at -2 °C (Figure 4.8 (a)) showed a notable decrease in the astaxanthin content throughout the storage period reaching as low as 5.6±1.2 µg/g during the 3rd week of storage. The 1% and 5% ME-supplemented groups showed higher astaxanthin contents at 16.8±11.1 µg/g and 18.1±2.0 µg/g, respectively. The samples stored at -18 °C (Figure 4.8 (b))

exhibited a pattern similar to those stored at -2 °C with the control group exhibiting a consistently lower astaxanthin content throughout the duration of storage compared with the ME-treated groups. On the 4th week of storage, the astaxanthin content of the control group was at 7.8±0.5 µg/g, while the 1% and 5% ME-supplemented groups were at 22.7±13.0 µg/g and 20.9±1.8 µg/g, respectively. The supplementation of ME into the feeds of the fish showed that it can mitigate the decrease in astaxanthin content in fish meat during storage.

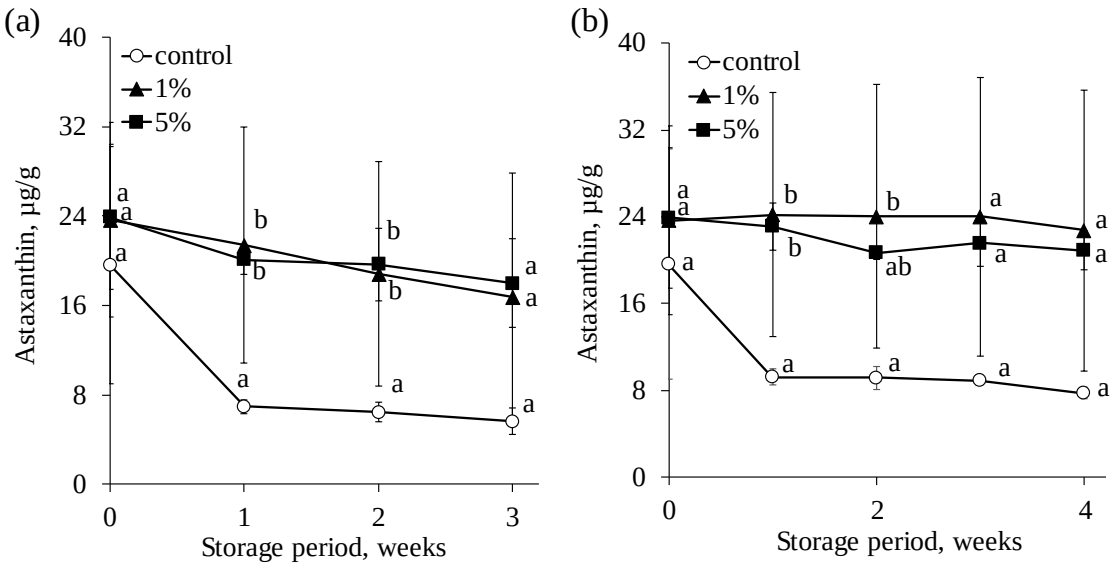

Figure 4.8. Effects of mushroom extract feed supplementation in the total astaxanthin content of minced *O. kisutch* meat during storage. Changes during storage at -2 °C (a); and -18 °C (b). Data are presented as mean ± standard deviation (n=3). Values with different superscript letters represent significant differences among treatment groups ($p < 0.05$) at each particular time point

4.4 Discussion

The results of the feeding experiment showed that the supplementation of ME to the diet of *O. kisutch* is a feasible strategy for the accumulation of ergothioneine in the fish system, with no adverse effects on its growth and pigmentation during culture. In addition, the significant increase in ergothioneine levels in the blood and meat of ME-supplemented fish suggests that salmonids may possess ergothioneine-specific transporters, thus making ME-feed supplementation during culture a more viable strategy for ergothioneine accumulation for the

subsequent control of lipid oxidation and astaxanthin degradation in fish meat during post-harvest storage. As previously reported, ergothioneine can only be synthesized by certain fungal and mycobacterial species and its presence in other organisms such as mammals only occurs via its acquisition through the diet[104]. Ergothioneine availability in organ systems varies and is influenced by the distribution and expression of an organic cation transporter 1 (OCTN1)[42] which was originally described to have a multi-specific function for certain substrates similar to ergothioneine[105]. Recent studies have revealed though that OCTN1 is highly specific to ergothioneine as its main substrate[42, 43].

A Basic Local Alignment Search Tool (BLAST) search on the National Center for Biotechnology Information Protein Database using the sequence for *Homo sapiens* OCTN1[106] as a query revealed that the salmonids *O. mykiss*[107] and *Salmo salar*[108] also possessed OCTN1 homologs, although these were still annotated as protein sequences with unknown functions. The high sequence similarity of *H. sapiens* OCTN1[106] and the BLAST hits from *O. mykiss*[107] and *S. salar*[108] could denote that the OCTN1 homologs from fish may have a function similar to that in humans and could thus play an important role in ergothioneine uptake. The organic compound selenoneine is a selenium analog of ergothioneine, which is a strong antioxidant isolated from blood and meat tissues of tuna[109]. It was reported by Yamashita et al. that the transport of this compound is mediated by the same transporter, OCTN1[110]. A similar study also revealed high expression levels of OCTN1 in the blood cells of selenoneine-supplemented zebrafish[110]. Additional studies have also quantified the concentration of selenoneine in the meat of different species of fish and recovered a significant level from swordfish, bigeye tuna, Pacific Bluefin tuna, albacore tuna, yellowfin tuna, and alfosino[111, 112]. These findings could suggest that OCTN1 is highly conserved across several fish species, and with its presence, follows the possibility of ergothioneine uptake in fish.

It was previously demonstrated that astaxanthin degradation can be mitigated by the presence of other antioxidants such as α-tocopherol and ascorbic acid present in a nanodispersion system[31]. The results of the subsequent storage experiments demonstrated that ergothioneine-rich ME suppressed lipid HPO accumulation and exhibited improved astaxanthin stability by delaying the rate of discoloration in the fish meat during low temperature storage.

The process of lipid peroxidation proceeds via a chain reaction mechanism which follows a bell-shaped reaction curve characterized by a lag at the initiation phase, peaking at the end of the propagation phase, and subsequently followed by the degradation phase[113]. This dynamic change in HPO accumulation and degradation was most notable in samples stored under the higher temperature tested in this study (-2 °C). At this temperature, the control group reached a peak in HPO accumulation as early as the 1st week of storage whereas ME-supplemented groups exhibited a delay in peak HPO accumulation which was observed during the 2nd week of storage. Furthermore, storage of meat at -18 °C showed that supplementation of 5% ME coupled with low temperature storage strongly suppressed HPO accumulation throughout the storage period suggesting additive effects of antioxidant supplementation and temperature control in mitigating HPO accumulation.

The visual evaluation of meat discoloration, the R^* value results of meat samples were quite notable during the storage duration. This could be attributed to the nature of astaxanthin degradation and the probable localization of ergothioneine in these experiments. The conjugated double bonds in the polyene chains of certain compounds such as astaxanthin act as chromophores, or the structural moiety responsible for its visible pigmentation when it absorbs and reflects light. Etoh et al.[114] identified some of the reaction products of the autoxidation of astaxanthin, and among

these, 13-apoastaxanthinone, which arises from the oxidation and cleavage of the double bond at C-13, was the major reaction product. Aside from 13-apoastaxanthinone, the other autoxidation products of astaxanthin still retain considerable portions of its unsaturated polyene moiety and could therefore still impart visual colorations. The autoxidation and degradation of astaxanthin are therefore less discernable through simple visual evaluations but can only be differentiated using more detailed methods such as HPLC analyses. Thus, visual perception of redness may not be directly correlated with the actual astaxanthin concentrations. It must be noted that these observations indicate the significant role of antioxidant localization in the retention of astaxanthin pigments in salmon meat as well as the prevention and suppression of lipid oxidation as suggested in the previous studies[115].

Although the 1% treatment group only had minimal ergothioneine content in blood and no detectable quantity was recovered in meat samples, a considerable effect against astaxanthin degradation was still demonstrated. These results may further confirm the findings from Chapter 2, wherein the crude extract of mushroom *P. cornucopiae* exhibited significantly higher antioxidative properties than pure ergothioneine alone. Thus, even though the recovery of ergothioneine in 1% feeding group is low to almost non-detectable, there could be other components from ME that may have contributed to the antioxidative actions in *O. kisutch* meat during low temperature storage through the mitigation of lipid oxidation. Hence, the dietary supplementation of crude extracts is not only an economical strategy of administering ergothioneine in fish but also other bioactive components present in the extract that could result in a synergistic protective property.

4.5 Summary

To minimize additional production costs, ergothioneine-rich ME supplementation need not be done throughout the whole duration of grow-out culture. In fact, ME feed daily supplementation at a rate of approximately 550 µg ergothioneine per kg body weight of fish just several weeks prior to harvest could probably lead to significant ergothioneine accumulation in the fish tissues and help preserve the fish meat quality during storage by preventing astaxanthin degradation and lipid oxidation, similar to the effects observed for ergothioneine supplementation to the diets of cultured yellowtail (*Seriola quinqueradiata*)[55]. In addition, the use of mushroom extract processing and trimming wastes offers a cheaper alternative of acquiring the potent antioxidant ergothioneine and other possible compounds with antioxidative and other bioactive properties as a dietary supplement for fish during grow-out culture. In light of this, further studies should focus on optimizing the delivery of ergothioneine in terms of supplement concentration and administration duration.

CHAPTER 5

Bioinformatic Identification of the Ergothioneine Transporter (ETT/OCTN1) genes in salmonids and gene expression analysis in *Oncorhynchus mykiss*

5.1 Introduction

Certain fungal species biosynthesize the hydrophilic antioxidant ergothioneine [33, 116, 117], but this compound cannot be synthesized by higher organisms such as vertebrates. Since the discovery of ergothioneine in 1909, the specific function of this compound in the human body has remained unclear, however several studies consider it as an intracellular antioxidant [116]. As a hydrophilic antioxidant, ergothioneine intracellularly functions as a singlet oxygen quencher and a non-radical species scavenger that prevents photooxidation and allows DNA repair of UV-damaged cells [37, 38], protects against lipid peroxidation, conserves endogenous antioxidants glutathione and α-tocopherol [39], acts as a hydroxyl radical scavenger and transition metal (ferrous or copper(II)) ion-dependent oxidation inhibitor, and slows reaction rates with superoxide or hydrogen peroxide [116]. Moreover, ergothioneine exists in a mostly concentrated state in the mitochondria suggesting that it functions as a cytoprotectant for specific mitochondrial materials such as DNA [118].

Given the important role of ergothioneine as an antioxidant in various cellular processes, understanding its uptake in organisms that cannot synthesize this compound is a crucial factor in efforts to utilize it as a feed additive for cultured commodities such as poultry, other livestock, and fish. A considerable number of studies have already been conducted elucidating the role of a specific transporter protein known as Organic Cation Transporter 1 (OCTN1) [44], which has also been coined as an Ergothioneine Transporter (ETT) by other researchers [41, 45], in the uptake of ergothioneine. The gene encoding for OCTN1 belongs to a larger family of transporter proteins broadly classified as the Solute Carrier Family 22, of which various gene homologs play a wide

range of functions in relation to transporting organic cations across cellular membranes [44]. The presence of various gene homologs for OCTN1 has been documented in a number of mammalian and avian species [106, 119, 120]. In fish, however, the identification and characterization of the gene encoding for ETT has only been reported in the zebra fish (*Danio rerio*) [121, 122]. Previous studies on the supplementation of ergothioneine into the diet of cultured salmonids have shown that this compound is readily taken up by salmonids, resulting in fish flesh with improved shelf life during storage [123]. Identifying the appropriate genes to study through molecular biology techniques is therefore an important step in understanding the dynamics of ergothioneine uptake in salmonids.

The considerable number of gene homologs in the SLC22 gene family in a wide range of species has posed as a challenge in determining which gene specifically encodes for the homolog whose main function is the transport of ergothioneine. For instance, the gene encoding for OCTN2 in humans has been characterized as a transporter that functions specifically with carnitine, with minimal to no ability to transport ergothioneine, despite having a relatively high rate of sequence homology [124]. Similar observations were also reported for the SLC22 homologs in zebra fish, wherein distinct and specific carnitine and ergothioneine transporters were identified and functionally characterized [121]. With the significant sequence information obtained through these previous studies, the identification of the specific ergothioneine transporter gene in salmonids can be achieved through the use of stringent bioinformatics analyses. Identifying the appropriate salmonid gene homologs *in silico* therefore becomes the most practical approach prior to further studies on ergothioneine transporters through molecular biology techniques. Thus, the specific aim of this study was to identify the most likely candidate for ETT homologs in three commonly cultured salmonid species, Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), and Atlantic salmon (*Salmo salar*), through bioinformatics. To further elucidate the role

of ETT in ergothioneine uptake in salmonids as demonstrated in the previous chapters, gene expression analysis was also carried out in *O. mykiss*, the model species for salmonids.

5.2 Materials and methods

5.2.1 Materials and chemicals

Rainbow trout (117±17 g average body weight; 18±1 cm average fork length) used for this feeding experiment were obtained and raised in TUMSAT Oizumi Research Station, Yamanashi Prefecture, Japan. The base diet used for feeding was a commercial diet named 3P rainbow trout training pellets purchased from Marui Industry Co., Ltd (Nagano Prefecture, Japan). The mushroom extract used as feeding supplement was the *Pleurotus cornucopiae* 10 °Bx extract obtained from Three B Co., Inc. (Hokkaido, Japan), as described in the previous chapter.

5.2.2 Access to sequences

Nucleotide and amino acid sequence information was obtained from the GenBank and Protein Data Bank (PDB) online databases of the National Center for Biotechnology Information (NCBI) curated by the National Institutes of Health (NIH) (https://www.ncbi.nlm.nih.gov/). As an initial step, sequences for SLC22 homologs for zebra fish (*D. rerio*) were obtained by accessing sequences with accession numbers NP_957143.1 (unknown function), CDM63451.1 (ergothioneine transporter - ETT), and XM_005170337 (carnitine transporter - CTT) detailed in the study of Pfeiffer, Bach, Bauer, Campos da Ponte, Schomig and Grundemann [121]. These sequences were chosen to serve as query sequences for the Basic Local Alignment Search Tool (BLAST - https://blast.ncbi.nlm.nih.gov/Blast.cgi), given that *D. rerio* is the only piscine species whose SLC22 homologs for specific functions (i.e. ergothioneine or carnitine transport) have been functionally characterized to date.

The *D. rerio* amino acid sequences for three SLC22 homologs were subsequently used to individually mine for homologs in the sequenced genomes of either *O. mykiss*, *O. kisutch*, or *S. salar* using the online Protein BLAST program (blastp). BLAST searches were limited to either *O. mykiss*, *O. kisutch*, or *S. salar* databases by specifying the organism taxonomic id (taxid) in the Search Set window of the blastp program. Homologs of ETT genes from other organisms (non-piscine) were subsequently obtained by using appropriate keywords (i.e. SLC22A4, OCTN1) in the search function of the main NCBI page. A preference for SLC22 homologs that have been functionally characterized as ergothioneine transporters were selected in order to ensure that downstream sequence analysis would rely on homologs that have been experimentally confirmed.

5.2.3 Phylogenetic analysis

Prior to phylogenetic tree construction, obtained sequences were aligned using Clustal Omega [125]. To determine the most probable functions of the SLC22 homologs obtained for salmonids, a phylogenetic tree was constructed from the amino acid sequences of the three salmonid species together with the SLC22 amino acid sequences from *D. rerio* using the Neighbor-Joining method with a bootstrap of 1000 replicates on the software MEGA 7 [126]. Another phylogenetic tree was also constructed using the same method with additional ETT sequences coming from non-piscine species whose functions as specific ETTs have been confirmed through experimental methods [120, 124].

5.2.4 Multiple sequence alignment (MSA)

To further confirm the putative functions of the various SLC22 homologs obtained for salmonids, a more detailed analysis of the multiple sequence alignments (MSA) of salmonid and *D. rerio* homologs was carried out using Clustal Omega [125]. Putative transmembrane domains (TMDs) for *D. rerio* and *O. mykiss* ETTs were predicted and visualized using the online software

MEMSAT2 [127] and Protter [128], respectively. This served as a point of comparison for identified TMDs in the human ETT as reported by Bacher et al.[124]. Conserved amino acid residues within predicted TMDs specific for ETTs were visualized using outputs from Clustal Omega.

5.2.5 Feeding experiments for ETT gene expression analysis

The feeding experiment was conducted at the TUMSAT Oizumi Research Station. The commercial feed was used as a based diet which was used for the control group, and the treated groups were fed the same base diet with the additional supplementation of *P. cornucopiae* extract at a concentration of 7% (w/w). The daily feeding rate was based on 1% of the average fish body weight and was broadcasted twice daily over a period of 7 weeks. After the feeding trial, *O. mykiss* blood and muscle samples were collected for both ETT gene expression analysis by RT-qPCR and ergothioneine quantification.

5.2.6 Ergothioneine analysis

Ergothioneine from blood samples were quantified according to the method of Nguyen et al.[97]. Briefly, 4 mL of distilled water was added to 1.5 mL of whole blood and placed in a water bath at 90 °C for 12 min while agitating every 3 min intervals. Samples were then cooled on ice for 3 min and 1 mL of an aqueous solution of methimazole (200 µg/mL) was subsequently added as an IS. The samples were centrifuged at 3000 x *g* for 15 min at 4 °C. The resulting supernatant was collected to which 12 mL absolute ethanol was subsequently added. The solution was then agitated and kept on ice for 30 min, and centrifuged at 3000 x *g* for 15 min at 4 °C. The resulting supernatant was collected and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water.

Flesh sample was extracted using the Bligh and Dyer method[61], with slight modifications. Instead of the addition of water, 0.88 % of KCl was added, and 1 mL of an aqueous solution of methimazole (200 µg/mL) was added as an internal standard prior to the homogenization. The water-methanolic layer was collected and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was dissolved in 2 mL distilled water.

A 20-µL portion of the sample solution was subjected to analysis. Quantitative analysis was carried out by HPLC analysis following the method of Nguyen et al.[97]. Briefly, ergothioneine from the sample extract was chromatographically separated using two reversed-phase C_{18} columns (ACE C18-AR 3 µm, 150 × 4.6 mm i.d.) set in a straight series, eluted with 10% methanol in deionized water with 0.1% acetic acid at a flow rate of 0.20 mL/min and monitored at 254 nm using a photodiode array detector (SPD-M10A Shimadzu, Kyoto, Japan). Separated ergothioneine was reacted with 0.23 M 2,2'-dipyridyl disulphide in 0.25 M HCl at a flow rate of 0.15 mL/min in a post-column reaction coil, wherein the resulting reaction product 2-thiopyridone was monitored at 343 nm (SPD-M10A). Varying concentrations of an *L*-(+)-ergothioneine standard were used to obtain a calibration curve. All data for this analysis are expressed as µg of ergothioneine per g or mL of sample.

5.2.7 Analysis of ETT gene expression in *O. mykiss* blood and muscle

Upon harvest, blood and muscle samples from fish were immediately collected, fixed in RNA*later*™ Stabilization Solution (ThermoFisher Scientific), and transported to the laboratory under chilled conditions for further processing. Upon arrival at the laboratory, total RNA from tissue samples were extracted using Sepasol-RNA I Super G (Nacalai Tesque) following the manufacturer's protocols. Extracted RNA was subsequently used for cDNA synthesis using an Applied Biosystems™ High-capacity cDNA Reverse Transcription Kit with RNase inhibitor

(ThermoFisher Scientific) following prescribed manufacturer's protocols. The resulting cDNA was used to determine relative gene expression levels of *O. mykiss* ETT normalized against the expression of the 18S rRNA housekeeping gene of *O. mykiss*. Primer sets used for the expression analysis are presented in Table 5.1. The resulting amplicon for the ETT primers targeted a 157 bp-long sequence while the 18S primer set targeted a 152 bp-long sequence. The ETT primers were designed based on the gene sequence identified to be the most probable ETT homolog in *O. mykiss* based on bioinformatics analyses. In addition, the target amplicon for these ETT primers spans transmembrane domains 10 and 11. Quantification of relative expression levels were carried out in a SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) reaction mixture on an Applied Biosystems 7300 Real-Time PCR System (ThermoFisher Scientific) following the standard program for qPCR reactions. Calculations for changes in expression levels were carried out using the ΔΔCt method.

Table 5.1. Primer sequences used for Real-time PCR analysis.

	Primer Sequence
Target gene (*O. mykiss* ETT) forward	5' - ATACCACCAAATCTGAGCTCTGTG - 3'
Target gene (*O. mykiss* ETT) reverse	5' - CTCTGGAGGCCATGGAACAG - 3'
Housekeeping gene (18s) forward	5'- GTGATAGGGATTGGGGATTGC - 3'
Housekeeping gene (18s) reverse	5' - CGATCCGAGGACCTCACTAAAC - 3'

5.3 Results

5.3.1 Sequence mining and phylogeny

Using three previously characterized SLC22 homologs from *D. rerio* [121] as individual amino acid query sequences for a pBLAST search, top homolog hits were obtained for *O. mykiss*, *O. kisutch*, and *S. salar* and are presented in Table 5.2. Sequence identity for these homologs ranged from 62-68 %, indicating a relatively high degree of sequence homology for SLC22 genes among piscine species.

Table 5.2. Accession numbers and percent identity with *D. rerio* of SLC22 homologs from salmonid species.

Accession number	Function	Nearest BLAST hit homolog accession numbers	Percent identity with *D. rerio*
Danio rerio SLC22 homologs[121]		***Oncorhynchus mykiss***	
NP_957143.1	Unknown	XP_021473182.1	63
CDM63451.1	Ergothioneine transporter	XP_021473178.1	67
XM_005170337	Carnitine transporter	XP_021473180.1	62
Danio rerio SLC22 homologs[121]		***Oncorhynchus kisutch***	
NP_957143.1	Unknown	XP_020320431.1	62
CDM63451.1	Ergothioneine transporter	XP_020320681.1	65
XM_005170337	Carnitine transporter	XP_020320007.1	63
Danio rerio SLC22 homologs[121]		***Salmo salar***	
NP_957143.1	Unknown	XP_013992315.1	68
CDM63451.1	Ergothioneine transporter	XP_014053491.1	66
XM_005170337	Carnitine transporter	XP_014053489.1	63

Phylogenetic analysis of *D. rerio* and salmonid sequences are shown in Figure 5.1, wherein the selected homologs formed three distinct clades—the ergothioneine transporters (ETT), carnitine transporters (CTT), and a clade of homologs with unknown function. Each of the three SLC22 homologs from *D. rerio* fell under separate clades, indicating a notable level of sequence divergence based on the known functions of each of these homologs. Individual SLC22 homologs from each salmonid species were also differentiated among the three clades thus suggesting that similar to *D. rerio,* individual salmonid SLC22 homologs may have distinct transporter functions specific to particular substrates such as ergothioneine and carnitine. The high bootstrap values for each of the distinct clades (100%) further indicates that the divergence observed among the SLC22 homologs and the distinct functions associated with this is strongly supported. Based on this phylogenetic tree, it appears that the salmonid homologs with accession numbers XP_021473178.1 (*O. mykiss*), XP_020320681.1 (*O. kisutch*), and XP_014053491.1 (*S. salar*) are

the most probable candidates as specific ETTs given that they fall under the same clade as the functionally characterized ETT from *D. rerio*.

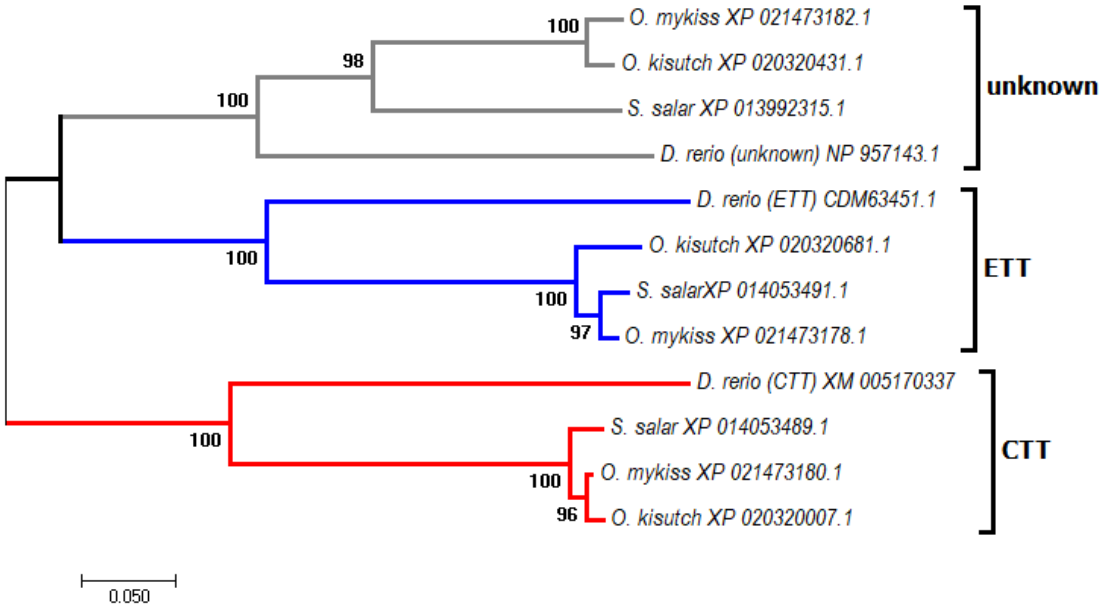

Figure 5.1. The evolutionary history of piscine SLC22 homologs was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.81394392 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 526 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Accession numbers of the sequences used for the analysis appear beside the name of each taxa.

To further confirm the phylogenetic placement of the various salmonid SLC22 homologs, especially those identified as putative ETTs, additional taxa representing known ETTs from non-piscine species (mammalian and avian) were included in another round of phylogenetic analysis. The resulting phylogenetic tree reveals that despite non-piscine ETTs forming a notably distinct sub-clade, this sub-clade still falls within a larger clade in which the *D. rerio* and putative salmonid ETTs are grouped, as shown in Figure 5.2. Again, the high bootstrap value for this clade (99 %) strongly suggests that the salmonid homologs with accession numbers XP_021473178.1 (*O.*

mykiss), XP_020320681.1 (*O. kisutch*), and XP_014053491.1 (*S. salar*) in all probability function as ETTs as well.

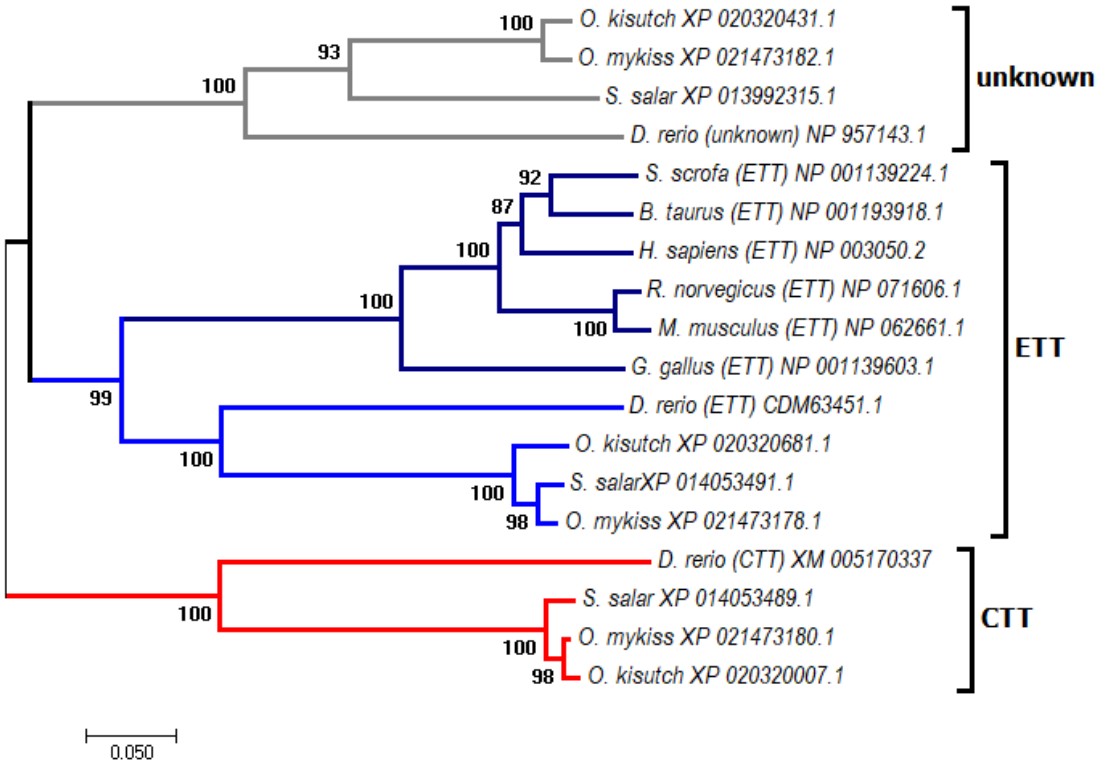

Figure 5.2. The evolutionary history of piscine and non-piscine SLC22 homologs was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.41633553 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 18 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 524 positions in the final dataset. Accession numbers of the sequences used for the analysis appear beside the name of each taxa.

5.3.2 Functional differentiation based on MSA

Given that of the three SLC22 homologs identified in *D. rerio*, only two have been assigned as specific carnitine or ergothioneine transporters, succeeding analyses of the SLC22 homologs from salmonids focused on those putatively identified as ETTs and CTTs. Multiple sequence alignment of putative salmonid CTTs and ETTs along with *D. rerio* ETT shows a relatively high

degree of sequence similarity between ETTs and CTTs based on numerous conserved domains throughout the amino acid sequences (Appendix 5.1).

Being known as a membrane-bound transporter protein, prediction of putative TMDs for *D. rerio* ETT was also carried out, revealing a total of 12 predicted TMDs. This observation was similar to the structure of previously characterized human ETT (Bacher et al.). The identified TMDs in *D. rerio* ETT were subsequently used to identify the corresponding TMDs in the putative salmonid ETTs and CTTs (Appendix 5.1). Further analysis of the individual TMDs revealed specific amino acid residues that were distinct for either CTTs or ETTs, thus highlighting sequence information that could help in differentiating these two types of SLC22 homologs. Transmembrane domains 5, 7, 8, 9, 10, and 12 have been previously identified as having important roles in differentiating substrate specificity between CTTs and ETTs [124]. Analysis of these TMDs in piscine CTT and ETT sequences (*D. rerio* and salmonids) points to specific amino acid residues that could contribute to the functional dichotomy of these two transporters (Figure 5.3).

For instance, among the most notable differences in amino acid residues in TMD 5 would be the amino acid at the 10th position wherein the hydrophilic side chain of phenylalanine (F) in ETT is replaced with a positively charged histidine (H) in CTT. Within the same TMD, the last residue (24th) in ETTs is characterized by a bulky phenylalanine (F) sidechain, which is subsequently replaced with a smaller alanine (A) sidechain in CTTs. In TMD 8, the non-polar sidechains of alanine (A) and tryptophan (W) at positions 19 and 20 in ETTs are replaced by serine (S) and threonine (T) in CTTs, respectively. Aside from the change in residue polarity at these positions, tryptophan (W) at position 20 in ETTs is also a structurally larger sidechain compared to the threonine (T) residue observed in the same position for CTTs. A similar occurrence is also observed in TMD 9 wherein threonine (T) at position 5 in ETTs is replaced with phenylalanine

(F) in CTTs. Other substitutions observed within the other identified TMDs also show distinct differences in terms of either side chain polarity, charge, or molecular bulk.

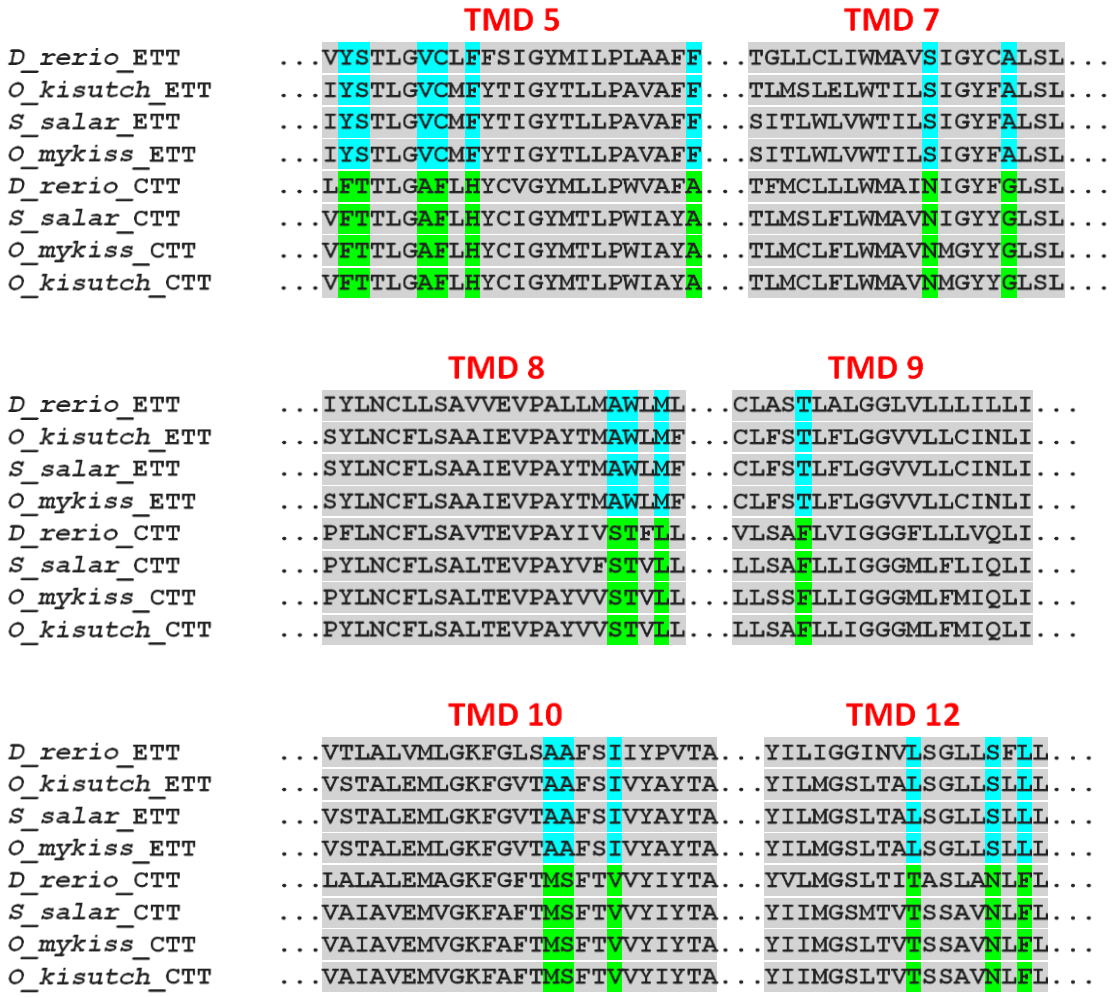

Figure 5.3. Multiple sequence alignment of selected putative TMDs from *D. rerio* and salmonids. Amino acid residues highlighted in green or blue connote unique residues between ETTs (blue) and CTTs (green).

To compare the features of the predicted TMDs identified in piscine ETT sequences, the topology of *D. rerio* ETT and the putative *O. mykiss* ETT (as representative of salmonid sequences) was visualized using the online platform Protter. Results from this analysis show nearly identical structural topology for the functionally characterized *D. rerio* ETT and the best candidate for *O. mykiss* ETT as compared to *O. mykiss* CTT with the most notable difference seen in the proximities of TMDs 6 and 7 (Figure 5.4). These results thus further highlight the probability that

the candidate salmonid ETTs identified through bioinformatics function similarly to typical ETTs with specificity for ergothioneine as substrate.

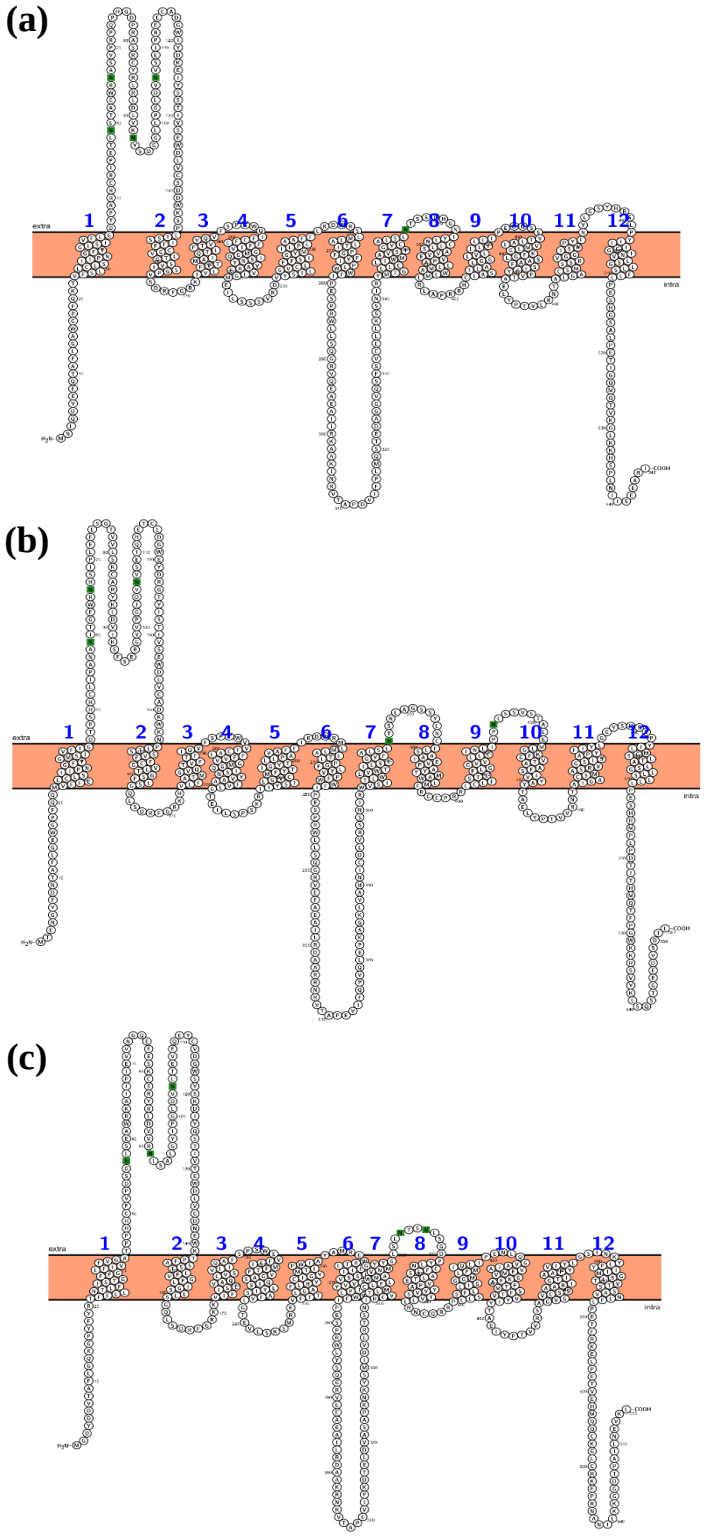

Figure 5.4. Predicted topology of (a) *D. rerio* (characterized) ETT, (b) *O. mykiss* (putative) ETT, and (c) *O. mykiss* (putative) CTT.

5.3.3 ETT gene expression and ergothioneine content of ME-fed *O. mykiss*

To determine the effects of ME feed supplementation on the relative expression levels of ETT gene expression in *O. mykiss*, RT-qPCR was employed, concomitant with quantification of ergothioneine accumulation in fish blood and muscles. Ergothioneine content in the blood and muscle tissues of the control and ME supplemented fish are shown in Figure 5.5. Similar to the results of the feeding experiments in the previous chapters (Chapters 4 and 5), blood ergothioneine concentrations in *O. mykiss* significantly increased after a feeding period of seven weeks with 7% ME feed supplementation (Figure 5.5(a)). The blood concentrations observed in this feeding trial were also notably higher than those from the previous experiments. Ergothioneine concentration in muscles also revealed a significant increase in ME supplemented fish compared to the control (Figure 5.5(b)).

The expression of the ETT gene in the corresponding tissues were analyzed and the qualitative and quantitative results of ETT gene expression of the control and the ME supplemented groups are presented in Figure 5.6 and Figure 5.7, respectively. An interesting opposite trend was observed, wherein ETT gene expression levels in the blood and muscles of ME supplemented fish were notably downregulated when compared to the control (Figure 5.7(a)). The downregulation of ETT gene expression in blood was much more pronounced with a nearly 86% decrease, than in muscle which exhibited a lower 47% decrease (Figure 5.7(b)). Another notable observation was that the magnitude of decrease in ETT gene expression levels appear to be of the same order as the increase in ergothioneine accumulation in the corresponding tissues.

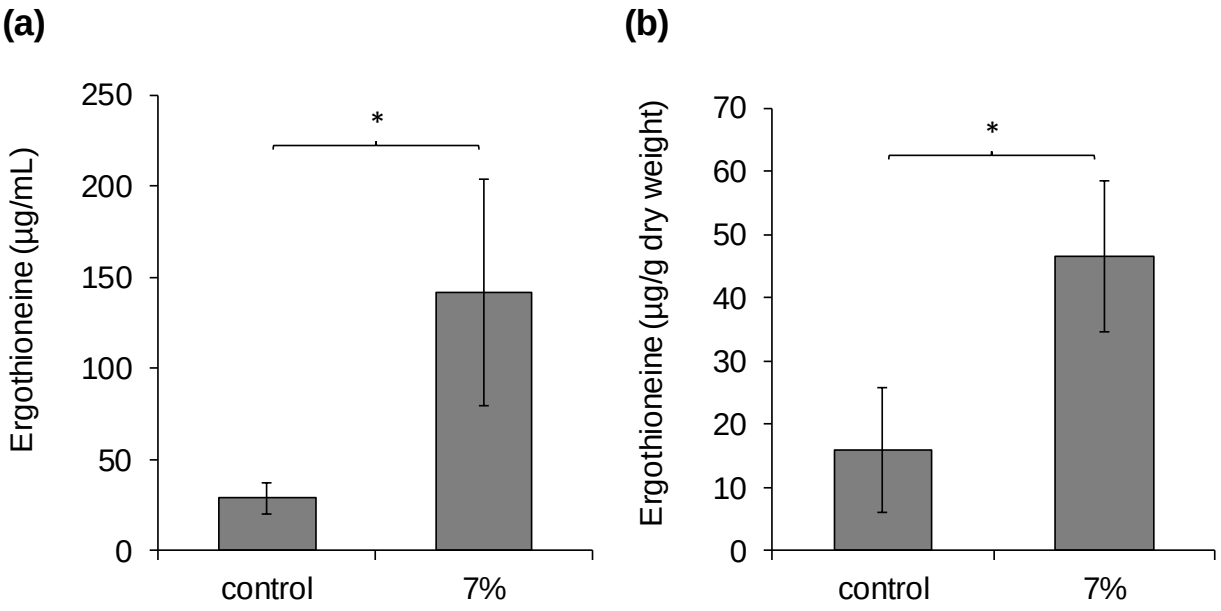

Figure 5.5. Ergothioneine content in blood (a); and meat (b) of *O. mykiss* after 7 weeks of feed supplementation with different concentrations of mushroom extract. Data are presented as mean ± standard deviation (n=3). Significant differences among treatment groups ($p < 0.05$) are indicated by asterisks.

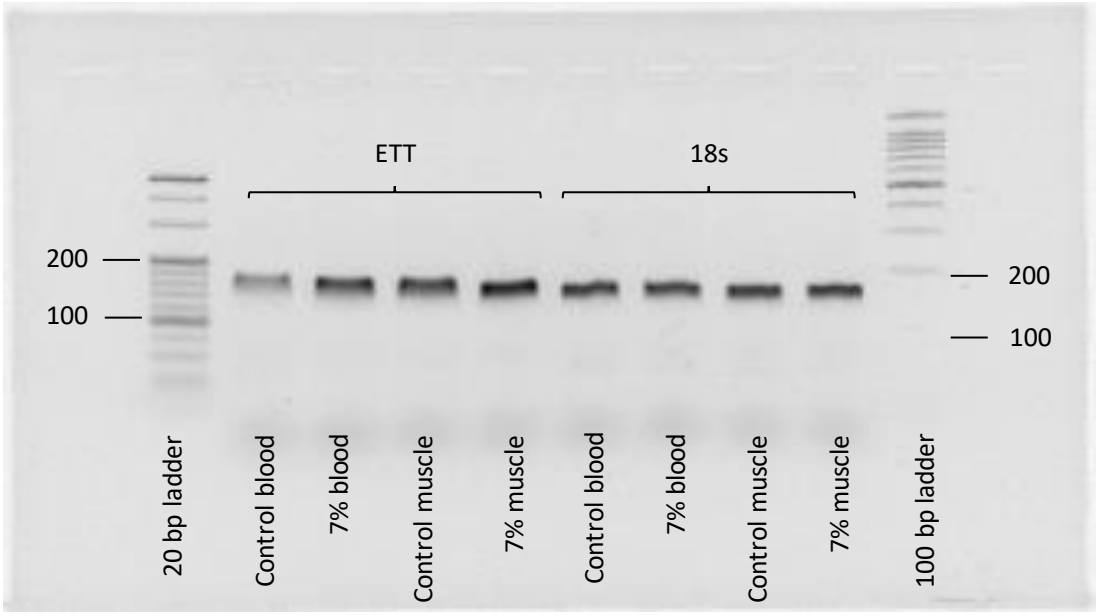

Figure 5.6. Expression of ETT genes in blood and muscle tissues of the non-supplemented (control) and the ME-supplemented *O. mykiss*.

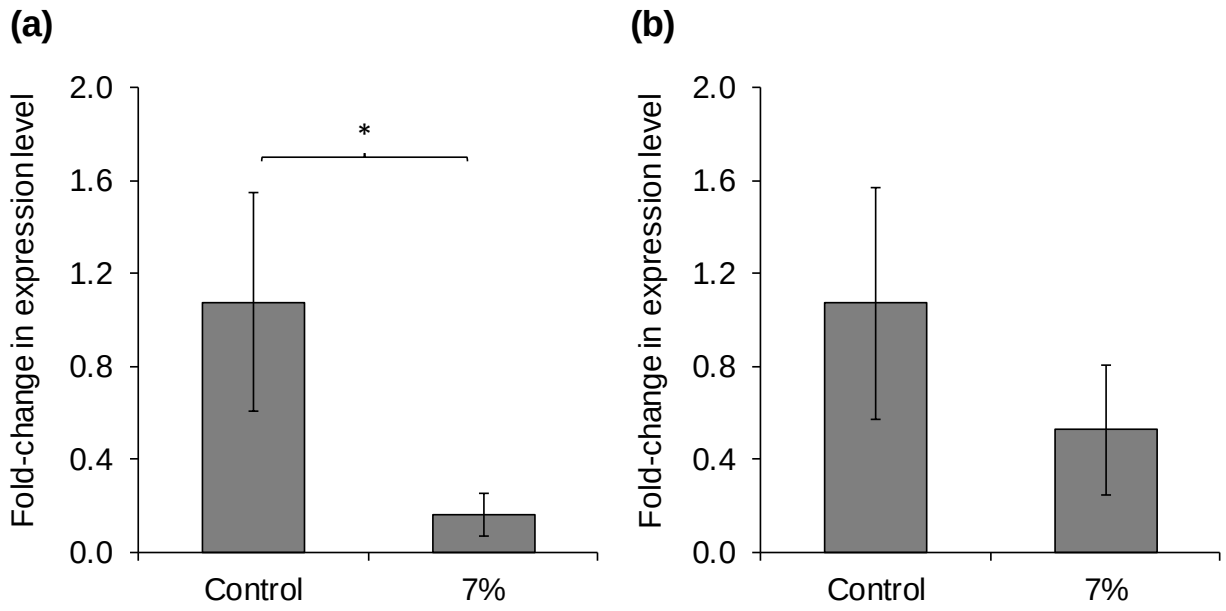

Figure 5.7. Expression levels of ETT gene in blood (a); and muscle (b) tissues after 7 weeks of feed supplementation with different concentrations of mushroom extract. Data are presented as mean ± standard deviation (n=6). Significant differences among treatment groups ($p < 0.05$) are indicated by asterisks.

5.4 Discussion

Bioinformatics has become an indispensably powerful tool in nearly all branches of biological research. Most recent developments have allowed not only the acquisition, but the processing and analysis of larger amounts of genetic information, from the genome of a single species to the vast metagenomes of entire ecological niches. In the context of gene discovery, bioinformatics plays an integral role not only in the initial identification, but also in the subsequent validation of selected genes of interest. Prioritizing genes (and encoded proteins) of interest through computational analysis from a larger family of homologous genes allows researchers to focus limited resources on specific genes that are to be further investigated through more detailed experiments [129]. Developments in bioinformatics techniques and technology have thus been harnessed to produce a variety of logic-based gene prioritization methods [130, 131], a framework of which has been applied to this study.

The SLC22 gene family to which ergothioneine and carnitine transporters belong is one example of a relatively large gene group that shares a considerable amount of homology among its members but also exhibit distinct and specific functions for each unique homolog [44]. This family contains a total of 15 plasma membrane bound proteins of which 13 have already been functionally characterized [44] including ETT (synonymous to OCTN1/SLC22A4) [106] and CTT (synonymous to OCTN2/SLC22A5) [132, 133].

Both ETT and CTT share a notable degree of sequence homology with human ETT having 77% identity and 82% similarity with human CTT. In the case of piscine homologs, both ETT and CTT from *D. rerio* shared nearly indistinguishable sequence similarity with human ETT thus making the initial identification of specific ETT and CTT candidates through bioinformatics rather challenging [121]. Specific ETT and CTT homologs in *D. rerio* were subsequently identified through detailed experimental procedures [121] and the resulting sequence information has thus served as an integral tool in identifying their corresponding homologs in other piscine species such as the salmonids involved in this study.

The putative salmonid ETTs and CTTs identified in this study still exhibited a considerable amount of homology with salmonid ETTs sharing 51% identity and 63% similarity with salmonid CTTs. Despite this, the availability of information for the specific ETT and CTT in another piscine taxa (*D. rerio*) has allowed for the construction of a fairly reliable phylogenetic tree that clearly distinguishes the various salmonid homologs according to their specific functions. The resulting phylogenetic trees showing the placement of piscine SLC22 homologs suggests that the distinct function of ETTs and CTTs are a result of divergent evolution. The grouping of non-piscine ETTs with those from *D. rerio* and salmonids also suggests that the ETTs and CTTs share common ancestry prior to the speciation of fish and land animals. These results are in agreement with wider

phylogenetic studies conducted for larger datasets of SLC22 homologs that postulate that these transporters evolved more than 450 million years ago [134].

Going beyond the classification of genes through phylogeny and the subsequent inference of particular functions, more detailed analyses in the form of multiple sequence alignments allow researchers to determine genes to prioritize for further experiments by pinpointing specific domains associated with specific functions[135]. In the case of ETTs and CTTs, specificity for particular substrates were found to be dictated by certain amino acid residues in particular TMDs[124]. In the current study, alignment of *D. rerio* and putative salmonid ETTs and CTTs allowed for the identification of TMDs in salmonid sequences based on predicted TMDs in the *D. rerio* ETT sequence. This alignment revealed several amino acid residues within the TMDs that were notably distinct between piscine ETTs and CTTs. Furthermore, when only the TMD sequences in salmonids were aligned and compared, it showed relatively lower sequence identities and similarities than entire ETT and CTT sequences.

Numerous studies on enzymes and transporter proteins have shown that even single amino acid substitutions could result in drastic changes in protein function, specificity, and kinetics. Such events have been documented in some SLC22 homologs. For instance, single amino acid substitutions in OCT2 have been shown to change the uptake kinetics of particular drugs [136]. Pfeiffer, Bach, Bauer, Campos da Ponte, Schomig and Grundemann [121], working more specifically with human ETT and CTT, demonstrated that substitution of ETT amino acid residues with corresponding residues from CTT resulted in ETT gaining the ability to transport carnitine. Parallel substitutions of CTT residues with those from ETT on the other hand did not enable ergothioneine transport by CTT. These observations led to the conclusion that the distinct functions of ETT and

CTT are due to differences in substrate binding and turnover movement as effected by differences in amino acid substitutions in their TMDs.

The effect of substrate availability can have a marked effect on the expression of genes encoding transporter proteins such as ETT. In the current study, increased dietary supplementation of ergothioneine through mushroom extracts resulted in the downregulation of the gene identified to encode ETT in rainbow trout. A similar trend was observed in mouse livers when fed with elevated levels of dietary ergothioneine[137]. These results reflect typical patterns observed for transporter proteins wherein transporters with a high-affinity for their substrates increase in expression when their substrates are scarce and vice-versa[138, 139]. Ergothioneine has been identified as the specific substrate for ETT and kinetic studies have shown the considerably high affinity of this transporter for ergothioneine[45]. Further *in vivo* evidence for the high affinity of ETT for ergothioneine can be partially gleaned from the results of the current experiment in that, despite the decrease of ETT gene expression in the blood and muscles of *O. mykiss*, increased accumulation of ergothioneine was still observed in these tissues. This observation is further supported by previous reports that demonstrated increasing ergothioneine uptake rates via ETT with increasing substrate concentrations[42]. The current results therefore suggest that ETT gene expression in salmonids is, to an extent, affected by substrate availability.

The current results demonstrating the expression of ETT in muscle tissues of *O. mykiss* as well as the detection of appreciable amounts of ergothioneine now provide stronger evidence of the feasibility of dietary supplementation of ergothioneine-rich mushroom extracts in maintaining salmonid flesh quality after harvest. With both biochemical and molecular tools at hand, improved feeding strategies should therefore be further investigated to determine optimum dietary inclusion rates taking into consideration the ability of salmonids to take up ergothioneine as well as the

effects of supplementation levels on the regulation of the transporter responsible for its uptake, ETT.

5.5 Summary

Based on the various bioinformatics analyses conducted for salmonid SLC22 homologs, a clearer picture of the most appropriate candidates for salmonid ETTs was thus obtained. The salmonid homologs with accession numbers XP_021473178.1 (*O. mykiss*), XP_020320681.1 (*O. kisutch*), and XP_014053491.1 (*S. salar*) have been identified as specific ETTs based on evolutionary phylogeny, sequence analysis, and topology comparison. Expression analysis of the gene encoding for ETT in *O. mykiss* further revealed that this transporter is expressed in muscle tissues, thus supporting previous studies that have postulated the potential of ergothioneine dietary supplementation in maintaining salmonid flesh quality after harvest.

CHAPTER 6

General Conclusion

Salmonid fish species are some of the most widely consumed fishery commodities in the world. The prominent orange-red meat pigmentation of salmonids is one of their most distinct characteristics, which influences the overall quality and market value of these fish as well. Given the economic importance of salmonids to the fisheries industry, research focusing on both the culture and post-harvest strategies for this commodity will have resounding effects in terms of improving its overall value.

This study was contextualized as a practical approach of utilizing natural bioactive products, such as ergothioneine-rich extracts from edible mushroom, for the stabilization of astaxanthin in aquatic products. The direct use of crude mushroom extracts is a cost-effective strategy that was considered since further purification of ergothioneine from crude mushroom extracts could entail additional processing costs which could diminish the practicality of this strategy.

The findings of the *in vitro* elucidation of the antioxidative properties of mushroom extract and its effects against lipid oxidation in a model liposome system validated the hypothesis that mushroom extracts can effectively control the progress of lipid oxidation in liposomes under oxidation-induced conditions. The antioxidative activity of mushroom extracts was able to protect astaxanthin from degradation as well. In addition, the presence of ergothioneine or mushroom extracts together with astaxanthin in liposome preparations exhibited additive synergistic antioxidative functions that could neutralize reactive radical species that initiate and promote lipid oxidation. Interestingly, the crude mushroom extracts were shown to have significantly higher antioxidative activities than pure authentic ergothioneine standards, suggesting the probable

presence of other bioactive compounds in crude mushroom extract with potent antioxidative properties. With these results as a basis, future studies on the elucidation of the other bioactive components and properties present in mushroom extracts could provide a comprehensive understanding of the antioxidative and protective functions of these extracts. Moreover, an in depth focus on the identification and characterization of the various other components in mushroom extracts could eventually lead to the discovery of other bioactive properties that go beyond antioxidative activity.

The *in vitro* addition of mushroom extracts to salmonid meat provided the preliminary evidence suggesting the viability of using mushroom extracts to preserve salmonid meat against oxidation during post-harvest storage. Moreover, the positive results of the feeding trial on *Oncorhynchus mykiss* in terms of feed acceptability, growth, and successful uptake of ergothioneine from the diet gave further credence to the feasibility of applying the protective functions of mushroom extracts in salmonid fish culture. Similar results obtained from the *Oncorhynchus kisutch* feeding trial further confirmed the dietary supplementation of mushroom extracts as a viable strategy. These experiments can, therefore, support the prospect of applying this strategy to a wider breadth of cultured salmonid species.

The positive results of mushroom extract dietary supplementation in controlling lipid oxidation and astaxanthin degradation in salmonid meat during post-harvest low temperature storage further validated the feasibility of applying this approach to utilize the antioxidative protective functions of crude mushroom extracts during post-harvest. In light of these, future studies on optimal mushroom extract concentrations as dietary supplements, feed delivery systems, and feeding durations should be done in order to translate these preliminary results into improved supplementation strategies and reduced costs during grow-out culture.

Ergothioneine is a potent antioxidant that is known to be commonly biosynthesized by certain fungal species which is taken up and accumulated in animals through their diet. Even with the discovery and characterization of ergothioneine-specific transporters that are ubiquitously expressed in several animal cells, the main and principal function of ergothioneine is still the topic of several debates. Aside from the effects of mushroom extract dietary supplementation on the control of lipid oxidation and astaxanthin degradation in fish meat during post-harvest storage, future studies on the possibility of other protective functions in fish physiology, as well as the other protective effects during post-harvest may be contextualized to further investigate the main physiological function of ergothioneine, as well as its possible synergistic relationship with other components in mushroom extracts.

The results of the data mining and bioinformatics analyses on the specific gene (SLC22A4/OCTN1/ETT) responsible for the absorption, transport, and accumulation of ergothioneine in salmonid species serves as a foundation for future studies on expression profiles of this gene in salmonid species given the lack of any published data regarding this aspect. The current results demonstrated that ETT is expressed in *O. mykiss* blood and muscle tissues with expression levels affected by substrate availability. These findings provide stronger molecular evidence of the feasibility of dietary supplementation of ergothioneine-rich mushroom extracts in maintaining salmonid flesh quality against oxidative damage and degradation. Moreover, functional characterization of this gene through heterologous expression will be a significant part of any future study in order to confirm the actual functions of these putative genes.

Overall, the utilization of natural bioactive products such as ergothioneine-rich extracts of edible mushroom species in the stabilization of astaxanthin through the control of the progress of

lipid oxidation in *O. mykiss* and *O. kisutch* meats has been demonstrated to be an effective post-harvest strategy in preserving the nutritional quality and market value of these commodities.

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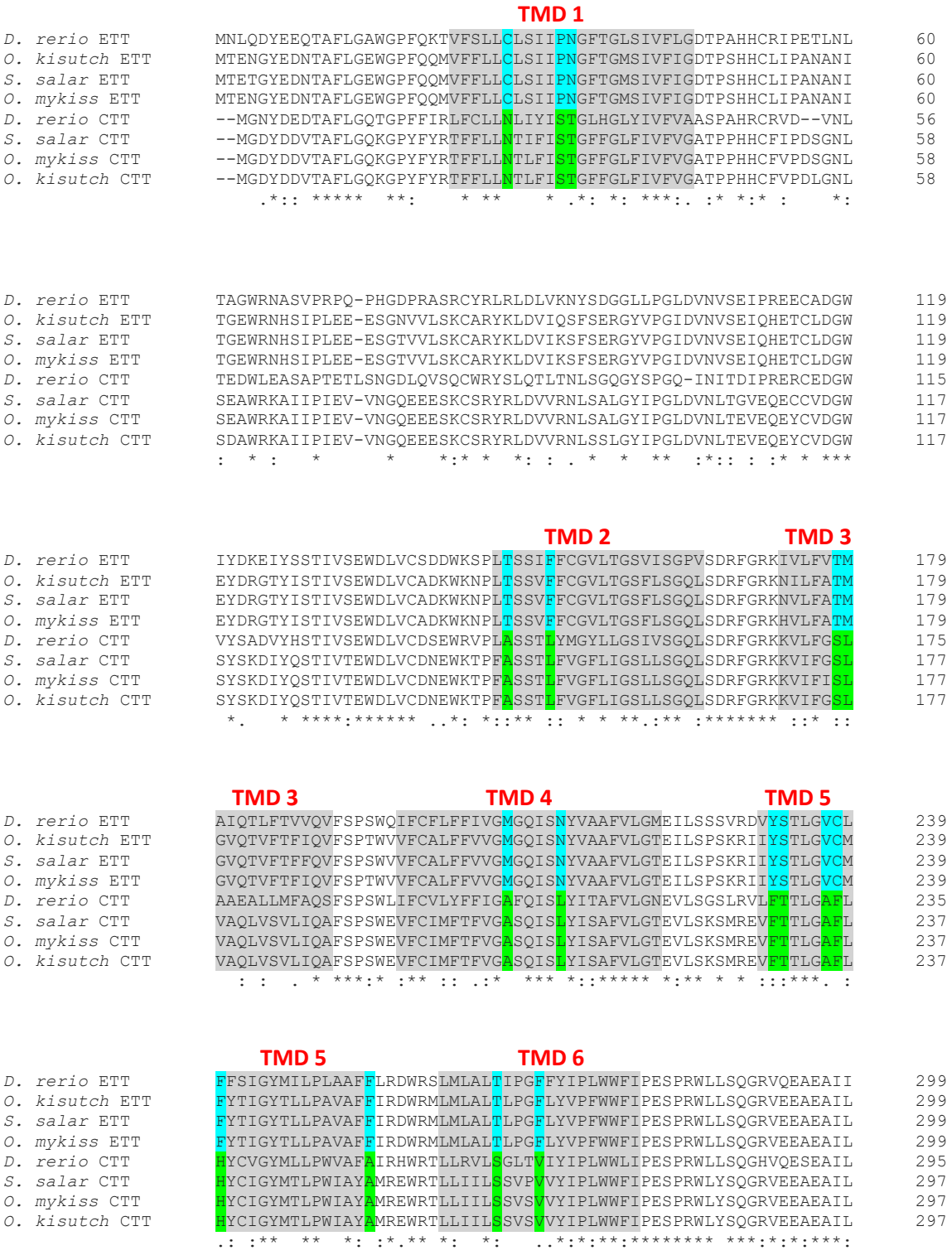
Appendices

Appendix 5.1 Multiple sequence alignment of *D. rerio* ETT and CTT with putative ETTs and CTTs from salmonids.



Appendix 5.1 (*continuation*). Multiple sequence alignment of *D. rerio* ETT and CTT with putative ETTs and CTTs from salmonids