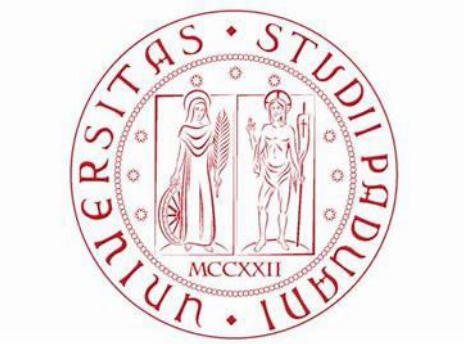

UNIVERSITÀ DEGLI STUDI DI PADOVA
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Second Cycle Degree (MSc)
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From Waste to Natural Preservation: An Experimental Study on Using Olive Oil Mill Wastewater Phenolic Compounds for Surimi Safety and Shelf-life Extension

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Ultimately, to all those who were the sunshine, bringing light into the darkest days...

This achievement is as much yours as it is mine.

Abstract

This study investigates the application of phenolic compounds extracted from Olive Mill Wastewater (OMW) as a natural preservative to extend the shelf life and enhance the quality of Red Mullet (*Mullus barbatus*) surimi. Given fish vulnerability and the dual antioxidant-antimicrobial properties of polyphenols, this research evaluates valorizing these compounds to mitigate microbial spoilage and lipid oxidation. Beyond improving safety, quality, and organoleptic characterization, this research contributes to environmental protection through waste valorization and the circular economy. The phenolic extract, derived via enzymatic hydrolysis and a multi-stage membrane system, was spray-dried and incorporated into surimi during mincing. The experimental design compared two processing protocols: washing-before-freezing versus washing-after-thawing, to evaluate their impact on stability. Samples were stuffed into collagen casings, steam-cooked, and stored under modified-atmosphere packaging (80% N_2 /20% CO_2) at 4 ± 1°C up to 60 days, with analyses at 0, 15, 30, 45, and 60 days. To determine quality and microbial growth (TVC, LAB, *Staphylococcus aureus*, yeasts, and molds) and physicochemical properties (pH, a_w, TVB-N, acidity, moisture, protein, and salt) were monitored. In conclusion, the wash-before-freezing protocol offers a superior preservation strategy by stabilizing the protein matrix and optimizing water retention over time. While the multi-stage hurdle system (washing, cooking, and MAP packaging) heavily suppressed bacterial growth across all samples, the incorporated OMW polyphenols acted as a quality upgrade by reducing free fatty acid accumulation and fishy odors. Ultimately, this work establishes a non-destructive, sustainable strategy that successfully meets the industrial demands for clean-label seafood preservation through agri-food waste valorization.

Chapter 1

1 Introduction

1.1 Seafood and the Global Nutrition Crisis

One of the critical emerging crises the world is facing is the challenge of ensuring adequate food supply and malnutrition. Due to the growing population, the need for safe and nutritious food has been increasing, especially for children. Based on recent studies, 372 million preschool-aged children are suffering from some form of malnutrition and deficiencies. A lack of nutrients undermines a person's ability and impairs growth and brain development while its disadvantages extend beyond these points. (FAO,2022) Therefore, overcoming this life-threatening challenge requires either increasing the production of safe food or, more sustainably, enhancing public awareness of the valuable food resources that already exist. On the other hand, by minimizing food waste and re-using nutritional compounds, not only contribute to tackle feeding challenges but also simultaneously address environmental concerns.

One of the most nutritious food sources that must be considered in resolving this issue is seafood. Fisheries and aquaculture, which have grown significantly in recent years, can play a key role in this regard. Global fish production, including both wild capture and aquaculture production, has reached to about 223.2 million tonnes in 2022. (FAO,2024)

1.2 Global Fish Consumption Trends: Europe and Italy

The global rise in Fisheries and aquaculture indicates the rising demand and consumption of food fish. As the FAO reported, since 1961, per capita fish consumption has increased

two and a half times (rising from 9.1 kg in 1961 to 20.7 kg in 2022), indicating that fish is being consumed more and has become one of the most popular animal protein choices. In terms of regional distribution, Asia with 70 percent, holds the first position in aquatic animal production while Europe, with 9 percent, is ranked in the second tier. Aquatic foods are rich in omega-3 fatty acids, minerals, and vitamins and they provide 15 percent of the global supply of animal protein and 6 percent of total protein worldwide.

Italy ranks among the top six EU countries in fish consumption (31.2 kg per year) and has the lowest proportion of overweight adults (46%) as well as a relatively low cardiovascular mortality rate (34%).Taken together, these indicators may be associated with higher fish consumption, along with other contributing factors. (Krešić et al., 2022)

Along with the high nutritional value of fish, the upward trend in public interest and increasing production support the idea that aquatic foods can help address malnutrition and food security challenges.

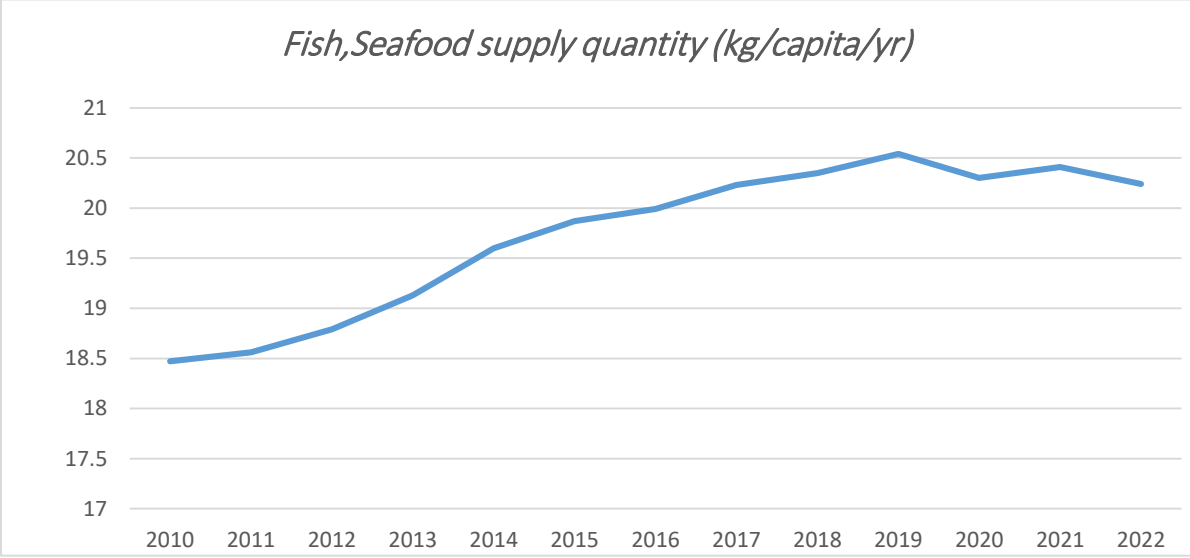

Figure 1.1 Per capita fish and seafood supply worldwide from 2010 to 2022 (kg per capita per year), based on FAOSTAT data

According to FAOSTAT data (2010–2022), the per capita food supply of fish increased from 18.4 kg to around 20.6 kg in 2019, after which it remained relatively stable with a slight decline Nevertheless, the overall global trend still indicates growth over the past decade.

1.3 Seafood as a Functional Food: Linking Consumption to Health Outcomes

As a globally accessible and nutrient-dense food source, fish and seafood play a critical role in sustaining health, preventing malnutrition (FAO,2025), and reducing the risk of chronic diseases. Fish and seafood are recognized as valuable sources of protein, fatty acids, and trace minerals, as well as vitamins such as A, D, and E .Beyond being a source of animal protein and beneficial fats, fish offers important micronutrients such as iodine, selenium and calcium, and, when consumed in its entirety, can also provide iron, zinc. (FAO,2025)

Previous experimental studies have indicated that the nutritional constituents of fish, particularly n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), may offer protective effects against cardiovascular disease (CVD) and cancers, and are essential for brain development and function. (Von Schacky, 2021)

Omega-3 fatty acids are indispensable nutrients that contribute to the health of multiple body systems, including the nervous, visual, immune, cardiovascular, integumentary, and connective tissues. Among these, DHA and EPA hold particular significance for brain and retinal development. DHA is more directly involved in maintaining optimal brain and eye function, whereas EPA is more closely associated with cardiovascular health, modulation of inflammation, and immune system activity. A lack of sufficient DHA and EPA can raise the likelihood of health problems, especially those affecting cognition and vision. (Von Schacky, 2021)

These compounds have also been shown to influence blood pressure (Del Brutto et al., 2016), lipid metabolism, and glucose regulation. (Rajaram et al., 2009) The combination of these bioactive compounds makes fish and seafood important dietary components that should be included in a healthy diet. At the same time, further scientific research is needed to fully understand and maximise their potential health benefits.

1.4 Promoting Sustainability in the Seafood Sector: From Underutilized Species to Circular Economy Models

1.4.1 Circular Economy

As the population and food demand grow, the importance of the circular economy and bioeconomy in supplying sustainable products also rises. One aspect of these strategies is meeting food demand while maintaining resources and minimizing negative environmental impacts, for instance by reducing food waste. (Pomoni et al., 2024) The concept of the circular economy was first defined by Pearce and Turner in 1990, who argued that since the Earth functions as a circular system of natural cycles, it demonstrates that the linear economy fails to meet demands and that the economy must therefore follow a circular approach. (Rizos et al., 2017)

The circular economy is a closed system, which, according to Van Berkum and Dengerink (2019), is portrayed by the 3Rs “Reuse–Recycle–Reduce,” rather than the “Take–Produce–Consume–Discard” model of the linear economy. (Van Berkum & Dengerink, 2019) This closed loop, which has an impact on the environment, society, and economy, aims to reduce waste by suggesting that the output of one system can be utilized as the input of a subsequent system. As proposed by Jurgilevich et al. (2016), who highlighted the application of the circular economy in the food system, the circular economy is described by the 4R theory “Reuse, Repair, Refurbishing, Recycling” in order to convert waste into a resource. (Jurgilevich et al., 2016; Pomoni et al., 2024)

1.4.2 Underutilized Species

As fishery demand is gradually increasing, the overfishing of certain well-known species has become a significant concern. Overfishing occurs when the balance between fish capture and natural reproduction is disrupted, leading to environmental and climate-related impacts. Therefore, this thesis focuses on neglected species, which are often overlooked and underutilised, as an approach to addressing this challenge.

The term “Underutilized Species” refers to species that are nutritionally valuable but are not widely consumed or have not received sufficient attention despite their nutritional and mineral content. Although these species are often used as bait or fertilizer, they hold

considerable potential in the human diet and environment, as well as in addressing the previously mentioned malnutrition concerns. (Masi et al., 2025)

These species are able to provide a diversified diet with high and rich nutrient content, while also serving as a sustainable solution to the current challenge of food security and the increasing food demand due to population growth. The benefits are not limited to these aspects; further advantages such as maintaining the fishery industry, economic benefits, and reducing the overexploitation of well-known products may also be identified. Hence, this strategy must be undertaken, as neglected species could be viewed as a promising future in the food system. (Hunter et al., 2019; Talucder et al., 2024)

Red mullet, which is used in our experiments, is often considered neglected species. Red mullet is rarely consumed in some countries and has not yet fully established its place in the human diet. In conclusion, this species is examined as it aligns with the aim of this study.

1.4.3 Case Study: Red Mullet (*Mullus barbatus*)

Red mullet (*Mullus barbatus*), belonging to the family Mullidae, is a demersal schooling fish that inhabits the Mediterranean Sea and the Black Sea. As shown in Figure 1.2, Red mullet exhibits external morphological features such as a red-coloured body with a silvery belly, yellow fins, and in some individuals two long barbels, sometimes referred to as antennas. This species has an elongated body, typically 10–20 cm in length, and can grow up to 30 cm, especially in females. Additionally, its average weight is approximately 0.5 kg. (Zonn et al., 2021) A notable point regarding this species is its high nutritional value, including a rich content of unsaturated fatty acids and a considerable amount of minerals. Mineral concentrations, such as Fe, Na, Mg, K, Ca, Ag, Sr, Li, and Zn, may differ depending on fish size and habitat. However, according to the findings of Nita et al. (2025), no statistically significant differences were observed in the fatty acid composition (SFA, MUFA, and PUFA) among different habitats. The average protein content was approximately 12 g per 100 g (ranging from 10.8 to 14.3 g), which is considered high, while the lipid content ranged from 13.2 to 16.8 g per 100 g. (Nita et al., 2025; Zonn et al., 2021)

Given these nutritional characteristics, the following case study focuses on the use of this fish for surimi production and on the evaluation of polyphenol supplementation as a strategy to enhance the product's added value and antioxidant properties. This approach aims to improve product quality while promoting the principles of the circular economy through the valorization of underutilized biological resources.

Figure 1.2 Red mullet (*Mullus barbatus*). Image by the author

1.5 Olive Oil: Types and Key Characteristics

The olive tree *Olea europaea* L. (Oleaceae family), native to the Mediterranean region, is widely cultivated for oil production in several countries, including Italy, Spain, and Greece. Differences in polyphenol content, quality, and nutritional attributes are mainly explained by geographic origin and subtypes.

Olive oil has several subtypes, including extra virgin olive oil (EVOO), virgin olive oil (VOO), refined olive oil, and pomace oil, which are classified according to their extraction, chemical, and thermal processing methods. EVOO contains a high polyphenol content and a low acidity level, together with lignans, which makes it the most beneficial olive oil subtype, with notable disease prevention potential. Refined olive oil is produced through chemical processing, which leads to a reduction in polyphenol content and consequently limited health effects. Pomace oil, which ranks lowest in polyphenol content among olive oil subtypes, is obtained by extracting the remaining oil from olive skins, seeds, and pulp. (Foscolou et al., 2018)

1.5.1 Importance of olive oil in human nutrition and Mediterranean diet

The Mediterranean diet is widely recognized as a combination of healthy food consumption and a healthy lifestyle. Two main components of the Mediterranean diet are fish and olive oil, as they provide valuable nutrients and health-promoting properties. Olive oil, the primary fat source in the Mediterranean diet, contains approximately 14.5% saturated fatty acids and about 85% unsaturated fatty acids, along with a considerable amount of polyphenols. In terms of fat composition, olive oil is rich in monounsaturated fatty acids and, compared to other vegetable oils, contains less linoleic acid and higher levels of oleic acid, while being free of trans fatty acids. The presence of bioactive compounds in olive oil explains the association between regular olive oil consumption and a reduced risk of certain diseases, such as cardiovascular disease. (Foscolou et al., 2018) Several studies have shown that polyphenols act as natural antioxidants, are resistant to oxidation, and exhibit anticancer effects, which contribute to the health-promoting properties of olive oil. Polyphenols are associated with several health benefits, including the prevention of diabetes mellitus, reduction of blood pressure, anti-allergic effects (Gorzynik-Debicka et al., 2018), and protection of LDL from oxidation. (Foscolou et al., 2018) Other beneficial effects of polyphenols include a reduced risk of breast and colorectal cancer, anti-inflammatory and autoimmune disease modulation, and improved digestive system function. (La Lastra et al., 2001)

1.5.2 Composition and Biological Activity of Olive Oil Phenolic Compounds

Around 98% of the total weight of olive oil consists of a glyceride fraction, with the majority being oleic acid (MUFA), and a minor unsaponifiable fraction (around 2%) composed of biologically active compounds, such as polyphenols, tocopherols, sterols, and carotenoids. Oleic acid has been associated with a lower incidence of cardiovascular disease by reducing LDL and maintaining HDL, compared to PUFA, which highlights olive oil’s superiority over other oils. In addition, olive oil has antioxidant properties, which are attributed to lipophilic compounds (tocopherols and carotenoids) and hydrophilic compounds (polyphenols). (Tripoli et al., 2005)

Phenolic compounds, secondary plant metabolites, are divided into several classes based on their chemical structure, namely phenolic acids (e.g., gallic and vanillic acids),

phenolic alcohols (e.g., tyrosol and hydroxytyrosol), flavonoids, lignans, and secoiridoids (e.g., oleuropein). Among these phenolic compounds, tyrosol, hydroxytyrosol, and secoiridoids (particularly oleuropein) are among the most abundant and are strongly associated with organoleptic and antioxidant properties. Bitterness and pungency are influenced by these compounds and their derivatives.

The biological activity of phenolic compounds depends on their chemical structure, meaning that more complex structures generally exert greater biological effects and health benefits. Of note, the concentration of these bioactive compounds can vary depending on the area of origin, cultivar, and fruit ripening stage.(Servili et al., 2009)

Regarding health-promoting properties, each phenolic compound exhibits different roles. For example, oleuropein, which occurs in olive fruit, is converted into oleocanthal during oil processing. Oleocanthal inhibits COX-1 and COX-2 activity in a dose-dependent manner; these enzymes are linked to inflammation and pain, and this action is similar to that of ibuprofen.(Servili et al., 2009)

As further examples, olive polyphenols prevent oxidation by neutralising free radicals and breaking oxidative chain reactions, thereby increasing shelf life, improving heat stability, and helping maintain nutritional quality. Reactive oxygen species (ROS) lead to oxidative stress, which contributes in the development of several diseases and may increase carcinogenic processes by damaging DNA. Moreover, oxidative stress has been linked to faster ageing and damage to the colonic mucosa, increasing the risk of inflammatory bowel diseases. (Servili et al., 2009)

1.6 Olive Mill Wastewater (OMW): From Waste to Value

As mentioned previously, olive oil is extracted from olive fruits, continuously producing extra virgin olive oil (EVOO) and virgin olive oil (VOO) . Unlike what is commonly believed, the final yields of the olive oil extraction process are not particularly high. Only approximately 20% of the olive fruit is converted into the oil phase, while about 30% remains as a semi-solid cake, and nearly 50% is released as olive mill wastewater (OMW). Consequently, although olive fruits are rich in phenolic compounds, around 98% of these compounds are transferred to OMW and the semi-solid fraction, with only about

2% remaining in the extracted oil, a fact that should be taken into consideration. (Kachouri et al., 2016)

Two modern extraction techniques are currently used: the two-phase and the three-phase processes. In the three-phase process, in contrast to the two-phase system, hot water is added during extraction, resulting in the generation of a considerable volume of OMW. The amount of OMW produced exceeds that of olive oil by more than twofold, with a typical oil-to-OMW ratio of approximately 1:2.5. For instance, in Italy alone, about 1.4 million m^3 of OMW is generated annually. (Cuffaro et al., 2023)

OMW is an aqueous liquor with a reddish-brown color and a pH ranging from 4 to 5, consisting of vegetation water, soft tissues of the olive fruit, and additional water used during processing. (Benaddi et al., 2023) Due to its high content of polyphenols and organic substances, OMW should be regarded as a valuable source of phenolic compounds rather than merely a waste product. The main phenolic compounds present in OMW are hydrophilic, including phenolic acids (e.g., gallic and vanillic acids) and phenolic alcohols (e.g., tyrosol and hydroxytyrosol). (Cuffaro et al., 2023)

Despite being rich in bioactive compounds and polyphenols (up to 10 g/L), OMW is considered an environmental pollutant due to its high toxicity on plants and environment, leading to ecological imbalance. (Cuffaro et al., 2023; Kachouri et al., 2016) As a result, the direct disposal of OMW into soil or water bodies is prohibited in several countries. (Benaddi et al., 2023) Taken together, these considerations indicate that the recovery of phenolic compounds from OMW not only shows strong potential for food-system and nutrition-related applications but also offers a strategy to address a serious environmental challenge.

1.7 The Shift from Synthetic to Natural: Addressing Current Limitations

To address this issue, studies demonstrate the efficacy of both conventional preservation methods and the use of additives to mitigate these spoilage issues. Currently, physical methods such as freezing, chilling, vacuum packaging, and modified atmosphere packaging (MAP) are widely utilized. However, these technologies face significant

limitations, including safety risks such as the growth of anaerobic pathogens (e.g., *Clostridium botulinum*) and economic barriers related to high operational costs. On the other hand, Carocho et al. (2014) argue that synthetic chemical additives have historically been employed to mitigate these risks and to preserve sensory attributes such as texture, flavor, and aroma. Despite their functional efficacy in extending shelf life, research indicates that prolonged exposure to these substances is increasingly associated with long-term health concerns, including allergenicity, gastrointestinal and liver disorders, and carcinogenic potential. This impact has created a critical research gap, highlighting the urgent need to explore safer, natural alternatives.(Carocho et al., 2014)

1.8 Natural Preservatives: Opportunities and Limitations

As Carocho et al. (2014) discussed, natural additives are increasingly preferred as consumer awareness has improved towards food safety, leading them to seek additives of natural origin over their synthetic counterparts. Moreover, natural additives are highly valued for their ability to prevent lipid peroxidation and microbial growth, while providing multifunctional properties in food systems. (Carocho et al., 2014, 2015)

However, compared to synthetic alternatives, natural extracts often require higher concentrations and cause higher production costs, which may influence their application. Beyond that, the inherent instability of these compounds making them highly susceptible to degradation when exposed to heat, light, air, and varying pH levels alongside undesirable organoleptic changes, remain significant limitations. These factors necessitate the development of advanced delivery systems or extraction techniques to ensure the safety and viability of natural preservatives in food matrices. (Kamal et al., 2025) While natural preservatives are promising, their success depends on overcoming challenges like seasonal supply, food matrix reactions, and extraction safety. Specifically, solvent extraction must be highly selective; otherwise, an unselective process will accidentally concentrate raw material contaminants alongside the desired bioactive compounds. (Socas-Rodríguez et al., 2021)

Despite these challenges, polyphenols stand out among natural alternatives due to their dual ability to inhibit both microbial activity and lipid oxidation, the two primary drivers of

fish spoilage. As natural antimicrobials, they are derived from three main sources: microorganisms (such as Nisin, Pediocin, and other Bacteriocins), animals (including Lysozyme from eggs, and Lactoperoxidase or Lactoferrin from milk), and plants (like Polyphenols and Essential Oils). Plant-derived antimicrobials are products of secondary metabolism, which help the plant resist stress and protect it from predators. (Carocho et al., 2015) Polyphenols serve as antimicrobial factors through several pathways. Primarily, the hydroxyl groups in polyphenols interact with bacterial cell membranes, leading to leakage of cell contents and further dissipation of the pH gradient and ATP pool. Another mechanism is that in Gram-positive bacteria, polyphenols interact directly with the peptidoglycan layer, resulting in cellular lysis, while in Gram-negative bacteria, phenolic acids penetrate the outer lipopolysaccharide and generate the same result. Beyond microbial effects, polyphenols inhibit the growth of fungi by damaging the mycelium and membrane permeability. Binding to essential enzymes, such as lipase and amylase, is another way for polyphenols to inhibit metabolic activities. (Kamal et al., 2025)

On the other hand, antioxidants play a key role in preventing the negative effects of oxidation, such as off-flavors, by inhibiting peroxidation in its initiation or propagation stages. According to previous investigations, antioxidants can be categorized into five types: primary antioxidants (radical scavengers), chelators, quenchers, oxygen scavengers, and regenerators.(Kamal et al., 2025) Among natural options, polyphenols show their antioxidant activity through several key mechanisms. Initially, they act as radical scavengers or chain breakers and donate a hydrogen atom or an electron to neutralize free radicals, inhibiting oxidative chain reactions. Beyond that, these compounds can form metal chelates with ions such as ferrous iron, preventing the formation of reactive hydroxyl radicals. Moreover, polyphenols can stimulate the activity of essential antioxidant enzymes, including catalase, peroxidase, and superoxide dismutase which stop the oxidation process.(Andrés et al., 2023)

Additionally, vitamins like ascorbic acid (Vitamin C) and tocopherols (Vitamin E) are used for their antioxidant activity. Other compounds, such as carotenoids, also act as effective additives in various products, ranging from oils and meats to dairy and baked goods. (Carocho et al., 2015)

1.9 Classification and Properties of Polyphenols

More than 8,000 different polyphenols have been identified, reflecting the variety and structural diversity of these compounds. (Kamal et al., 2025) Regarding their classification, several articles categorize them into distinct classes based on different criteria. For instance, Cardona et al. (2013) focus on their structural complexity, such as the number of phenolic rings and substituting groups, and classify them into flavonoids and non-flavonoids. Moreover, Kamal et al. (2025) classify polyphenols based on their source, chemical structure, and functions, dividing them. (Cardona et al., 2013)

Table 1.1. Comparison of different classes of polyphenol

Criterion / Category	Classification / Backbone	Key Features / Characteristics	Reference
Structural Complexity	Flavonoids / Non-flavonoids	Based on the number of phenolic rings and substituting groups	(Cardona et al., 2013)
Source & Structure	Phenolic acids, Flavonoids, Stilbenes	Linkage type and botanical origin	(Kamal et al., 2025)
	Phenolic Acids	found in free form in fruits/vegetables and bound in grains/seeds like Gallic acid, Ferulic acid, Caffeic acid	(Kamal et al., 2025)
	Flavonoids	The largest group, includes Catechins, Quercetin, Anthocyanins, Isoflavones	(Kamal et al., 2025)
	Stilbenes	Resveratrol, found in red wine. has potent anti-carcinogenic properties.	(Kamal et al., 2025)
	Lignans (Dibenzylbutane)	Low molecular weight with phytoestrogen-like activity, examples include Secoisolariciresinol, Matairesinol	(Kamal et al., 2025)

1.10 Natural Phenolic Compounds as Preservatives in Aquatic Products

Phenolic compounds are found in herbal extracts such as green tea, grape seed, bayberry, and apple as well as in essential oils like thyme, oregano, rosemary, and verbena, all of which serve as effective natural food preservatives. Several studies investigate the antimicrobial and antioxidant effect of tea polyphenols on aquatic food products particularly fish in terms of shelf-life extraction. (Kamal et al., 2025)

Sun et al. (2017) claimed that apple polyphenols may serve as a natural antioxidant to extend the shelf-life and quality of grass carp (*Ctenopharyngodon idellus*) surimi during storage at 4 °C. The surimi treated with polyphenols showed delayed lipid oxidation and significantly maintained texture and gel strength compared to the non-treated samples. (L. Sun et al., 2017) It has been reported by Li et al. (2012) that Chinese bayberry extract, due to the antimicrobial effects derived from its phenolic compounds, can extend the shelf-life of surimi even when stored at room temperature by inhibiting the growth of *Serratia marcescens* and *Pseudomonas aeruginosa*. Notably, its combination with tea polyphenols improved the inhibitory effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. These natural extracts can be applied as alternatives to sodium nitrite and potassium sorbate, maintaining surimi freshness without affecting consumer health.(J. Li et al., 2012) In another study, Huang et al., 2018 investigated the effects of essential oils (EOs) such as oregano, thyme, and cinnamon on various meat products, including pork bacon, pork filet, chicken filets, and salmon. In this experiment, samples were immersed for 2 minutes in the EOs, followed by storage at 4 °C. The results demonstrated that the EO treatments delayed lipid oxidation and inhibited microbial growth, which prevented the increase of TVB-N. (Z. Huang et al., 2018)

Chapter 2

2 Literature Review

The idea of utilizing agri-food waste as a natural additive to enhance food value has become a hot topic in recent years. Previous studies have shown that agri-food waste, including by-products from olive oil, wine, coffee, fruit, and dairy processing, contains phytochemicals and bioactive compounds that can be reused as valuable resources while also reducing waste and generating commercial value.(Papaioannou et al., 2022)

Studies have shown that Large-scale olive-mill wastewater (OMW) imposes severe environmental challenge because it carries an extremely high organic load (Biological Oxygen Demand and Chemical Oxygen Demand). Its rich phenolic content is toxic to aquatic and terrestrial microorganisms, leading to soil and groundwater contamination when the waste is spread or discharged untreated. Also, the acidic nature and offensive odors of this dark waste stream emphasize the urgent need for effective treatment strategies for safe disposal.(Caporaso et al., 2018)

2.1 Olive oil mill water waste

The application of extracted phenols from OMW as ingredients for functional foods (Caporaso et al., 2018), in the preparation of functional beverages (Zbakh & El Abbassi, 2012) and natural preservatives for food safety (Fasolato et al., 2016) has been widely investigated. The quantitative and qualitative profile of phenolic compounds depends on the olive cultivar, geographical origin, and the specific extraction methods employed.

According to the investigation by Ianni et al. (2022) on the phenolic profile of EVOO from the Moraiolo cultivar, the extract is characterized by a high content of secoiridoids, particularly oleocanthal and oleacein. These compounds contribute to the potent antioxidant properties associated with olives from this region. Additionally, the presence of oleuropein aglycone serves as a significant antimicrobial compound. Although the levels of hydroxytyrosol and tyrosol are typically lower in EVOO, their concentration increases significantly in OMW due to their hydrophilic nature. Given that the OMW used in this study originated from the same geographical region and cultivar (Moraiolo, Umbria) as investigated by Ianni et al. (2022), it can be inferred that the extract contains a similar phenolic distribution. As a result, the antimicrobial and antioxidant effects observed in our samples are likely attributed to the high presence of secoiridoids and their derivatives, such as hydroxytyrosol and tyrosol.(Ianni et al., 2022)

2.2 Extraction Techniques for Olive Mill Waste: Benefits and Limitations

The recovery and removal of phenols from OMW to valorize by-products can be carried out through several extraction methods. These include solvent extraction like the liquid-liquid method, using ethanol, methanol, chloroform or ethyl acetate as a solvent (Fattoum et al., 2023), the Cloud point method (Gortzi et al., 2008; Katsoyannos et al., 2006); and membrane process methods (Ianni et al., 2021), which involve three distinct techniques: Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis which can be performed either singly or in series to obtain better results. Finally, adsorption method using resin, such as Amberlite, (Fernandes et al., 2024) and advanced methods like supercritical CO_2 and ultrasound-assisted and microwave-assisted are also common.

Solvent extraction, particularly in the form of liquid-liquid extraction, is one of the methods that have been investigated in terms of phenol recovery. In this article, ethyl acetate was selected; despite achieving a high efficiency in recovering efficiency of phenol (total 90%), the requirement of large volumes of organic solvent and generation of hazardous waste have hindered its application. (Rahmanian et al., 2014)

As highlighted in the study by Fattoum et al. (2023), the phenolic concentration and antioxidant activity of extracts can differ significantly based on the extraction method and the solvents utilized. As demonstrated in Table2.1 among the solvent methods, the mixture of methanol-chloroform (1:1) and the liquid-liquid extraction method using ethyl acetate yielded the highest phenolic concentrations. However, the study on the antioxidant activity of these extracts illustrated that the strongest activity was acquired in the methanolic extract. (Fattoum et al., 2023)

Table 2.1. Antioxidant activity (IC_{50}) of various solvent extracts; Adapted from(Fattoum et al., 2023)

Solvent	IC50 (mg L−1)m±SD*
Ethanol	25.88±0.59
Methanol	15.75±0.73
Chloroform	N/Q
Chloroform/methanol	23.52±0.70
Ethyl acetate	26.68±1.24

*Data are expressed as mean ± SD (n=3) /NQ: Not Quantifiable.

Even though using hydro-ethanolic—a mixture of 85% ethanol (a food-grade solvent) and the aqueous phase of the waste —is considered a highly appropriate solvent and has shown a decent preservation of phenols and polar antioxidant stability for 18 weeks (Galanakis et al., 2010), the process parameters must be controlled as extraction efficiency depends strongly on pH, time, solvent-to-waste ratio, and number of stages. To overcome these limitations, Cloud Point Extraction (CPE) has emerged as a clean and non-toxic alternative with the aim of reducing solvent usage. According to the findings of (Gortzi et al., 2008) and (Katsoyannos et al., 2006), while only a 4-12% volume of surfactants such as Genapol X-080 or Triton X-114 was utilized, recovery rates of 89.5% and higher than 96% were achieved, respectively. Notably, the elusive feasibility of the separation step (to isolate the trapped phenolic compounds from surfactants) and the necessity of a fat removal step remains downsides to these studies.

In the adsorption technique, the OMW effluent is passed through a column packed with Amberlite resin, allowing phenolic compounds to bind to the resin surface, followed by a desorption step using a selective solvent to recover the phenols for valorization. However, this method has several key limitations, such as low and incomplete phenol extraction efficiency (only 42%), the requirement for a high volume of solvents for desorption, being time-consuming and costly, and the uncertain durability of the resin, which could cause safety concerns. (Fernandes et al., 2024)

Membrane-based techniques, widely applied in the literature, enable selective separation based on molecular size. Microfiltration (MF) and ultrafiltration (UF) are used as a pre-treatment step for high-molecular-weight compounds. In contrast, nanofiltration (NF) and reverse osmosis (RO) are used for specific phenol classes, although they may be less effective than UF. UF membranes (e.g., regenerated cellulose (Galanakis et al., 2010); or 25-kDa polysulfone (Cassano et al., 2011)) allow smaller antioxidants to pass and maintain the antioxidant properties of the permeate while removing heavier fragments. The key advantages of this approach include its high efficiency -as (El-Abbassi et al., 2012) achieved almost 100% compound recovery after 8 hours with Direct Contact Membrane Distillation (DCMD) - its time-efficiency, the fact that it does not demand any solvents, and its cost-effectiveness. Additionally, in the last few years, the compatibility of these methods with the food, water, and pharmaceutical industries has made it a common and promising approach, particularly for products aiming to extend shelf-life using natural preservatives, sustain antioxidant and/or antimicrobial activity, and achieve higher stability. These aims have been followed in distinct food matrices, such as oils, dressings, fresh or processed/cured meats, biscuits, seafood, and fish products.(Ianni et al., 2021)

However, these methods may be affected by membrane fouling and operational costs. Despite these limitations, enzymatic treatment followed by a three-phase membrane extraction process (Microfiltration, Ultrafiltration, and Reverse Osmosis, remain a suitable choice for the present study due to their effectiveness in concentrating bioactive compounds.

Table 2.2. Summary of phenolic recovery yields across different extraction and membrane technologies

Extraction/Treatment Method	Technique / Material	Results and Efficiency	Reference
Membrane	Membrane Distillation (PTFE)	100% separation	(El-Abbassi et al., 2012)
	Ultra/Nanofiltration / Reverse Osmosis	0.5 to 30 g/L Polyphenol Concentration	(Rahmanian et al., 2014)
	Microfiltration/ ultrafiltration/ reverse osmosis consisting of a polymeric hydranautics membrane	Retentate containing 464.870 mg/L free low MW polyphenols	(Rahmanian et al., 2014)
	Nanofiltration/ microfiltration/ osmotic distillation/ vacuum membrane distillation	Recovery of 78 % of the initial content of polyphenols	(Rahmanian et al., 2014)
Adsorption	Amphoteric Resin	99% recovery Hydroxytyrosol And total phenolic compounds in OMW 42 %	(Rahmanian et al., 2014) / (Fernandes et al., 2024)
	Amberlite XAD7 / XAD16	77% recovery Hydroxytyrosol	(Rahmanian et al., 2014)
Cloud Point	CPE (Triton X-114)	> 96% recovery	(Katsoyannos et al., 2006)
	CPE (Genapol X-080)	89.5% recovery	(Gortzi et al., 2008)
Solvent	Ethyl Acetate (LLE)	> 90% Total Phenols / 85.46% Hydroxytyrosol	(Rahmanian et al., 2014)
	Chloroform/Methanol (Mix)	1.07±0.10 g EAG/L	(Fattoum et al., 2023)
	Ethanol (Maceration)	0.66±0.13 g EAG/L	(Fattoum et al., 2023)
	Methanol (Maceration)	0.15±0.01 g EAG/L	(Fattoum et al., 2023)

2.3 Literature Review on the Application of OMW Phenols in Food Systems

The exploration of OMW as a source of bioactive compounds is not a recent development; it has been a subject of intensive study for several years, aimed at obtaining higher efficiency in phenol recovery and application. Extracted phenols from OMW has been widely applied for different products such as vegetable oil, milk (Servili et al., 2011)

cheese (Roila et al., 2016), bakery products (Galanakis et al., 2018 b) and meat products (Balzan et al., 2017; Galanakis, 2018 a) especially aquatic products. Hence, the following sections provide a detailed literature review of extracted phenols applications within each food matrix.

2.3.1 Dairy Products

Besides the effect of fortification with phenolic compounds, a study (Servili et al., 2011) targeted the synergistic effect of OMW-derived phenolics on starter cultures and probiotic strains (*S. thermophilus*, *L. delbrueckii*, and *L. plantarum*), while evaluating the sensory profile with the Paired Comparison Preference Test. OMW-derived phenolics were obtained through enzymatic treatment followed by a three-phase membrane extraction process (Microfiltration, Ultrafiltration, and Reverse Osmosis). These extracts were incorporated into protein-rich dairy matrices to develop a milk-based beverage, which was monitored during 30-day storage at 4 °C. After a comprehensive multi-step methodology, the results showed that the extracted phenols can be successfully applied in the development of functional milk beverages without impairing the fermentation process, probiotic viability, or overall sensory quality.

Pseudomonas fluorescens, the primary agent responsible for spoilage and discoloration in Mozzarella cheese, was targeted using phenolic extracts from the Moraiolo olive cultivar to evaluate their potential as a natural preservative. Owing to the high concentrations of hydroxytyrosol and tyrosol in this OMW extract, the results illustrated a clear dose-dependent antimicrobial effect. While lower doses (5 mg/mL) inhibited merely 15% of the strains, a concentration of 8 mg/mL successfully achieved a 100% mortality rate. (Roila et al., 2016)

2.3.2 Bakery Products

Regarding shelf-life extension in complex food matrices, (Galanakis et al., 2018 b) conducted a comparative experiment on two bakery products: bread and rusks. What distinguishes this study is the comparison between emulsified and non-emulsified polyphenol formulations over a storage period of 20 days for bread and 12 weeks for rusks. Antioxidant activity was measured using the ABTS+ radical scavenging assay,

while microbial growth (Total coliforms, Yeasts-Moulds, *Bacillus spp.,* and *E. coli*) was monitored via standard methods. The results showed a 5-day extension in the shelf-life of bread, while the emulsified phenolic-enriched rusks lasted for 12 weeks, which is double the duration of the blank sample. Consequently, the study concluded that emulsification methods yield higher efficiency compared to non-emulsified formulations, confirming the antioxidant and antimicrobial effects of OMW phenolics.

2.3.3 Meat and Meat-Based Products

In order to compare the efficiency of OMW phenols with commercial synthetic antioxidants, Roila et al. (2022) performed a comparative analysis of beef burgers. The study included a control group, a group formulated with a commercial antioxidant, and a group formulated with phenols extracted from OMW (via membrane filtration followed by spray-drying with maltodextrin). After storage for 7 days at 4°C under retail-like light conditions, the antioxidant effect was measured by lipid oxidation (TBARS), and antimicrobial activity was evaluated against the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas fluorescens*. They also tracked burger microbiology on days 0, 2, 5, and 7 for TVC, *Enterobacteriaceae*, LAB, *Pseudomonas*, and staphylococci, checked for *Salmonella*, and modeled the growth.

The study confirmed the higher antioxidant capacity of the extracted phenols compared to the commercial antioxidant, showing 62% lower lipid oxidation than the control on day 7, along with lower final microbial counts and longer lag phases in the extract-treated burgers. These findings highlight the potential of OMW extract as a natural antioxidant and antimicrobial agent to improve beef burger shelf-life. (Roila et al., 2022)

Unlike studies that apply OMW extracts as a direct additive to minced meat, Roila et al. (2018) investigated the impact of dietary supplementation on the shelf-life of chicken breast. Chickens were fed with varying levels of phenols for 20 days, followed by a 7-day refrigerated storage analysis of the breast fillets. Interestingly, despite the different methodology, the study yielded consistent results, showing a reduction in peroxide values and TBARS. This supports the antioxidant properties of these compounds even when used in dietary supplementation. (Roila et al., 2018)

Thermal stability and the production of cholesterol oxidation products (COPs), which are associated with food safety, are two crucial factors in food safety and additive stability. Considering this, (Balzan et al., 2017) evaluated whether phenols recovered from OMW could replace chemical additives in fresh pork sausages and protect the product during refrigerated storage and after cooking. The experiment was run in three replicates: a control and two formulated batches with varying phenol concentrations. Then, sausages were left unpackaged under alternating light at 2±2 °C, sampled at 0, 7, and 14 days; the corresponding samples were also cooked at 200 °C to an internal temperature of 85 °C before analysis. Oxidation markers, including peroxide value, TBARS, and cholesterol oxidation products in raw and cooked sausages, were measured with standard chemical methods, and sensory discrimination was checked.

Findings have illustrated that adding the phenolic extract significantly lowered pH and reduced lipid and especially cholesterol oxidation markers, which were 4-fold lower in raw sausages and 17-fold lower in cooked sausages. Although heat and storage caused phenol loss, the residual phenols still kept oxidative markers low, with TBARS remaining below 1 mg MDA/kg in the treated products. Despite the distinguishable taste of the phenol sausages, they were not described as unpleasant by panelists, while the control was tasted as oilier and slightly rancid. Overall, the paper concludes that phenols from OMW were effective antioxidants for fresh pork sausages and a plausible clean-label ingredient, with 0.075 g/100 g presented as an effective level.

2.3.4 Fish and Fish-Based Products

Indicating the versatility of OMW phenols across distinct protein sources and application strategies, Fasolato et al. (2016) evaluated these compounds as a natural preservative for gilt-head seabream and chicken breast, using spraying and dipping (1 min) methods, respectively. Following storage and microbial monitoring, the results showed a prolonged lag phase for spoilage bacteria in the fish. In the chicken samples, the treatment delayed the growth of *Pseudomonas* and *Enterobacteriaceae*, reduced lipid oxidation (TBARS), and resulted in a two-day shelf-life extension.(Fasolato et al., 2016)

Expanding the use of OMW phenols in fish, which is considered a highly perishable product, represents a significant step toward mitigating lipid oxidation and microbial growth, thereby extending shelf-life and ensuring both food safety and quality. Hence, in the study by Miraglia et al. (2016), 72 fresh salmon steaks were divided into three groups: a control, and two groups immersed in either 1.5 g/L or 3 g/L extract. This was followed by packaging under a modified atmosphere (70% CO_2, 25% N_2, and 5% O_2) and storage in the dark at 4°C for 2 hours, 3 days, and 6 days.

Antioxidant activity (DPPH) and lipid oxidation (TBARS) were measured alongside microbiological quality, pH, colour, phenolic composition, and alpha-tocopherol levels. A reduction in microbial growth was observed by evaluating the total viable count and *Enterobacteriaceae*. Moreover, the 3 g/L treated group produced the lowest TBARS values, supporting the improvement of fresh salmon quality. Although a slight color alteration toward yellow was observed at the beginning, the paper concluded that the extract slows lipid oxidation and enhances hygienic quality during storage, with a stronger effect seen at the higher concentration. (Miraglia et al., 2016)

Highlighting the difference in the antioxidant potency of polyphenols, researchers compared the antioxidant activity and the ability to reduce lipid oxidation of four different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid), first *in vitro* and then in mackerel mince and a menhaden oil-in-water emulsion. The results showed that tannic acid was the most effective preservative among the tested phenols. (Maqsood & Benjakul, 2010)

To justify the concentrations used in food matrices like surimi, it is crucial to consider the intrinsic lethal potency of the extract. Fasolato et al. (2015) addressed this by determining the MBC of purified phenols (PEOW) against 18 different strains, including spoilage bacteria, food-borne pathogens, and starter cultures from genera such as *Staphylococcus*, *Listeria*, *Escherichia*, *Salmonella*, *Pseudomonas*, *Lactobacillus*, and *Pediococcus*. Although MIC concentrations could not be recognized due to turbidity, Gram-positive bacteria, particularly *Listeria monocytogenes* and *Staphylococcus aureus*, showed the lowest resistance, with MBCs ranging from 1.5 to 3 mg/mL. In contrast, Gram-

negative strains were generally less sensitive, with MBCs around 6 to 12 mg/mL. Notably, *Pediococcus pentosaceus* was the most resistant lactic acid bacterium, surviving up to 12 mg/mL. In conclusion, PEOW exhibits promising bactericidal activity, especially against Gram-positive food-borne pathogens, making it a viable candidate for food-model studies. (Fasolato et al., 2015)

In contrast with the positive results observed in the dietary supplementation of broilers in the study by Roila et al. (2018), supplementing rainbow trout (*Oncorhynchus mykiss*) failed to enhance the shelf-life or oxidative stability of the fish meat. Although the trout were fed a phenol-rich diet derived from OMW, the fillets naturally deteriorated over a 13-day storage period.(Bordignon et al., 2022) This ineffectiveness might be attributed to either an insufficient dosage (the maximum 600 mg/kg level being too low for high-lipid fish) or the delivery method (dietary supplementation). To tackle these limitations, direct application methods such as spraying or dipping could be more effective in cases like surimi, as they provide a higher local concentration directly into the meat.

In conclusion, the effectiveness of this bioactive compound is linked to diverse parameters, such as the sample preparation protocol, whether the sample is fresh or cooked, the composition and type of food matrices, and storage conditions. Based on previous literature, this study selected a specific approach to overcome existing limitations and gaps. Despite earlier findings, the efficacy of this approach in restructured fish products like surimi, which involves intensive washing and freezing cycles, remains unexplored. This study fills this gap by incorporating spray-dried phenolic extracts directly into the Red Mullet surimi matrix, taking into account the distinct fatty acid profile and enzymatic composition of this fish.

Chapter 3

3 Aims of the Study

Given the antioxidant and antimicrobial properties of phenols, this study aims to evaluate the application of OMW-derived polyphenols in surimi produced from *Mullus barbatus.* Specifically, the primary objective is to set up the operational procedures for surimi production, comparing the interaction between different processing approaches, such as washing before freezing (Process-WB) versus washing after thawing (Process-WA), and to conduct a comprehensive nutritional characterization of the final product. Furthermore, this research seeks to evaluate the impact of polyphenol incorporation on inhibiting microbial activity and preventing lipid oxidation within these processes. Ultimately, the study aims to define the product's behavior during its shelf life through a comprehensive microbiological assessment to determine its final quality and preservation limits. To achieve this, a comprehensive microbiological assessment was designed, focusing on critical categories: total spoilage populations, including mesophilic and psychrotrophic bacteria, which drive undesirable quality changes over time; lactic acid bacteria (LAB) as indicators of specific spoilage pathways; and Staphylococci to evaluate hygiene and biological safety. In addition, yeasts and molds were monitored as key factors of spoilage, as they can proliferate under refrigerated storage. By monitoring these specific parameters, alongside physicochemical characteristics, lipid oxidation, and qualitative observations (visual and odor), this study aims to determine the ultimate potential of natural phenolic extracts, supported by the optimal washing process, in enhancing the overall quality and safety of surimi.

Chapter 4

4 Material and Methods

4.1 List of material

4.1.1 Extraction of OMWW

A crude phenolic-rich extract was obtained from fresh olive mill wastewater produced from Moraiolo olives harvested in Umbria (Central Italy). Prior to filtration, the olive vegetation water underwent a hydrolytic enzymatic treatment with a pectinase/hemicellulase preparation (O-Max S, OE Italia S.r.l., Marsala, Italy) at 20 °C for 12 h. The treated olive vegetation water was then processed through a pilot-scale membrane system consisting of three successive steps—microfiltration, ultrafiltration, and reverse osmosis—as previously described by (Ianni et al., 2021). To obtain a more stable powder formulation, the resulting phenolic extract was mixed with maltodextrin (Glucidex 19, Lestrem, France) at a 1:1 ratio (d.w.) and subsequently spray-dried.

4.1.2 Media Preparation

To provide an optimal growth environment for the selected microorganisms, specific culture media were used in this study. Plate Count Agar (PCA) was employed for determining the Total Viable Count (TVC). In this work, PCA media were incubated at two distinct temperatures: 30 °C to target mesophilic bacteria and 4 °C to evaluate Psychrotrophic bacteria. Mannitol Salt Agar (MSA) and De Man, Rogosa, and Sharpe Agar (MRS) were used for the enumeration of *Staphylococcus aureus* and Lactic Acid

Bacteria (LAB), respectively. Finally, yeasts and molds were enumerated using Oxytetracycline Glucose Yeast Extract Agar (OGA).

The dehydrated media were weighed according to the manufacturer's instructions (PCA: 23.5 g/L, MSA base: 111 g/L, OGA: 30g/L, MRS: 67g/L) and prepared following standard laboratory procedures. The powders were suspended in distilled water and sterilized by autoclaving at 121 °C for 15 minutes, with the exception of MSA, which was autoclaved at 118°C for 5 minutes. After sterilization, the media were cooled to approximately 45–50 °C and, under a laminar flow hood, approximately 20 mL of medium was poured into sterile Petri dishes, which were left to solidify at room temperature under the hood. The prepared plates were labelled and stored in a refrigerator at 4 °C.

Table 4.1. Specifications of culture media used for microbiological analysis

Culture Media	Manufacturer (Source)	Target Microorganism	Function / Principle	Incubation Conditions	Reference
Plate Count Agar (PCA)	Microbiol Diagnostici, Italy	Mesophilic bacteria	General enumeration of aerobic bacteria Total Viable Count (TVC)	30◦C for 72 h	(ISO 4833-2, 2013)
Plate Count Agar (PCA)	Microbiol Diagnostici, Italy	Psychrotrophic Bacteria	Enumeration of cold-tolerant spoilage bacteria	4◦C for 10 days	(ISO 17410, 2019)
Since De Man, Rogosa, and Sharpe Agar (MRS)	Microbiol Diagnostici, Italy	Lactic Acid Bacteria (LAB)	Enriched with nutrients to support the growth of (LAB),	30◦C for 48 h	(ISO 15214, 1998)
Mannitol Salt Agar (MSA)	Microbiol Diagnostici, Italy	*Staphylococcus aureus*	An indicator of skin-borne contamination and hygiene during handling	37◦C for 24–48 h	Commercial protocol
Oxytetracycline Glucose Yeast Extract Agar (OGA)	Condalab, Spain	Yeast and Mold	The oxytetracycline prevents bacterial growth.	25◦C for 3–5 days	(ISO 21527-1, 2008)

4.1.3 Reagents and Solutions

All reagents used in this study were of analytical grade. Unless otherwise specified, distilled or deionized water of equivalent purity was employed to prepare all solutions.

Table 4.2. Specifications of Reagents and Solutions used for chemical analysis

Reagent / Solution	Analysis Method	Purpose	Concentration / Preparation
Perchloric acid	TVB-N	Extraction	6 g / 100 mL (0.6 mol/L)
Sodium hydroxide	TVB-N	Alkalization	20 g / 100 mL
Boric acid	TVB-N	Absorption (receiver solution for volatile bases)	3 g / 100 mL
Hydrochloric acid	TVB-N	Titration	0.01 mol/L (automatic distillation solution)
Tashiro Indicator	TVB-N	Endpoint detection	2 g Methyl red + 1 g methylene blue in 100 mL ethanol 95%
Phenolphthalein	TVB-N	Indicator	1 g / 100 mL in 95% ethanol
pH Buffer solutions	pH measurement	Calibration	pH 4.01 (Red) and 7.00 (Green)
KCl Standard	Water activity	Calibration	0.50 mol/kg in H2O (0.984 a_w)
Sodium Sulfate	Protein extraction	Boiling point elevator (Digestion)	Solid powder (15 g per sample)
Copper(II) Sulfate Pentahydrate	Protein extraction	Catalyst (Digestion)	Solid powder (0.8 - 1.0 g per sample)
Sulfuric Acid	Protein extraction	Digestion reagent	98% Concentrated (20 mL per sample)
Sodium Hydroxide / Soda	Protein extraction	Alkalization (Distillation)	(40% w/w solution)
Boric Acid	Protein extraction	Absorption (Receiver solution)	3 g / 100 mL
Hydrochloric acid	Protein extraction	Titration	0.1 N Standard solution
Nitric acid	Nacl determination	Acidifying agent	65% Concentrated
Ferric ammonium alum	Nacl determination	Indicator	-
Silver nitrate	Nacl determination	Precipitating agent	0.1 N Standard solution
Standard salt solution	Nacl determination	Control	0.1 N Standard solution

Ammonium thiocyanate	Nacl determination	Titration	0.1 N Standard solution
Petroleum ether	Fat Extraction	Extraction solvent	-
Potassium hydroxide (KOH)	Acidity	Titration	0.5 N Standard solution
Diethyl ether	Acidity	Solubilization	Solvent mixture (Ethanol/Ether 1:2 v/v)
Ethanol	Acidity	Solubilization	Solvent mixture (Ethanol/Ether 1:2 v/v)

4.1.4 Apparatus and Equipment

Table 4.3. Equipment used for analysis

Equipment / Apparatus	Model / Specification	Manufacturer / Country	Application
pH Meter	Digital Portamess	Knick, Germany	pH measurement
Water Activity Meter	AQUALAB 4TEV (Dew Point)	METER Group, USA	a_w measurement
Homogenizer High-speed Blender	ULTRA-TURRAX T 25 basic (8,000 – 45,000 rpm)	IKA-WERKE, Germany	Sample homogenization
Meat Grinder	C 15B	LA FELSINEA SRL, Italy	Fish mincing
Thermo-sealing and MAP system	VGP Series (Vacuum Gas Packaging)	Orved S.p.A., Musile di Piave, Italy	Tray Sealer
Oven	combi-oven steamed	UNOX ChefTop	Cooking
Lab Blender (BagMixer®)	Interscience	France	Sample homogenization for microbial and chemical analysis
Analytical Balance	Precision: 0.1 g	BEL Engineering, UK	Weighing samples and reagents
Burette	5 mL (0.01 mL graduation)	-	Precise titration
Kjeldahl Tubes	High-quality Glass	-	Holding extract during distillation
Magnetic Stirrer	RT Basic-12	Thermo Scientific, USA (Assembled in South Korea)	Stirring

Protein digestion paper	PERFECTE 2 Extra Rapida (Ø 12 cm, 90 gsm)	Gruppo Cordenons SpA, Italy	Digestion
Freeze-dryer	Lyovapor™ L-200.	Büchi, Switzerland	Sample lyophilization
Solvent Extraction Unit	Universal Extractor E-800	Büchi, Switzerland	Fat Extraction
Kjeldahl Digestion unit	DK 20 heating digester	VELP Scientifica, Italy	Protein Extraction
Fume scrubber system	K-415	Büchi, Switzerland	Fume neutralization during protein digestion
Steam Distillation Unit	UDK 127 (Semi-automatic)	VELP Scientifica, Italy	Protein distillation / TVB-N distillation
Laboratory Drying Oven	Type M 120 - VF	MP Instruments s.r.l., Italy	Moisture content determination / Sample drying
Laboratory Centrifuge	SL 16	Thermo Electron LED GmbH, Germany	Centrifugation for salt determination
Laboratory Hot Plate	Type CK 11	Gerätewerk Matrei reg. Gen.m.b.H	Sample heating
Double-door commercial refrigerator	Salumajo 1400	Majolo S.r.l.,Italy	Sample storage

4.1.5 Fish source

Red Mullet (*Mullus barbatus*), a fish species belonging to the Mullidae family, was obtained from the wholesale fish market of Chioggia (VE) Italy. The fish were transported to the laboratory in polystyrene boxes containing ice, and processed within 24h of capture. All sample preparation steps for *M. barbatus* were carried out in different trials at the Department of Comparative Biomedicine and Food Science, University of Padua, between May 2025 and April 2026.

4.2 Method

4.2.1 Protocol for surimi preparation

A total of 15 kg fish (*Mullus barbatus*) per each trial, was obtained and eviscerated, skinned, and trimmed to isolate edible muscle tissue, which was kept below 10 °C until mincing. The cleaned material, weighing approximately 7.3 kg, was divided into two 3.65 kg batches: control surimi and a phenolic-enriched surimi. Mincing was subsequently carried out using a cutter (C15B, La Felsinea S.r.l., Italy), during this step the ingredients listed in Table 4.4 were added according to the formulation of each batch.

Table 4.4. Composition of ingredients used for control and phenolic-enriched surimi batches.

Ingredient	Control (kg)	Phenols (kg)
Fish (edible portion)	3.65	3.65
Glycerol	0.0365	0.0365
Ice	0.73	0.73
Phenols (spray-dry)	-	0.0183
Maltodextrin (DE-19)	0.0179	-

The control batch utilized, maltodextrin, 1% glycerol, and 20% ice to prevent protein denaturation, while the phenolic-enriched formulation replaced maltodextrin with phenolic extract.

4.2.2 Comparative Protocols for Surimi Preparation: Pre- and Post-Freezing Washing Cycles

Since one of the primary objectives of this study was to evaluate the most efficient protocol for sample preparation, the preparation process was carried out following two

distinct approaches: washing after freezing (WA) and washing before freezing (WB). This preliminary phase was conducted at the Department of Comparative Biomedicine and Food Science, University of Padova, in November 2024, utilizing alternative fish matrices—specifically scorpionfish, hake, and perch—to optimize and compare the sample preparation methods.

In summary, while red mullet remained the definitive standard for final assessment, the inclusion of alternative species served as an essential tool during the preliminary set-up phase to optimize mechanical washing cycles and surimi preparation protocols, thereby ensuring methodological consistency throughout the entire study period.

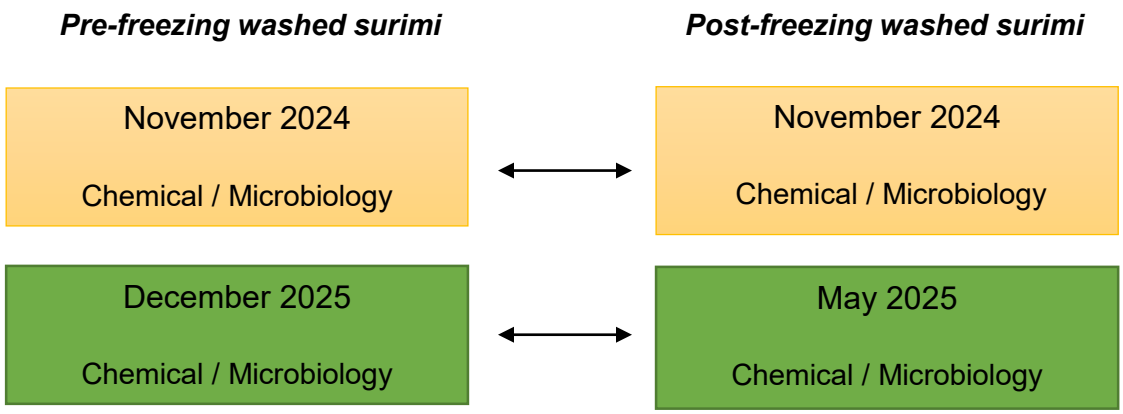

Figure 4.1. Schematic workflow of the surimi experimental design, comparing the two approaches (pre-freezing vs. post-freezing washing cycles) across different sampling periods

In the "washing-after-freezing" protocol, aliquots were first packed and frozen at –20 °C in a blast chiller for 30 days. The frozen samples were allowed to thaw at 4 °C for 24 h and three washing cycles were performed using demineralized water (fish: water ratio 1:4). Each washing step lasted 20 min and in the final washing cycle, Sodium chloride (0.6%) was added to enhance myofibrillar protein extraction, adapted from the method described by Balange & Benjakul, (2009). The washed mass was wrapped in food-grade gauze and mechanically pressed to remove excess water. (Figure 4.2)

Figure 4.2. Mechanical press used to remove excess water from surimi samples.

Subsequently, the material was stuffed into 2.5 cm collagen casings (Figure 4.3) and cooked in a steam oven (40 °C for 2 h, followed by heating to an internal temperature of 85 °C, maintained for 5 min). After cooling to 3 °C, the casings were removed under laminar flow and samples were portioned into 25 g units.

Figure 4.3. Filling collagen casings with material.

Figure 4.4. Cooking and cooling process

In the "washing-before-freezing" protocol, the same operations were performed; however, washing and pressing were completed prior to freezing the mass at –20 °C for 30 days.

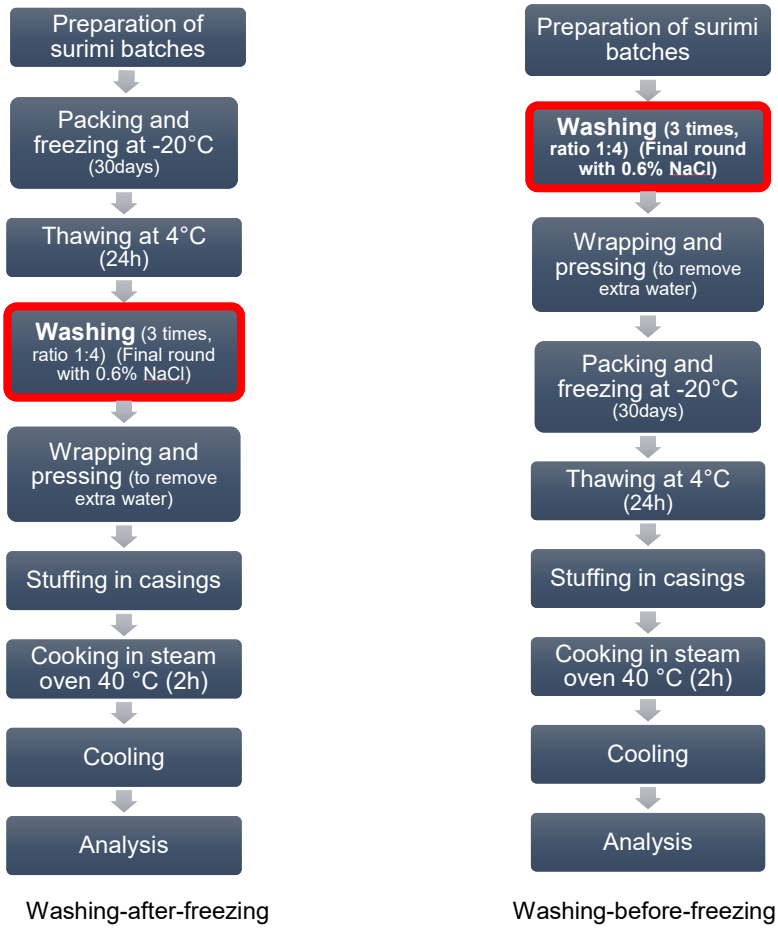

Figure 4.5. Flow diagram of the experimental process for surimi for two different technological approaches: washing after freezing and washing before freezing

4.2.3 Modified Atmosphere Packaging (MAP) and Storage Condition

Following cooling, the casings were removed under laminar flow conditions, and the samples were portioned into 25 g units and placed in polypropylene trays (162 × 116 × 60 mm; barrier thickness: 0.54 mm; Gollin Srl, Italy). Subsequently, the samples were labelled and divided into two experimental groups: the polyphenol-treated group (F) and the untreated control group.

The polypropylene trays were heat-sealed with PET/PE/EVOH/PE film (12 + 50 µm; anti-fog, anti-UV; Gollin 5025N) after gas flushing (80% N_2 / 20% CO_2) using an ORVED VGP system. (Figure 4.6) The storage stability study was conducted using initially vacuum-packed samples placed inside white plastic trays to ensure uniform exposure. These trays were maintained under controlled environmental conditions at 4 ± 1 °C within a double-door commercial upright refrigerator (Salumajo 1400, Majolo S.r.l.) equipped with wire mesh shelving to facilitate optimal air circulation. (Figure 4.7) To simulate the required photoperiod consisting of 9 h of light and 13 h of darkness, the refrigerator was retrofitted with a lighting system equipped with Master TL-D Food Philips 58 W/79 lamps, providing illumination in the 450–480 nm wavelength range suitable for meat and fish products. These fixtures were positioned directly above each shelf to ensure uniform light distribution across all samples. Sampling was systematically performed at predetermined intervals throughout the storage period, specifically on days 0, 15, 30, 45, and 60. At each designated time point, the respective vacuum-packed matrices were collected from the refrigerated unit and immediately transferred to an ultra-low temperature freezer at –80 °C to halt all metabolic and enzymatic activities prior to analysis.

Figure 4.6. Vacuum sealing of samples using an ORVED VGP packaging machine

Figure 4.7. Storage conditions of the samples

4.3 Analysis

4.3.1 Microbiological Analysis

Each surimi sample was weighed, transferred into a Stomacher bag containing 90 mL of Maximum Recovery Diluent (MRD) and homogenized for 1 minute using a BagMixer. Tenfold serial dilutions ($10^{-}1$ to $10^{-}6$) were prepared from the homogenate. Aliquots (0.1 mL) from each dilution were then plated onto the appropriate culture media. The plates were incubated at the temperatures and for the durations specified by the manufacturer's instructions.

Microbiological analyses were performed according to international standard methods, including ISO 4833-2 for total viable count, ISO 17410 for psychrotrophic bacteria, ISO 15214 for lactic acid bacteria, and ISO 21527-1 for yeast and mold enumeration. According to the specifications outlined in Table 4.1; PCA plates were incubated for 48-72 h at 30 °C for mesophilic bacteria and at 4 °C for 10 days for psychrotrophic bacteria, MSA plates were incubated at 37 °C for 48 h, and MRS plates at 30 °C for 48 h. Since lactic acid bacteria require anaerobic or microaerophilic conditions, MRS plates were placed in a BD GasPak™ EZ container, this system utilized a GasPak sachet to deplete oxygen and generate CO_2, thereby creating an optimal growth environment. (Figure 4.8) Yeast and mould growth on OGA plates was monitored for up to 5 days at room temperature to detect low-abundance contamination.

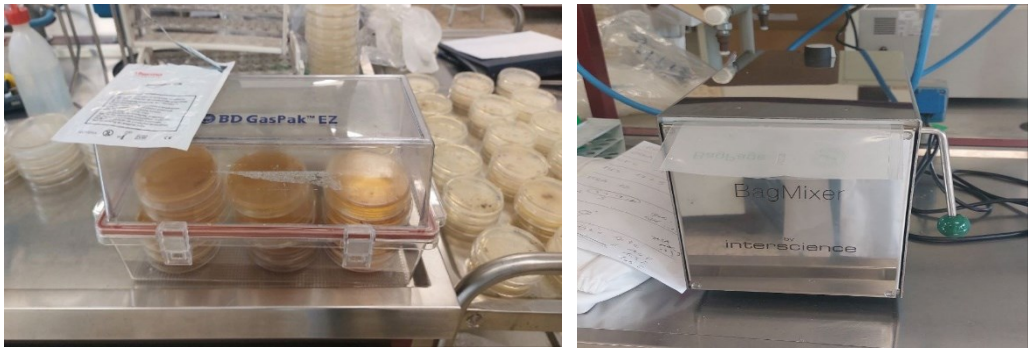

Figure 4.8. Laboratory equipment: Anaerobic BD GasPak™ EZ container (Left); BagMixer® sample homogenizer (Right)

To calculate the number of Colony Forming Units (CFU) per gram of sample, plates containing between 10 and 300 colonies are selected. The weighted mean formula is applied in accordance with ISO 7218:

$$N = \frac{\sum c}{\mathrm{V} \times (n_1 + 0.1 \times n_2) \times d}$$

Where:

N: Number of microorganisms per gram of sample.

$\sum c$: Sum of the colonies counted on all plates from two consecutive dilutions.

V: Volume of the inoculum applied to each plate (0.1 ml).

n_1 : Number of plates counted from the first dilution.

n_2 : Number of plates counted from the second dilution.

d: Dilution factor of the first dilution considered.

In addition to the microbiological analysis, preliminary visual and olfactory observations were casually noted during storage to track changes in color and odor between the control (C) and polyphenol-treated (F) groups, without constituting a formal sensory analysis.

4.3.2 Chemical Analysis

An overview of the analytical parameters and the established standard protocols applied in this work is presented in Table 4.5.

Table 4.5. Standard methods utilized for the physicochemical analysis of surimi samples

Analytical Parameter	Methods
pH	AOAC Official Method 981.12: 2000
a_w	ISO 21807: 2004

Moisture Content	AOAC Official Method 950.46: 1990
Total Volatile Basic Nitrogen	Commission Regulation No 2074/2005: 2005
Protein	AOAC Official Method 928.08
Fat	AOAC Official Method 960.39
Acidity	AOAC Official Method 940.28
Salt	AOAC Official Method 937.09

4.3.2.1 Determination of pH

The pH of the surimi samples was measured using a digital pH meter according to the AOAC Official Method 981.12 (AOAC, 2000c; Kilemile & M. Runyogote, 2024) Approximately 2.5 g of the material was weighed into a plastic tube, and deionized water was added at a 1:10 ratio (w/v). Subsequently, the mixture was homogenized for 30 s at 11000 rpm using a vertical shaft homogenizer (ULTRA-TURRAX T25 basic, IKA-WERKE) After homogenization, each sample was filtered through fluted filter paper. The pH meter (KNICK Portamess) was calibrated using standard buffer solutions at pH 4.01 and pH 7.00 (HACH LANGE GMBH). Finally, the pH of the samples was then measured and recorded.

Figure 4.9. Experimental setup for sample preparation, including the ULTRA-TURRAX T25 basic homogenizer (Left) and the KNICK Portamess pH meter (Right)

4.3.2.2 Determination of Water Activity

For water activity analysis, samples were prepared by weighing approximately 1 g of the material into disposable plastic sample cups. The water activity (a_w) was determined using an AQUALAB 4TEV Dew Point Water Activity Meter (Decagon Devices, USA), based on the dew point principle according to ISO 21807. (Bal-Prylypko et al., 2025; International Organization for Standardization, 2004) The device had been calibrated using a 0.984 a_w standard salt solution (0.50 mol/kg KCl in H2O, Steroglass, Italy). Each sample cup was then placed into the chamber, and the measurement was recorded.

Figure 4.10. AQUALAB 4TEV Dew Point Water Activity Meter by Decagon Devices.

4.3.2.3 Determination of Moisture Content

Moisture content was determined gravimetrically by drying the samples in a ventilated oven, according to the AOAC Official Method 950.46. (AOAC, 1990) Initially, the ceramic crucibles were dried in an oven at 110 °C for 1 h to remove any residual moisture or impurities. To prevent the absorption of ambient humidity, the crucibles were transferred to a desiccator and allowed to cool to room temperature before their empty weight was recorded. Approximately 5–10 g of the sample was then placed into each crucible and dried in an oven at 100 °C for 24 hours. Subsequently, the crucibles were transferred back to the desiccator for 30 min to cool. Finally, the dry weight was measured and recorded, and the moisture content was calculated using the following equation:

$$\boldsymbol{UR}(\%) = \frac{\boldsymbol{P} - \boldsymbol{p}}{\mathbf{P}} \times \mathbf{100}$$

Where:

P: the initial weight of the sample (g)

$\boldsymbol{p}$**:** the weight of the sample after drying (g)

Figure 4.11. Experimental equipment for moisture content determination: drying oven and glass desiccators

Figure 4.12. Weighing the ceramic crucibles containing dried surimi samples

4.3.2.4 Determination of Total Volatile Basic Nitrogen (TVB-N)

The concentration of Total Volatile Basic Nitrogen (TVB-N), an essential freshness indicator for meat and fish products, was measured using the official steam distillation method according to EU regulations. (Commission Regulation (EC) No 2074/2005, 2005) Volatile nitrogenous bases were extracted from the samples using a 0.6 mol/L perchloric acid solution. Subsequently, the alkalized extract underwent steam distillation, and the volatile components were trapped in an acidic receiver solution. The TVB-N concentration was ultimately calculated based on the volume of acid consumed during titration and expressed in mg/100 g of sample matrix.

The following equipment was utilized for sample preparation and analysis: a meat grinder for fish mince homogenization, a high-speed blender (8,000–45,000 rpm), fluted filter paper (150 mm diameter) for rapid filtration, and a 5 mL burette with 0.01 mL graduations for titration.

As the initial step of sample preparation, approximately 2 g of fish muscle tissue was weighed and placed in a labeled glass jar. Then 18 mL of perchloric acid was added (at a 1:9 w/v ratio), and the mixture was homogenized using a high-speed blender for 2 minutes, followed by filtration. The resulting extract could be stored for up to 7 days at 2-6 °C prior to analysis.

Figure 4.13. Sample preparation steps: labeled glass jars for sample storage (Left) and the filtration process (Right)

As illustrated in Figure 4.14, semi-automatic distillation was conducted using a VELP UDK 127 distillation unit. A glass Kjeldahl tube was charged with 15 mL of the extract,

supplemented with 2–3 drops of phenolphthalein and 2 mL of sodium hydroxide to ensure alkalization. Concurrently, a receiver Erlenmeyer flask containing 20 mL of boric acid solution and 3–5 drops of Tashiro's indicator was prepared. Following a 10 min distillation period, the volatile bases trapped in the receiver solution were determined by titration with a 0.01 mol/L standard hydrochloric acid solution.

Figure 4.14 VELP Scientifica UDK 127 Distillation Unit (VELP Scientifica, Italy)

To ensure the accuracy of the final results, the titration was monitored using a pH meter until the endpoint of 5.0 (±0.1) was reached. This approach was adopted because the color change of the Tashiro's indicator was not sufficiently distinct within the specific sample matrix to serve as the sole endpoint indicator.

Additionally, a blank trial was conducted following the same distillation and titration procedure; however, the fish extract was replaced with 15 mL of perchloric acid solution to account for any potential nitrogenous traces in the reagents.

The Total Volatile Basic Nitrogen (TVB-N) was calculated using the following equation:

$$TVB - N\left(in\frac{mg}{100g}\right) = \frac{(V_1 - \mathrm{V_0}) \times 0.14 \times 2 \times 100}{M}$$

Where:

V_1 = Volume of 0.01 mol/L hydrochloric acid solution used for the sample (mL)

V_0= Volume of 0.01 mol/L hydrochloric acid solution used for the blank (mL)

M = Weight of the fish sample (g)

0.14 = The weight of nitrogen in mg equivalent to 1 mL of 0.01 mol/L HCl

2 = The dilution factor derived from the sample preparation process

4.3.2.5 Determination of Protein

For protein determination, the Kjeldahl method was employed according to the AOAC Official Method, 928.08.(AOAC, 2000a; Yaranoğlu et al., 2023). Samples were first placed in a lyophilizer using a Lyovapor™ L-200 freeze-dryer. After 24 hours of lyophilization at a condenser temperature of -54.8 °C and a chamber pressure of 0.500 mbar, the freeze-dried were ground into a fine powder.

Figure 4.15. Lyophilization of fish samples using a laboratory freeze dryer (Lyovapor™ L-200, BUCHI)

Following this step, 0.35 g of each ground sample was weighed onto nitrogen-free weighing paper using an analytical balance. Care was taken during handling to prevent skin oil contamination.

The wrapped paper samples were transferred into a Kjeldahl digestion unit, 15 g of sodium sulfate and 0.9 g of copper(II) sulfate pentahydrate as a catalyst were added to each tube. Under a fume hood, 20 mL of concentrated sulfuric acid (98%) was carefully dispensed into each tube using a semi-automatic dispenser.

Subsequently, the tubes were placed in the digestion block to initiate the digestion process for protein extraction. The unit was connected to a fume scrubber system to collect and neutralize the hazardous acid fumes released while the temperature was gradually increased to 400 °C.

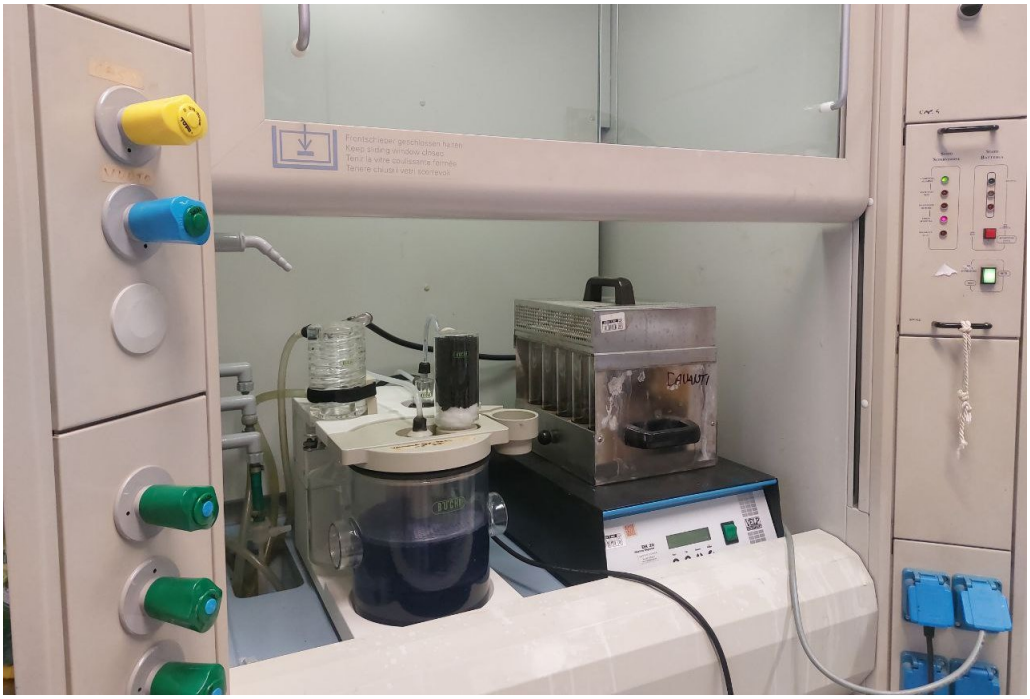

Figure 4.16. The Kjeldahl digestion setup inside a laboratory hood, showing the mineralizer (VELP Scientifica) and the connected fume scrubber system (BUCHI)

Upon completion of the digestion process, the tubes were allowed to cool for 30 min, and a small volume of deionized water was carefully added under a fume hood. Each glass tube was then transferred to the semi-automatic distillation unit (UDK 127, VELP Scientifica). A receiving Erlenmeyer flask with 30 mL of boric acid solution was placed at the outlet. During distillation, a sodium hydroxide solution (40% w/w) was automatically dispensed by the system to alkalize the sample, thereby releasing ammonia into the boric acid solution. After a 5 min distillation period, the distillate was titrated against a standard

hydrochloric acid solution (0.01 mol/L). The titration was monitored using a calibrated pH meter to ensure accuracy until the endpoint was reached (pH 4.6).

Figure 4.17. The ammonia distillation process using the VELP UDK 127 unit (Left), and Digested samples showing the characteristic clear blue-green color (Right)

4.3.2.6 Determination of lipid

To determine the total lipid content of the samples, cellulose extraction thimbles were first labeled, and their bottom sections were filled with quartz sand to increase the extraction surface area. Afterwards, 5 g of the ground sample was weighed into each thimble, and covered with an additional layer of quartz sand. (Figure 4.18) In parallel, the empty glass extraction beakers were weighed to record their tare weight. The lipid extraction was performed using a Soxhlet apparatus according to the AOAC Official Method 960.39, adapted for the Buchi Universal Extractor E-800 (AOAC, 2007). The prepared thimbles were loaded into the apparatus, and the extraction beakers, each containing 80 mL of petroleum ether, were attached to the unit to initiate the automatic extraction process.

Figure 4.18. Fat extraction setup: Sample preparation station including extraction thimbles, and quartz sand (Left); The Buchi Universal Extractor E-800 unit during the extraction process (Right)

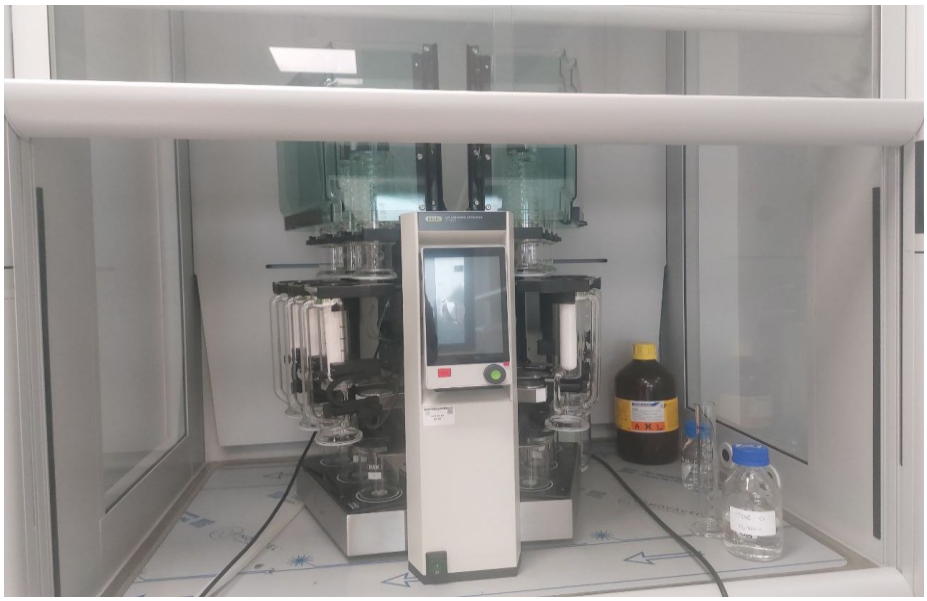

Figure 4.19. Front view of the Buchi Universal Extractor E-800

The Universal Extractor E-800 automatically performed three distinct operational phases: extraction, rinsing, and solvent recovery. The extraction phase lasted 4 h, during which the hot solvent percolated through the samples for 30 cycles to dissolve the lipids.

Subsequently, a 30 min rinsing phase was carried out to remove residual lipid from the thimble using condensed solvent. Following the solvent recovery phase, the beakers containing the extract lipids were placed in a drying oven at 50 °C for 2 h to evaporate any remaining moisture or solvent. The beakers were then cooled to room temperature in a desiccator. Based on the final weight of the beakers, the total lipids content was calculated using the following equation:

$$Fat\ content\ (\%) = \frac{W2 - W1}{Ws} \times 100$$

Where:

W_1= weight of the empty glass beaker (g)

W_2= weight of the beaker containing the extracted fat after drying (g)

W_s= weight of the initial sample (g)

4.3.2.7 Determination of Free Fatty Acids (FFA) content

For evaluation of the acid value of the extracted fat, according to the Official Method 940.28, (AOAC, 2012), initially, 0.15 g of the lipid sample was weighted into an Erlenmeyer flask, followed by the addition of 30 mL of an ethanol/ diethyl ether solution (1:2 v/v). Prior to the titration against a 0.5 N potassium hydroxide (KOH) solution, a few drops of phenolphthalein were added as an indicator. The solution was titrated until a persistent pink color emerged, signaling the endpoint. The volume of titrant consumed was recorded, and the acid value was calculated according to the following equation:

$$FFA\ (\%\ Oleic\ acid) = \frac{V \times N \times 28.2}{p}$$

Where:

V= the volume of KOH used for titration (mL)

N= normality of the KOH solution

P= the fat sample initial weight

28.2= the equivalent weight factor for oleic acid

4.3.2.8 Determination of Salt (NaCl)

Relying on the classic Volhard back-titration principle, the sodium chloride concentration was measured according to the AOAC Official Method 937.09 (AOAC, 2000b). For the quantification of sodium chloride concentration, 2.5 g of each freeze-dried sample was precisely weighed into a 50 mL plastic Falcon tube, and 25 mL of deionized water pre-heated to 70 °C was added. The mixture was homogenized using a rotor-stator homogenizer (ULTRA-TURRAX T25 basic, IKA-WERKE) at 11,000 rpm for 1 min. Subsequently, an additional 25 mL of deionized water was added to the tube. The Falcon tubes were counterbalanced and centrifuged (SL 16, Thermo Scientific) at 2,000 rpm for 5 min at 4 °C to precipitate the insoluble fraction and obtain a clear supernatant.

Figure 4.20. The laboratory centrifuge (SL 16, Thermo Scientific)

A 10 mL aliquot of the supernatant was pipetted into a 100 mL beaker, followed by the addition of 1 mL of 65% nitric acid, 0.5 mL of ferric ammonium sulfate indicator, and 10 mL of 0.1 N silver nitrate. Concurrently, a reference trial was prepared following the same procedure, substituting the sample with 10 mL of a 0.1% standard sodium chloride solution. The mixtures were heated to a boil on a hot plate, after which the inner walls of the beakers were rinsed with deionized water. The solutions were then filtered and

subsequently titrated against a 0.1 N ammonium thiocyanate solution until a persistent orange-brown color emerged, signaling the endpoint.

Figure 4.21. Determination of chlorides: Sample boiling step on the hot plate (Left); the endpoint of the titration (Right)

4.4 Statistical analysis

Data were analyzed using the non-parametric Mann–Whitney U two-tailed test, as implemented in XLSTAT (Addinsoft, Paris, France). The test was used to compare the effects of the main experimental factors on the investigated parameters. Specifically, comparisons were performed between samples washed before freezing (WB) and after freezing (WA), between polyphenol-supplemented (F) and control (C) samples, and among the different storage times during shelf-life studies (T0, T15, T30, T45, and T60). Pairwise comparisons between sampling times were carried out to evaluate temporal changes during storage. Statistical significance was established at $p < 0.05$ for all statistical analyses and graphical representations.

Chapter 5

5 Result and Discussion

The chapter is structured into three main sections: the first section details the comparative results between technical preparation methods, followed by a comparison between the treated groups and the control group, while the final section reports the influence of storage time on shelf life.

5.1 Comparative Analysis of Technical Preparation Methods

To compare the effect of different technical preparation methods (wash-after-freezing and wash-before-freezing), selected physicochemical parameters were determined. Among all parameters, a highly significant difference ($p = 0.004$ and $U = 342.5$) in pH values was observed. Statistical analysis showed that the mean pH for Process-WB (7.07 ± 0.38) was significantly higher than that of Process-WA (6.91 ± 0.35). As depicted in Figure 5.1, Process-WB shifts the system toward a more neutral/alkaline state compared to Process-WA. Our findings indicate that the pre-freezing washing cycle (Process-WB) effectively removes water-soluble organic acids, particularly lactic acid accumulated post-mortem as well as acidic sarcoplasmic proteins, which following this elevates the pH toward a neutral state. This findings are in baseline agreement with Zhang et al (2022), which similarly demonstrated that the pH value of surimi undergoes a statistically significant increase during successive washing steps, shifting from 6.32 in the unwashed matrix (W0) to 6.83 after three washing cycles (W3) ($p < 0.05$) .On the other hand, the reduction in pH observed in the Process-WA samples can be attributed to the continuation of post-

mortem glycolysis and the progressive accumulation of lactic acid within the unwashed fish mince during its initial frozen storage phase prior to the delayed washing step. Furthermore, because the myofibrillar proteins in the WB group were protected from acid-induced denaturation during freezing, they remained structurally intact until cooking. Upon thermal processing, these intact proteins underwent optimal denaturation, exposing a greater number of hidden alkaline amino groups to the matrix, which effectively sustained a higher pH. Conversely, the prolonged exposure of unwashed meat to endogenous enzymes in WA compromised the protein buffering capacity, resulting in a lower and less stable pH profile during subsequent refrigerated storage.

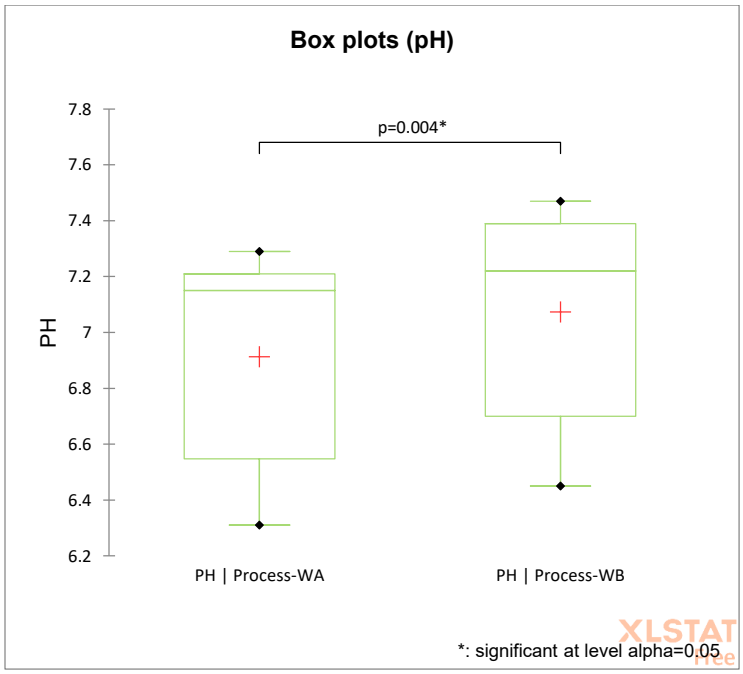

Figure 5.1. pH of prepared surimi as affected by different technical processing methods (Process-WA vs. Process-WB).

In line with pH changes, the total protein content exhibited a significantly higher value in the Process-WA samples compared to the Process-WB group. The results of the non-parametric Mann-Whitney U test (as shown in Figure 5.2) confirmed a highly significant difference between the preparation methods ($U = 656.5$, $p < 0.0001$), with the mean protein percentage of Process-WA (22.767 ± 0.973%) being markedly higher than that of Process-WB (20.531 ± 1.576%).

The higher protein retention observed in the Process-WA (wash-after-freezing) samples can be scientifically explained by the lack of removal of water-soluble sarcoplasmic proteins during the prior freezing stage. This absence of an immediate washing step allows endogenous enzymes to remain active and subsequently lower the pH, which induces severe structural unfolding and denaturation of both myofibrillar and sarcoplasmic proteins. While retaining these two protein fractions increases the total protein percentage, this structural change eventually weakens the final gel-forming ability of the prepared surimi. This finding is in accordance with the results obtained by Li et al . (2016), on catfish (*Clarias lazera*) surimi, which similarly illustrated that refrigerated storage without prior washing induces severe protein denaturation and a significant reduction in protein solubility. In conclusion, performing the washing cycle in the first place effectively removes myoglobin and water-soluble sarcoplasmic proteins, whose presence would otherwise compromise the color quality, induce off-odors, and impair the final formation of a strong surimi gel network.

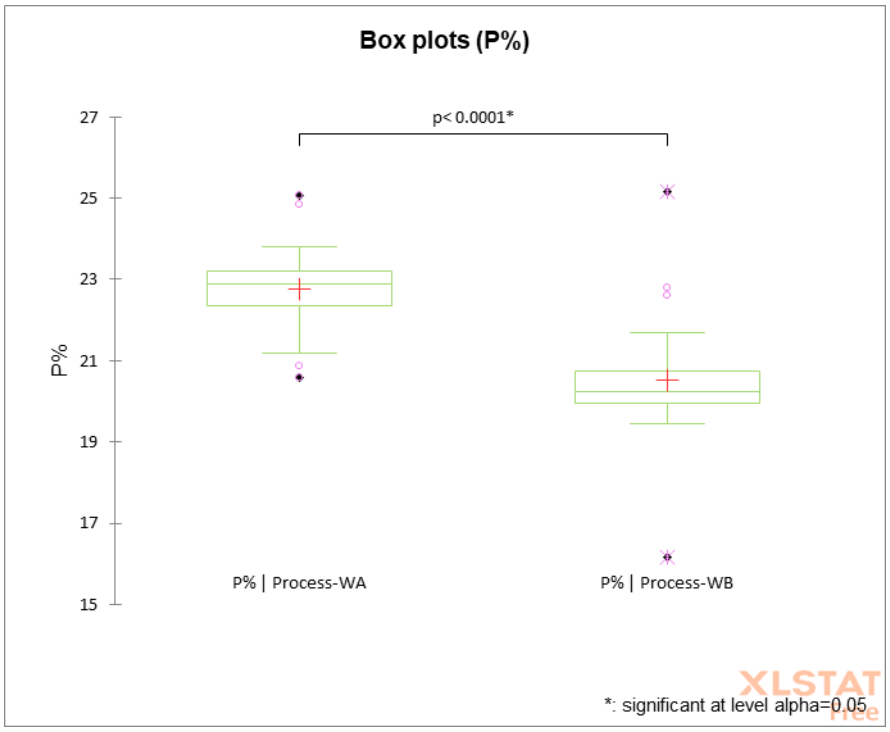

Figure 5.2 Total protein percentage (P%) of prepared surimi as affected by different technical processing methods (Process-WA vs. Process-WB).

As discussed, the lower protein content in the wash-before-freezing process inherently creates more relative space within the muscle matrix for water retention. At the same time, the elevated pH observed in the Process-WB samples plays a role in enhancing the Water Holding Capacity (WHC) of the surimi. This phenomenon is driven by the generation of a net negative charge on the myofibrillar proteins, which promotes the structural unfolding of the protein chains to create sufficient internal space for water. Figure 5.3 indicates the results of the non-parametric Mann-Whitney two-tailed test, showing a highly significant difference ($U = 314$, $p = 0.002$) in moisture content between the preparation methods .The mean moisture percentage of the Process-WB samples ($71.694 \pm 3.556\%$) was markedly higher than that observed in the Process-WA group ($67.639 \pm 6.348\%$).

This statistical confirmation supports the previously discussed biochemical mechanism and is in agreement with the results of Ramadhan et al. (2014), who showed that increasing the washing process with salt significantly elevated the WHC. In light of these results, while the presence of maltodextrin stabilizes the protein network structure, Process-WB inherently elevates moisture levels, which can be theoretically attributed to a higher WHC. This trend can also be attributed not only to a higher WHC but also to the preservation of free and immobilized water fractions within the intact cellular matrix, as these water components were not depleted by the thawing-induced drip loss that occurs prior to washing in the Process-WA approach.

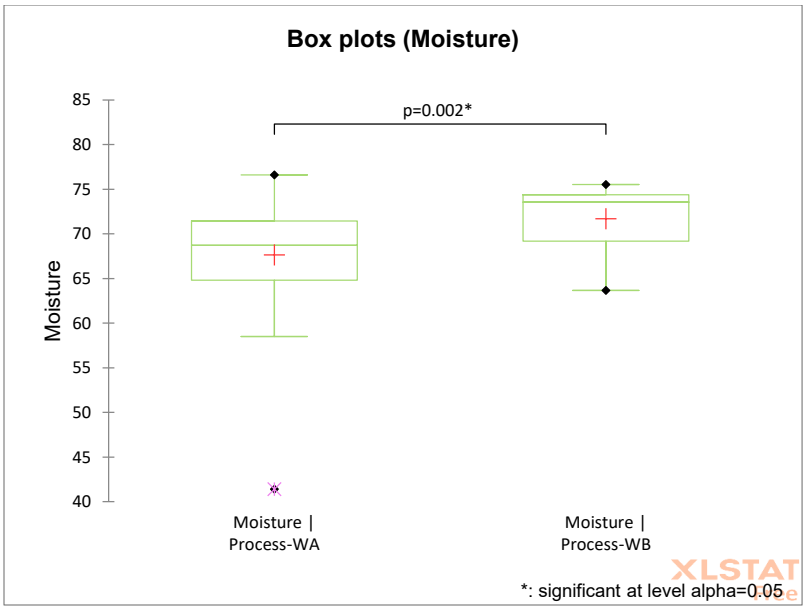

Figure 5.3 Moisture content of prepared surimi as affected by different technical processing methods (Process-WA vs. Process-WB).

Regarding the remaining parameters, no statistically significant differences ($p > 0.05$) were observed between the two technical preparation methods.

5.2 Efficacy of OMW Treatment: Treated and Control Group Analysis

Regarding the efficacy of the phenol-treated group, a significantly lower measurable salt content was observed compared to the control group ($U = 630$, $p = 0.001$). Specifically, the group treated with phenolic compounds exhibited a lower salt content (0.320 ± 0.069%) than the control group (0.398 ± 0.090%). Based on the findings of Balange and Benjakul (2009), on mackerel (*Rastrelliger kanagurta*) surimi, this lower salt retention in polyphenol-treated samples can be scientifically justified by the formation of a reinforced myofibrillar protein network. The incorporated polyphenols act as molecular cross-linkers that bind tightly to the protein active sites, thereby inducing steric hindrance and masking the ionic binding sites with their phenolic rings. (C. Sun et al., 2026) This rapid, non-covalent cross-linking promotes a premature aggregation and structural compaction of the protein matrix. Consequently, the accessibility of salt (NaCl) to the protein core is restricted, suppressing its capacity to effectively unfold and solubilize the actomyosin filaments during the final salting stage.

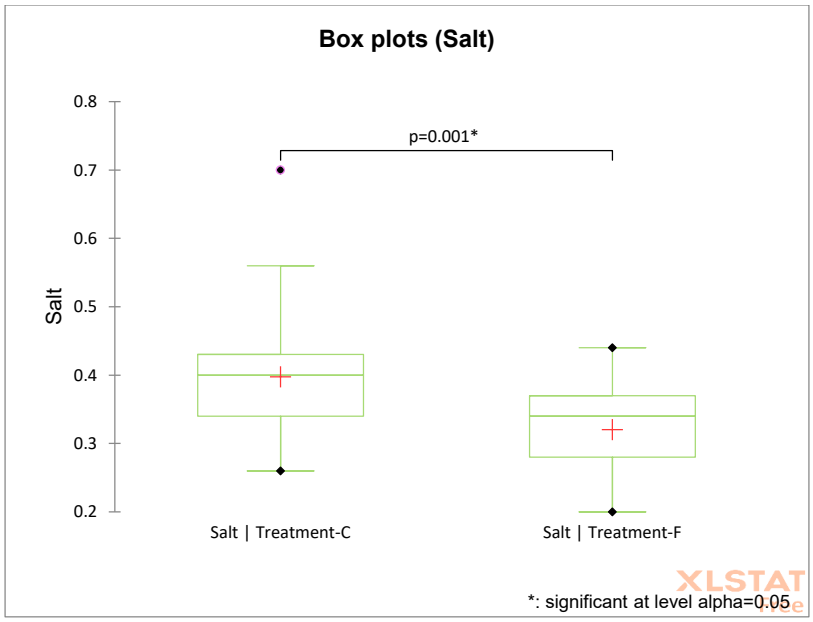

Figure 5.4 Salt content of prepared surimi as affected by phenolic compound treatments (Treatment-F) compared to the control group (Treatment-C) (p = 0.001).

This salt finding paves the way to understand the changes in water activity (a_w). In fact, the results confirm a significant shift in a_w between the phenolic treatment and the control group (U = 408.500, p = 0.007). As shown in Figure 5.5, the treated group exhibited a higher a_w (0.993 ± 0.004) than the control group (0.991 ± 0.002).

According to the polyphenol-protein interactions discussed by Lešnik et al. (2025), the increase in water activity (a_w) upon the addition of Olive Mill Wastewater (OMW) extract can be attributed to the water-mediated hydrogen bond bridges formed between polyphenols and proteins. This phenomenon drives a dynamic influx of water into the binding sites and the subsequent displacement of trapped water into a free state. These findings can be interpreted in the present work to scientifically justify the higher a_w value observed in the phenolic-treated group. Besides that, OMW inherently contains hydrophilic organic matter, and organic acids. These components may alter the osmotic balance within the matrix, leading to a further liberation of bound water.

In summary, the polyphenolic compounds in OMW likely interacted with the myofibrillar proteins via water-mediated pathways, restructuring the water network and displacing a fraction of bound structural water into mobile, free water states within the food matrix.

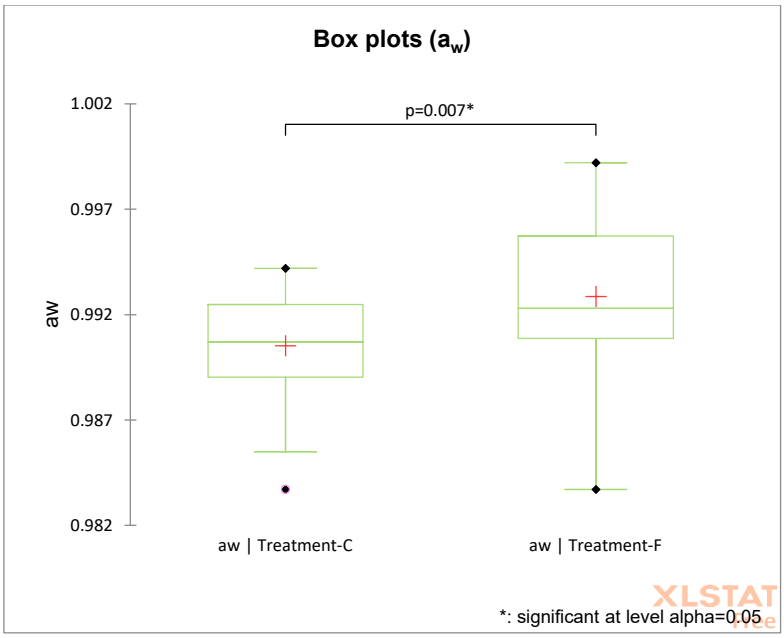

Figure 5.5 a_w content of prepared surimi as affected by phenolic compound treatments (Treatment-F) compared to the control group (Treatment-C) (p = 0.007).

Based on our preliminary visual and olfactory observations, which did not constitute a structured sensory evaluation, there were no clear changes in the appearance of either the control or phenol-treated groups, except for the odor. (Figure 5.6) In the phenol-treated groups, the fishy odor was noticeably lower than in the control group. This qualitative observation is in agreement with Huang et al. (2022), who reported that phenolic acids (such as gallic acid and ferulic acid, which are also found in OMW) help eliminate protein-bound fishy odors in sea bass. However, it is worth noting that more advanced analytical methods, such as volatile organic compound (VOC) analyses, should be utilized in future studies to precisely define and characterize the subtle changes in the overall aroma profiles. In summary, these preliminary observations suggest that phenolic compounds might help reduce fishy odor, though further structured testing is required to confirm consumer acceptability.

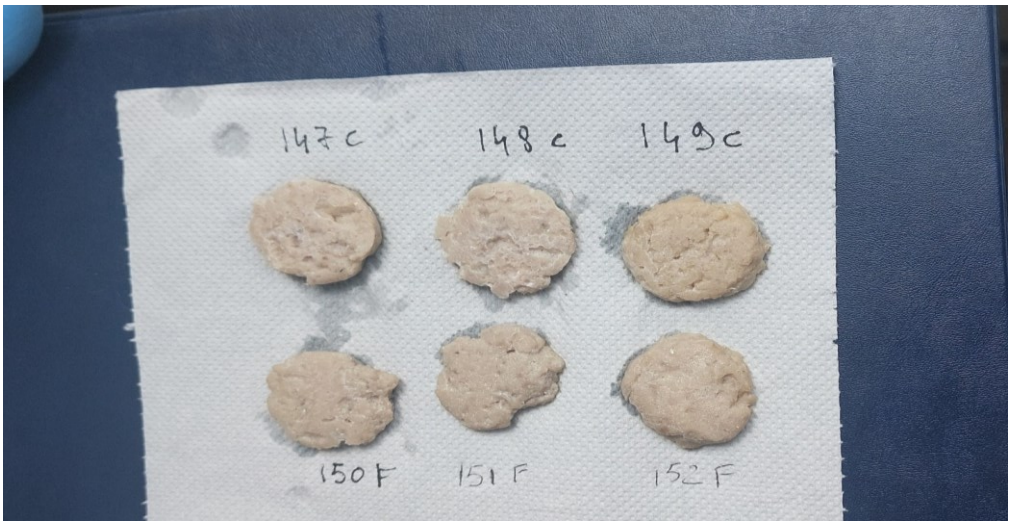

Figure 5.6 Preliminary visual appearance of control and phenol-treated surimi samples during the storage period, showing no distinctive alterations between the experimental groups.

As for the other evaluated parameters, their values remained unaffected and showed no statistical deviation from the untreated control. However, to evaluate the impact of the treatment on different microbiological parameters, a non-parametric pairwise Mann-Whitney U test (two-tailed) was performed. This test was used to compare the distributions of five bacterial and microbial populations between the control (C) and treatment (F) groups. The results of the analysis, summarized in the chart in Figure 5.7, indicated no statistically significant differences between the two experimental conditions for any of the tested microbial targets. Specifically, the asymptotic significance (p-value) for mesophilic bacteria ($U = 778.0$, $p = 0.812$), molds and yeasts (OGA) ($U = 777.0$, $p = 0.606$), lactic acid bacteria (MRS) ($U = 798.0$, $p = 0.975$), psychrotrophic bacteria ($U = 790.0$, $p = 0.887$), and *Staphylococcus* (MSA) ($U = 179.5$, $p = 0.970$) were all well above the standard significance threshold of 0.05.

This observation demonstrates that the treatment did not exert a significant inhibitory or stimulatory effect on the growth and survival of these microbial groups under the tested conditions. This lack of significant difference can be explained by the effectiveness of preparation methods and MAP packaging alongside optimal storage conditions.

As proven by literature, modified atmosphere packaging (MAP), utilizing carbon dioxide (CO_2) (Lee et al., 2022) and nitrogen (N_2) either in combination or solely, substantially extends the shelf life of food products, including meat (Bingol & Ergun, 2011), by inhibiting bacterial growth.(McMillin, 2020) It has long been established that under anaerobic conditions, carbon dioxide and nitrogen can extend the shelf life of fresh products by up to 7-fold and 2-fold, respectively, compared to air. (Enfors et al., 1979)

This anaerobic environment inhibits aerobic bacteria from growing; however, our results show that this stability depends on a combination of factors rather than gas composition alone. The precise selection of the polymer film and a constant storage temperature of 4 °C collectively contributed to maintaining stable storage conditions. This barrier system was so effective that it exerted a maximum inhibitory impact even within the control group, masking the need for any additional synergistic effect between the packaging and the polyphenol treatment.

Furthermore, the initial microbiological quality played a crucial role in this extended lag phase. The application of the thermal cooking procedure during surimi preparation successfully inactivated and minimized the vast majority of the initial microflora on day zero. This thermal reduction in the initial microbial population, combined with the low storage temperature of 4 °C, severely restricted the metabolic activity and replication of the bacteria. The collective impact of these hurdles successfully maintained the total bacterial counts below 5 log CFU/g throughout most of the storage period, which remains well below the acceptable safety and spoilage threshold of 6–7 log CFU/g established for meat products. (ICMSF, 1986) Therefore, this integrated approach can be presented as a highly effective and optimal preservation strategy for surimi-based products.

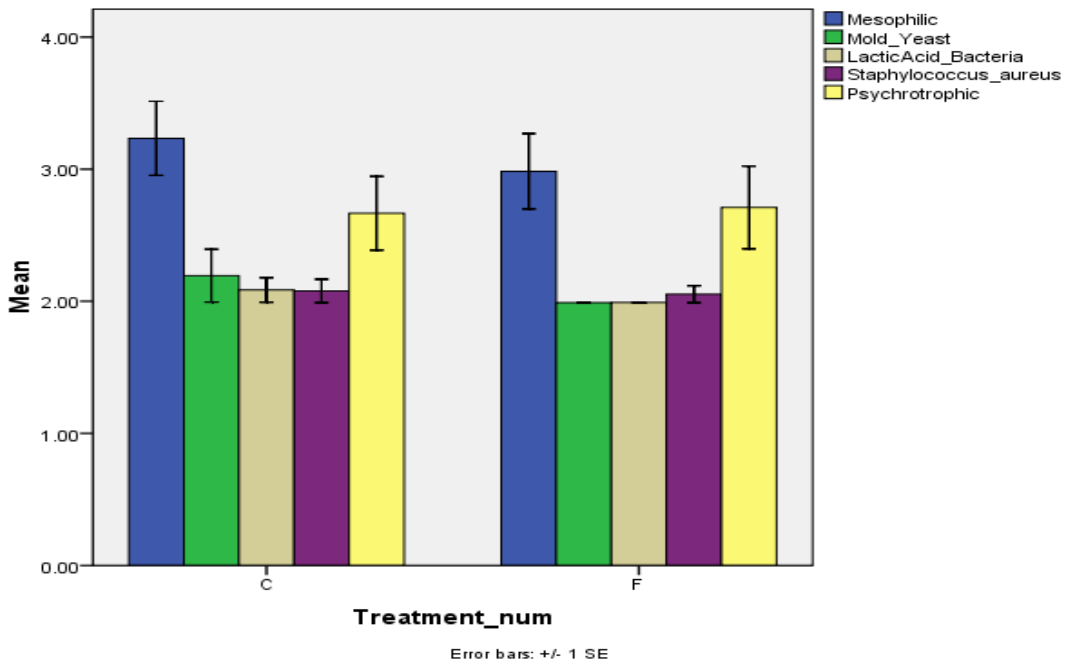

Figure 5.7 Microbial counts of surimi-based products under control (C) and polyphenol treatment (F) conditions (LOD = 2.0 log CFU/g. Samples with 0 colonies plotted as 1.99 log CFU/g.)

The success of this integrated approach in controlling microbial proliferation directly correlates with the suppression of chemical spoilage markers. As comprehensively reviewed by Kamal et al. (2025), preserving aquatic food products remains a critical challenge due to highly unsaturated lipid profiles and rapid post-mortem degradation, which typically occurs through three main pathways: microbial proliferation, lipid oxidation, and protein degradation. In a standard spoilage scenario, native Gram-positive and Gram-negative bacteria rapidly penetrate tissues, while psychrophilic bacteria utilize non-protein nitrogen compounds during refrigerated storage to cause unpleasant odors and color changes.

To monitor this degradation, the accumulation of Total Volatile Basic Nitrogen (TVB-N) serves as a primary biochemical index of muscle food spoilage, marking the progressive loss of freshness during storage. Following glucose depletion, bacterial and endogenous enzymes shift to proteolytic degradation, reducing trimethylamine-N-oxide (TMAO) into volatile trimethylamine (TMA) and deaminating amino acids to liberate free ammonia (NH3). Concurrently, bacterial decarboxylation converts free amino acids into toxic

biogenic amines.(Bekhit et al., 2021; Hopkins et al., 2020) In the present work, however, because the initial microbial population was successfully restricted by the thermal cooking process and barrier systems, the subsequent evaluation of TVB-N and microbial loads did not show any significant indicators of deterioration. This lack of volatile nitrogen accumulation provides solid biochemical evidence demonstrating that our surimi preparation setup was highly effective in preventing both microbial and enzymatic spoilage.

5.3 Shelf Life Analysis over Storage Time and Microbial Spoilage

With regard to the effect of storage time on the overall quality of both surimi batches, the changes during the 60-day storage period were evaluated using a pairwise Mann-Whitney U test. As illustrated in Figure 5.8, the baseline salt content at T0 was 0.331 ± 0.050%. Although the salt level showed slight fluctuations throughout the storage period, statistical analysis revealed that the only significant difference occurred between T0 and T30 ($p = 0.008$), where the salt content reached its peak at 0.386 ± 0.056%. No significant differences ($p > 0.05$) were observed among the other storage time intervals.

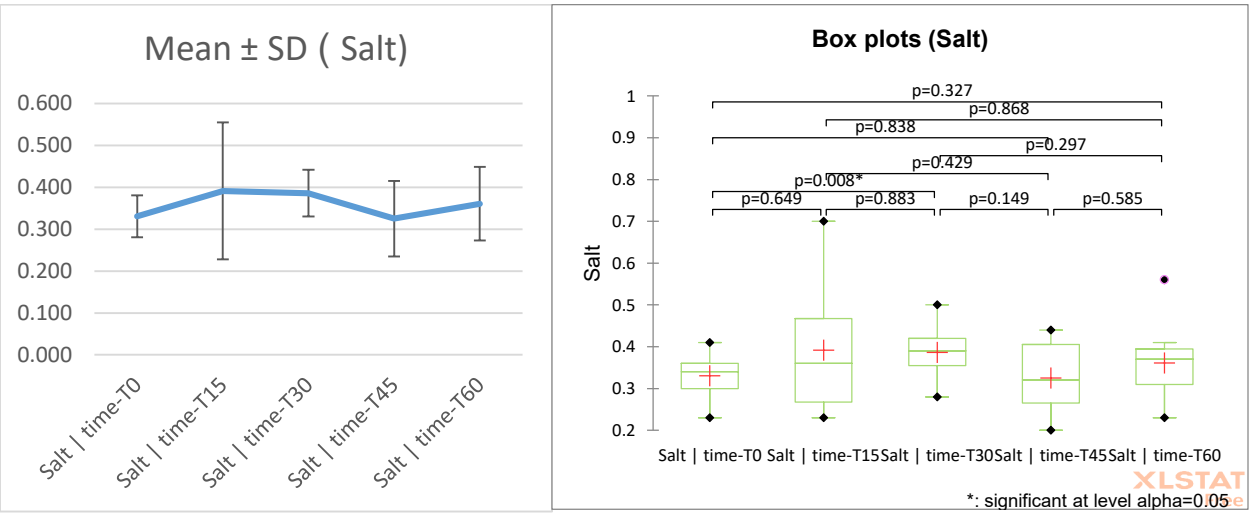

Figure 5.8 Dynamics of salt content in surimi samples during the 60-day refrigerated storage period. The left panel illustrates the Time-based trend based on Mean ± SD, and the right panel presents the detailed box plots along with pairwise comparison p-values. Asterisk (*) denotes a statistically significant difference ($p < 0.05$).

However, it is important to note that due to the significant amount of missing data at T15, the statistical reliability of that specific time point might be limited. Alternatively, from a biochemical standpoint, this peak observed at T30 could be attributed to the leakage of trapped water (syneresis/drip loss) from the protein network. Although surimi lacks the organized cellular and fibrous structure of fresh meat, the phenomenon of syneresis follows the same molecular rules in both systems. As described by Mizrahi (2010), syneresis in food gels induces physical instability, which in this case acts as a carrier to release the bound NaCl ions into the free-water phase.

Statistical analysis of the moisture content during the 60-day storage period, as shown in Figure. 5.9, indicated that the highest mean was at T0 (72.385 ± 2.949%). The moisture level dropped to 67.096% at T15 (showing high variation and an outlier) before stabilizing around 69% at T30 and T45. According to the pairwise comparison matrix, the only significant difference was found between T0 and T30 ($p = 0.012$), confirming a notable decrease after 30 days. Other intervals, such as T0 vs. T15 ($p = 0.093$) and T0 vs. T60 ($p = 0.097$), showed no significant differences, proving that the moisture content remained stable through the rest of the storage period.

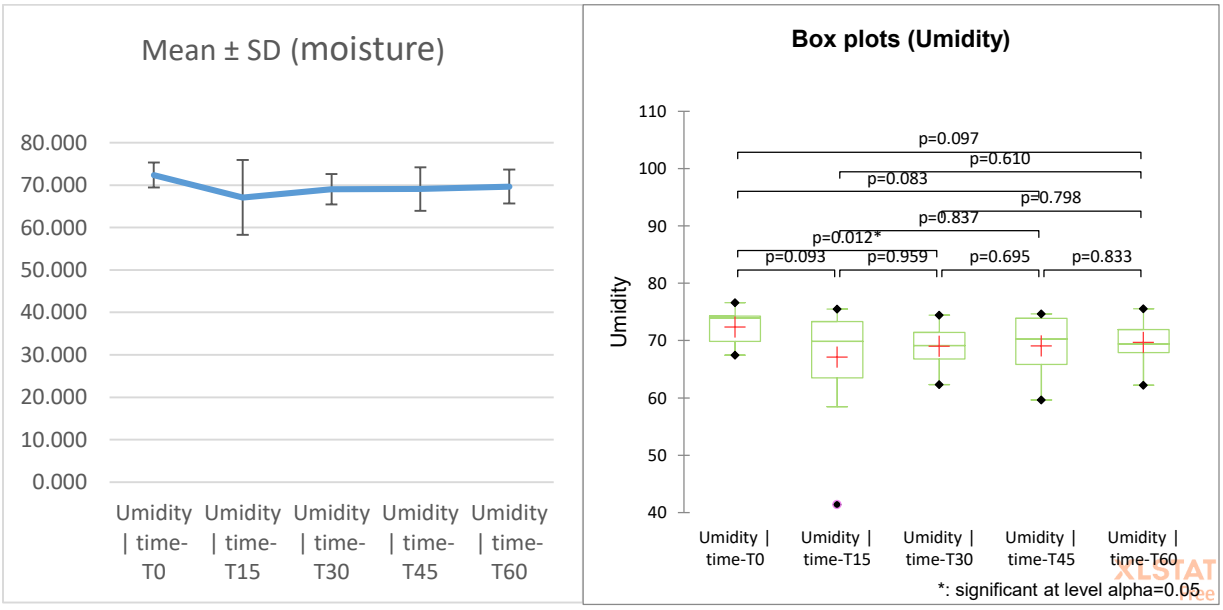

Figure. 5.9. Dynamics of moisture content in surimi samples during the 60-day refrigerated storage period. The left panel illustrates the Time-based trend based on Mean ± SD, and the right panel presents the detailed box plots along with pairwise comparison p-values. Asterisk (*) denotes a statistically significant difference ($p < 0.05$).

During refrigerated storage, the gradual alterations in the cooked myofibrillar protein network can diminish its water-holding capacity, leading to the gradual release or dissociation of bound water from the gel matrix, as observed around T30. Our findings regarding total protein content (P%) changes during the 60-day storage are presented in Figure 5.10. According to the summary statistics, the highest mean protein content was observed at Day 0 (T0) with 22.645 ± 1.593%. As the storage time progressed, the protein percentage showed a gradual and steady decline, reaching its lowest value at Day 60 (T60) with 21.307 ± 1.099%. The slight decrease in protein percentage over time can be attributed to the gradual release of water and soluble nitrogenous compounds from the cooked gel network during refrigerated storage.

The pairwise comparison matrix revealed that this decrease was statistically significant only between T0 and T60 ($p = 0.030$). No statistically significant differences ($p > 0.05$) were found between any other consecutive storage intervals. This indicates that while the initial thermal cooking process induced a complete denaturation of the myofibrillar proteins into a structured gel, the subsequent degradation during storage was not severe enough to rapidly destroy this matrix. Furthermore, this structural preservation can be scientifically attributed to the protective effect of the added maltodextrin. Acting as a water-binding and textural stabilizing agent, maltodextrin effectively maintained protein stability and restricted matrix collapse for nearly two months. These interactive dynamics between protein stability and moisture alterations are consistent with the cryoprotective mechanisms previously described by Chen et al. (2022).

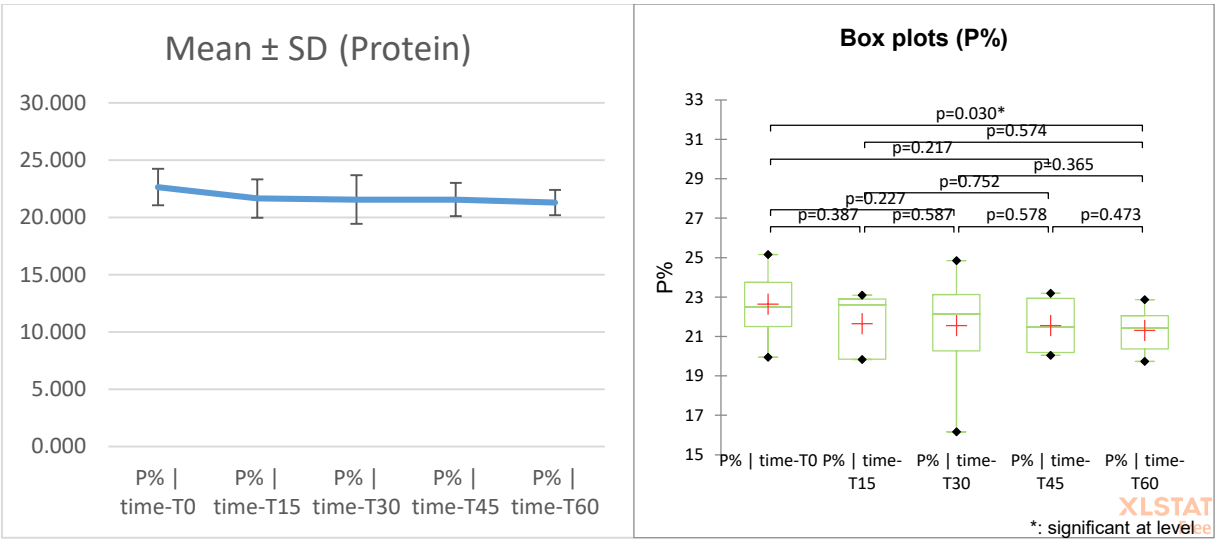

Figure. 5.10. Dynamics of protein content in surimi samples during the 60-day refrigerated storage period. The left panel illustrates the Time-based trend based on Mean ± SD, and the right panel presents the detailed box plots along with pairwise comparison p-values. Asterisk (*) denotes a statistically significant difference ($p < 0.05$)

The changes in acidity percentage (A%) of the surimi samples during the 60-day refrigerated storage period are presented in Figure 5.11. The baseline acidity started at 1.771 ± 0.008 at Day 0 (T0). Pairwise comparisons revealed a highly significant increase by Day 15 (T15 = 1.777%; $p < 0.0001$) compared to T0 . As storage time progressed, the acidity percentage generally increased, with higher values observed at the later sampling points, reaching its highest levels by the end of the storage trial (T60).

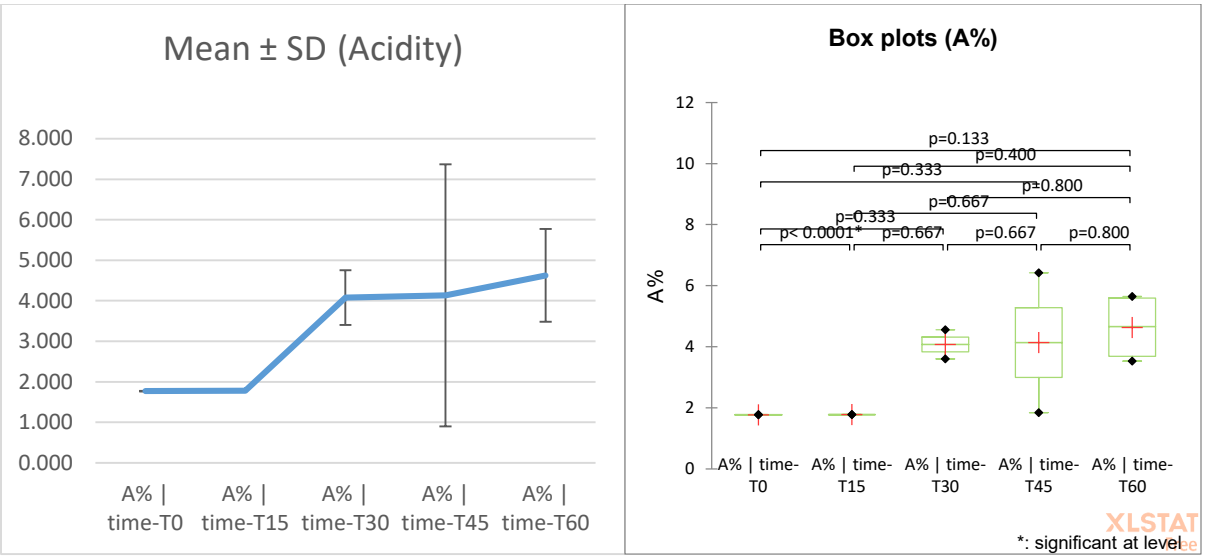

Figure. 5.11. Dynamics of acidity in surimi samples during the 60-day refrigerated storage period. The left panel illustrates the Time-based trend based on Mean ± SD, and the right panel presents the detailed box plots along with pairwise comparison p-values. Asterisk (*) denotes a statistically significant difference ($p < 0.05$).

However, due to the limited sample size at certain time points in this experiment, a formal inferential statistical analysis could not provide fully reliable conclusions. Therefore, we reported the direct observation of raw data trends as a complementary analysis to interpret these results alongside the statistical analysis.

The raw data evaluating acidity percentage over time highlighted differences in the evolution of acidity between the control and phenol-treated samples during storage. According to the line chart presented in Figure 5.12, both groups exhibited nearly identical acidity levels at the baseline (T0). However, as storage progressed, acidity generally increased, with noticeable differences emerging at intermediate sampling points. At T30, the control sample (C1e = 4.56%) showed a higher acidity level than the phenol-treated sample (F1E = 3.60%). This difference was most pronounced at T45, where the control sample (C2k) reached a peak value of 6.42%, while the phenol-treated sample (F2j) remained relatively stable at 3.65%. By the final sampling point (T60), acidity values converged between the two groups, with the control samples recording 5.30% and 5.61%, and the phenol-treated samples reaching 5.53% and 5.54%.

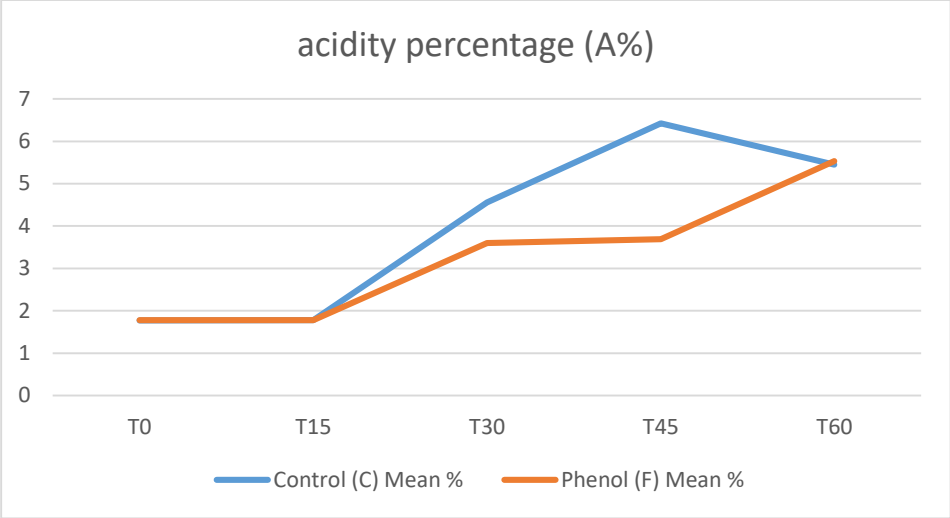

Figure. 5.12. Comparative Time-based trends in acidity percentage (A%) between the control (C) and phenolic-treated (F) surimi batches during the 60-day refrigerated storage period

Overall, these descriptive findings suggest that while both groups ultimately experience an increase in acidity by the end of the storage period, the control samples appear to undergo a faster and more intense acidification process during the intermediate phases (T30 and T45) compared to the phenol-treated samples. Although the limited number of samples is not sufficient for a comprehensive statistical conclusion, this finding highlights the potential of phenolic compounds in maintaining stable acidity levels during storage. This is consistent with existing literature, such as Wu et al.(2023), who found that antioxidant powders rich in phenolic compounds can lower the acid value in surimi over time.

6 Conclusions

The primary objective of this study was to establish and optimize the technical set-up for surimi preparation from Red Mullet (*Mullus barbatus*), evaluating the effectiveness of mechanical washing cycles and processing techniques. Concurrently, this work investigates the potential of utilizing agri-food byproducts, specifically phenolic compounds extracted from OMW, as a natural preservative rather than treating them merely as waste. Ultimately, it demonstrates that the shelf life, physicochemical properties, and microbial stability of the final product are driven by a multi-layered interaction of these processing steps, active treatments, and packaging systems, with the polyphenolic treatment serving as one integrated contributing factor.

Regarding technical preparation, the physicochemical and statistical data demonstrate that the wash-before-freezing method (Process-WB) is scientifically superior to the wash-after-freezing approach (Process-WA). Although Process-WA shows a higher total protein content, keeping sarcoplasmic proteins for a long time causes free fatty acid accumulation and a following pH drop, which potentially compromises the final gel-forming ability. In contrast, Process-WB results in a significantly higher moisture percentage and an elevated pH. Mainly, this moisture is effectively bound and entrapped within the alkaline-shifted protein matrix. Because this water is restricted within the structured protein network and remains unavailable for bacterial growth, it does not compromise the product's shelf life. Based on this evidence, it can be concluded that Process-WB provides a more stable product with better quality.

In terms of polyphenolic efficacy, although the phenolic compounds did not directly extend the surimi shelf life due to the highly effective baseline hurdle technologies, their contribution was demonstrated by an improved aroma profile through the reduction of fishy odor, alongside the mitigation of free fatty acids (FFAs). This qualitative improvement, combined with lower salt retention and controlled free fatty acid (FFA) development, markedly enhanced the final product’s overall quality. In other words, an ideal antioxidant and quality enhancer should limit the formation of lipid oxidation products or protein breakdown products (such as TVB-N). Therefore, the lack of significant

differences in these evaluated chemical indices confirms the non-destructive nature of the phenolic treatment and the selected technical protocol in preserving the fundamental quality of the surimi matrix.

Ultimately, this study provides an optimal preparation and packaging framework for the seafood industry to minimize initial microbial proliferation and early-stage structural damage. These findings pave the way for utilizing natural antioxidants as a non-destructive strategy to refine the sensory profiles and structural stability of surimi, successfully meeting the growing consumer demand for sustainable and clean-label seafood products.

7 Limitation and Recommendations for future research

A limitation of this study was the relatively small sample size of Red Mullet collected during specific trial periods. This constraint makes it challenging to fully generalize the statistical evaluations for certain experiments. Also another limitation of this study was that the combined, multi-stage processing conditions including the washing cycles, cooking step, packaging, and storage environment, provided an overly optimized environment that heavily suppressed bacterial growth across all groups. Because microbial proliferation did not occur even in the control samples, evaluating the true, isolated antimicrobial efficacy of the added phenolic compounds was highly challenging. Therefore, further research is needed to investigate these samples under storage conditions that deliberately allow for microbial challenge. This approach will provide a more precise understanding of how effectively these antimicrobial phenolic compounds function in the surimi matrix. In addition, the lipid extraction step should be optimized using Accelerated Solvent Extraction (ASE) to minimize oxidative degradation and enhance antioxidant recovery. Furthermore, investigating the gel-forming ability and water-holding capacity (WHC) of the surimi would be highly beneficial to comprehensively validate the functional potential of the wash-before-freezing (Process-WB) method. Future studies should incorporate volatile organic compound (VOC) analyses to

objectively characterize the changes in the surimi aroma profile and validate these preliminary olfactory observations. Finally, aligned with the growing demand for sustainable food systems, future studies should evaluate the feasibility of utilizing green and advanced extraction techniques, such as supercritical CO_2 extraction and ultrasound-assisted extraction, to develop a comprehensive, efficient, and safe preservation methodology.

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