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Enhancing storage stability of smoke-flavored horse mackerel filets using natural extracts as preservatives

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The Atlantic horse mackerel (*Trachurus trachurus*) is a globally favored fish due to its abundance, nutritional value, and affordability, but it faces quality preservation challenges. To address this, this study aimed to enhance its value by creating low-salt smoked products with natural bioactive compounds from seafood and forest sources. The fish filets were divided into four groups: one as a control, and the others were treated with various bioactive extract solutions, specifically pine bark, mussels, and microalgae. After 15 days of storage at 4°C, significant differences in properties were observed. Moisture and salt had an inverse relationship, with decreasing moisture and pH over time. Oxidation levels remained acceptable, although sensory quality was affected by storage. Microbiological analysis uncovered high contamination levels in certain samples at specific points in time, although no pathogens such as *Salmonella* spp. or *Listeria monocytogenes* were detected. While microalgae extract was the most powerful antioxidant, its performance was hampered by the poor sensory scores. On the other hand, pine bark extract was the most acceptable from a sensory point of view and revealed some antimicrobial inhibition. Using natural antioxidants provides an appealing solution for consumers seeking products with clean labels.

KEYWORDS

bioactive extracts, salt reduction, smoked fish, pine bark extract, mussel extract, microalgae extract

1 Introduction

Fisheries and aquaculture products play a vital role in human food, and it is estimated that about 15% of the world's ingested animal protein comes from fish (FAO, 2012, 2017). Portugal is one of the largest consumers of fish in the world (first consumer of fish in European Union and third worldwide), with abundant fish species on its coast, but little valued both commercially and by the final consumer, leading to the need for simultaneous import of significant volumes of other species (Almeida et al., 2015; Silva et al., 2020). Fish is greatly perishable but an essential foodstuff due to its protein and unsaturated fatty matter contents. Horse mackerel (*Trachurus trachurus*), a medium-fat species abundant in the northeast Atlantic (Adeyemi et al., 2013), plays a significant role in the world's marine fisheries (Karoui and Hassoun, 2017).

Currently, the food industry's quest for extended shelf life, in conjunction with consumers' preferences for healthy, safe, and convenient food options, has spurred the exploration of innovative preservation methods. Within the food sector, the employment of antioxidants ranks among the frequently employed means to manage lipid oxidation. Various traditional techniques for delivering antioxidants have been utilized for this objective, including direct mixing for minced products and methods such as spraying, glazing, or injection for whole muscle pieces, as discussed in a study (Baptista et al., 2020). Antioxidants, whether natural or synthetic, play a crucial role in slowing down or preventing the oxidative breakdown of substances, especially unsaturated fatty acids, during processing and storage. These antioxidants need to efficiently hinder oxidation at lower concentrations, maintain stability throughout processing and storage, and should be free from any undesirable odors, tastes, or toxicity. Additionally, antioxidants contribute to prolonging shelf life without adversely affecting sensory attributes or nutritional content (Rathod et al., 2021).

Numerous plant extracts have been used for food applications (Pazos et al., 2006; Khan et al., 2009; Ucak et al., 2011). Recent efforts focus on the positive role of antioxidant molecules in plant extracts. Thus, successful applications have widely been carried out on marine oils (Thorisson et al., 1992; Hamilton et al., 1998), minced fish (Ramanathan and Das, 1992; Boyd et al., 1993) and filets (He and Shahidi, 1997; Khalil and Mansour, 1998). In works published so far, bark extracts are a good source of phenolic compounds (Jerez et al., 2007; Aspé and Fernández, 2011) with different extraction processes using various solvents, affecting the composition and biological activity of the extracts (Tümen et al., 2018). Bark extracts of *Pinus pinaster* have a mixture of many substances used to treat a wide range of degenerative diseases through their antioxidative, anti-inflammatory, antitumor, antiatherogenic, antiviral, and antimicrobial properties. They have cardiovascular and cholesterol-lowering benefits and increase microcirculation by increasing capillary permeability (Tümen et al., 2018). Nowadays, there is great interest centered on the potential benefits of adding bioactive compounds to food products due to their known antioxidant and antimicrobial activities, arousing scientific interest mainly due to the high number of microorganisms resistant to emerging antibiotics (Balasundram et al., 2006; Jerez et al., 2007; Seabra et al., 2012; Chupin et al., 2015), and Atlantic pine bark extracts becoming in some way interesting for the pharmaceutical and food industries, mainly by patenting them as a source of procyanidins under the trade names of Pycnogenol®, Oligopin® as well as Flavangenol®, Mármol et al. (2019), Dziedziński et al. (2021), and Alonso-Esteban et al. (2022). Some studies published on using Pycnogenol in meat products (Ahn et al., 2004, 2007; Hameş-Kocabaş et al., 2008) reported a reduction of *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella typhimurium* and delayed growth of *Listeria monocytogenes* and *Aeromonas hydrophila*. In studies carried out by Iglesias et al. (2010), adding maritime pine bark extracts to fish muscle reduced the formation of lipid oxidation products.

Regarding seaweeds, highly productive photosynthetic organisms, their great metabolic and physiological diversity makes them a sustainable source of various products with commercial interest. In recent years, much interest has been focused on the biotechnological potential of macro and microalgae. As a natural source of bioactive compounds, algae have a wide range of biological activities, including antimicrobial, antioxidant, antitumor, antiviral, and anti-inflammatory, besides other health benefits (Mendes et al., 2013; Fleita et al., 2015; Michalak and Chojnacka, 2015; Pane et al., 2015; Sousa et al., 2016). Until now, the antimicrobial potential of algae has generally been tested *in vitro*, providing reliable quantitative estimates of minimum inhibitory concentration (MIC) values for many samples (Pina-Pérez et al., 2017). Compounds reported as present in algae include phlorotannins, terpenoids, phenolic compounds, acrylic acid, steroids, cyclic polysulfides, ketones and halogenated alkanes, and also fatty acids that act as bactericidal agents (Watson and Cruz-Rivera, 2003). *Tetraselmis* species are sources of polyunsaturated fatty acid (PUFAs), vitamin E, carotenoids, chlorophyll, tocopherols, and polyphenols (Pérez-López et al., 2014). Several algae species are commercially cultivated in some countries. The biomass produced is used as a source of products for application in the food, pharmaceutical, medical, nutraceutical, cosmetic and aquaculture industries. Its balanced nutritional composition (source of carbohydrates, proteins of high biological value, fatty acid profile, vitamins, and minerals) and the presence of minor bioactive compounds (antioxidant pigments such as beta-carotene, chlorophyll, lutein, zeaxanthin, fucoxanthin or astaxanthin) are important characteristics to be explored in the development of new foods with added value (Sousa et al., 2008; Nova et al., 2020). Seaweeds and microalgae are excellent sustainable marine resources for developing innovative food products that privilege health, nutrition, and environmental sustainability (Nova et al., 2020). In addition, as the marine environment contains about half of the global biodiversity and abundant waste related to its exploration, using marine species to produce bioactive peptides may contribute to sustainable development. It may also represent economic gains (Cunha and Pintado, 2022).

Peptides with antimicrobial activity have aroused scientific interest mainly due to the high number of microorganisms resistant to emerging antibiotics (Bahar and Ren, 2013). Therefore, marine species have often been described as a source of bioactive peptides. The mussels belong to this group with their peptides associated with bioactive properties, including antioxidant, anti-hypertensive, antimicrobial, anticancer, anti-inflammatory, anticoagulant, antidiabetic, and antiviral properties (Je et al., 2005; Jung et al., 2007; Jung and Kim, 2009; Balseiro et al., 2011; Kim et al., 2012; Neves et al., 2016; Cunha et al., 2021). Beyond this, other peptides have been isolated from the *Mytilus* sp. with broad-spectrum activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria (Grienke et al., 2014). In the work by Cunha et al. (2021), a more efficient hydrolysate was produced from the mussel *Mytilus galloprovincialis*, with higher protein/peptide levels and increased antioxidant and antihypertensive activity. It should also be noted that the discarded mussels used to produce functional ingredients for the food, cosmetic and pharmaceutical industries can contribute to the valorisation of world waste in a circular economy context (Cunha et al., 2021). The aim of this study was the valorisation of low

commercial value and abundant fish species on the Portuguese coast, such as Atlantic horse mackerel (*T. trachurus*), by developing smoked-flavored products with reduced salt content and fortified with natural bioactive compounds extracted from forest by-products (pine bark extract (*P. pinaster* Aiton subsp. *atlantica*) and seafood (mussel extract, *M. galloprovincialis*, with peptides <3 kDa and microalgae extract, *Tetraselmis* sp. with peptides <3 kDa).

2 Materials and methods

2.1 Extraction of phenolic compounds

Phenolic compounds were extracted from three distinct sources: pine bark, mussels, and microalgae. Due to the unique characteristics of each of these materials, specific extraction techniques were employed, which are detailed in the subsequent sections.

2.1.1 Pine bark

Pine bark was collected from *P. pinaster* Aiton subsp. *atlantica* trees in the northern region of Portugal (Vila Nova de Cerveira, Viana do Castelo, Portugal) was collected from trees ∼22–25 years old. The collection process involved making a circular cut in a specific area of the main trunk of freshly cut trees, ∼1.30 cm above ground level. To prepare the extract, the pine bark samples underwent a thorough cleaning process, which included multiple washes with distilled water to remove impurities such as dirt, lichens, and resin. Subsequently, the cleaned samples were dried at 40°C for 48 h. The resulting dried material was then finely ground and sieved with an amplitude of 0.2 for 1 min using the Analysette 3 PRO machine (Fritsch, Germany) to select particles within the diameter range of 200–850 μm. These samples were stored in airtight bags in a cool, dry, and dark environment until further use. The pine bark extract was obtained through microwave-assisted extraction, utilizing the ETHOS X microwave extraction system with the SK-12 medium-pressure rotor (Milestone, Italy). The extraction process was conducted for 30 min, employing microwave irradiation at a power of 1,600 W and a temperature of 110°C. A sample of 2.5 g of pine bark sample was placed into an extraction vessel within the equipment. Subsequently, 50 ml of a solvent mixture [water: ethanol (50:50)] was added to the vessel, resulting in a solid-to-liquid ratio of 1:20 (w:v). Upon completion of the extraction, the resulting extract was cooled and diluted to a final volume of 200 ml using the extraction solvent. It was then frozen at −80°C and subjected to lyophilisation using the Alpha 1-2 LDplus lyophiliser (Christ, Germany) under vacuum conditions for 48 h. The lyophilised extract was subsequently dissolved in water to achieve the desired concentration.

2.1.2 Mussel

The extraction of mussel peptides was performed according to Cunha et al. (2021) procedure. First, mussel biomass was mixed with ultrapure water in a ratio of mussel:water of 1:2 (w:v) and pH was adjusted to 7.5. Then, 2.7% (v/w) of subtilisin (New Cell Supreme 4000L, was supplied by NewEnzymes) was added, and the mixture was incubated at 40°C for 3 h in an orbital shaker (Thermo Scientific™ MaxQ™ 6000). The pH was verified and adjusted to 7.5 every 15 min. To stop the hydrolysis reaction, the samples were incubated at 90°C for 10 min to inactivate the enzymes. Samples were centrifuged (30 min, 5,000×g), and the supernatant was collected.

2.1.3 Microalgae

The *Tetraselmis* hydrolysate was prepared following a method previously described for the microalgae *Nannochloropsis oceanica* (Cunha et al., 2023). Briefly, the microalga (spray-dried) was mixed with deionised water in a 1:10 (w/v) ratio and hydrolysed with 4.7% cellulase (New Pro 16L, was supplied by NewEnzymes) for 2 h at 50°C. Then, 5% subtilisin (New Cell Supreme 4000L, was supplied by NewEnzymes) was added, and the hydrolysis proceeded at 40°C for 2 h. All the hydrolysis steps occurred with a controlled pH of 7.5 and an agitation of 125 rpm. The enzymatic reaction was stopped by increasing the temperature to 90°C for 10 min. The water-soluble extract (supernatant) was then separated by centrifugation (20 min, 5,000×g).

2.1.4 Filtration

The water-soluble hydrolysates produced from the microalgae and the mussels were submitted to an ultrafiltration (UF) process on a tangential flow filtration system (Cogent® μScale; Merckmillipore) recurring to a cut-off membrane threshold of 3 kDa. The <3 kDa fraction was then frozen for further application.

2.2 Sample preparation/fish processing

About 180 whole Atlantic horse mackerel (*T. trachurus*), size T3 (200–400 g), were donated by the local company Docapesca—Portos e Lotas, SA (Matosinhos, Portugal) and transported to Viana do Castelo research laboratory in refrigeration boxes. The same day, fishes were beheaded, eviscerated, fileted manually without removing the skin layer, and immediately washed with tap water. Two filets were obtained from each fish, and all filets were immersed in a brine of salt (2%) and liquid smoke (5%, RudinSmoke Euro 100, Ruitenberg) and kept for 16 h at 4°C. A filet:brine ratio of 1:1 (w/v) was used. Then, they were removed and drained for 10 min, followed by drying in a mechanical smoker (Maxi AFOS MK, England), without smoke production, at 70°C for 4 h with a final thermal shock of 90°C for 1 h. Smoke-flavored filets were divided into four groups with distinct treatments: sprayed (13 μl/cm^3) bioactive extract solutions (100 mg/ml), mussel extract solution (sample code MuE), *M. galloprovincialis*, with peptides <3 kDa; microalgae extract solution (sample code MiE), *Tetraselmis* sp. with peptides <3 KDa; and pine bark extract solution (*P. pinaster* Aiton subsp. *Atlantica*; sample code PBE), and one without any further spraying after processing, acting as a control sample (sample code C). The smoke-flavored fish were cooled in a blast chiller (Mercatus, Italy) until 3°C, vacuum-packed (thickness 90 μm; Permeability: O_2−50 cm^3/m^3 dbar; CO_2−150 cm^3/m^2 dbar; N_2−10 cm^3/m^2 dbar; Water vapor transmission−2.8 g/m^2 d) and stored at 4–5°C for 15 days. Quality changes

were studied by monitoring the physicochemical, microbiological and sensory properties weekly. Antioxidant and anti-hypertensive activities were determined at day 0. Each sample was composed of three filets homogenized for the analytical determinations. Triplicate measurements were conducted for each parameter.

2.3 Physicochemical analysis

Moisture content was assessed through a drying process in a vacuum oven (Memmert UFP 600, Schwabach, Germany) at a temperature of 103°C until a constant weight was obtained according to the AOAC procedures 925.40 (AOAC, 1995). Water activity was measured using a water-activity meter (LabTouch-aw meter Novasina, Switzerland). The pH values were measured using in the fish muscle a pH meter (CRISON pH 25+) with a glass probe. The sodium chloride (NaCl) content was determined following the guidelines outlined in AOAC method 937.09 (AOAC, 1995), which employs Volhard's method. This method involves back titration using potassium thiocyanate. Before titration, an excess volume of silver nitrate solution was added to the solution containing chloride ions (specifically fish chlorides), forming a silver chloride precipitate. The excess silver ions were then titrated with potassium thiocyanate in the presence of ammonium iron (III) sulfate. The peroxide values (PV) were determined in 25 g of the blended sample using the iodometric method with visual endpoint detection, as described in ISO 3960:2017. Additionally, the thiobarbituric acid reactive substances (TBARs) were determined in a 15 g portion of the sample through the spectrophotometric method specified in Ke et al. (1984). Each sample was composed of three filets homogenized for performing the analytical determinations. Triplicate measurements were conducted for each parameter.

2.4 Antioxidant and anti-hypertensive activity

The Oxygen radical absorbance capacity (ORAC) assay for measuring the antioxidant activity was performed in a black 96-well microplate (Nunc, Denmark), and the fluorescence was evaluated for 80 min in a multi-detection plate reader (Synergy H1; Biotek Instruments, Winooski VT, USA) as previously described by Cunha et al. (2021). The ORAC activity was expressed as μmol TE (Trolox equivalent)/g. ABTS scavenging assay was performed as described previously by Cunha et al. (2021). The reaction was executed in a 96-well microplate, and the absorbance was measured at 734 nm in a multi-detection microplate reader (Synergy H1; Biotek Instruments, Winooski VT, USA) controlled by Gen5 Biotek software (version 3.04). The results were expressed as μmol TE (Trolox equivalent)/g. The antihypertensive potential was evaluated by the capacity of inhibiting the angiotensin-converting enzyme (ACE). The assay was performed as described before by Cunha et al. (2021) in a black 96-well microplate (Nunc, Denmark) with a multi-detection microplate reader (Synergy H1; Biotek Instruments, Winooski VT, USA) controlled by Gen5 Biotek software (version 3.04). The results were expressed as the extracts' inhibitory% of ACE enzyme (iACE) at a 50 mg/ml concentration.

2.5 Microbiological analysis

A fish package was opened weekly for microbiological analysis, and a 30 g sample of smoke-flavored fish was aseptically taken from different filet parts, homogenized for 90 s in a stomacher and decimally diluted. Total Viable Counts were performed on pour plates according to EN ISO 4833-1:2013; Psychrotrophic microorganisms according to ISO 17410:2001; Enterobacteriaceae counts according to ISO 21528-2:2017 and Yeasts and Molds according to NP 3277-1:1987. *Salmonella* and *L. monocytogenes* were detected according to ISO 6579-1: 2017 and ISO 11290-1:2017, respectively.

2.6 Sensorial evaluation

The set of three smoke-flavored samples with added extract (100 mg/ml—MuE, MiE and PBE) and one control sample (C) without added extract was submitted to the sensory assessment performed in a test room (ISO 8589:2007 ISO, 2007) using a quantitative descriptive analysis—QDA® (Meilgaard et al., 2016). A portion of fish filet of each treatment was placed on coded white plates and presented to the panelists at room temperature (20°C). A panel of seven semi-trained members was selected among the ESTG-IPVC collaborators with experience in fish/seafood sensory analysis. Previously to the smoked fish sensory evaluation, judges received extra training on salty and bitter taste, as well as on smoked odors and off-flavors and defined the main attributes, scales and verbal anchors. The defined attributes, such as characteristic brightness, color, smoke, fish odor, cohesiveness, oiliness, dryness, salty taste, acid flavor, bitter taste, and smoke flavor, were rated on a 9-point intensity scale. A final question was added to the score sheet to assess global appreciation of the product, using a scale from 1 to 5, in which the values 5 and 4 corresponded to samples without defects, being the product acceptable, and the values 3, 2, and 1 to defective samples and therefore an unacceptable product. Sensory analysis was performed at 0, 7, and 15 days of storage.

2.7 Statistical analysis

The mean and standard deviation were calculated to perform a data analysis for all physicochemical analysis results. A variance analysis (ANOVA) was performed to detect any significant differences ($p < 0.05$) between treatments and over the storage period, if $p < 0.05$, a Tukey's HSD *posthoc* test was used. A Principal Component Analysis (PCA) was performed using sensory data, assessing which organoleptic properties were the most significant to explain the effect of treatments and storage. PCA is a technique for reducing the dimensionality of datasets increasing interpretability while minimizing information loss through variables and samples correlation with principal component axes (Jolliffe and Cadima, 2016). This allows better visualization of the identified groups,

thus improving the interpretation of the relative variations of the different formulations and the similarities or differences between the samples. Statistics were analyzed using TIBCO Statistica Ultimate Academic 14.0.0 for Windows (TIBCO Software Inc., California, USA). Data mining was carried out with principal component analysis (PCA) to investigate differences between treatments and to correlate the main characteristics and their changes along storage time. PCA was performed using the Autobiplot.PCA function was built in R language by Alves (2012).

3 Results and discussion

3.1 Physicochemical characterization and changes during storage

Results of analytical determination of moisture content, water activity (a_w) and NaCl content carried out on the different samples/treatments are shown in Table 1. Given the high heterogeneity of the fish filet samples, these parameters were determined with a reasonable sample size ($n = 9$ for each condition) and carried out when opening the packages for further analysis. Significant differences ($p < 0.05$) were observed in all parameters when comparing with control samples with MuE, MiE, and PBE: the control sample showed lower moisture content and water activity and higher NaCl concentration. The application of the extract by spraying it onto the surface of the filets likely led to an increase in the moisture content of the treated samples when compared to the control group, as expected. This is because moisture content, water activity, and salt content are closely interconnected parameters. Moisture content represents the quantity of water present in food and its constituents. In contrast, the water activity parameter signifies the amount of water in foods available to engage in degradation processes, such as microbial spoilage. Higher water activity values tend to elevate the likelihood of accelerated microorganism growth, as documented by St. Angelo et al. (1996). Concerning moisture levels, Cardinal et al. (2001) suggested maintaining a content below 65% for smoked fish. In this investigation, all moisture values remained below this recommended threshold, with the highest recorded moisture content being 57.97% in samples treated with PBE. Kolodziejska et al. (2002) and Goulas and Kontominas (2005) also reported similar observations of notably lower moisture levels in various mackerel samples.

Regarding salt content, it's worth noting that the control sample displayed higher values, approximately double those of the other samples. This disparity can be explained by the fact that the control sample did not undergo the spraying process with solutions containing bioactive compounds. Furthermore, when evaluating the ratio of NaCl to water, it becomes evident that the control sample exhibits significantly higher values due to the absence of extract spraying. This variation can also be attributed to the substantial variability within the filets, leading to differences in the absorption of NaCl within the brine. This variability is further underscored by the notable standard deviation values observed among the samples. Reducing the salt content in food products is a global objective. Many individuals following a sodium-restricted or health-conscious diet tend to avoid consuming smoked fish due to its high sodium content. As highlighted in the study by Rybicka et al. (2022), which investigated the production of smoked mackerel filets with reduced sodium chloride (NaCl) content, the sodium chloride values reported in their research were similar to those obtained in this study, albeit with higher moisture content.

Figure 1 illustrates the pH values of the samples during their refrigerated storage. On the first day of storage, the pH of the samples fell within the range of 6.39–6.76, with PBE samples displaying the higher values and the control samples having the lower ones. Notably, at this initial sampling point, there were no significant differences ($p > 0.05$) between the MuE samples (6.57 $\pm$ 0.02) and MiE samples (6.55 $\pm$ 0.02). However, the pH of all treated samples decreased after 7 days of storage, with the MuE sample presenting the lowest pH value (6.20 $\pm$ 0.03), which was statistically distinct from the other samples. In contrast, the pH of the control samples increased on the same day. The pH value of the PBE sample did not differ significantly from that of the control sample at this stage. By day 15, the pH of the samples continued to decline, except for the MuE samples. At this juncture, the PBE sample exhibited the highest pH value (6.38 $\pm$ 0.03), demonstrating significant differences ($p < 0.05$) when compared to the Control and MiE samples. This decline in pH values observed in all samples after 15 days of storage ($p < 0.05$) is likely attributed to the expected increase in activity of lactic acid bacteria, which typically exert a buffering effect on the food substance, as noted by Iacumin et al. (2017).

Eyo (2001) emphasized that pH indicates the degree of microbial spoilage in fish, noting that some proteolytic microbes produce acid from carbohydrate decomposition, thereby elevating the acidity of the medium. In our study, total viable counts (TVC) were higher at the 15-day mark in the control and MiE samples, exhibiting the lowest pH values (Figure 1). Moreover, it's important to note that the decline in pH values could also be attributed to the increased presence of free fatty acids resulting from lipolysis. This biochemical reaction has the potential to adversely affect the taste, flavor, odor, color, texture, and appearance of food products, along with diminishing their nutritional value, as described by Gotoh and Wada (2006).

To assess the impact of the treatments on the oxidative stability of the smoke-flavored fish samples during refrigerated storage, peroxide value (PV) and thiobarbituric acid reactive substances (TBARs) were evaluated, as detailed in Table 2. These two methods operate on different principles: PV assesses the formation of hydroperoxides as a result of fat and oil oxidation, and TBARs evaluate the products generated due to the oxidation of unsaturated fatty acids in fat and oil, as well as other thiobarbituric acid reactive substances, effectively gauging the secondary breakdown products of lipid peroxidation, as reported by Gotoh and Wada (2006). PV serves as an indicator of the initial oxidation stage in fish muscle, and it plays a crucial role in fish spoilage during storage. Peroxides, the primary products in the early stages of lipid oxidation, are unstable and eventually break down into volatile low molecular weight compounds such as ketones, acids, aldehydes, and alcohol. These breakdown products can impart an unusual taste and odor to food products, as Ucak et al. (2011) documented. As indicated in Table 2, the peroxide value (PV) levels initially exhibited an increase

TABLE 1 Physicochemical quality parameters (Moisture, a_w and NaCl and NaCl:Water ratio) of the smoke-flavored horse mackerel filets: C, control sample; MuE, mussel extract; MiE, microalgae extract; PBE, pine bark extract.

Sample	Moisture content (g/100 g)	a_w	NaCl (g/100 g)	NaCl:Water ratio (g/kg)
C	48.88 ± 1.34^{c}	0.930 ± 0.017^{c}	2.75 ± 0.30^{a}	56.26 ± 6.38^{a}
MuE	54.59 ± 0.97^{b}	0.946 ± 0.010^{b}	1.33 ± 0.68^{b}	24.36 ± 14.68^{b}
MiE	53.10 ± 2.96^{b}	$0.954 \pm 0.006^{b,a}$	$1.27 \pm 0.29^{b,c}$	23.92 ± 6.52^{b}
PBE	57.97 ± 2.75^{a}	0.961 ± 0.007^{a}	0.76 ± 0.16^{c}	13.11 ± 3.27^{b}

[a,b,c]Items in the same column with different superscripts are significantly different ($p < 0.05$).

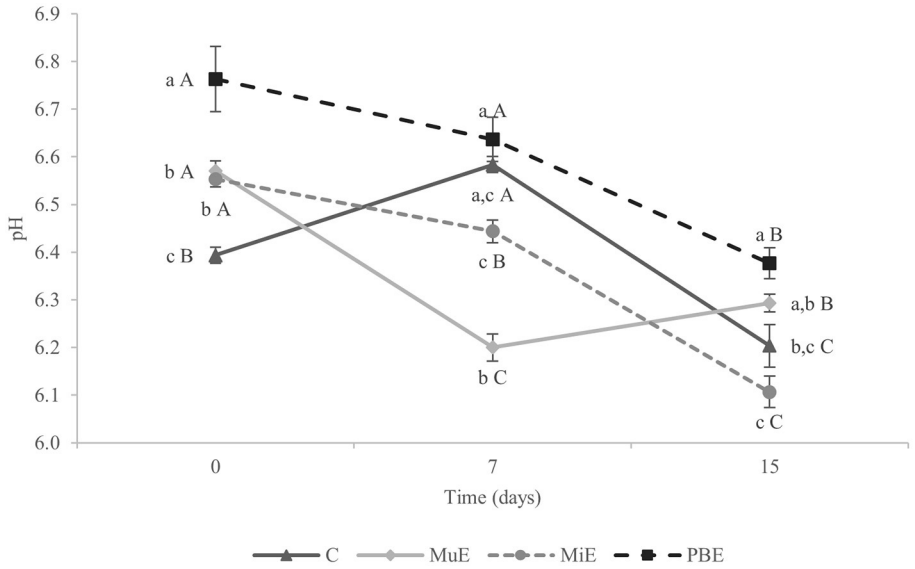

FIGURE 1
pH values of smoke-flavored horse mackerel filets during 15 days of storage: C, control sample; MuE, mussel extract; MiE, microalgae extract; PBE, pine bark extract. a,b,c—Items in the same storage time with different superscripts are significantly different ($p < 0.05$); A, B, C—Items in the same sample along the time with different superscripts are significantly different ($p < 0.05$).

and then subsequently decreased over time, with the exception of the MiE sample. These patterns align with findings reported in previous studies by Nair et al. (1976), Ke et al. (1977), and Al-Bulushi et al. (2005). The initial increase in PV observed in the samples during the first 7 days can be attributed to lipid oxidation processes. The subsequent decrease in PV may be attributed to the decomposition of hydroperoxides, which are primary products of oxidation, into secondary lipid oxidation products, as well as their interaction with proteins, as noted by Boselli et al. (2005) and Ozogul et al. (2017). Notably, samples that had extract added showed consistently lower PV values throughout the entire study period compared to the control group, and these differences were statistically significant ($p < 0.05$), except for the 15-day time point for the MiE sample. In the case of the PBE sample, a significant decline in PV was observed after it reached its peak values, suggesting the instability of peroxides and their susceptibility to forming secondary products, in line with the observations made by Danowska-Oziewicz and Karpińska-Tymoszczyk (2005). These results are consistent with findings from other studies (Alçiçek, 2011; Kumolu-Joh and Ndimele, 2011; Tenyang et al., 2020), where extracts of plant origin were applied to smoked fish products. According to Raeisi et al. (2016), the maximum acceptable limit for peroxide value (PV) in food products is typically set between 10 and 20 mEq O_2/kg of fat. In this study, all samples maintained peroxide content within the acceptable range for human consumption, with the maximum value for the control sample being 15.97 ± 0.06 mEq O_2/kg fat. Furthermore, the samples with the addition of extract remained well within the recommended limit of 15 mEq O_2/kg of fat as advised by the Codex Alimentarius Commission (FAO/WHO, 2001).

In the case of thiobarbituric acid reactive substances (TBARs) values, there was an initial decrease followed by an increase over time, except for the PBE sample, which exhibited a consistent decline throughout the entire storage period. Malondialdehyde, a compound that reacts with TBA to form TBARs, can create amidine bonds by crosslinking amino acids and interact with various components in fish, including nucleosides, nucleic acids, phospholipid amino acids, and other aldehydes produced during lipid oxidation. The extent of this interaction can vary depending on the fish species, as noted by Nair et al. (1986). This interaction could explain the initial decrease in TBARs levels from 0 to 7 days. As storage progresses, the rate of consumption of hydroperoxides may exceed the rate of their formation (Undeland et al., 1999), leading to the formation of other oxidation products detectable as

TABLE 2 Biochemical parameters (TBARs and PV) of smoke-flavored horse mackerel filets during 15 days of storage: C, control sample; MuE, mussel extract; MiE, microalgae extract; PBE, pine bark extract.

	Time (days)		
	0	7	15
PV (mEq.O_2/Kg fat)			
C	2.76 ± 0.17^{aC}	15.97 ± 0.06^{bA}	14.27 ± 0.07^{aB}
MuE	1.98 ± 0.01^{bA}	5.89 ± 0.36^{cB}	5.06 ± 0.27^{cB}
MiE	1.53 ± 0.01^{cB}	13.15 ± 0.04^{aA}	13.57 ± 0.38^{aA}
PBE	0.51 ± 0.02^{dC}	13.73 ± 0.60^{aA}	10.73 ± 0.23^{bB}
TBARs (mg malondialdehyde/kg sample)			
C	7.89 ± 0.10^{bA}	3.40 ± 0.05^{bC}	6.19 ± 0.04^{bB}
MuE	5.83 ± 0.10^{cA}	1.13 ± 0.02^{dC}	2.22 ± 0.01^{cB}
MiE	10.90 ± 0.85^{aA}	2.47 ± 0.03^{cC}	7.14 ± 0.02^{aB}
PBE	7.36 ± 0.24^{bA}	5.99 ± 0.08^{aB}	1.50 ± 0.01^{dC}

a,b,c,d—Items in the same column with different superscripts are significantly different ($p < 0.05$).

A, B, C—Items in the same row with different superscripts are significantly different ($p < 0.05$).

TBARs. This phenomenon may account for the observed results in the samples, except for the PBE sample. Since TBARs can also react and degrade over time, their levels may not consistently increase, which could explain the decrease observed in the samples. Throughout the entire storage period, the peroxide value exhibited an increase from the initial value, and this increase was statistically significant ($p < 0.05$) for all samples. In contrast, TBARs values displayed an inverse trend, with a significant decrease ($p < 0.05$) observed between the beginning and end of the storage period. Additionally, it was noted that at 0 and 15 days, the TBARs values of the samples containing extracts were lower than those of the control samples, except for the MiE sample. Among the extracts tested, the PBE extract appears to have had the most effective prevention against lipid oxidation, with decreasing values of TBARs over the 15 days. In contrast, the results presented by MiE and MuE extracts seem to indicate that their ability to prevent lipid oxidation decreased after 7 days of storage. This study also investigated the effectiveness of natural extracts in slowing down lipid oxidation during the shelf life of hot-smoked catfish, and the results were promising, as reported by Kumolu-Joh and Ndimele in 2011. According to the statistical analysis of variance, significant differences were observed at all sampling points and among various sample treatments ($p < 0.05$). It's important to note that the formation of aldehydes in fish is typically attributed to enzymatic reactions. However, in the context of hot smoking, their generation primarily results from processes such as thermal degradation, Maillard reactions, and modifications in lipid composition. These reactions occur when fish are subjected to thermal treatments like heating, baking, or smoking, as described by Bienkiewicz et al. (2022).

Moreover, the intricate nature and composition of fish fats, liquid smoke, and the extracts themselves can contribute to the absence of clear patterns in lipid oxidation, as measured by parameters such as peroxide value (PV) and thiobarbituric acid reactive substances (TBARs), in processed foods. While PV and TBARs serve as valuable biochemical indicators of spoilage, additional research is needed to establish their correlations with sensory attributes and microbiological aspects.

The combination of antioxidant extracts, salting, drying techniques, and vacuum packaging (thus minimizing oxygen exposure) has shown a significant reduction in primary lipid oxidation. For instance, Swastawati et al. (2020) examined the application of liquid smoke nanocapsules to catfish filets roasted at 90°C for 4 h. They discovered that using nanocapsules could effectively inhibit oxidation during storage, as PV and TBA results remained below the established thresholds for up to 10 days of storage.

In another study conducted by Fellenberg et al. (2020), the objective was to determine the antioxidant effects of natural extracts on the oxidative quality and color changes in marinated rainbow trout filets during storage at 4°C. The findings suggested that natural antioxidants could offer an alternative approach to extending the shelf life of trout filets, particularly during the initial 6 days of storage at 4°C.

3.2 Antioxidant and anti-hypertensive activity of smoke-flavored horse mackerel filets

The results of antioxidant activity determined by ORAC and ABTS methods are presented in Table 3. Among the filets, the one treated with microalgae extract (MiE) demonstrated the most favorable outcomes in terms of antioxidant activity, as determined by the ORAC and ABTS methods, with values of 45.20 ± 1.84 and 12.90 ± 0.64 μM TE/g initial sample, respectively. In comparing the MiE sample with the other samples, statistically significant differences in the results of the ABTS method were observed. However, there were no significant differences in the results obtained from the ORAC method between the MiE and MuE samples, with values of 45.20 ± 1.84 and 37.65 ± 2.64 μM TE/g, respectively. Additionally, the smoked-flavored horse mackerel filet exhibited an interesting antihypertensive profile, with an ACE (angiotensin-converting enzyme) inhibition of 14.41 ± 0.8 at a 50 mg/ml concentration. Nonetheless, the tested extracts notably enhanced their biological potential, with the microalgae extract achieving the most significant anti-hypertensive profile at 64.43 ± 4.3 iACE inhibition (50 mg/ml). These results suggest that the studied extracts hold substantial potential for developing innovative food products that emphasize functionality, convenience, nutrition, and health benefits.

3.3 Microbiological quality of smoke-flavored horse mackerel filets during storage

The microbiological values of studied samples at 0, 7, and 15th day of storage are shown in Figure 2. The initial bacterial counts for Total Viable Bacteria (TVC) and Psychrotrophic bacteria (Figures 2A, B) in the control sample were 3.9×10^2 and 1

TABLE 3 Results obtained for the Anti-hypertensive and Antioxidant activity in smoke-flavored horse mackerel filets.

	iACE inhibition (50 mg/ml)	ORAC (μM TE/g initial sample)	ABTS (μM TE/g initial sample)
C	14.41 ± 0.8[a]	15.20 ± 6.36[a]	6.57 ± 0.28[a]
MuE	18.61 ± 0.6[a]	37.65 ± 2.64[b]	8.52 ± 0.18[a,c]
MiE	64.43 ± 4.3[b]	45.20 ± 1.84[b]	12.90 ± 0.64[b]
PBE	16.55 ± 1.7[a]	12.34 ± 3.60[a]	9.56 ± 1.02[c]

[a,b,c]Items in the same column with different superscripts are significantly different ($p<0.05$).

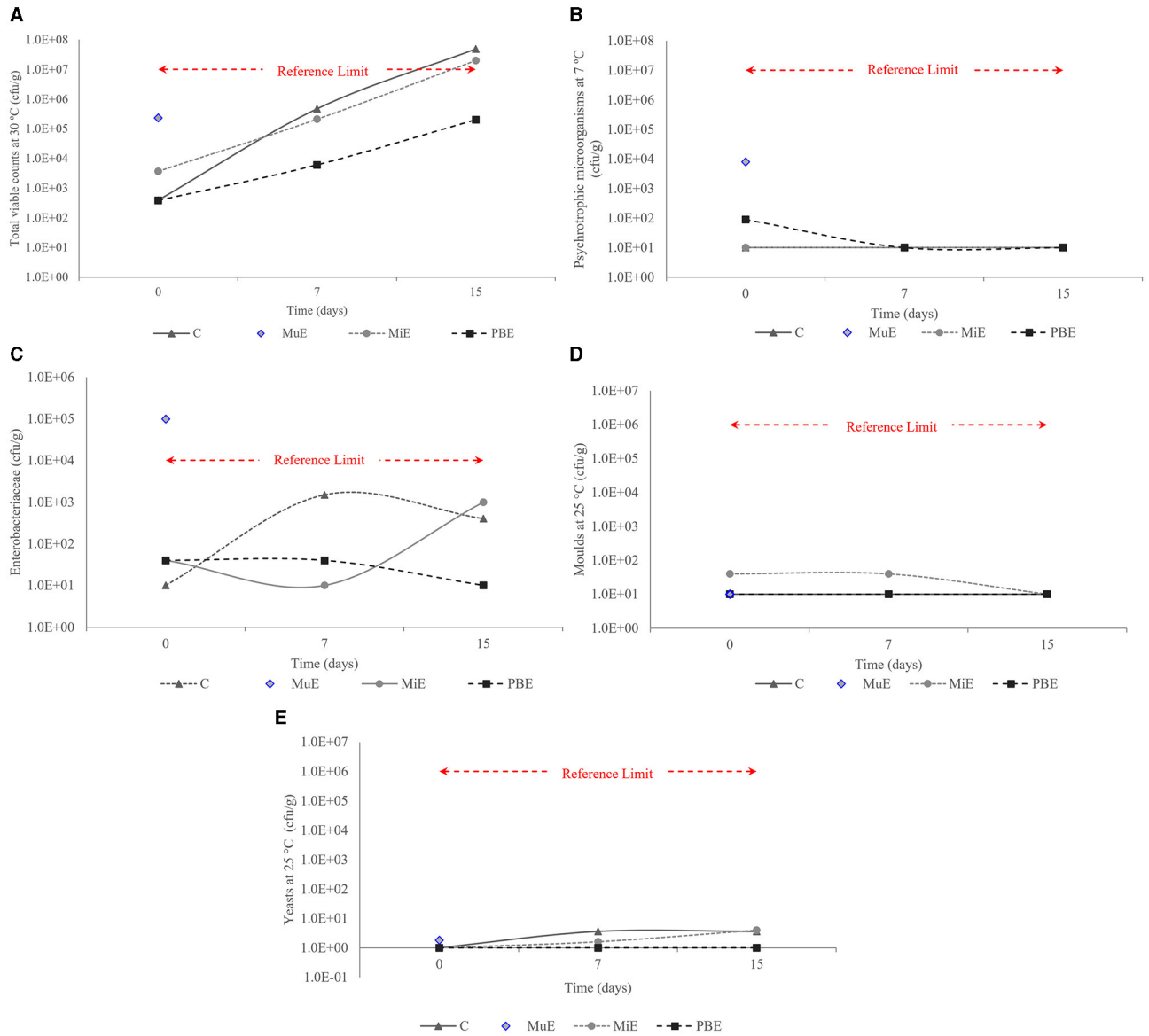

FIGURE 2
(A) Total viable counts; **(B)** Psychrotrophic bacteria at 7°C; **(C)** Enterobacteriaceae counts; **(D)** Molds counts at 25°C; and **(E)** Yeast counts at 25°C at 30°C during storage.

$\times 10^1$ cfu/g, respectively. In the MuE sample, the counts were 2.3×10^5 for TVC and 8×10^3 cfu/g for Psychrotrophic bacteria. The MiE sample had counts of 3.7×10^3 for TVC and 1×10^1 cfu/g for Psychrotrophic bacteria, while the PBE sample had counts of 3.8×10^2 for TVC and 9×10^1 cfu/g for Psychrotrophic bacteria. For ready-to-eat products, the recommended limit value for Total Viable Bacteria is typically $<10^7$ cfu/g (HPA, 2009; INSA, 2019). Considering that the TVC counts in the Control and MiE samples, after 15 days of storage, as well as the numbers of Enterobacteriaceae (Figure 2C) in the

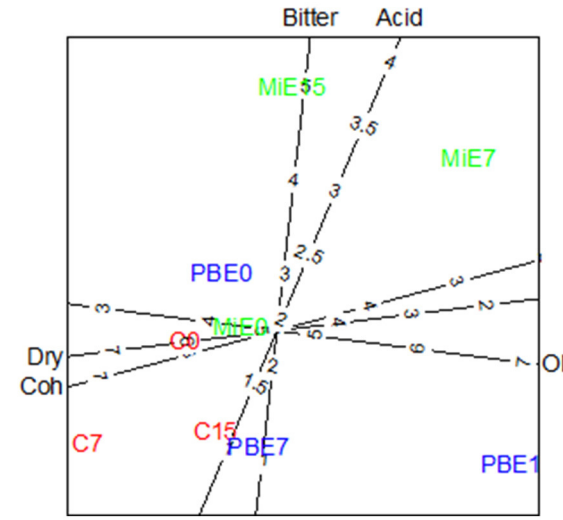

FIGURE 3
Principal component analysis of sensory data (PC1 vs. PC2) were C is control formulation; MiE—formulation with the application of seaweed extract; PBE—formulation with the application of pine bark extract, for sampling times 0, 7, and 15 days.

MuE samples at day 0 (initial time), exceeded the recommended limit value for ready-to-eat products, the decision was made to discontinue the study at this sampling point. Additionally, since the numbers of Enterobacteriaceae in the MuE samples exceeded the recommended limit value for ready-to-eat products, no further microbiological assays were conducted beyond this point. In all samples, a notable increase in bacterial populations was observed over time, except in the case of the PBE sample. Specifically, between day 0 and day 7 of shelf life, the population increased by 1.47 logarithms (approximately 15 times the initial population) when PBE was used. In contrast, in the control sample (C), the observed increase was 2.2 logarithms (equivalent to 22 times the initial population). This suggests that the use of PBE had a partial to moderate bacteriostatic effect on the growth of mesophilic bacteria, potentially extending the product's shelf life compared to the other samples. Some studies have suggested that compounds found in pine bark, such as tannins and flavonoids, can inhibit the growth of both gram-positive and gram-negative bacteria, including mesophiles (Torras et al., 2005). These extracts also contain various phenolic compounds like catechins, taxifolin, and phenolic acids, which have been demonstrated to possess antimicrobial activity (Iravani and Zolfaghari, 2014). The increase in Psychrotrophic bacteria counts from 0 to 7°C is associated with issues related to the preservation of cold-stored foods (Erkmen and Bozoglu, 2016). When a significant number of psychrotrophic bacteria are detected, it indicates shortcomings in the preservation process (González-Fandos et al., 2004).

In the present study, there was no observed increase in the psychrotrophic bacterial population over time in any of the samples, and relatively low and stable levels were maintained, well below the maximum reference value of 10^7 cfu/g. Based on these findings, there was no significant effect from any compound on the psychrotrophic bacteria. However, it's important to note that no definitive conclusions can be drawn due to the low quantities of psychrotrophic bacteria detected. The Enterobacteriaceae counts exhibited an abnormal variation in population over time. The distribution of these bacteria did not follow a normal (uniform) distribution but rather a sporadic distribution, where each spot corresponds to establishing a bacterium that has developed into a colony. This development could have occurred due to exposure or cross-contamination, typically at the surface of the fish filet samples processed in this study. Another possible explanation for the results obtained could be sampling variability, which refers to variations in the preparation of the mother suspension despite taking precautions to obtain a sample that was as representative as possible (including parts with more or fewer stains) and batch variability, as the samples originated from different fish with potentially different and variable flora depending on exposure and handling.

The decrease in Enterobacteria numbers in PBE samples may be attributed to various hurdle factors, such as pH reduction, in combination with the bactericidal and antioxidant properties of phenolic compounds present in the smoke, as suggested by Zaki et al. (2021). The results indicate the presence of Enterobacteriaceae in the samples but do not provide a comprehensive assessment of their evolution over time. The recommended limit for Enterobacteriaceae in ready-to-eat products is typically $<10^4$ cfu/g (HPA, 2009; INSA, 2019). The yeast and mold counts (Figures 2D, E) observed in this study did not exceed the recommended value of $<10^6$ cfu/g (HPA, 2009) throughout the storage period for all samples. The increase in yeast counts may have been influenced by the vacuum packaging of the samples, as the absence of oxygen prevents the proliferation of yeasts and molds. It's noteworthy that *Salmonella* and *Listeria* were not detected during storage. These results suggest a correlation between sensory analysis and the microbiological analysis of the horse mackerel filets, supporting the overall safety and quality of the products.

3.4 Sensory quality of smoke-flavored horse mackerel filet

Sensory analysis is widely recognized as a crucial tool for evaluating fish quality within the industry (Warm et al., 2001; Loutfi et al., 2015). The sensory attributes of fish quality are closely intertwined with the chemical and biochemical composition of the fish and how they evolve during storage (Du et al., 2001; Gómez-Guillén et al., 2009; Messina et al., 2016). Therefore, sensory analysis plays a significant role in estimating the shelf life of fish products (Leroi and Joffraud, 2000; Martinez et al., 2007). In Figure 3, the Principal Component Analysis (PCA) output represents the sensory analysis results provided by the panel of evaluators, capturing 51.4% of the total information. It's important to note that sensory analysis was not concluded for MuE samples due to the presence of off-odors reported by the panelists. Additionally, in accordance with microbiological determinations, MuE samples exhibited a higher level of contamination by Enterobacteriaceae on day 0, exceeding

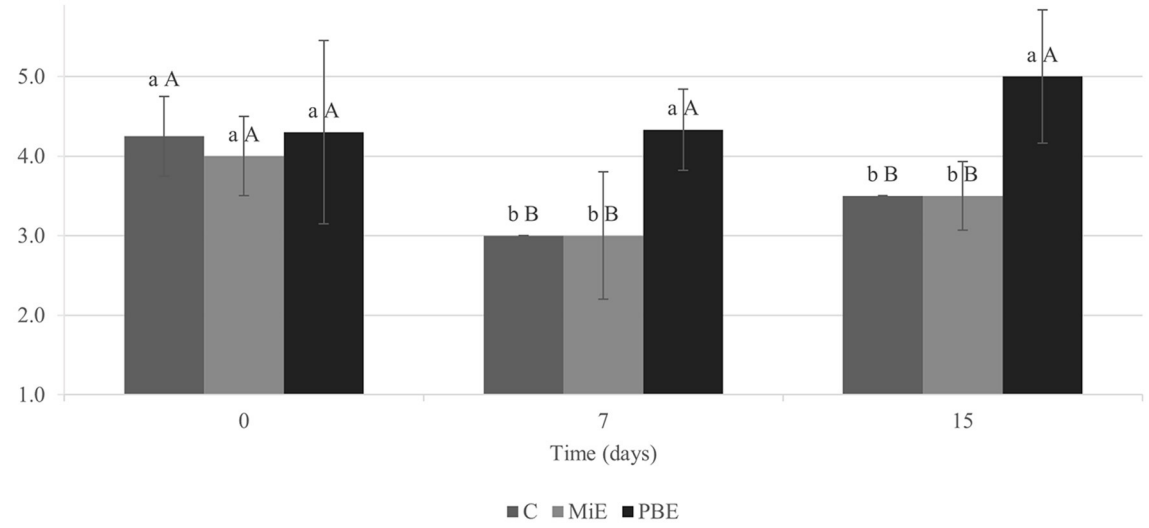

FIGURE 4
Global acceptance. C, control formulation; MiE, formulation with the application of seaweed extract; PBE, formulation with the application of pine bark extract, for sampling times 0, 7, and 15 days. a,b Items in the same study time with different superscripts are significantly different ($p < 0.05$). A, B Items in the same sample with different superscripts are significantly different ($p < 0.05$).

the recommended limit value established by the Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market (HPA, 2009) by more than 1 log.

The sensory data analysis employed the "Autobiplot.PCA" function, which applies a biplot to PCA displays. In Figure 3, the biplot is depicted on the plane of principal components 1 and 2, summarizing nearly 58% of the total information contained in the sensory data. These results shed light on which sensory attributes played a crucial role in discriminating between samples, with bitterness, acidity taste, and attributes related to texture such as dryness, cohesiveness, and oiliness standing out.

The biplot can be interpreted by drawing a perpendicular line from a specific variable axis to the sample position, allowing for multiple variable readings to be made within a single orthogonal plane. For instance, if we take samples C0, MiE0, and PBE0, they project orthogonally to attributes such as oily (≈4.0), acidity (≈2.4), and bitterness (≈3). This type of PCA analysis facilitates the interpretation of results in terms of the original sensory attributes and sample values, thus avoiding the complexities associated with interpretations in terms of latent variables and relative values.

The PCA plot in Figure 3 also reveals a grouping of samples based on storage time. Samples MiE7 and MiE15 are discriminated and projected toward the upper side of the plot. This separation is attributed to higher scores in attributes related to bitterness and acidity taste, which increase over the storage period. In contrast, samples treated with PBE for 7 and 15 days were characterized by the panelists as more oily and less dry or cohesive than the control (C). Among them, PBE15 and MiE7 were considered significantly oilier than the others. The control samples were generally perceived as drier, with higher cohesiveness and a saltier taste (Supplementary Table 1). This observation aligns with expectations, as these attributes tend to be highly correlated.

Figure 4 illustrates the overall appreciation of the products using a 5-point scale, where panelists were asked to assess the overall suitability of the products. In this scale, scores equal to or <3 points were considered indicative of the existence of a defect, while values of 4 and 5 indicated that the product met quality requirements. The threshold between the presence or absence of a defect, when considering average values, was set at 3.5. The results indicate that there were no statistically significant differences ($p > 0.05$) among the samples collected at the first sampling point (C0, MiE0, and PBE0). The average score for these samples was approximately 4, suggesting that the products were considered satisfactory or good. At the second sampling point (7 days of storage), panelists noted significant differences ($p < 0.05$), primarily between the control (C) and MiE samples. However, PBE-treated samples were consistently rated as good or satisfactory across all sampling points. Samples with 15 days of storage exhibited a similar pattern in panelist evaluations. Therefore, it can be inferred that the PBE treatment was more suitable for this product, as the samples treated with PBE appeared to be more stable over the duration of the study.

4 Conclusions

Consumers are increasingly demanding safe and natural products, and reducing salt in foods is a global objective. This drive has led to the exploration of new preservation techniques that enhance microbial quality and safety while minimizing the impact on nutritional and sensory qualities. In this context, natural compounds have garnered significant attention from both research and industry due to their potential to offer quality and safety benefits with minimal health implications. Additionally, the use of natural ingredients aligns with the principles of food sustainability. However, salt remains a crucial component for

ensuring microbial stability in products like smoked-flavored fish. This study has demonstrated that natural extracts possess antioxidative and antimicrobial properties that can delay oxidative rancidity, thereby extending the shelf life of smoked-flavored fish. At the outset, MiE samples exhibited the highest antioxidant activity as determined by the ORAC and ABTS methods. However, an increase in total viable counts was observed in these samples by the end of the 15-day storage period, although it remained below the recommended limit levels at days 0 and 7. MuE samples demonstrated the lowest PV value at day 15, but these extracts were associated with off-odors detected by sensory panelists. After 15 days of storage, the only acceptable samples in both microbiological and sensorial analysis were those with the application of PBE extract, with this result seeming to indicate that this extract was more effective, resulting in a product with a longer shelf life, that is, of at least 15 days. While TBARs and PV remained below detection levels throughout the study, it is essential to acknowledge that extended storage can potentially lead to rancidity, which can adversely impact the sensory attributes of the samples.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

DB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. PN: Investigation, Methodology, Project administration, Writing – review & editing. SC: Investigation, Methodology, Project administration, Writing – review & editing. VM: Formal analysis, Writing – review & editing. ÉF: Investigation, Validation, Writing – original draft, Writing – review & editing. RP-P: Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. CB: Formal analysis, Investigation, Writing – original draft. MP: Formal analysis, Supervision, Writing – review & editing. AG: Conceptualization, Data curation, Supervision, Writing – review & editing. MV-V: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1296265/full#supplementary-material

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