Assessment of lipid oxidation and microbial decontamination of sardine (*Sardina pilchardus*) fillets processed by plasma-activated water (PAW)

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ABSTRACT

Plasma activated water (PAW) is emerging as a mild and environmentally friendly technology for microbial and chemical decontamination of food. The aim of this work was to evaluate the degree of oxidation of lipids of sardine tissue treated by PAW generated with a pulsed corona discharge. The effect of PAW on the natural microflora of the fish was also evaluated. Under the adopted experimental conditions, PAW was able to reduce the number of mesophilic aerobes and *Pseudomonas* spp. by 0.22 and 0.20 log units, respectively, but no increase in shelf life was observed at chilling temperatures compared to water washing. A loss of polyunsaturated fatty acids (PUFAs) and an increase in volatile oxidation products derived from the cleavage of PUFA hydroperoxides were observed at the longest treatment duration. Twelve cholesterol oxidation products (COPs) were identified in sardine lipids, but no significant differences in total COPs content were observed between PAW processed and control samples. Free radical mediated oxidation pathways led to the most abundant COPs, but a significant contribution of non-radical pathways was also observed. Further studies are needed to better understand the low efficiency of PAW in microbial decontamination of proteinaceous materials.

1. Introduction

The food industry is constantly searching for milder, more selective and less energy-consuming processes to meet the growing consumer desire for "fresh-like" food and awareness of environmental issues. Several non-thermal technologies (high pressure, pulsed electric fields, ultrasound, cold irradiation, UV and pulsed light) have been investigated and evaluated as alternatives to conventional thermal processes for food safety and preservation to prevent the negative effects of time/temperature combination on the sensory and nutritional properties of food and to increase the overall energy efficiency of production lines (Jadhav, Annapure, & Deshmukh, 2021). Recently, plasma generated

under "mild" conditions (room temperature or near room temperature, atmospheric pressure) has been shown to be a powerful medium for decontamination of food, food processing equipment and packaging materials (Ganesan, Tiwari, Ezhilarasi, & Rajauria, 2021). In particular, the use of plasma-activated water (PAW), produced by treating water with a plasma source, has been proposed to overcome the limited penetration depth and low surface coverage of direct cold atmospheric plasma treatments (CAP). Since the first report of inactivation of *Staphylococcus aureus* inoculated on strawberries (Ma et al., 2015), the decontamination potential of PAW has been investigated in many raw materials and foods (fruits, vegetables, meat and meat products, eggs, tofu, button mushrooms) (Oliveira, Fernández-Gómez, Álvarez-Ordóñez, Prieto, & López, 2022), but few studies have focused

on its application in the preservation of marine and freshwater fish. Soaking in PAW has been shown to extend the shelf-life of Asian sea bass (*Lates calcarifer*) steaks (Chaijan et al., 2022) and to successfully inactivate *Shewanella putrefaciens* in Yellow River carp (*Cyprinus carpio*) fillets (Liu, Zhang, Meng, Bai, & Dong, 2021) and foodborne pathogens (*L. monocytogenes, S. Typhimurium*) in grass carp (*Ctenopharyngodon idella*) (Esua, Cheng, & Sun, 2020). PAW ice and PAW glazing were able to significantly delay microbial growth and loss of quality indices in fresh shrimp during storage (Herianto et al., 2022; Liao et al., 2018), while Zhao et al. (2021) investigated the decontamination efficacy of the combination of PAW and sonication in mackerel fillets. Recently, Kim et al. (2022) showed that activation of rearing water by CAP was effective against *Aeromonas hydrophila* without causing significant physiological damage to the fish (*Cyprinus carpio haematopterus*).

The decontamination ability of PAW is attributed to the synergy between the acidic environment and a plethora of short-lived (peroxynitrous acid/peroxynitrite, superoxide and hydroxyl radicals, singlet oxygen, ozone) and long-lived (hydrogen peroxide, nitrite) chemical species capable of exerting strong oxidative stress on microbial cell membranes and internal structures (oxidative DNA damage, protein and membrane lipid peroxidation) (Zhao, Patange, Sun, & Tiwari, 2020). However, the reactive oxygen and nitrogen species (RONS) can trigger the onset and/or accelerate the progress of lipid oxidation, especially when conditions are favourable due to lipid characteristics (high degree of unsaturation) and/or substrate properties (high lipid content and/or low water activity). This can lead to the formation of off-flavours, loss of nutritionally valuable components (polyunsaturated fatty acids, proteins, vitamins) and the production of risky or toxic substances, which could limit the application of plasma technologies in food processing. In particular, short-lived reactive oxygen species (superoxide and hydroxyl radicals, singlet oxygen) exhibit strong oxidising ability, but their chemical and biological role in plasma-activated media is still controversial due to their half-life (Gao, Francis, & Zang, 2022). Long-lived species (hydrogen peroxide, ozone) are expected to contribute significantly to lipid oxidation during contact of PAW with solid foods. Recently, the role of peroxynitrite as a strong oxidant has been highlighted, which can be continuously regenerated from the long-lived species hydrogen peroxide and nitrite and can provide a reservoir for the strong oxidants nitric oxide and hydroxy radicals (Herianto, Hou, Lin, & Chen, 2021).

Lipid oxidation is a major problem in seafood processing and storage because the depot (triacylglycerols) and structural lipids (phospholipids) of fish, crustaceans and molluscs are extremely rich in long chain polyunsaturated fatty acids (PUFAs) (Pacetti et al., 2015). Secci and Parisi (2016) recently reviewed the main factors influencing lipid oxidation of fish from farm to fork and the measures to prevent oxidative spoilage throughout the fish supply chain. The combined use of plasma treatment and plant extracts rich in antioxidants was proposed to counterbalance the oxidative pressure caused by reactive species generated in plasma. Chaijan et al. (2022) developed a new preservation approach by pre-treating Asian sea bass steaks with PAW before coating them with whey protein isolate enriched with raw ginger extract, while Olatunde, Benjakul, and Vongkamjan (2020) claimed that the combined use of CAP and ethanolic coconut husk extract, either in free or liposomally encapsulated form, could extend the shelf life of chilled sea bass slices with less lipid oxidation than CAP treated. Gavahian, Chu, Mousavi Khaneghah, Barba, and Misra (2018) recently reviewed the oxidative effects of cold plasma technologies on food lipids, but currently available data on oxidative degradation of tissue lipids from seafood processed with PAW are scarce and limited to global indices of primary and secondary oxidation products (peroxide value, thiobarbituric acid reactive substances) (Chaijan et al., 2022; Herianto et al., 2022; Liao et al., 2018; Liu et al., 2021; Zhao et al., 2021).

For the above reasons, we aimed to provide new information on the oxidative degradation of fish lipids and microbial decontamination upon PAW treatment. Ready-to-use and ready-to-eat fish fillets have recently become widely available to consumers and have therefore been used as a model for assessing PAW effects. In particular, sardine (*Sardina pilchardus*) is the most important fish species caught in the Mediterranean Sea (158,166 t, representing 22.4% of total landings, average 2018–2020) (FAO, 2022) and plays an important role in the culinary traditions of countries located along the sea. Non-volatile (hydroperoxides; cholesterol oxidation products, COPs) and volatile (short-chain alcohols, aldehydes, and ketones) substances were determined to obtain a comprehensive assessment of the oxidation conditions of sardine lipids.

2. Materials and methods

2.1. Plasma treatments and lipid extraction

PAW was prepared by exposing 500 ml of sterile distilled water (SDW) to a pulsed corona discharge generated by a high-voltage generator (AlmaPulse, AlmaPlasma s.r.l., Bologna, Italy) with a peak voltage of 18 kV and a pulse repetition frequency of 5 kHz for 1 min with stirring. The total dissipated power was 209.25 ± 78.21 W. The CAP is generated in the air gap (5 mm) between the tip of the stainless-steel working electrode and the liquid surface. The reaction chamber was not hermetically sealed, so that the plasma was generated in the ambient air in a static state. An overview of the equipment for making PAW and an enlargement of the reaction chamber can be found in the supplementary material (Fig. S1). PAW parameters (pH, electrical conductivity, hydrogen peroxide, nitrites, ozone) were checked according to methods described in Laurita et al. (2021) to verify compliance with the specifications given by the plasma source developers. The initial water temperature was in the range of 19.9–21.7 °C, measured with a digital handheld thermometer with temperature probe (VWR International S.r. l., Milan, Italy). The plasma discharges caused an increase in water temperature of 1.5–2.0 °C at the longest exposure times (30 min). Therefore, no cooling systems were adopted in the prototype used in our

experiments.

Headed, gutted and filleted fresh sardines caught in the Adriatic Sea were purchased from a local farm (Ecopesce S.r.l., Cesenatico, FC, Italy) and kept refrigerated during transport to the laboratories of the Department of Agricultural, Food and Environmental Sciences (D3A) of the Università Politecnica delle Marche, where they were immediately processed. Samples of approximately 45 g of fish fillets were taken at random from the bulk and immersed in 150 ml of PAW (treatments) and SDW (controls) for 10, 20 and 30 min with gentle agitation. The fillets were then removed from the liquid medium, blotted with sterile absorbent paper to remove excess water, and subjected to lipid extraction and microbial analysis. Three replicates were performed for each treatment time and separately for chemical and microbiological analyses. Total lipids were extracted from the PAW-treated samples and controls by a modification of the "cold" (room temperature) extraction procedure originally developed by Bligh and Dyer (Christie & Han, 2010).

2.2. Analysis of the total FA composition

Fatty acid methyl esters (FAMEs) were previously prepared by acid-catalysed transesterification of crude lipids (Christie & Han, 2010) and analysed by gas chromatography (GC) using a Trace 1300 instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TG-Polar capillary column 60 m × 0.25 mm i. d., 0.20 μm film thickness (Thermo Fisher Scientific, Waltham, MA, USA) and a flame ionisation detector (FID), under the operating conditions described in Haddad, Mozzon, Strabbioli, and Frega (2010). A standard mixture of 37 fatty acid methyl esters (FAMEs) provided by Supelco, (Bellefonte, PA, USA) was used for peak identification. The total FA composition (weight percent of total FA) was calculated using the peak area normalisation method.

2.3. Determination of FA oxidation products

The total amount of primary lipid oxidation products (hydroperoxides) was measured by iodometric titration, according to the procedure described in the Official Methods and Recommended Practices of the American Oil Chemists' Society (AOCS, 2003).

Secondary volatile oxidation products were collected from the static headspace of crude lipids by solid phase microextraction (SPME), using a Supelco (Bellefonte, PA, USA) "Gray fibre" (Divinylbenzene/Carboxen/Polydimethylsiloxane). More specifically, a 10 ml glass vial was filled with 0.5 g of crude lipid extract and the fibre was exposed to headspace at 40 °C for 45 min. The volatiles were then analysed by GC-MS on a Varian 3900 instrument coupled to a Saturn 2100 T ion trap mass detector (Varian Analytical Instruments, Walnut Creek, CA) and equipped with a DB-5 type capillary column. Chromatographic peaks were identified by: (i) comparison of electronic impact (EI; 70 eV) fragmentation patterns and linear retention indices (RIs) with data published in the NIST 2020 Mass Spectral Library (National Institute of Standards and Technology); (ii) analysis and interpretation of chemical ionisation (CI; reagent gas: methanol) mass fragmentation patterns; (iii) comparison with MS data and RIs of pure analytical standards available in our laboratories (Mozzon, Foligni, & Mannozzi, 2020).

2.4. Analysis of the unsaponifiable fraction

About 250 mg of the crude lipids were accurately weighed and saponified overnight with 10 ml of methanolic potassium hydroxide 1 mol/l at room temperature and protected from light. 5α-cholestane (1 ml of a 1000 mg/l solution in hexane) and 19-hydroxycholesterol (25 μl of a 500 mg/l solution in hexane/isopropanol 3:2 v/v) were previously added to the lipid samples as internal standards for quantification of total cholesterol and COPs, respectively. The saponified mixture was transferred to a separating funnel with 10 ml water. Three extractions

were performed with 10 ml diethyl ether each and the organic layers were collected in a clean separatory funnel. The combined extracts were washed three times with 10 ml water each, filtered through anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator at 35 °C (BÜCHI Labortechnik AG, Flawil, Switzerland). Total unsaponifiable matter was finally dissolved in 1 ml *n*-hexane and divided into a 100 μl fraction, for cholesterol determination, and a 900 μl fraction, for COPs analysis, as described in Foligni et al. (2022). Total unsaponifiables and COPs were analysed using a Trace 1300 gas chromatograph equipped with a FID and coupled to an ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A Dual Detector Microfluidics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to split the injected sample 1:1 between the mass spectrometer (qualitative analysis) and the FID (quantitative analysis). Details of the operationals conditions were described in Foligni et al. (2022). Seven pure analytical standards (7α-hydroxycholesterol; 7β-hydroxycholesterol; 5α,6α-epoxycholestan-3β-ol; 5β,6β-epoxycholestan-3β-ol; cholestane-3β,5α,6β-triol; 25-hydroxycholesterol; 7-ketocholesterol) were available to confirm the identification of COPs. All solvents, reagents, and pure analytical standards were provided by Merck Life Science S.r.l. (Milan, Italy), except for 19-hydroxycholesterol, which was supplied by Vinci-Biochem S.r.l. (Florence, Italy).

2.5. Microbiological analyses

The PAW-treated and control fillets (10 g) were immediately suspended in sterile 0.1g/100 ml peptone-water solution (90 ml) and homogenised using a Stomacher® 400 instrument (Seward Ltd., Worthing, UK). Appropriate tenfold serial dilutions of the homogenates were then performed and incubated on appropriate media (Oxoid Ltd, Basingstoke, UK) to count the viable cells of the following microbial groups: (i) total aerobic mesophilic microorganisms, after aerobic incubation at 30 °C for 48 h on standard plate count agar; (ii) *Enterobacteriaceae*, after incubation on violet red bile glucose agar at 37 °C for 24 h; (iii) *Pseudomonas* spp, after incubation on Pseudomonas agar base with CFC (cetrimide, fucidin, cephalosporin) selective supplement at 25 °C for 48 h; (iv) β-glucuronidase positive *E. coli*, after incubation on chromogenic coliforms agar at 37 °C for 24 h.

2.6. Data analysis

Analytical data were analysed using JMP® Version 10 (SAS Institute Inc., Cary, NC, USA). Two-way (factorial) ANOVA was used to test and estimate the effect of the independent factors (dipping medium, dipping time) and their interaction on the measured variables; one-way ANOVA and Tukey-Kramer's honest significant difference (HSD) test were used to compare the experimental variables between groups. The level of significance was set at $p < 0.05$.

3. Results and discussion

Different types of water (sterile distilled, deionised, osmotised, tap water) have been tested by several authors for the production of PAW (Herianto et al., 2021; Zhao, Patange, et al., 2020). To avoid the introduction of other variables (physicochemical parameters of water), we used SDW, which is free from minerals, organic matter and microorganisms and has shown optimal efficacy in deactivating microbes in several studies (Zhao, Patange, et al., 2020). In freshly prepared PAW, the levels of long- and short-lived reactive species were 1.45 ± 0.39, 13.98 ± 3.47 and 0.31 ± 0.06 mg/l for hydrogen peroxide, nitrites, and ozone, respectively. The accumulation of RONS during plasma activation was accompanied by a decrease in the pH of SDW from 6.12 ± 0.3 to 3.64 ± 0.8 units and an increase in conductivity from 8.87 ± 0.59 to 76.1 ± 0.7 μS/cm. The pH and RONS values were consistent with PAW ice produced by a dielectric barrier discharge (DBD) plasma source for

shrimp storage (pH 3.04; hydrogen peroxide 2.15 ± 0.02 ppm) (Liao et al., 2018), and with PAW produced by a plasma jet source (pH 3.11 ± 0.02; hydrogen peroxide 13.43 μmol/l; nitrites 420 μmol/l) and reported by Zhao et al. (2021) as such and in combination with sonication against native microbiota and inoculated bacterial species in mackerel fillets. Much higher concentrations of hydrogen peroxide were found by Chaijan et al. (2022) in PAW used for soaking Asian sea bass steaks (100 mg/l, determined by test strips) and by Herianto et al. (2022) in PAW produced by direct piezoelectric discharge and used as a glazing agent for shrimp (15.6–60.7 mg/l).

3.1. Effect of PAW on the oxidation degree of the saponifiable lipids

Tissue lipids ranged from 3.37 to 5.50 g/100 g fresh weight (Table 1). The most represented FAs were docosahexaenoic acid (C22:6 Δ4,7,10,13,16,19; DHA) (13.46–26.19%) and eicosapentaenoic acid (C20:5 Δ5,8,11,14,17; EPA) (16.41–22.22%). Long-chain PUFAs belonging to the n-3 family accounted for a total of 33.30–48.31% of the total FAs. Palmitic acid (C16:0) (12.73–17.87%) and palmitoleic acid (C16:0 Δ9) (11.39–16.02%) were the most abundant saturated (SFAs) and monounsaturated (MUFAs) fatty acids. The total FA composition of tissue lipids from seafood treated with cold plasma has been analysed by several authors as an indirect marker for assessing the degree of lipid oxidation. A decrease in MUFA and/or PUFA was observed in slices of Asian sea bass and in pre-packaged Atlantic mackerel fillets exposed to direct plasma treatment by a DBD source (Albertos et al., 2017; Olatunde et al., 2020; Singh & Benjakul, 2020), but no data are currently available on the effects of PAW on the FA composition of seafood lipids. Statistical analyses showed a significant effect of the dipping medium (Dm) on the most represented FAs: PAW treatments caused a decrease in DHA contents, while SFA (C14:0, C16:0) percentages were higher in PAW-soaked fillets than in SDW-soaked ones (controls) (Table 1). In contrast, dipping time (Dt) had no significant effect on the FA composition of sardine lipids.

PV was used as a measure of the primary lipid oxidation products in the sardine fillets studied (Table 2). PVs were in the range of 3.2–7.0

Table 1

Lipid content (g/100 g fresh weight; mean ± SD, n = 3), cholesterol content (mean ± SD, n = 3) and fatty acid composition (% w/w as methyl esters; mean ± SD, n = 3) of sardine fillets soaked in PAW (Pn samples) and sterile distilled water (Cn samples). The numbers (10, 20, 30) indicate the soaking times in minutes.

	Controls			Treatments[c]			ANOVA[a]		
	C10	C20	C30	P10	P20	P30	Dt	Dm	Dt × Dm
Crude lipids	3.37 ± 0.40	5.50 ± 1.24	3.50 ± 0.09	4.21 ± 0.44	5.21 ± 1.20	4.95 ± 0.78			
Cholesterol (mg/100 g fat)	981 ± 128	972 ± 63	1067 ± 66	965 ± 45	1009 ± 54	974 ± 106			
Cholesterol (mg/100 g FM)	33 ± 1	53 ± 9	37 ± 3	41 ± 2	52 ± 9	54 ± 6			
FA[b]	3.37 ± 0.40	5.50 ± 1.24	3.50 ± 0.09	4.21 ± 0.44	5.21 ± 1.20	4.95 ± 0.78			
C10:0	0.04 ± 0.01[ab]	0.02 ± 0.01[b]	0.04 ± 0.04[ab]	0.01 ± 0.01[b]	0.02 ± 0.01[b]	0.12 ± 0.04[a]	*		*
C12:0	0.72 ± 0.11[ab]	0.59 ± 0.45[ab]	0.15 ± 0.13[b]	0.49 ± 0.06[ab]	0.76 ± 0.31[ab]	1.21 ± 0.18[a]			*
C13:0	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01			
C14:0	7.69 ± 1.85[ab]	6.79 ± 1.84[ab]	5.75 ± 0.43[b]	8.86 ± 0.76[ab]	9.08 ± 1.85[ab]	11.70 ± 1.78[a]		*	
iso-C15:0	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
anteiso-C15:0	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00			
C14:1	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.03		*	
C15:0	0.45 ± 0.16	0.42 ± 0.03	0.43 ± 0.03	0.62 ± 0.00	0.55 ± 0.04	0.62 ± 0.08		*	
C15:1 Δ10	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01			
C16:0	12.73 ± 2.79	13.77 ± 1.12	14.49 ± 3.66	17.87 ± 1.44	15.71 ± 0.08	17.83 ± 1.68		*	
iso-C17:0	0.18 ± 0.04	0.21 ± 0.06	0.15 ± 0.01	0.24 ± 0.06	0.21 ± 0.06	0.27 ± 0.02			
anteiso-C17:0	0.11 ± 0.05	0.30 ± 0.03	0.14 ± 0.07	0.17 ± 0.08	0.15 ± 0.00	0.16 ± 0.01			
C16:1 Δ9	11.81 ± 2.31	11.63 ± 1.51	11.39 ± 2.19	14.69 ± 0.52	13.91 ± 1.06	16.02 ± 1.54		*	
C17:0	0.34 ± 0.01	0.57 ± 0.23	0.32 ± 0.01	0.41 ± 0.09	0.41 ± 0.13	0.47 ± 0.01			
C17:1 Δ10	0.17 ± 0.06	0.24 ± 0.04	0.19 ± 0.04	0.15 ± 0.05	0.13 ± 0.04	0.14 ± 0.02			
C18:0	2.10 ± 0.60	2.62 ± 0.07	2.49 ± 0.33	1.82 ± 0.63	2.16 ± 0.47	2.52 ± 0.44			
C18:1 Δ9	3.01 ± 0.16	4.82 ± 0.18	6.53 ± 2.59	4.78 ± 1.33	4.28 ± 1.31	3.74 ± 0.08			
C18:1 Δ11	4.01 ± 0.16	4.52 ± 0.57	4.10 ± 0.69	4.20 ± 0.66	4.26 ± 0.47	4.53 ± 0.28			
C18:2 Δ9,12	1.06 ± 0.04	1.16 ± 0.04	1.00 ± 0.03	1.19 ± 0.25	1.05 ± 0.25	1.10 ± 0.01			
C18:3 Δ6,9,12	0.46 ± 0.12	0.46 ± 0.06	0.40 ± 0.01	0.45 ± 0.08	0.44 ± 0.05	0.46 ± 0.01			
C20:0	0.20 ± 0.03	0.27 ± 0.15	0.22 ± 0.05	0.19 ± 0.01	0.25 ± 0.06	0.25 ± 0.06			
C18:3 Δ9,12,15	0.37 ± 0.03	0.48 ± 0.11	0.34 ± 0.05	0.47 ± 0.19	0.41 ± 0.18	0.28 ± 0.11			
C20:1 Δ11	0.26 ± 0.12	0.61 ± 0.20	0.71 ± 0.22	0.59 ± 0.16	0.48 ± 0.14	0.36 ± 0.04			
C21:0	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.01			
C20:2 Δ11,14	0.23 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.23 ± 0.03	0.22 ± 0.05	0.19 ± 0.02			
C20:3 Δ8,11,14	0.51 ± 0.15	0.46 ± 0.03	0.44 ± 0.05	0.37 ± 0.01	0.43 ± 0.03	0.42 ± 0.06			
C22:0	1.68 ± 0.54	1.42 ± 0.09	1.10 ± 0.26	1.11 ± 0.08	1.33 ± 0.24	1.26 ± 0.40			
C20:4 Δ5,8,11,14	0.09 ± 0.02	0.10 ± 0.04	0.09 ± 0.04	0.07 ± 0.01	0.07 ± 0.05	0.23 ± 0.18			
C22:1 Δ13	0.11 ± 0.02	0.17 ± 0.08	0.21 ± 0.06	0.16 ± 0.01	0.16 ± 0.04	0.13 ± 0.01			
C22:1 Δ11	0.95 ± 0.04[a]	1.06 ± 0.07[a]	1.13 ± 0.06[a]	0.90 ± 0.06[ab]	0.95 ± 0.11[a]	0.60 ± 0.11[b]		**	*
C20:5 Δ5,8,11,14,17	22.22 ± 5.49	20.78 ± 2.11	16.44 ± 2.68	16.41 ± 1.07	18.22 ± 2.41	17.40 ± 2.79			
C23:0	0.06 ± 0.01	0.04 ± 0.04	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.05 ± 0-01			
C22:2 Δ13,16	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.00			
C24:0	1.38 ± 0.05	1.31 ± 0.01	1.52 ± 0.19	1.20 ± 0.25	1.33 ± 0.04	1.10 ± 0.13			
C24:1 Δ15	1.26 ± 0.04	1.22 ± 0.12	1.32 ± 0.28	1.00 ± 0.34	1.20 ± 0.03	1.03 ± 0.07			
C22:5 Δ7,10,13,16,19	2.65 ± 0.40	2.69 ± 0.38	2.37 ± 0.40	2.56 ± 0.00	2.56 ± 0.00	2.17 ± 0.32			
C22:6 Δ4,7,10,13,16,19	23.08 ± 0.19[ab]	20.90 ± 2.13[ab]	26.19 ± 5.19[a]	19.11 ± 5.20[ab]	19.10 ± 2.76[ab]	13.46 ± 1.82[b]		*	
SFA	27.70 ± 3.63[ab]	28.38 ± 1.39[ab]	26.84 ± 2.96[b]	33.09 ± 1.45[ab]	32.03 ± 1.24[ab]	37.63 ± 3.75[a]		**	
MUFA	21.65 ± 2.41	24.34 ± 1.80	25.65 ± 5.47	26.58 ± 2.33	25.47 ± 0.99	26.67 ± 0.99			
PUFA	50.66 ± 6.05	47.28 ± 0.41	47.52 ± 8.43	40.34 ± 3.78	42.50 ± 0.25	35.71 ± 4.74		*	
n-3 PUFA	48.31 ± 5.73	44.84 ± 0.47	45.33 ± 8.32	38.02 ± 4.14	40.28 ± 0.17	33.30 ± 4.82		*	

[a] Significance of the factors at P < 0.05 (*) and P < 0.01 (**) levels. Factors are dipping time (Dt), dipping medium (Dm), and their interaction (Dt × Dm).

[b] Cm:n Δx, m = number of carbon atoms; n, number of double bonds; x, position of double bonds; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3 PUFA = Σ(C18:3 Δ9,12,15 + C20:5 Δ5,8,11,14,17 + C22:5 Δ7,10,13,16,19 + C22:6 Δ4,7,10,13,16,19).

[c] Means marked with a different letter are significantly different at an alpha level of 0.05 according to an HSD test.

Table 2
Content of non-volatile lipid oxidation products (mean ± SD, n = 3) of crude lipids extracted from sardine fillets soaked in PAW and sterile distilled water SDW). P10-30 are PAW-soaked fillets for 10–30 min; C10-30 are SDW-soaked fillets for 10–30 min.

	Controls[c]			Treatments[c]			ANOVA[a]		
	C10	C20	C30	P10	P20	P30	Dt	Dm	Dt × Dm
PV (meq O_2/kg fat)	3.2 ± 0.4	6.9 ± 0.6	7.0 ± 1.1	5.0 ± 0.4	6.3 ± 2.0	7.0 ± 1.3	*		
7α-OH[b] (μg/g fat)	15.4 ± 5.6	15.8 ± 6.0	26.4 ± 10.9	16.3 ± 1.4	12.8 ± 1.4	13.4 ± 0.1			
6β-OH (μg/g fat)	7.7 ± 0.9	9.0 ± 3.4	7.6 ± 1.1	9.3 ± 1.1	8.2 ± 4.8	11.3 ± 0.1			
6α-OH (μg/g fat)	2.0 ± 1.4	1.8 ± 1.4	3.2 ± 0.5	1.1 ± 0.3	1.4 ± 0.1	1.5 ± 0.0			
7β-OH (μg/g fat)	23.4 ± 7.8	25.0 ± 12.8	38.6 ± 15.1	21.6 ± 2.3	24.9 ± 8.5	30.9 ± 0.7			
4β-OH (μg/g fat)	1.4 ± 0.6	1.5 ± 0.2	1.4 ± 0.5	0.7 ± 0.1	0.8 ± 0.8	1.3 ± 0.0			
5,6β-epoxy (μg/g fat)	14.6 ± 4.3	9.8 ± 1.7	15.6 ± 2.1	8.5 ± 0.2	9.5 ± 5.3	12.8 ± 0.3			
5,6α-epoxy (μg/g fat)	9.3 ± 2.4	4.7 ± 1.1	5.5 ± 1.5	4.2 ± 0.2	4.5 ± 2.6	6.2 ± 0.1			
5-OH (μg/g fat)	1.6 ± 0.4	0.9 ± 0.2	1.7 ± 0.2	1.1 ± 0.2	0.5 ± 0.2	0.7 ± 0.0			
4α-OH (μg/g fat)	1.5 ± 0.6	1.2 ± 0.0	1.8 ± 0.1	1.2 ± 0.0	1.1 ± 0.4	1.3 ± 0.0			
3,5,6-triol (μg/g fat)	4.1 ± 0.2	2.3 ± 1.4	3.2 ± 1.3	2.3 ± 0.6	3.0 ± 0.6	3.5 ± 0.1			
Triol (μg/g fat)	0.6 ± 0.3	0.9 ± 0.1	1.0 ± 1.0	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.0			
7-keto (μg/g fat)	37.9 ± 1.3	20.5 ± 3.2	33.4 ± 5.8	21.3 ± 2.3	25.2 ± 9.1	31.5 ± 0.7			*
Total COPs (μg/g fat)	118.8 ± 23.3	93.2 ± 30.5	139.1 ± 17.5	88.6 ± 5.2	93.1 ± 31.1	115.6 ± 1.9			
Total COPs (μg/100 g FM)	395.5 ± 30.9	531.8 ± 283.8	485.7 ± 48.5	374.2 ± 60.3	465.8 ± 50.0	635.9 ± 10.5			

[a] Significance of the factors at P < 0.05 (*) and P < 0.01 (**) levels. Factors are dipping time (Dt), dipping medium (Dm), and their interaction (Dt × Dm).
[b] COPs abbreviations as in Table S2 (Supplementary Material). FM, fresh matter.
[c] Means marked with a different letter are significantly different at an alpha level of 0.05 according to an HSD test.

meq O_2/kg lipids, which was comparable to the levels of hydroperoxides previously reported in PAW-processed mackerel fillets (Zhao et al., 2021). Although lipids most sensitive to oxidative degradation (n-3 PUFAs) were significantly reduced by the PAW treatment, the amount of FA hydroperoxides did not reflect the effect of the dipping medium. Conversely, soaking time significantly affected the degree of primary peroxidation of the FA hydrocarbon chains.

The loss of n-3 PUFAs detected in PAW-soaked samples was consistent with the content of volatile oxidation products derived from the cleavage of FA hydroperoxides. Approximately 40 volatiles were identified or tentatively identified in the headspace of sardine oils collected with a divinylbenzene/carboxen/polydimethylsiloxane SPME fibre. Matching of MS data and chromatographic behaviour of volatiles with proprietary libraries was used for peak identification. Chemical ionisation experiments were performed on some samples to obtain more information about the structure of the unknown compounds. This is because the ionisation conditions are milder than the electron impact, which increases the relative abundance of the molecular ion and facilitates the identification of the molecular weight of the unknown components. A summary of the mass spectral data and chromatographic retentions used for peak identification can be found in the supplementary material (Table S1). As previously reported by Giogios et al. (2009), the headspace of sardine lipids was characterised by a large number of long-chain alkanes and alkenes, but key markers of lipid peroxidation were also detected. Most of the volatiles originate from the β-cleavage of hydroperoxide isomers of n-3 PUFAs, which is reflected in their structures, namely conjugated trienes (2,4-heptadienals, 3,5-octadien-2-one), C6–C7 alkenals (2-hexenal, 4-heptenal), cyclic (2-ethylfuran) and C5 volatiles (2-pentenal, 2-penten-1-ol, 3-penten-2-one). In particular, 2,4-heptadienals could be formed from 16-OOH of DHA and 14-OOH of EPA, while 2-alkylfurans were formed from the corresponding (E)-2-alkenals (Gómez-Cortés, Sacks, & Brenna, 2015). The origin of the straight-chain alkanals (hexanal, nonanal, octanal) could be traced to the cleavage of n-6 (13-OOH of linoleic acid) and n-9 (10-OOH, 11-OOH of oleic acid) FA hydroperoxides (Xu, Yu, Li, Chen, & Wang, 2017). Both treatment and soaking time strongly affected the amount of secondary volatile oxidation products derived from n-3 PUFAs (4-heptenal, 2,4-heptadienal isomers, 3,5-octadien-2-one), which contributed most to the aroma of the fillets (Table 3). Also n-nonanal increased significantly in PAW-treated fillets depending on dipping time.

It is noteworthy that hexanal is often chosen as a typical marker when volatiles are used to measure the extent of lipid oxidation of foods. However, in fish oils, due to their FA composition which is very rich in PUFAs, other products such as (E,E)-2,4-heptadienal and 4-heptenal have been characterised as very strong odorants that contribute to the unpleasant fishy aftertaste (Gómez-Cortés et al., 2015; Xu et al., 2017))

Table 3
Levels of volatile oxidation markers (GC-FID Area counts × 10^{-3}; mean ± SD, n = 3) sampled by SPME in the headspace of sardine lipids from PAW-processed fillets (P samples) and controls dipped in distilled water (C samples). 10–30 are the dipping times (min).

	Controls[b]			Treatments[b]			ANOVA[a]		
	C10	C20	C30	P10	P20	P30	Dt	Dm	Dt × Dm
furan, 2-ethyl-	122 ± 63	274 ± 167	406 ± 221	402 ± 203	358 ± 184	615 ± 23			
3-penten-2-one, (E)-	95 ± 1	211 ± 127	326 ± 223	259 ± 49	284 ± 148	286 ± 30			
2-pentenal, (E)-	177 ± 113	225 ± 179	396 ± 146	233 ± 18	289 ± 18	278 ± 7			
2-penten-1-ol, (Z)-	429 ± 255	421 ± 313	349 ± 15	347 ± 5	877 ± 14	648 ± 38			
hexanal	118 ± 27	304 ± 183	420 ± 395	279 ± 29	314 ± 141	447 ± 28			
2-hexenal, (E)-	65 ± 2	112 ± 45	250 ± 119	124 ± 10	147 ± 45	302 ± 78	*		
4-heptenal, (Z)-	78 ± 16[b]	227 ± 198[b]	335 ± 300[b]	268 ± 30[b]	254 ± 152[b]	3623 ± 1462[a]	**	*	*
2,4-heptadienal, (E,Z)-	335 ± 284[b]	765 ± 281[b]	552 ± 335[b]	853 ± 332[b]	807 ± 293[b]	5233 ± 240[a]	***	***	***
n-octanal	23 ± 5	24 ± 11	20 ± 14	29 ± 11	45 ± 4	44 ± 10		*	
2,4-heptadienal, (E,E)-	640 ± 213[b]	1364 ± 828[b]	1139 ± 621[b]	1292 ± 122[b]	2082 ± 26[b]	5888 ± 1537[a]	**	**	*
3,5-octadien-2-one, (E,E)-	2931 ± 40[b]	4793 ± 621[b]	4577 ± 980[b]	6970 ± 110[b]	6955 ± 1998[b]	14,045 ± 912[a]	**	**	**
n-nonanal	227 ± 86[b]	352 ± 4[b]	569 ± 405[b]	277 ± 7[b]	1298 ± 570[b]	4249 ± 1063[a]	**	**	**

[a] Significance of the factors at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) levels. Factors are dipping time (Dt), dipping medium (Dm), and their interaction (Dt × Dm).
[b] Means marked with a different letter are significantly different at an alpha level of 0.05 according to an HSD test.

and should be considered as more effective markers of lipid oxidation. Although some attempts have been made to assess the contribution of volatile compounds to the overall flavour profile and to identify important markers of oxidation (Gómez-Cortés et al., 2015; Xu et al., 2017)), several critical factors (odour detection thresholds, internal standards used to quantify volatiles, interaction effects between volatiles) might limit the effectiveness of the quantitative parameters proposed to weigh the contributions of volatiles to sensory perception.

3.2. Effect of PAW on the oxidation degree of cholesterol

Cholesterol is a key component of animal cell membranes, necessary for regulating their fluidity and functionality. The total cholesterol content of sardine fillets ranged from 965 to 1067 mg/100 fat (corresponding to 33–54 mg/100 g fresh weight) (Table 1), which is consistent with previously reported data on *S. pilchardus* caught in the Mediterranean Sea (Cardenia, Rodriguez-Estrada, Baldacci, & Lercker, 2013). No significant differences in total cholesterol content were found between controls and treated samples, nor in the levels of other oxidation-sensitive substances identified in the unsaponifiable matter of sardine lipids (Mozzon, Pacetti, Frega, & Lucci, 2015): squalene (31–39 mg/100 g fat; 1.3–1.8 mg/100 g fresh weight) and α-tocopherol (1.9–5.1 mg/100 g fat; 0.1–0.2 mg/100 g fresh weight).

The presence of a double bond in position 5,6 of the B-ring makes cholesterol as sensitive as MUFAs to autoxidation and photooxidation reactions, producing a range of polar (epoxy, hydroxy and keto derivatives) and non-polar (dienes, dienones, enones) products. The former have been studied for decades due to their proven toxicity and association with various degenerative diseases (Maldonado-Pereira, Schweiss, Barnaba, & Medina-Meza, 2018). To the authors' knowledge, only Pérez-Andrés et al. (2020) reported the ability of a cold plasma source (DBD) to degrade pure cholesterol in a model system, but no data on degradation products were presented.

Twelve cholesterol oxidation products (COPs) have been identified or tentatively identified in sardine lipids. The presence of 7-hydroxycholesterol epimers, 5,6-epoxycholesterol isomers, 7-ketocholesterol and cholestane-3β,5α,6β-triol was confirmed by spiking with available pure analytical standards, while the presence of less common COPs was established by careful interpretation of mass fragmentation patterns and comparison with MS and chromatographic retention data published in the literature. A summary of the MS and chromatographic retention data used for peak identification can be found in the supplementary materials (Table S2). The peaks at RRTs (relative retention times to 5α-cholestane on a DB-5 type column) 1.27 and 1.34 were assigned to stereoisomers of cholest-4-ene-3,6-diol. The presumed molecular ion at m/z 546, corresponding to the monohydroxy derivatives of cholesterol, was confirmed by the presence of fragments at m/z 531 ([M-15]$^+$), 456 ([M-90]$^+$) and 441 ([M-90-15]$^+$), due to the loss of the methyl group and/or the trimethylsilylhydroxy group (TMSOH). The abundant fragment ion at m/z 403, resulting from the loss of two methyl groups and the side chain (C$_8$H$_{17}$), confirmed the localisation of the two hydroxy groups in the ring system and was suggested by Grandgirard, Martine, Joffre, Juaneda, and Berdeaux (2004) to be characteristic of the EI fragmentation patterns of 4-ene-6-hydroxy structures. The RRT value of 1.27 was consistent with those reported by the same authors for 6β-hydroxycholesterol; therefore, the peak at RRT 1.34 was tentatively attributed to 6α-hydroxycholesterol. The mass spectra of the peaks at RRT 1.39 and 1.46 revealed a prominent ion at m/z 147, the origin of which is not yet known and which has been reported as a marker for cholestene-3,4-diols (Breuer, 1995), and a group of fragment ions at m/z 129 (A-ring fragment), 417 [M-Aring]$^+$ and 327 [M-90-Aring]$^+$, characterising the A-ring cleavage of TMS derivatives of Δ^5-steroids. Based on these fragmentation patterns and the chromatographic behaviour reported by Grandgirard et al. (2004), the peaks at RRT 1.39 and 1.46 were tentatively assigned to 4β- and 4α-hydroxycholesterol, respectively. A cholestenediol structure was also suggested by the mass spectra of the

component at RRT 1.45, which showed intense peaks at m/z 456 and 441, associated with the loss of TMSOH and a further loss of a methyl group from a molecular ion of 546 Da. It was suggested that this was 6-cholestene-3,5-diol formed together with 4-cholestene-3,6-diols (peaks at RRTs 1.27 and 1.34), by a concerted ene addition of singlet oxygen to the Δ^5 unsaturation of cholesterol. The presence of a 5-hydroxy derivative of cholesterol has been previously suggested in photooxidised butter (Luby, Gray, Harte, & Ryan, 1986), while the precursor 5α-hydroperoxide has been described as the major oxidation product of cholesterol in photooxidised cell membranes (Bachowski, Thomas, & Girotti, 1988). The peak at RRT 1.56 showed a fragmentation pattern very similar to that of pure cholestane-3β,5α,6β-triol and was tentatively assigned to a cholestane triol stereoisomer.

Total COPs ranged from 88.6 to 139.1 μg/g fish lipids, corresponding to 374.2–635.9 μg/100 g fresh weight and 15.6–25.0 μg/g dry weight (average water content of fillets was 75.8 ± 1.8 g/100 g) (Table 2). These values were lower than those reported by Barreira et al. (2023) (39.53 ± 2.14 μg/g dry weight) and Ferreira et al. (2017) (61.2 ± 2.8 μg/g dry weight), but higher than the COP levels in raw sardines reported by de Carvalho et al. (2021) (11.5 ± 0.1 μg/g dry weight) and Cardenia et al. (2013) (62–371 μg/100 g fresh weight). The calculated percentage of cholesterol oxidation ranged from 0.92 to 1.21, slightly higher than the values reported by Cardenia et al. (2013) (0.1–0.9%) for sardine fillets stored at 4 °C for 4 h in the dark and exposed to light. The experimental data showed no significant effect of PAW treatment on cholesterol oxidation of sardine fillets. However, the total content of COPs in untreated and PAW-soaked fillets could pose a risk to human health, according to the threshold of toxicological concern (TTC) for unclassified compounds reported in Cardenia et al. (2013). The same authors emphasised that storage could be a more critical factor for the formation of COPs than processing. In fact, 4 h of exposure to light at 4 °C was sufficient to increase the total COPs of sardine fillets by 6 times.

The reaction mechanisms involved in the formation of COPs have been largely elucidated (Iuliano, 2011). Free radical-mediated oxidation pathways start with hydrogen abstraction from allylic positions (predominantly C7) and evolve via a chain-reaction mechanism that eventually leads to 7-hydroxy and 7-keto derivatives of cholesterol. Singlet oxygen reacts directly with the Δ^5 double bond through a concerted "ene" addition mechanism. Accordingly, the oxygen is inserted at one of the two carbon atoms of the double bond, which is shifted to an allylic position. The photosensitised oxidation of cholesterol mainly produces the 6-ene-5α-hydroperoxide, which is converted to the more stable epimers of 4-ene-6-hydroperoxide, producing 5- and 6-monohydroxy derivatives. The double bond in ring B can also be the target of peroxide-containing reagents (lipid hydroperoxides, peroxyl radicals, peroxyacids, hydrogen peroxide), which act as donors of a single oxygen atom to form 5,6-epoxides. Under acidic conditions, epoxides can undergo hydrolytic epoxide-ring opening to form 3,5,6-cholestane triols. Ozone can also react directly with Δ^5 unsaturation to form epoxides via the unstable intermediate 1,2,3-trioxolane. The qualitative and quantitative profile of COPs could therefore provide useful information on the preferred mechanism of oxidation and help to clarify the relationships between causes (plasma reactive species) and effects (COPs). In particular, 7-hydroxy and 7-keto derivatives are expected to be the major COPs, and our data confirmed this general behaviour (65–71% of total COPs), indicating a major contribution of the free radical-mediated oxidation pathway. The dominance of the β-epimer among the 7-hydroxy compounds has been frequently reported, and it was confirmed by our experimental data (ratio 7β-OH/7α-OH = 1.3–2.3). The very low amount of 4-OH-5-ene isomers confirmed a large asymmetry in the behaviour of the two allylic positions (C4 and C7) with respect to Δ^5 unsaturation. Epoxidation also contributed significantly to cholesterol degradation (14–20% of total COPs). As briefly summarised above, both free radical and non radical-mediated pathways may be involved in the formation of 5,6-epoxides, depending on which lipid (FA hydroperoxides and peroxyl radicals) and non-lipid species (hydrogen peroxide,

peroxynitrite, ozone) may trigger the oxidative cascade. The presence of epimeric 4-en-6-hydroxycholesterol and 5-hydroxycholesterol supports the contribution of photosensitised, non-radical-mediated oxidation of cholesterol. The 5,6β-epoxy/5,6α-epoxy ratio was in the range of 1.6–2.9, lower than the values reported by Iuliano (2011) (3–11).

3.3. Effect of PAW on microbial decontamination of sardine fillets

Indicators of faecal contamination (*Enterobacteriaceae*, *E. coli*) were below the detection limit (10 CFU/g) in all samples. The total number of mesophilic aerobes and *Pseudomonas* in the processed samples (PAW-dipped and SDW-dipped) ranged from 2.35 to 2.72 and 1.65–1.92 Log CFU/g, respectively, demonstrating the freshness of the raw materials. After 4 days of storage at 4 °C, the bacterial counts increased to 3.74–4.19 and 3.74–4.18 Log CFU/g, for mesophilic aerobes and *Pseudomonas* respectively (Fig. 1). Under the selected experimental conditions (soaking time 10–30 min; solid/liquid ratio 1:3 w/v), both the soaking medium and the contact time affected microbial load (Table 4). At the longest treatment time (30 min), the total number of mesophilic aerobes and *Pseudomonas* was 0.22 and 0.20 Log CFU/g lower, respectively, in fillets soaked with PAW than in fish soaked with SDW. Unexpectedly, after 4 days of storage at 4 °C, the vital counts were higher in the samples treated with PAW than in the corresponding controls soaked in SDW for the same time. The available data on the efficacy of PAW in microbial decontamination of fish and fish products are very limited and do not demonstrate significant and overall efficacy of the treatment. Liu et al. (2021) reported a 1.03 log reduction of *Shewanella putrefaciens* inoculated onto carp fillets after 6 min immersion in PAW, while up to 1.21 log reductions of *L. monocytogenes* and 1.44 log reductions of *S. Typhimurium* were measured in PAW-soaked grass carp fillets (Esua et al., 2020). However, Zhao et al. (2021) observed that PAW treatments of mackerel fillets (dipping time 10 min; solid/liquid ratio 1:3 w/v) did not significantly reduce the natural microbiota (total mesophilic and total psychrotrophic bacteria) and inoculated bacterial species (*E. coli*, *Listeria innocua*, *Pseudomonas fluorescens*) compared to immersion in water. Zhao, Ojha, Burgess, Sun, and Tiwari (2020) also reported no significant decontamination effect after 30 min immersion of mackerel cubes inoculated with 6.6 Log CFU/g *P. fluorescens* in PAW prepared from SDW activated by plasma jet. Chaijan et al. (2022) found no

Table 4
Analysis of variance[a] applied to a full factorial linear model in which dipping time (Dt; 10–30 min) and dipping medium (PAW vs SDW) are the factors potentially influencing total aerobic mesophilic bacteria (TVC 30 °C) and *Pseudomonas* spp (Log CFU/g samples, n = 3).

Factors	After soaking		After 4 days of storage at 4 °C	
	TVC 30 °C	*Pseudomonas* spp	TVC 30 °C	*Pseudomonas* spp
Dt	***	*	***	**
Dm	**		***	**
Dt × Dm	***	**	**	*

[a] Significance of the factors at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) levels.

differences in bacterial counts (total viable count, total psychrotrophic bacteria, *Pseudomonas* spp.) between PAW-soaked Asian sea bass steaks and controls dipped in distilled water (solid/liquid ratio 1:3 w/v; contact time 120 s). The same authors indicated that treatment with PAW was able to slow down microbial growth during storage at 4 °C, thus extending the shelf life of the product by three times. PAW ice was also able to significantly retard microbial growth in shrimp and extend their storage time (Herianto et al., 2022; Liao et al., 2018). A comprehensive understanding of microbial inactivation mechanisms is paramount for an emerging technology, especially to assess whether the technology will effectively kill microbial cells or could sublethally injure them. Reactive oxygen species (singlet oxygen, hydroxyl radicals, hydrogen peroxide) have been shown to damage cell wall and membrane. Impaired membrane integrity promotes the flux of reactive species into the cellular environment, resulting in severe damage to intracellular components such as DNA. The physical effects of PAW on microbial cells, including pH, UV photons and mechanical waves, have also been described (Zhao, Patange, et al., 2020).

Recently, different types of organic substances (bovine serum albumin, beef extract, peptone, fish gelatin, fish oil, fish homogenate) have been tested for their ability to reduce the bactericidal efficacy of PAW and a "quenching" effect of the matrix against the PAW reactive species has been suggested by several authors (Xiang et al., 2019; Zhao, Ojha, et al., 2020). In our experiments, the pH of PAW increased from 3.64 to 6.36–6.65 after 10 min of contact with fish fillets and to 6.78–6.80 after 30 min of contact, which was consistent with the decreased ability of
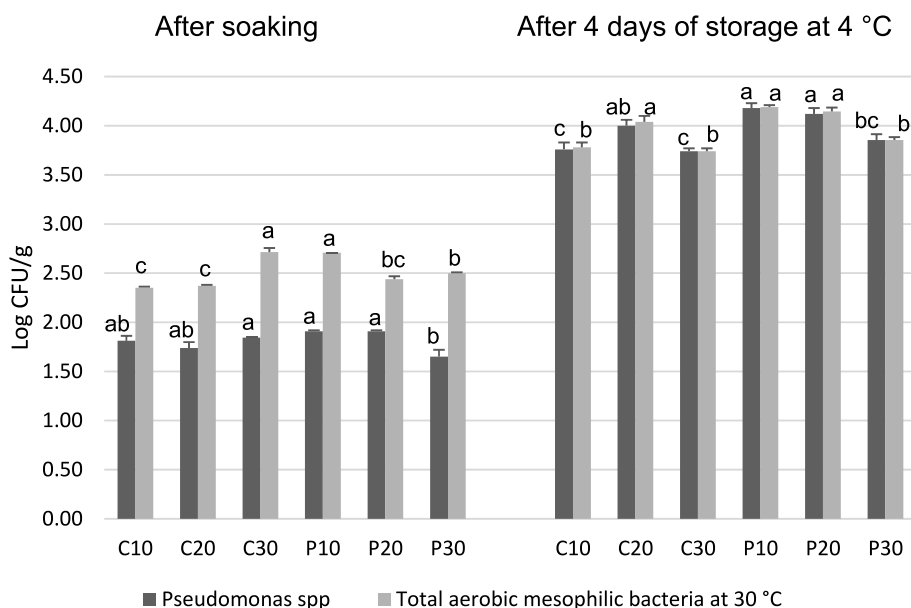

Fig. 1. Total aerobic mesophilic bacteria at 30 °C and *Pseudomonas* spp counts (Log CFU/g samples; mean ± SD, n = 3) in sardine fillets subjected to different treatments. Pn are soaked in PAW; Cn are soaked in sterile distilled water; 10–30 are soaking times in minutes. Means marked with a different letter are significantly different at an alpha level of 0.05 according to an HSD test.

plasma discharge to lower the pH of PAW added by fish gelatin (Zhao, Ojha, et al., 2020) and with the increase in the pH of PAW in a dose-dependent manner after the addition of beef extract and peptone (Xiang et al., 2019). Therefore, a scavenging effect of amino acids and proteins extracted by liquid media from fish tissues during soaking was hypothesised, but further studies are needed to verify the behaviour of PAW reactive species during the contact with proteinaceous materials.

4. Conclusions

Oxidation of lipids has been reported in various types of foods processed with PAW. However, the assessment of the degree of oxidation was limited to the analysis of global indices for primary and secondary oxidation products (peroxide value and thiobarbituric acid reactive substances, respectively) and, to a lesser extent, the total FA composition. More advanced analytical techniques for direct quantification of chemical species derived from FA and cholesterol oxidation have not yet been performed in seafood. In particular, headspace sampling by SPME and direct GC–MS analysis of oxygenated volatile compounds derived from β-cleavage of primary hydroperoxides have proven to be sensitive and reliable methods to assess the progress of lipid oxidation in food. Due to the varying levels of toxicity, identification and quantification of the chemical species derived from the oxidative degradation of cholesterol is essential to evaluate the safety of the technology. This is a crucial step for the successful introduction of PAW in food processing and its scale-up to from pilot plant to industrial production.

Under the adopted experimental conditions, the PAW soaking of sardine fillets was able to reduce the natural microflora to a very small extent (0.22 and 0.20 log units for the total number of mesophilic aerobes and *Pseudomonas* spp, respectively), but no improvements in shelf-life at refrigeration temperatures were observed. At the longest treatment duration (30 min), signs of PAW-induced oxidative degradation of fish lipids were observed (loss of PUFAs; increase in secondary volatile oxidation products derived from the cleavage of FA hydroperoxides), but the qualitative and quantitative composition of the COPs fraction was not significantly affected by contact with PAW. The composition of COPs showed a major contribution from oxidation pathways mediated by free radicals, but a significant contribution from non-radical pathways triggered by non-lipid reactive species of PAW (ozone, hydrogen peroxide, peroxynitrite) was also observed.

However, the properties of PAW are influenced by many critical parameters that directly affect the type and concentration of RONS generated: plasma source, energy input (power, voltage, frequency), feeding gas and flow rate, pressure (atmospheric, vacuum chamber), water volume, plasma-liquid interface contact mode (in-water, above water, aerosol, bubble), activation time. The mode of application introduces additional variables (immersion time, stirring speed, temperature), which makes comparing experimental data on microbial decontamination and clarifying the relationship between the reactive species and the chemistry of lipid oxidation in food a major challenge.

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CRediT authorship contribution statement

Massimo Mozzon: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. **Cinzia Mannozzi:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **Roberta Foligni:** Investigation, Writing – review & editing. **Andrea Osimani:** Investigation, Writing – review & editing. **Filippo Galdenzi:** Investigation, Visualization. **Romolo Laurita:** Resources, Investigation, Writing – review & editing. **Silvia Tappi:** Resources, Investigation, Writing – review & editing, Project administration. **Marco Dalla Rosa:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115401.

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