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Effect of Aqueous and Lipophilic Mullet (*Mugil cephalus*) Bottarga Extracts on the Growth and Lipid Profile of Intestinal Caco-2 Cells

Antonella Rosa,* Angela Atzeri, Monica Deiana, M. Paola Melis, Debora Loru, Alessandra Incani, Barbara Cabboi, and M. Assunta Dessì

Dipartimento di Biologia Sperimentale, Sezione di Patologia Sperimentale, Università degli Studi di Cagliari, Cittadella Universitaria, SS 554, Km 4.5, 09042 Monserrato, Cagliari, Italy

ABSTRACT: The importance of $n-3$ polyunsaturated fatty acid ($n-3$ PUFA) intake has long been recognized in human nutrition. Although health benefits, $n-3$ PUFA are subject to rapid and/or extensive oxidation during processing and storage, resulting in potential alteration in nutritional composition and quality of food. Bottarga, a salted and semi-dried mullet (*Mugil cephalus*) ovary product, is proposed as an important source of $n-3$ PUFA, having high levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In this work, we investigated the extent of lipid oxidation of grated bottarga samples during 7 months of storage at −20 °C and room temperature under light exposure. Cell viability, lipid composition, and lipid peroxidation were measured in intestinal differentiated Caco-2 cell monolayers after 6−48 h of incubation with lipid and hydrophilic extracts obtained from bottarga samples at different storage conditions. The storage of bottarga did not affect the $n-3$ PUFA level, but differences were observed in hydroperoxide levels in samples from different storage conditions. All tested bottarga extracts did not show a toxic effect on cell viability of differentiated Caco-2 cells. Epithelial cells incubated with bottarga oil had significant changes in fatty acid composition but not in cholesterol levels with an accumulation of EPA, DHA, and 22:5. Cell hydroperoxides were higher in treated cells, in relation to the oxidative status of bottarga oil. Moreover, the bottarga lipid extract showed an *in vitro* inhibitory effect on the growth of a colon cancer cell line (undifferentiated Caco-2 cells).

KEYWORDS: Bottarga, $n-3$ PUFA, lipid peroxidation, Caco-2 cells, cholesterol, fatty acids

1. INTRODUCTION

The chemical composition of edible fish and fish derivatives is widely studied for its high content of health-beneficial, long-chain, $n-3$ polyunsaturated fatty acids ($n-3$ PUFA), namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[1,2] Many studies have suggested that $n-3$ PUFA have an important role in human health, in the treatment of various diseases and disorders.[1,3] Lipid oxidation in foods is one of the major degradative processes responsible for changes in flavor, color, and texture during manufacturing procedures and prolonged storage; the oxidation of unsaturated lipids results in significant generation of cytotoxic and genotoxic compounds.[4] Although health benefits, $n-3$ PUFA are subject to rapid and/or extensive oxidation by exposure to air, light, or heat during processing, resulting in potential alteration in nutritional composition and quality of food.[5−7] Several PUFA oxidative degradation products have been measured in fish derivatives, such as lipid hydroperoxides and volatile secondary lipid oxidation products.[5,7,8] The salted and semi-dried mullet (*Mugil* genus) ovary product is regarded as a food delicacy and is produced in several countries in the world: in the western Mediterranean area as "bottarga", in Greece as "avgotaracho", and in Japan as "karasumi".[9,10] Among the manufacturers of bottarga, Sardinia, an insular region of Italy, is nowadays reaching a leader position, thanks to the high quality of bottarga produced. Sardinian bottarga is the final product of a number of treatments (salting and drying) on the whole roe of mullets (*Mugil cephalus*).[11] Nowadays, different procedures exist, from the more traditional to the industrial ones; in the latter, drying is conducted in rooms with controlled humidity and temperature. The final product can be sold as whole ovaries under vacuum packaging or grated in jar. In recent works,[11−13] we have studied the major lipid classes and the fatty acid and alcohol composition of commercial products of whole and grated bottarga samples manufactured in Sardinia. The total lipid content of bottarga samples was estimated to be in the range of 220−325 mg/g of edible portion. Among the major lipid classes, a high quantity of wax esters (approximately 50−65% of lipid classes), triacylglycerols, phospholipids, and cholesterol in its free and esterified forms was found; EPA and DHA amounted to 13−25% of total fatty acids.[11−13] Furthermore, the samples showed a relatively high content of free fatty acids (FFA) because of hydrolysis processes induced on the original lipid matrix by the manufacturing procedures and to a minor extent by storage conditions.[11,12] Despite the low degree of susceptibility to oxidation of $n-3$ PUFA when esterified in wax esters,[14] levels of conjugated diene fatty acid hydroperoxides in commercial grated and whole bottarga samples have been found.[11,12] These compounds are important nonradical intermediates of PUFA peroxidation with many undesirable biological effects.[4,15] Excessive lipid hydroperoxide concentrations in the gut can contribute to the impairment of mucosal detoxification pathways and enterocyte dysfunction, leading to the development of digestive tract disease conditions, such as inflammation and colon cancer.[15] Furthermore, bottarga

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samples during prolonged storage (i.e., under domestic or local market/supermarket conditions) are subject to non-enzymatic browning,[16] a process that has been reported to have controversial effects on food quality[17] and human health.[18] The beneficial health effect of $n-3$ PUFA in humans is strictly correlated to their modification in the cell membrane structure and their ability to affect numerous cellular functions.[19] To our knowledge, no data concerning the effect of the lipid fraction of mullet bottarga on cell viability and lipid profile have yet been published. In this work, a sample of grated bottarga was investigated for lipid composition and oxidative status immediately after preparation and during 7 months of storage at −20 °C in the dark (normally used for domestic storage) and room temperature under light exposure (conditions frequently adopted in local markets/supermarkets). Lipid oxidation was evaluated by the determination of the conjugated diene fatty acid hydroperoxides (HP) level; bottarga color change was also monitored. The lipid and aqueous fractions, with the latter containing different classes of metabolites,[20] were then isolated from bottarga samples and tested in differentiated Caco-2 cell monolayers, as an intestinal epithelial cell model, for the evaluation of the effect on cell viability, lipid composition, and lipid peroxidation in relation to storage conditions. Experimental studies have recognized that diets rich in $n-3$ PUFA slow the development of prostate, endometrial, breast, and colorectal cancer.[19,21] In this work, we also studied the effect of bottarga lipid extracts on viability in a cancer cell line (undifferentiated Caco-2).

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Cholesterol, triolein, trilinolein, oleyl oleate, cholesteryl palmitate, fatty acids, fatty alcohols, fatty acid methyl ester standards, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and desferal (deferoxamine mesylate salt) were purchased from Sigma-Aldrich (Milan, Italy). All solvents used, of the highest available purity, were also from Sigma-Aldrich. The reagents 20% BF_3 in MeOH and 1,1,1,3,3,3-hexamethyldisilazane (HMDS)/trimethylchlorosilane (TMCS)/pyridine (3:1:9) were purchased from Supelco (Bellefonte, PA). *cis,trans*-13-Hydroperoxyoctadecadienoic acid (*c,t*-13-HPODE) and *cis,trans*-9-hydroperoxyoctadecadienoic acid (*c,t*-9-HPODE) were obtained from Cascade (Cascade Biochem Ltd., London, U.K.). Cell culture materials were purchased from Invitrogen (Milan, Italy). AlamarBlue was obtained from Biosource Europe (Nivelles, Belgium). All of the other chemicals used in this study were of analytical grade.

2.2. Bottarga Sample and Storage Conditions. A grated bottarga sample of mullet was kindly supplied by the company "Stefano Rocca S.r.l." located in Sardinia (Italy). Ingredients reported in the label were mullet roe and salt. The freshly prepared sample (control) was immediately subjected to lipid extraction and analysis. Portions of the bottarga sample were then stored for 7 months in closed jars at −20 °C in the dark and at room temperature under light exposure. Samples were subjected to lipid component analysis at different time points (0, 1, 2, 3, 5, and 7 months). The browning process of bottarga samples was monitored by digital photography.

2.3. Preparation of Bottarga Extracts. The aqueous and lipophilic extracts used for the Caco-2 cell line study were obtained from bottarga stored at two different time and temperature conditions to study the effects in relation to a different peroxidative status and browning process. The extracts were obtained from bottarga within a short time after preparation (35 days at −20 °C in the dark, yellow sample) and during the 7th month of storage at room temperature under light exposure (more drastic oxidative conditions, browned sample). The extraction was performed using the method described by Folch et al.[22] by the addition of 12 mL of $CHCl_3$/MeOH (2:1, v/v) solution. After the addition of 4 mL of H_2O and centrifugation at 900*g* for 1 h, the $CHCl_3$ fraction (lipophilic extract) was separated from the MeOH/H_2O mixture (aqueous extract). Total lipids in the $CHCl_3$ fraction were quantified from the method by Chiang et al.[23] Lipophilic and aqueous extracts were dried under vacuum, and the residues were dissolved in EtOH and water, respectively.

2.4. Cell Cultures. The Caco-2 cell line was obtained from ECACC (Salisbury, Wiltshire, U.K.). Caco-2 cells have been obtained from a human colon adenocarcinoma; after confluence, these cells spontaneously differentiate into enterocytes.[24] Subcultures of the Caco-2 cells were grown in T-75 culture flasks with passage from a trypsin−ethylenediaminetetraacetic acid (EDTA) solution. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 2 mM L-glutamine, and penicillin (100 units/mL)−streptomycin (100 μg/mL) at 37 °C in 5% CO_2.

2.5. Cytotoxic Activity in Differentiated Caco-2 Cells: AlamarBlue Test. The cytotoxic effect of aqueous and lipophilic mullet bottarga extracts was evaluated in small intestine enterocytes (differentiated Caco-2) by the alamarBlue test.[25] For cytotoxicity experiments, Caco-2 cells were seeded in 24-well plates at a density of 10^5 cells/mL. Culture medium was replaced 3 times a week for 21 days until complete differentiation of cells. Cells were used in the experiments at a passage number of 50−66. Prior to the addition of extracts, cell culture medium was removed, Caco-2 cells were washed with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}, and then fresh medium was added. Cells were subsequently exposed to various concentrations of the aqueous (corresponding to 0.1−4 mL of MeOH/H_2O fraction obtained from 60 mg of edible portion) and lipophilic (0.2−1 mg/mL, in EtOH solution) extracts in complete culture medium and incubated for 24 h. For the lipophilic extract, an equivalent volume of EtOH was added to the controls. The cell medium was removed and replaced with 1 mL of alamarBlue solution [10% (v/v) in culture medium without FCS] in each well. Cells were incubated for 4 h at 37 °C, to allow for viable cells to convert the alamarBlue oxidize blue form to the reduced pink form. After incubation, the absorbance was measured at 570 and 600 nm with an auto microplate reader (Infinite 200, Tecan, Austria). The percentage of alamarBlue reduction was calculated and compared to the controls.

2.6. Cytotoxic Activity in Undifferentiated Caco-2 Cells: MTT Assay. The cytotoxic effect of lipophilic mullet bottarga extracts was evaluated in cancer Caco-2 by the MTT assay.[19,26] Cells were used in the experiments at a passage number of 50−57. Caco-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL in 100 μL of medium and cultured overnight. Prior to the addition of extracts, the cell culture medium was removed, Caco-2 cells were washed with PBS containing Ca^{2+} and Mg^{2+}, and then fresh medium was added. Cells were subsequently exposed to various concentrations of the lipid (50−500 μg/mL, in EtOH solution) extracts or an equivalent volume of EtOH for the controls and incubated for 24 h. An 8 μL portion of MTT solution (5 mg/mL of PBS) was then added and left for 4 h at 37 °C. The medium was aspirated, and 100 μL of dimethyl sulfoxide (DMSO) was added to the wells. The color development was measured at 570 nm with an Infinite 200 auto microplate reader. The absorbance is proportional to the number of viable cells. Evaluation of dead cells was also performed by microscopic observation.

2.7. Fatty Acid Profile Modulation in Differentiated Caco-2 Cells. Caco-2 cells were plated in Petri dishes at a density of about 10^6 cells/10 mL of complete medium and were used for fatty acid profile modulation experiments at 21 days post-seeding (passage number of 50−69); culture medium was replaced 3 times a week until complete differentiation of cells. Prior to the addition of the aqueous and lipophilic bottarga extracts, the cell culture medium was aspirated, cells were

washed with PBS containing Ca^{2+} and Mg^{2+}, and then fresh medium was added. The differentiated Caco-2 cells were treated with aqueous (corresponding to a 100 μL/mL of $MeOH/H_2O$ fraction obtained from 60 mg of edible portion) and lipophilic (100 μg/mL, in EtOH solution) extracts for 6−48 h. An equivalent volume of EtOH was added as a control to cells treated with lipophilic extracts; the maximal final concentration of EtOH was 0.5%. After treatment, the cells were scraped and centrifuged at 1200*g* at 4 °C for 5 min. After centrifugation, pellets were separated from supernatants and used for lipid extraction and analyses.[27]

2.8. Lipid Extraction and Preparation of Cholesterol, Fatty Alcohols, Fatty Acids, and Hydroperoxides. Total lipids were extracted from bottarga samples at different time points (0, 1, 2, 3, 5, and 7 months) and from differentiated Caco-2 cell pellets using the Folch et al. procedure[12,22,27] and quantified.[23] Separation of cholesterol, fatty alcohols, fatty acids, and oxidative products was obtained by mild saponification[27] as follows: 7 mL of the $CHCl_3$ fraction, containing the lipids, from each sample was dried down and dissolved in 5 mL of EtOH and 100 μL of desferal solution (25 mg/mL of H_2O), 1 mL of a water solution of ascorbic acid (25% w/v), and 0.5 mL of 10 N KOH were added. The mixtures were left in the dark at room temperature for 14 h. After the addition of 10 mL of *n*-hexane and 7 mL of H_2O, samples were centrifuged for 1 h at 900*g*. The hexane phase containing the unsaponifiable fraction (cholesterol and fatty alcohols) was collected, and the solvent was evaporated. A portion of the dried residue was dissolved in 1 mL of MeOH and injected into the high-performance liquid chromatography (HPLC) system. An aliquot of dried fatty alcohols was converted to trimethylsilyl ethers by a mixture of TMCS, HMDS, and anhydrated pyridine (1:3:9, v/v/v) (200 μL) for 2 h at room temperature, before being applied to capillary gas chromatography (GC). After the addition of a further 10 mL of *n*-hexane to the mixtures, samples were acidified with 37% HCl to pH 3−4 and then centrifuged for 1 h at 900*g*. The hexane phase (saponifiable fraction) with free fatty acids and conjugated diene fatty acid hydroperoxides (HP) was collected, and the solvent was evaporated. A portion of the dried residue was dissolved in 300 μL of CH_3CN with 0.14% CH_3COOH (v/v), and aliquots of the samples were injected into the HPLC system. An aliquot of dried fatty acids was methylated with 1 mL of 14% BF_3 in MeOH[28] for 30 min at room temperature. After the addition of 4 mL of *n*-hexane and 2 mL of H_2O, samples were centrifuged for 20 min at 900*g*. The hexane phase with fatty acid methyl esters was collected, and the solvent was evaporated. The residue was dissolved in 100 μL of *n*-hexane, and aliquots of the samples were injected into the GC system. The recovery of fatty acids, fatty alcohols, and cholesterol during the saponification was calculated using an external standard mixture prepared by dissolving 1 mg of triolein, trilinolein, oleyl oleate, cholesterol, and cholesteryl palmitate in 5 mL of EtOH and processed as samples. All solvent evaporation was performed under vacuum.

2.9. HPLC Analyses. Analyses of cholesterol and unsaturated fatty acids were carried out with an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector. Cholesterol, detected at 203 nm, was measured with the use of a Chrompack column (Chrompack, Middelburg, the Netherlands), Inertsil 5 ODS-3, 150 × 3 mm, and MeOH as the mobile phase, at a flow rate of 0.4 mL/min.[12,27] Analyses of unsaturated fatty acids and HP, detected at 200 and 234 nm, respectively, were carried out with a XDB-C_{18} Eclipse (150 × 4.6 mm, 3.5 μm particle size) (Agilent Technologies) equipped with a Zorbax XDB-C_{18} Eclipse (12.5 × 4.6 mm, 5 μm particle size) guard column (Agilent Technologies), with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (75:25:0.12, v/v/v), at a flow rate of 2.3 mL/min. The temperature of the column was maintained at 37 °C. The identification of cholesterol, fatty acids, and HP was made using standard compounds and the second derivative, as well as conventional UV spectra, generated with the Agilent Chemstation A.10.02 software. Calibration curves of all of the compounds were constructed using standards and were found to be linear, with correlation coefficients >0.995.

2.10. GC Analyses. Fatty acid methyl esters and trimethylsilyl ethers were measured on a gas chromatograph Hewlett-Packard HP-6890 (Hewlett-Packard, Palo Alto, CA) with a flame ionization detector and equipped with a cyanopropyl methylpolysiloxane HP-23 FAME column (30 m × 0.32 mm × 0.25 μm) (Hewlett-Packard). Nitrogen was used as a carrier gas at a flow rate of 2 mL/min. The oven temperature was set at 175 °C; the injector temperature was set at 250 °C; and the detector temperature was set at 300 °C. The fatty acid methyl esters and trimethylsilyl derivatives of fatty alcohols were identified by comparing the retention times to those of standard compounds. The composition of individual fatty acid and alcohol was calculated as a percentage of the total amount of fatty alcohols and acids (g %) using the Hewlett-Packard A.05.02 software.

2.11. Statistical Analyses. Graph Pad INSTAT software (GraphPad software, San Diego, CA) was used to calculate the means and standard deviations of two, three, or four independent experiments involving triplicate analyses for each sample. Evaluation of the statistical significance of differences was performed using one-way analysis of variation (ANOVA) and the Bonferroni post test.

3. RESULTS

3.1. Lipid Composition and Oxidation of Bottarga Samples. The lipid fraction was extracted from the grated bottarga control sample (total lipids, 343.7 ± 7.9 mg/g of the edible portion), and fatty alcohol, fatty acid, and cholesterol concentrations were analyzed together with the levels of conjugated diene fatty acid hydroperoxides (HP). Figure 1 shows the fatty acid composition (expressed as a percentage of total fatty acids) (Figure 1A) and chromatographic profile (Figure 1B) of the control bottarga sample by GC. Bottarga showed a concentration of approximately 17% of saturated fatty acids (mainly 16:0 and 18:0, 11.5 and 2.4%, respectively), 38% of monounsaturated fatty acids (mainly 16:1 *n*−7 and 18:1, 19 and 11.3%, respectively), and 35% of polyunsaturated fatty acids (mainly 22:6 *n*−3 and 20:5 *n*−3, 18.4 and 6.7%, respectively). From HPLC, the total cholesterol was measured as the mean content of 7.3 mg/g of the edible portion (22.5 μg/mg of total lipids). Furthermore, the highly unsaturated *n*−3 fatty acid content in the edible portion was detected as follows: 33.5 mg/g of DHA, 10.5 mg/g of EPA, and a minor amount of 22:5 *n*−3 (6.1 mg/g), as reported in Table 1. As found in previous investigations[11−13] only saturated (C_{14}−C_{18} chain length, 77%) and monounsaturated (16:1 and 18:1 isomers, 15%) fatty alcohols were found; in particular, the samples were characterized by a high amount of 16:0 (57%) and 16:1 *n*−7 (about 10%). The grated bottarga sample was divided into two portions, and then samples were stored for 7 months at −20 °C in the dark and room temperature under light exposure. The sample placed at room temperature showed a browning process, evident from 1 month, higher than that observed for the sample stored at a refrigerated temperature, which exhibited after 7 months a color similar to that of the control sample (yellow color) (Figure 2). Figure 3 shows the values of EPA, DHA, cholesterol (expressed as μg/mg of edible portion) and HP (nmol/mg of edible portion) measured in the bottarga control sample (Ctrl) and after 1, 2, 3, 5, and 7 months of storage at −20 °C (Figure 3A) and room temperature in the light exposure (Figure 3B). Stored samples did not exhibit a significant variation of *n*−3 PUFA and cholesterol levels with respect to the control; nevertheless, a significant HP increase (control value of 0.06 ± 0.01 nmol/mg of edible portion and 0.18 ± 0.04 nmol/mg

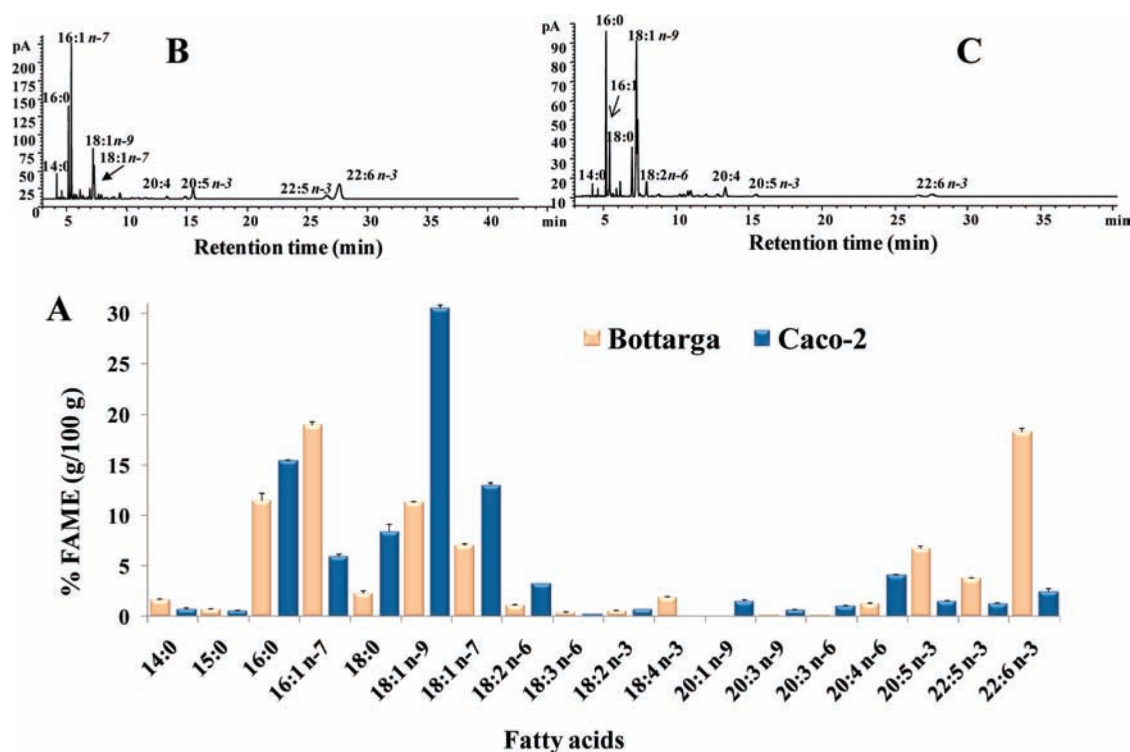

Figure 1. Composition, expressed as a percentage of total fatty acids (%), of fatty acids from the bottarga control sample and differentiated Caco-2 control cells by (A) GC (n = 6) and corresponding GC chromatographic profiles of (B) bottarga and (C) Caco-2 cells.

Table 1. Unsaturated Fatty Acid Compositions by HPLC of Bottarga and Caco-2 Control Samples[a]

fatty acid	bottarga mg/g	bottarga μg/mg of lipid	Caco-2 cells (μg/plate)
16:1 $n-7$	34.89 ± 0.66	101.50 ± 1.91	34.24 ± 9.60
18:1 $n-7$ + 18:1 $n-9$	32.71 ± 1.49	95.16 ± 4.35	253.88 ± 52.77
18:2 $n-6$	1.87 ± 0.36	6.20 ± 1.19	23.53 ± 6.30
18:3 $n-3$	2.29 ± 0.08	6.65 ± 0.24	2.11 ± 0.44
18:3 $n-6$	0.49 ± 0.03	1.44 ± 0.09	0.21 ± 0.08
18:4 $n-3$	3.21 ± 0.09	9.33 ± 0.26	
20:3 $n-3$ + 20:3 $n-6$	2.04 ± 0.13	5.93 ± 0.39	8.27 ± 1.69
20:3 $n-9$			6.45 ± 0.93
20:4 $n-6$	2.69 ± 0.04	7.82 ± 0.11	22.34 ± 3.58
20:5 $n-3$	10.50 ± 0.17	30.56 ± 0.49	11.40 ± 2.65
22:4 $n-6$			1.26 ± 0.26
22:5 $n-3$	6.15 ± 0.20	17.88 ± 0.59	6.53 ± 1.19
22:6 $n-3$	33.53 ± 0.30	97.55 ± 0.89	17.16 ± 3.10

[a] Data are the mean ± standard deviation of six samples.

of lipid) was observed from 1 month for both storage conditions, with higher HP values observed in the sample stored at −20 °C.

3.2. Cytotoxic Activity of Bottarga Extracts in Differentiated Caco-2 Cell Monolayers. The aqueous and lipophilic extracts, obtained from grated bottarga samples stored for 35 days at −20 °C in the dark (yellow sample) and 7 months at room temperature in the light (browned sample) were evaluated for cytotoxicity (alamarBlue test) in differentiated Caco-2 cell cultures. Figure 4 shows the viability, expressed as a percentage of the control, induced in epithelial intestinal cells after 24 h of incubation in the presence of different concentrations of mullet bottarga extracts. All of the extracts did not exert a significant reduction in cell viability at all tested concentrations.

3.3. Lipid Profile of Differentiated Caco-2 Cells Exposed to Bottarga Extracts. The aqueous and lipophilic extracts, obtained from bottarga stored for 35 days at −20 °C in the dark and 7 months at room temperature in the light, were tested in intestinal epithelial cell monolayers (differentiated Caco-2) for the evaluation of the effect on lipid composition and peroxidation, in relation to storage conditions. After 6−48 h of incubation with the bottarga extracts, the cell lipid fraction was extracted and the variations of the levels of fatty acids, fatty alcohols, cholesterol, and HP were analyzed with respect to control cells. Figure 1 shows the fatty acid composition (expressed as a percentage of total fatty acids) (Figure 1A) and chromatographic profile (Figure 1C) of differentiated Caco-2 control cells, obtained by GC. Caco-2 cells showed a concentration of approximately 25% of saturated fatty acids (mainly 16:0 and 18:0), 51% of monounsaturated fatty acids (mainly 18:1 isomers and 16:1 $n-7$), and 15% of polyunsaturated fatty acids (mainly 20:4 $n-6$ and 18:2). From HPLC, the total cholesterol was measured in control cells as a mean content of 133 ± 12.29 μg/plate; furthermore, the cell content of the most abundant unsaturated fatty acids was detected as follows: 253.9, 34.2, 23.5, and 22.3 μg/plate for 18:1 isomers, 16:1, 18:2, and 20:4, respectively, with a minor amount of DHA, EPA, and 22:5 $n-3$ (DPA), as reported in Table 1. Caco-2 control cells showed a HP value of 0.79 ± 0.18 nmol/plate. Fatty alcohols were not detected in the unsaponifiable fraction of Caco-2 control cells. Figure 5 shows the values

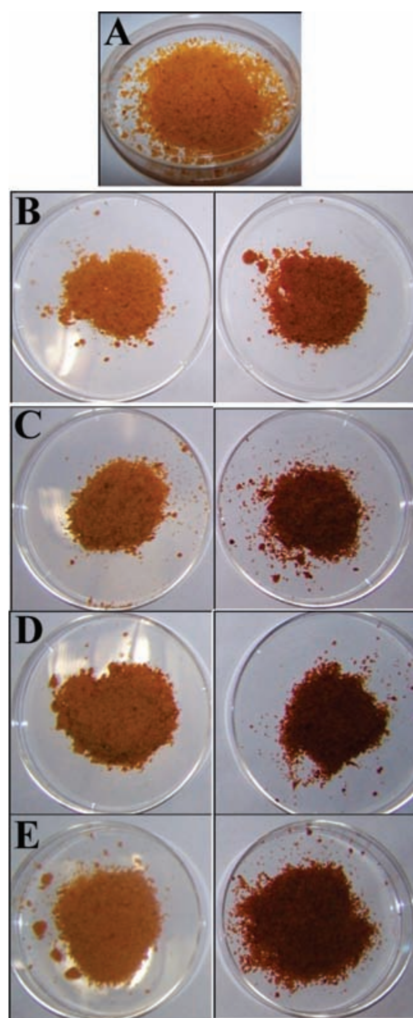

Figure 2. (A) Bottarga control sample and bottarga samples stored for (B) 1, (C) 2, (D) 3, and (E) 7 months at (left side) −20 °C and (right side) room temperature under light exposure.

(expressed as a percentage of the control) of the fatty acids 20:4, 18:1, 20:3 $n-9$, 16:1, DPA, EPA, DHA, HP (Figure 5A), and cholesterol (Figure 5B) measured in differentiated Caco-2 cells after 6, 18, 24, and 48 h of incubation in the presence of the lipid fraction (100 μg/mL) obtained from the bottarga sample stored for 35 days at −20 °C in the dark. Epithelial cells incubated with bottarga oil showed a significant change in fatty acid composition. Bottarga lipid extract induced a marked increase of DHA, EPA, and DPA cell levels (also 18:1 increase, although to a minor extent) from 6 h of incubation, whereas the treatment did not seem to affect the levels of cholesterol and other fatty acids. A significant increase of HP levels (bottarga extract HP value of 2.2−2.6 nmol/mg of lipid) was also observed in treated cells from 18 h of incubation. Figure 6 shows the values of fatty acids, HP, and cholesterol measured in differentiated Caco-2 cells after 24 and 48 h of incubation in the presence of the lipid fraction (100 μg/mL) obtained from the bottarga sample stored for 7 months at room temperature in the light. Also, the lipid fraction obtained from the browned bottarga sample induced a marked increase in the $n-3$ fatty acid level but not in the cholesterol level. A significant increase of the oxidative product HP was also observed (bottarga extract HP value of 3.0−4.8 nmol/mg of

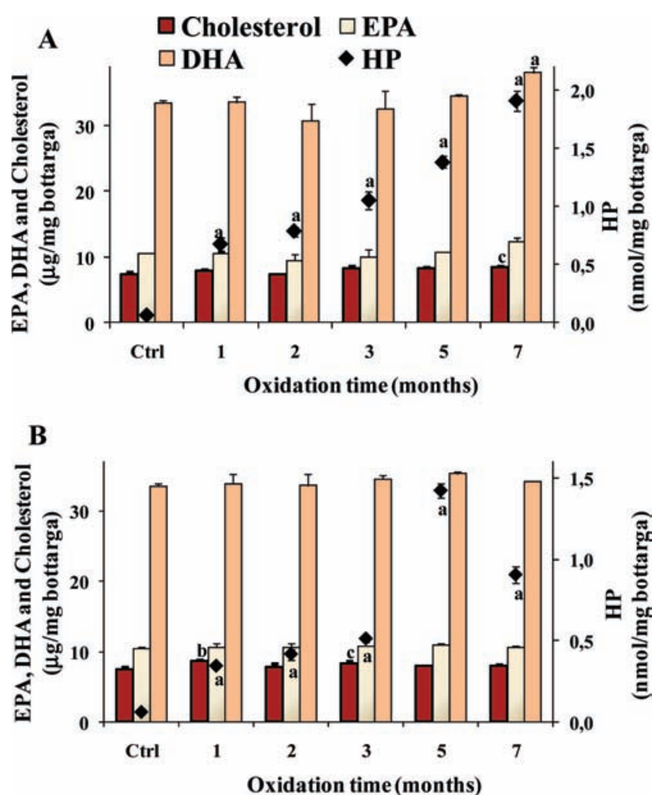

Figure 3. Values of 20:5 $n-3$ (EPA), 22:6 $n-3$ (DHA), cholesterol (expressed as μg/mg of edible portion), and conjugated diene fatty acid hydroperoxides (HP) (nmol/mg of edible portion) measured in the bottarga control sample (Ctrl) and after 1, 2, 3, 5, and 7 months of storage at (A) −20 °C and (B) room temperature under light exposure. a, $p < 0.001$; b, $p < 0.01$; and c, $p < 0.05$ versus Ctrl ($n = 4$).

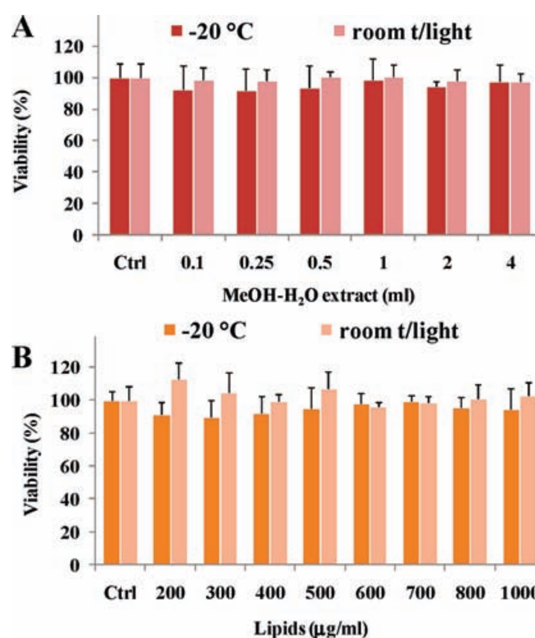

Figure 4. Viability, expressed as a percentage of the control, induced by 24 h of incubation with different amounts of the (A) aqueous (MeOH−H_2O) and (B) lipophilic extracts in the human differentiated Caco-2 cell culture (alamarBlue assay) ($n = 9$).

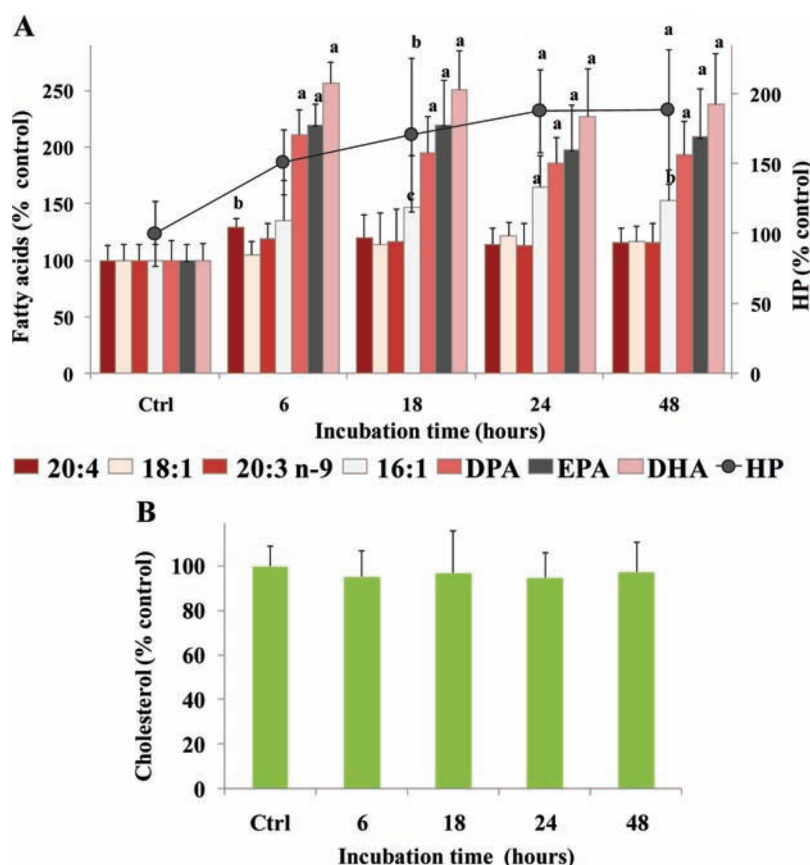

Figure 5. Values (expressed as a percentage of the control) of (A) fatty acids 20:4, 18:1, 20:3 $n-9$, 16:1, 22:5 $n-3$ (DPA), 20:5 $n-3$ (EPA), 22:6 $n-3$ (DHA), conjugated diene fatty acid hydroperoxides (HP) and (B) cholesterol measured in differentiated Caco-2 control cells (Ctrl) and after 6, 18, 24, and 48 h of incubation in the presence of the lipid fraction (100 μg/mL) obtained from the bottarga sample stored at −20 °C. a, $p < 0.001$; b, $p < 0.01$; c, $p < 0.05$ versus Ctrl ($n = 9$).

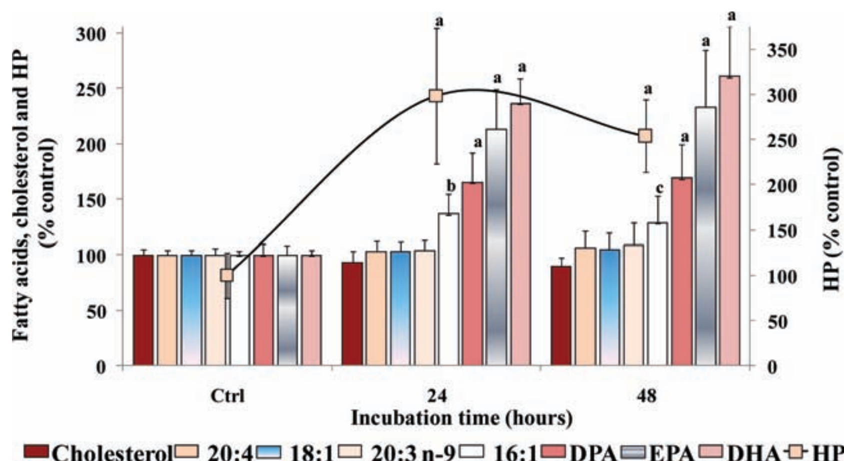

Figure 6. Values (expressed as a percentage of the control) of the fatty acids 20:4, 18:1, 20:3 $n-9$, 16:1, 22:5 $n-3$ (DPA), 20:5 $n-3$ (EPA), 22:6 $n-3$ (DHA), cholesterol, and conjugated diene fatty acid hydroperoxides (HP) measured in differentiated Caco-2 control cells (Ctrl) and after 24 and 48 h of incubation in the presence of the lipid fraction (100 μg/mL) obtained from the bottarga sample stored at room temperature under light exposure. a, $p < 0.001$; b, $p < 0.01$; c, $p < 0.05$ versus Ctrl ($n = 9$).

lipid) in Caco-2-treated cells, showing HP values superior to those observed during incubation with the oil obtained from the bottarga sample stored at −20 °C. Levels of fatty alcohols were measured in the unsaponifiable fraction of Caco-2 cells supplemented with both bottarga oils; the mixture of fatty alcohols was characterized by a high amount of 16:0 (>60%), 16:1 $n-7$ (about 9%), and 18:0 (7%), with a composition similar to that of the bottarga sample (data not shown). The 24 h of incubation of intestinal cells with the aqueous extracts (100 μL/mL) obtained from bottarga samples stored at −20 °C and room temperature in the light did not affect the levels of fatty acids, cholesterol, and HP, with treated cells showing values of these components similar to those of control cells (Figure 7).

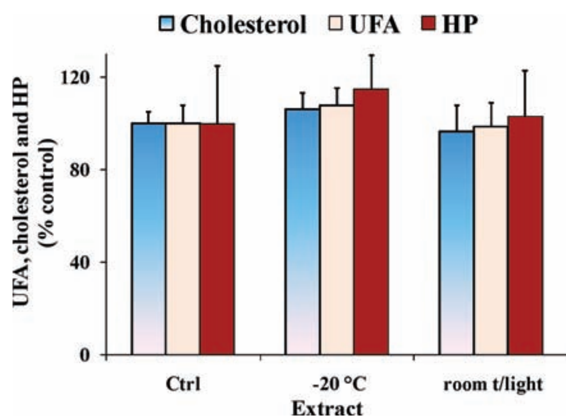

Figure 7. Values (expressed as a percentage of the control) of total unsaturated fatty acids (20:4, 18:1, 20:3 $n-9$, 16:1, 22:5 $n-3$, 20:5 $n-3$, and 22:6 $n-3$) (UFA), cholesterol, and conjugated diene fatty acid hydroperoxides (HP) measured in differentiated Caco-2 control cells (Ctrl) and after 24 h of incubation in the presence of the MeOH−H_2O fraction (100 μL/mL) obtained from bottarga samples stored at −20 °C and room temperature under light exposure (n = 9).

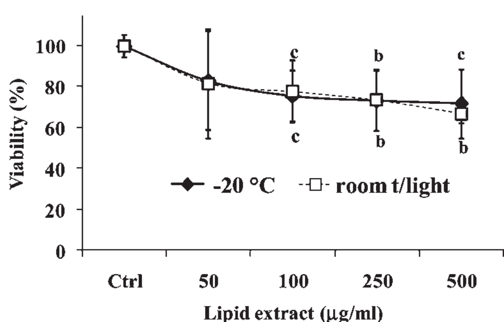

Figure 8. Viability, expressed as a percentage of the control, induced by 24 h of incubation with the lipid extracts (50−500 μg/mL) obtained from bottarga samples stored at −20 °C and room temperature under light exposure in human colon cancer Caco-2 cells (MTT assay). b, $p < 0.01$; c, $p < 0.05$ versus Ctrl (n = 12).

3.4. Cytotoxic Activity of Bottarga Lipophilic Extracts in Undifferentiated Caco-2 Cells. The lipophilic extracts, obtained from grated bottarga samples stored at the two different conditions (−20 °C in the dark and room temperature in the light) were also evaluated for cytotoxicity (MTT assay) in cancer Caco-2 cell cultures. Figure 8 shows the viability, expressed as a percentage of the control, induced in undifferentiated Caco-2 cells after 24 h of incubation in the presence of different concentrations of lipid bottarga extracts. Both the extracts showed a similar pattern, exerting a significant reduction in cell viability from the concentration of 100 μg/mL. At the highest concentration tested (500 μg/mL), a 30% decrease in cell viability was observed for both extracts.

4. DISCUSSION

The gastrointestinal tract is constantly exposed to dietary oxidized food compounds produced during the reactions that occur during processing and storage of foods; after digestion, a part of them is absorbed into the lymph or directly into the bloodstream.[4] The dietary intakes of highly unsaturated fats and of several foods form an important contributor to hydroperoxides and low-molecular-weight breakdown products (aldehydes, ketones, alcohols, etc.) in the human stomach and intestines.[4] Excessive lipid hydroperoxide concentrations in the gut lead to the enterocyte dysfunction and development of digestive tract disease conditions, such as inflammation and colon cancer.[15] The consumption of fish or fish derivatives containing $n-3$ PUFA has been found to be clinically beneficial to human health. The health benefits of dietary $n-3$ fatty acids include reduced susceptibility to mental illness, protection against heart disease, improved brain and eye function in infants, and alleviation of rheumatoid arthritis symptoms.[1,3] Despite nutritional benefits, $n-3$ PUFA are extremely sensitive to lipid oxidation, resulting in potential alteration in nutritional composition and quality of food during processing, storage, and distribution.[5−8] Among fish roe products, mullet roe is proposed as a rich source of $n-3$ PUFA, containing high amounts of EPA and DHA.[10−13] Sardinia island is characterized as having an ancient tradition in producing this delicacy, and Sardinian bottarga has nowadays an exceptional reputation in the market for its quality. Bottarga may be regarded as a natural source of health-beneficial $n-3$ PUFA, more stable than other sources, such as fish oils and their ethyl ester derivatives, because of the fact that a significant amount of $n-3$ PUFA in mullet roe is wax ester components,[10−13] and it was reported that wax esters enriched in $n-3$ fatty acids have a low degree of susceptibility to oxidation.[29] Nevertheless, levels of HP in commercial grated and whole bottarga samples have been found.[11,12] Several factor could affect the oxidative status of this food: the quality of the raw material, the type of salting and drying procedure adopted, the storage conditions (time, temperature, and light exposure) of raw roe and cured product, and the physical state of the matrix (as whole or grated bottarga).

The main objective of this work was to study, for the first time, the effect of the bottarga oil on cell viability, lipid composition, and lipid peroxidation in differentiated Caco-2 cell monolayers as an intestinal epithelial cell model, also in relation to the food oxidative status and browning process. The differentiated Caco-2 cells, retaining many of the morphological and functional characteristics (such as brush border microvilli, tight junctions, and dome formation) of normal small intestine polarized enterocytes, are extensively used in the prediction of intestinal drug absorption and in studies of toxicity of dietary oxidized lipids.[15,24] Freshly prepared grated bottarga was stored for 7 months at −20 °C in the dark and room temperature under light exposure. Both storage conditions did not significantly affect the $n-3$ PUFA level in bottarga samples, because of the low susceptibility to oxidation of these compounds when esterified in wax esters.[12,14] Nevertheless, a significant increase in the HP content with respect to the control sample was observed at both storage conditions. Lipid HP are the primary nonradical intermediates of PUFA peroxidation, and in the presence of metal ions or oxygen or at high temperature, these compounds break down to form secondary oxidation products (epoxyhydroperoxides, ketohydroperoxides, and cyclic peroxides), which could decompose to low-molecular-weight breakdown products (aldehydes, ketones, alcohols, esters, hydrocarbons, furans, and lactones) or condense to polymers.[4,7] Bottarga stored at −20 °C showed a higher HP level than the sample stored at room temperature, because of the higher stability of these compounds at a low temperature. The refrigerated temperature also showed to preserve bottarga from the non-enzymatic browning process; in fact, only the bottarga sample at room temperature exhibited a severe browning reaction. Non-enzymatic browning in mullet roe is the consequence of a series of reactions of amines, amino acids, peptides, and

proteins with reducing sugars and oxidized lipids (Maillard reaction and protein−lipid interaction), and the temperature of storage is well-known to exert an important role in this process.[16,17,30] The processing of mullet roe to obtain bottarga led to the increase of the level of free fatty acids and free amino acids with a high degree of browning.[16] Moreover, saturated and unsaturated aldehydes from HP decomposition are able to participate in amino acid−carbonyl compound interactions.[16,17,30] From our observations, the storage temperature resulted in a determining factor able to influence the kinetic of formation/degradation and, thus, the stability of HP and the browning process in bottarga samples. The aqueous and lipophilic fractions were then extracted from bottarga samples, having differences in the oxidative status and browning process and tested in intestinal cells to determine the potential generation of cytotoxic compounds because of degradation processes. Extracts were obtained from bottarga stored for 35 days at −20 °C in the dark (less marked deteriorative process) and during the 7th month of storage at room temperature in the light (higher HP level and browning process). The aqueous fraction was prepared because many low-molecular-weight metabolites, released by degradation processes (amino acids from proteolysis, organic acids, choline from lipolysis, biogenic amines, and trimethylamine) are soluble in H_2O.[20] All tested extracts did not show a toxic effect on intestinal epithelial cells (differentiated Caco-2). Moreover, both lipid extracts were able to induce significant modifications in differentiated Caco-2 cell fatty acid composition with a selective increase in cellular levels of $n-3$ PUFA, indicating a process of absorption of these important bioactive components. It has been recently found that wax esters, whose acidic moiety has been enriched in $n-3$ fatty acids, are easily absorbed and metabolized by rats.[14] Our results clearly showed that HP levels were higher in treated Caco-2 cells than control cells, depending upon the HP level in bottarga oil, although the measured values were not sufficiently high to cause toxicity in intestinal epithelial cells. Differentiated Caco-2 cells incubated with the bottarga oil did not show an increase of the cholesterol level compared to control cells, probably because of the $n-3$ PUFA-induced downregulation of transporter proteins that regulated intestinal cholesterol.[31] The hydrophilic extracts did not induce any change in the cellular lipid profile, with values of fatty acids, cholesterol, and HP identical to the control.

In this work, we also studied the effect of bottarga lipids on the viability in the cancer Caco-2 cell line. A growing body of literature supports the contention that bioactive food components containing $n-3$ PUFA are important in suppressing colon cancer.[19,21] Moreover, several investigations have shown the growth inhibitory effect and antiproliferative properties of fish oils, algal oils, $n-3$ PUFA mixtures, and pure EPA and DHA in human intestinal cancer Caco-2 cells.[19,21,32] According to the investigators, it has been shown that lipid peroxidation may play a potential role in $n-3$ PUFA-induced growth inhibition and cytotoxic effects. The results of the present study clearly indicated the cytotoxic effect of mullet bottarga lipids on undifferentiated cancer Caco-2 cells; nevertheless, this effect did not seem to be correlated to the oxidative status of the extract.

In conclusion, the present study has shown that the mullet bottarga may be considered an important natural source of $n-3$ PUFA. Mullet bottarga lipids seemed to be able to affect $n-3$ PUFA composition in intestinal epithelial cells and viability in colon adenocarcinoma cells. Taking into consideration that lipid peroxidation in intestinal cells, indicated by HP concentration, increased with an increasing bottarga HP level, a correct preparation and proper storage are certainly necessary to preserve the nutritional properties of this traditional food of Sardinia.

Further studies are necessary to assess the *in vivo* biodisponibility of bottarga $n-3$ PUFA and to clarify their potential antitumor properties.

AUTHOR INFORMATION

Corresponding Author

*Telephone: ++39-070-6754127. Fax: ++39-070-6754032. E-mail: anrosa@unica.it.

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