

Article

Omic and Biochemical Profiling of European Anchovy (*Engraulis encrasicolus*) Fillets Under Sub-Chilled Storage

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Abstract

Sensory, proximate composition, microbiological analyses and fatty acid profiling, combined with omic analyses, were employed to examine qualitative changes in the fillet of fresh European anchovies (*Engraulis encrasicolus*), caught in September of years 2020, 2021, and 2022 in the North Aegean Sea (Greece), and stored for 72 h in a salt-containing ice slurry (sub-chilled conditions). Sensorially, the quality of the whole fish decreased significantly ($p < 0.05$) at the end of the storage period. However, total microbial viable counts remained relatively stable ($\leq 10^5$ CFU), as did the proximate components of the fillet, as well as the fatty acid profile of the total fat, with the main differences noted only in the distribution of the DHA and EPA in the lipid classes. Histamine levels remained at trace levels during storage and, from the proteomic analysis of the 2021 sample, significant differences were observed only in actin $\alpha 1a$, out of 27 major muscle proteins examined. The 16S rRNA gene analysis indicated important inter-annual changes in the anchovy's microbiota, with no obvious pattern regarding the presence or changes in relative abundance of genera with spoilage potential during the storage period. Overall, the results suggest that short-term sub-chilled storage preserves to a large extent the nutritional and biochemical integrity of the anchovy fillets.

Keywords: European anchovy; spoilage microbiota; proximate composition; biogenic amines; proteomics; fatty acid analysis



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1. Introduction

Freshness is the most important quality of fish products, and its loss is associated with both internal and external factors. Internal factors include autolytic and oxidative processes, i.e., the release of endogenous enzymes that result in both protein and lipid degradation [1]. Microbial proliferation is the main external factor for loss of freshness [2], and it can act in

tandem with the autolytic and oxidative processes to accelerate tissue degradation, leading also to the production of endotoxins, rendering unsafe the consumption of the fish [3]. Furthermore, the onset and extent of the autolytic and microbially driven spoilage depend on the species, with the flesh texture and its chemical composition (moisture, protein and fat content) playing an important role in the process [4]. The geographical origin of the fish is also an important factor, as different environmental conditions influence the diet composition and feeding regime of the fish, as well as the qualitative and quantitative characteristics of the local marine microbiota, and thereby the physiological condition of the fish [4,5]. Important as well is the post-harvest handling of the fish, with the storage temperature being the most critical factor [1,6].

As fish are highly perishable, extending their shelf life remains a significant challenge. This is particularly important when the preference for fresh, locally caught seafood is culturally driven. In that case, the consumers' perceptions of fishery products are an important parameter influencing their marketability. Such is the case for the European anchovy (*Engraulis encrasicolus*), a species of high commercial and economic importance in the Mediterranean region, where it constitutes an integral part of the local diets, with a large part of the catch sold and consumed fresh [7].

The European anchovy (referred to as anchovy therein) is a small pelagic fish of the Engraulidae family, found on the continental shelf of the Eastern Atlantic, from the southern coasts of Norway to South Africa, and throughout the Mediterranean, Black, and Azov Seas [8]. They constitute heavily exploited fisheries, especially in the Mediterranean and Black Seas. According to the most recent data (average of years 2022 and 2023), the combined catches in these two areas amounted to approximately 400,000 tonnes, of which 282,000 tonnes are from the Black Sea alone. For the same period, the total Mediterranean catches amounted to 78,300 tonnes, of which over 52,000 tonnes were from the Adriatic Sea and Eastern Mediterranean and the remaining 26,000 tonnes from the Western and Central regions of the Sea [9]. In the eastern Mediterranean, the most important anchovy fishing grounds are in the northern part of the Aegean Sea (Thracian Sea) [10]. Currently, five genetically distinct anchovy populations are recognized in the Mediterranean, i.e., that of the Aegean Sea, of the Adriatic Sea (two distinct populations in the northern and southern parts of the Sea, respectively), of the western Mediterranean, and of the Alboran Sea [11,12], which are also distinct from the Black Sea anchovy population [13].

In addition to its economic importance, anchovy, as an omnivorous planktivore, plays a crucial role in the flow of energy between primary producers (i.e., phytoplankton) and higher trophic levels [14]. Within this flow of energy, of particular interest are the highly unsaturated fatty acids of the *n*-3 series, specifically the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, with their well-known health benefits. Seafood, particularly marine fish, is the primary source for these beneficial fatty acids in the human diet [15]. The cumulative content of EPA and DHA in the total fat of Mediterranean anchovies has been reported to range from 30 to 40% [16].

The main fishing gear for anchovy fisheries in Greece and throughout the Mediterranean and Black Seas is the purse seine. However, fishing practices and post-harvest handling of the catch may differ between regions. The purse seine fleet of Kavala (Region of Eastern Macedonia-Thrace, Greece) has, in recent years, participated in a Fisheries Improvement Project (FIP) aimed at Marine Stewardship Council (MSC) certification [17] and, therefore, serves as a reference concerning the fishing and post-harvest practices for anchovy in Greece. Specifically, on board, the fish are transferred directly from the net to basins containing ice and seawater (40–50%). After 10–15 min in the ice-water bath, the fish are placed in watertight expanded polystyrene (EPS) containers (10 kg capacity) with a small quantity of ice and seawater ($\approx$ 1 kg ice and 0.5 L water). As purse seiners

usually operate at night and the distance from the port to the fishing grounds rarely exceeds 30 nautical miles, the fish are landed at the fishing harbor of Kavala in the early morning hours, 3–5 h after the catch. The fish are then auctioned either to local retailers (fish shops) or to wholesalers. At the local fish shops, the fish are usually sold and consumed on the same day. The fish distributed to distant markets in Greece or neighboring countries are made available to consumers on the following day, at the earliest. To ensure the freshness of the fish destined for distant markets, the fish are transferred to new EPS containers (7 kg of fish/box) along with a slurry consisting of two kg of ice in one L of a refrigerated 3.5% NaCl solution in tap water, resulting in uniform, sub-chilled ($<0^{\circ}\text{C}$) storage conditions. The tightly sealed containers are then transported to their final destinations in refrigerated trucks. Based on experience and feedback from customers of the packing plant at the fishing harbor of Kavala, this procedure ensures that the fish retain a fresh-like appearance for two to three days post-harvest, before visible alterations become obvious (e.g., bloody operculum, occasional belly bursting), and the marketability of the product is reduced.

Presently, published information on the efficacy of ice, with or without salt, for the short-term storage of fresh anchovies is limited to four studies, using different protocols, with each one also being different from that described above for the anchovies from Kavala. Specifically, in two studies concerning European anchovies from the Atlantic, the fish were either stored in ice only (1 kg ice to 7 kg fish) and kept at 1°C for up to 12 days [6], or in 25% *w/w* ice, with or without the addition of 5% *w/w* NaCl, and kept at 4°C for up to 12 days [18]. In the two other studies, in which anchovies from the Mediterranean were used, the fish were stored either in flake ice (unspecified quantity of fish and ice) and kept at 4°C for up to 7 days, with daily replacement of ice [19], or with flake ice (unspecified amount in 10 kg of fish) and kept at 0°C for up to 7 days with occasional re-icing [16]. Furthermore, it is unclear whether any of these protocols are actually used in large-scale commercial processes, as is the case with the Thracian Sea anchovies from the port of Kavala. Additionally, the varying environmental conditions (biotic and abiotic) prevailing in the areas from which the fish originated cannot be overlooked, as these can influence the physical condition of the fish and potentially affect the rate of spoilage [20–22].

In this study, we tested the hypothesis that short-term (72 h) sub-chilled storage of Thracian Sea anchovies can effectively inhibit microbial proliferation and autolytic processes. We anticipated this method would preserve muscle integrity and nutritional value, despite observable visual deterioration of the product. To test this hypothesis and to evaluate and understand potential quality changes taking place, we applied conventional analytical chemical, biochemical, and microbiological methods in combination with next-generation technologies (microbiome and proteomic analyses). Additionally, to examine potential year-to-year variations in fish quality, the analyses were performed in the same month of three consecutive years (September 2020, 2021, and 2022). Exceptions to this are the proteomic and biogenic amines analyses, which, in the case of proteomics, were performed only on the 2021 sample, and in the case of biogenic amines, on the 2021 and 2022 samples only.

2. Materials and Methods

2.1. Sampling

Anchovy samples were collected on board a commercial fishing vessel (purse seine) operating in the western part of the Gulf of Kavala, Greece (W-NW of Thasos Island, Figure S1), on the nights of 8 September 2020, 13 September 2021, and 15 September 2022. The fish, approximately 10 kg, packed by the vessel's crew in closed EPS cases containing flake ice and seawater, were transported to the laboratory immediately after landing, i.e., approximately 3–4 h after capture. In order to reproduce the conditions used in the packing

plant at the fishing harbor of Kavala, 7 kg of fish were transferred to new EPS cases to which a mixture consisting of 1 L of a refrigerated (1 °C) 3.5% NaCl solution in tap water and 2 kg of flake ice was added, thus lowering the sample temperature to approximately −1 °C. The samples were then stored for a further 72 h in a cold room at 1 °C. The remaining freshly caught fish (D0 sample) were used for sensory, proximate, calorimetric, fatty acid, classical microbiological, microbiome, and proteomic analyses. Specifically, one fillet from each fish (minimum three, maximum six, depending on the analysis) was removed for proximate, fatty acid profile, lipid class, biogenic amine, and calorimetric analyses. These samples were pre-weighed before being frozen in liquid nitrogen and stored at −80 °C. For the typical microbiological analyses, whole fresh fish were used. Microbiome analyses were performed on fresh fillet samples and, separately, for the head along with the viscera. The same sampling procedure was followed for the fish stored for 72 h (D3 samples). The temperature of the fish was monitored regularly through the storage period to ensure that it remained below −0.5 °C.

2.2. Sensory Analysis

To assess changes in the sensory quality attributes of the sub-chilled anchovies during the specified storage period, the Quality Index Method developed by Massa et al. [23] was used. A panel of five trained assessors examined four fish at each time point: freshly caught anchovies (D0) and anchovies stored sub-chilled for 72 h (D3). The final score was calculated as the sum of all parameters described in the method.

2.3. Proximate Analysis and Calorimetry

Moisture, crude fillet protein, crude fat, and ash were determined according to the AOAC methodology [24], and all values were expressed as g/100 g of fillet. The calorific value (energy density) of the fillets was measured via adiabatic bomb calorimetry with an automated IKA C5000 Bomb Calorimeter, (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). For the analysis, one (1) gram of lyophilized fillet was used, and values were recorded as kJ/kg dry weight and, after accounting for the moisture of the pre-weighted fillets, were expressed as kJ/kg wet weight. The analysis was performed on fillets from three fish ($n = 3$).

2.4. Microbiological Analysis

Two to three whole fish (total weight ca. 25–30 g), from each day of storage (D0 and D3), were placed in Stomacher bags along with sterilized Buffered Peptone Water solution (Oxoid Ltd., Basingstoke, Hampshire, UK), at a ratio of 1:10 w/vol. Homogenization was performed in a Stomacher Lab-blender 400 (Seward Ltd., Worthing, West Sussex, UK) for 1 min at high speed. Aliquots of the homogenized samples (1 mL) were subjected to 10-fold serial dilutions, which were plated in triplicate on the non-selective Tryptic Soy Agar (TSA) for aerobic mesophilic bacteria and on the Cephaloridine, Fucidin, Cetrimide Agar (CFC plus CFC supplement) for *Pseudomonas* spp. The plates were subsequently incubated at the temperature/time conditions recommended by the manufacturer (Oxoid). Specifically, the incubation conditions for the mesophilic bacteria (TSA agar) were 48–72 h at 25 °C and for *Pseudomonas* spp. (CFC agar) 48 h at 25 °C.

2.5. Microbiome and Bioinformatic Analyses

For the extraction of microbial DNA for microbiome analysis, essentially the protocol described in [25] was used. Specifically, fillets from five fish from each storage day (D0 and D3) were placed in Stomacher bags along with the double volume of sterilized double-distilled water (2 mL H₂O/g of fillet) and were homogenized in a Stomacher Lab-blender 400 (Seward) for 2 min at medium speed. The homogenate was transferred to 50 mL tubes

and was centrifuged at $124\times g$ (800 rpm) for 1 min at 20 °C in an Eppendorf 5810 R refrigerated centrifuge equipped with an A-4-62 rotor (Eppendorf AG, Hamburg, Germany). From the supernatant, two aliquots of 8 mL each were then transferred to 10 mL tubes and subsequently centrifuged for 15 min at $2500\times g$, following which the pellet was resuspended in 3 mL of sterilized ddH₂O. The DNA was extracted from 200 µL of the suspension using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Following purification, the DNA concentration was measured with a Qubit[™] 4 Fluorometer (Invitrogen[™], Thermo Fisher Scientific, Waltham, MA, USA), using the Qubit[™] dsDNA BR Assay Kit, and its integrity was evaluated using 1% agarose gel electrophoresis. DNA samples were stored at −20 °C until library construction. The same procedure for DNA extraction was followed for head and viscera (H/V) samples.

Total bacterial composition in the fillet (F) and head-viscera (H/V) samples was assessed through 16S rRNA amplicon (V3–V4 hypervariable regions of the 16S rRNA gene) sequencing, following the instructions of Illumina's "16S Metagenomic Sequencing Library Preparation" (15044223 B) protocol, and paired-end sequencing (2 × 300 cycles) was performed on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) platform, as previously described [26].

Raw 16S rRNA amplicon sequences were imported into the QIIME2 platform (amplicon-2024.10) [27] and were processed with the DADA2 plugin [28]. Quality control and generation of the Amplicon Sequence Variants (ASVs) was conducted using the 'denoised-paired' method of DADA2. ASVs were classified up to the genus taxonomy level using a V3–V4 region-specific classifier trained on the SILVA v138.1 database [29]. Taxonomy-based filtering was applied to remove non-bacterial and unclassified sequences. Data visualization was performed in R v4.5.2 [30] by combining functions of the phyloseq [31], ggplot2 [32], and ampvis2 [33] packages. The 'amp_heatmap' function of ampvis2 was used to generate a heatmap of relative abundances of bacterial taxa across samples. Rarefaction curves were generated using the 'ggrare' function from the ranacapa package [34] to assess sequencing depth and sampling completeness. Alpha diversity analysis was conducted with the 'estimate_richness' function of phyloseq. Good's coverage was calculated for each sample based on the ASV count table. Beta diversity analysis was performed based on the Bray–Curtis dissimilarity matrix generated from ASV abundance data. Principal Coordinates Analysis (PCoA) was conducted using the ampvis2 package.

2.6. Biogenic Amines

Standard amine compounds of putrescine, cadaverine, spermidine, spermine, and histamine were obtained from Sigma-Aldrich (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) in the form of hydrochloride salt powder. Standard aqueous solutions (0.1 mg/mL) of free amines were prepared by dissolving their salts in HPLC-grade water. The benzoyl derivatization of the standard amine solutions was performed as described in [35]. For the extraction of biogenic amines from the muscle tissue of anchovy from the 2021 and 2022 samples, 5 g of tissue from three fillets ($n = 3$) were homogenized with T 25 basic ULTRA-TURRAX[®] disperser (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) in 20 mL of 6% TCA and subsequently centrifuged for 30 min ($3200\times g$, 4 °C). The supernatant was filtered through a Whatman No. 2 filter paper (Cytiva, Freiburg im Breisgau, Germany), and the volume was adjusted to 25 mL with HPLC-grade water. Benzoyl derivatization was performed as described above for the standard compounds, using 2 mL of the extracts. Biogenic amines were detected and quantified using a JASCO HPLC system equipped with a PU-980 Intelligent Pump, an LG-1580-02 Ternary Gradient Unit, an MD-1515 Multiwavelength Detector, an 860-CO column oven (JASCO Corporation, Hachioji, Tokyo, Japan), and a ZORBAX Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA,

USA). For the elution of the biogenic amines, the flow rate was set at 1.0 mL/min, and the gradient elution program with water (A) and acetonitrile (B) was as follows: 0 min 30% B, 8 min 40% B, 11 min 70% B, and maintained at this concentration until the end of the run (13 min). Detection of amines was monitored at 254 nm. Calibration curves for each standard benzoylated amine (0, 5, 10, 20 µg/mL solutions) were used to quantify the corresponding amines in the muscle tissue of anchovy. Sensitivity was assessed based on signal-to-noise (S/N) ratios, and the limits of detection (LOD) and quantification (LOQ) were calculated using the equations $LOD = 3 \times (S/N)$ and $LOQ = 10 \times (S/N)$, respectively. The LOD and LOQ values were determined to be 0.15 and 0.50 µg/mL (0.15 ppm and 0.5 ppm), respectively. All amines exhibited good linearity, with correlation coefficients (R^2) ≥ 0.99 .

2.7. Proteomic Analysis

Due to a lack of samples and limited resources, the proteomic analysis was limited to the 2021 sample. Dorsal white muscle tissue from three fish ($n = 3$) of each storage day was snap-frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle under cryogenic conditions. Approximately 250 mg of tissue powder was dissolved in lysis buffer {4% *w/w* SDS, 0.1 M Tris-HCl, 0.1 M DTE, pH 7.6} and lysis was performed in a water bath sonication apparatus. After complete solubilization, centrifugation was performed for 10 min at 12,000 rpm, and cellular debris was discarded. The supernatant was precipitated with a combination of ice-cold methanol and ice-cold chloroform for 24 h at -20°C . The precipitate was centrifuged for 30 min at 3000 rpm, and the protein pellet was dried under vacuum. Extracted proteins were resuspended in lysis buffer, and protein concentration was determined using the Bradford assay [36]. The protein extracts were reduced in 0.1 M DTE for 30 min and subsequently alkylated by addition of 0.05 M iodoacetamide in 8 M urea and 0.1 M Tris-HCl pH 8.5 for 25 min at room temperature in the dark. Afterward, trypsin solution was added at a trypsin-to-protein ratio of 1:100, and digestion was performed overnight at 36°C . The next day, peptides were subjected to spin filtration (Amicon Ultra 30 kDa centrifugal filter units, Millipore, Molsheim, France) and the peptide solution was vacuum-dried. The pellet was re-dissolved in 100 µL of HPLC phase A (99.9% water with 0.1% formic acid). The final peptide mixture was cleaned from impurities using a Millex syringe-driven filter unit (Merck KGaA, Darmstadt, Germany) and subsequently subjected to nanoLC-MS/MS analysis.

Peptide separation was performed using a reversed-phase analytical C-18 column (75 µm $\times$ 50 cm; 100 Å, 2-µm-bead-packed Acclaim PepMap RSLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Ultimate-3000 nanoLC system (Dionex, Thermo Fisher Scientific) connected online to an LTQ-Velos Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Initially, 6 µL of peptide mixture was loaded onto a C-18 pre-column at a constant flow rate of 5 µL/min in phase A. After 4 min of desalting, the valve was switched online, allowing the sample to pass through the analytical column previously equilibrated with phase A. Total elution time for all runs was 360 min using a 2–35% gradient of phase B (99.9% acetonitrile, 0.1% formic acid) at a constant flow rate of 300 nL/min. Different desalting times and gradient profiles were evaluated to optimize peptide recovery and chromatographic separation according to the biochemical characteristics of the sample's proteins.

Mass spectra were collected using an Orbitrap Elite mass spectrometer fitted with a nano-spray ion source. The instrument was operated in data-dependent acquisition mode using XCalibur™ v2.2 SP1.48 software (Thermo Scientific). Full-scan MS data were acquired in the 300–2000 *m/z* range at a resolution of 60,000 with a maximum injection time of 100 ms. Data-dependent tandem mass spectrometry for the 10 most intense precursor

ions per survey scan was performed using higher-energy collision dissociation (HCD) fragmentation in the Orbitrap at a resolving power of 15,000 and a normalized collision energy of 36%. An additional MS/MS acquisition method targeting the 20 most intense ions per scan was also performed, and the resulting datasets were merged for downstream analysis. Fragment ions were analyzed in the Orbitrap at a resolving power of 15,000 and a maximum injection time of 120 ms. Internal calibration was performed using m/z 445.120025 as lock mass. Dynamic exclusion of 45 s was applied to minimize repetitive sequencing of the same peptide ions. The resulting MS/MS data were processed using Proteome Discoverer software (version 1.4.0.388, Thermo Scientific). MS2 spectra were searched with the SEQUEST engine against the *Engraulis encrasicolus** protein database retrieved from UniProtKB. Search parameters included up to two missed tryptic cleavages, oxidation of methionine as a variable modification, peptide mass tolerance of 10 ppm, and fragment ion tolerance of 0.05 Da. Validation of peptide spectral matches was performed using the Percolator algorithm based on q -values at a false discovery rate (FDR) of 0.5%. Additional peptide filtering was applied based on Xcorr versus peptide charge values (maximum Delta Cn set at 0.05). Xcorr thresholds of 2.2 and 3.5 were applied for doubly and triply charged peptides, respectively. The minimum accepted peptide length was set to six amino acids.

2.8. Fatty Acid Analysis

Total fillet fat was extracted according to the Folch method [37], and one milligram was used for the transesterification of fatty acids, as described in [38]. Fillet fatty acid composition was determined by gas chromatography on a Hewlett Packard 5890-Series II chromatograph equipped with an SGE-BPX 70-0.25 (60 m $\times$ 0.25 mm $\times$ 0.25 μ m) capillary column and an FID detector, as previously described [39]. For peak identification, reference compounds (37 Component F.A.M.E. mix standard, Sigma-Aldrich) were analyzed under the same conditions as the samples. The contribution of each identified fatty acid was expressed as the percentage of its peak area to the total area of all peaks eluted in each chromatogram. Peak areas were calculated with the Hewlett-Packard GC ChemStation software (Agilent). The identity of the peaks of interest was further confirmed by GC-MS using a HP6890 chromatograph equipped with a DB-1ms column (30 m $\times$ 2.5 mm $\times$ 0.25 μ m) and coupled to an Agilent HP MSD 5973 mass spectrometer.

2.9. Lipid Class Separation and Analysis

For the separation of the lipid classes, a two-solvent Thin Layer Chromatography (TLC) system was used according to the protocol described in [40]. Briefly, 20 $\times$ 20 cm TLC plates (Silica Gel on TLC Al foils, Sigma-Aldrich) were used for the separation and isolation of the polar and neutral lipid classes. A total of 12 mg of total fat was spotted on the plates (12 spots/plate). The first solvent, consisting of methyl acetate, isopropanol, chloroform, methanol, and 0.25% aqueous KCl at a ratio of 25:25:25:10:9, respectively, was left to migrate 8 cm from the origin. At that point, the plate was removed from the TLC chamber, was air-dried and placed in a chamber containing the second solvent (isohexane, diethyl ether, and acetic acid at a ratio of 80:20:1, respectively) and was left to migrate for another 8 cm. After air drying, the lanes from both ends of the plate were cut off, stained with a solution containing 3% cupric acetate in 8% phosphoric acid, and were used as markers for the migration of the different lipid classes in the non-stained part of the TLC plate (Figure S2). For the identification of the different lipid classes, their migration pattern was compared to that reported in the literature using similar separation systems [40–43]. The classes analyzed in the neutral fraction were the triacylglycerols (TAG) and the free fatty acids (FFA). In the polar fraction, the analyzed classes were

phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), as well as the phosphatidylcholine (PC) and sphingomyelin (SM); however, as the latter two could not be efficiently separated by the system used, they are collectively referred to as SM-PC, with the contribution of the PC fatty acids considered most important, as this is the most abundant phospholipid in cellular and organelle membranes [44]. Subsequently, the corresponding areas of the plate were cut off, the silica gel was scraped off, transferred to 20 mL of chloroform: methanol solution (2:1), and stored at -20°C for 16 h. Following centrifugation to remove the silica gel, the solvent was evaporated in a rotary evaporator, and the lipids were resuspended in chloroform: methanol (2:1) and processed for fatty acid methylation and gas chromatography as described above. Total neutral and total polar lipids were similarly excised from the areas of the plate between the origin and the first solvent front (polar lipids) and between the first and second solvent fronts (neutral lipids).

2.10. Statistical Analysis

Statistical significance of storage-day differences in the sensory, proximate composition, microbiological, biogenic amines, proteomic, and fatty acid analyses was tested using One-Way ANOVA. Data were initially tested for normal distribution (Shapiro–Wilk test and Q-Q plots) and homogeneity of variance (Levene’s test). Samples conforming to the Null Hypothesis ($p > 0.05$) were analyzed with Fisher’s One-Way ANOVA and the Tukey post hoc test. For samples or variables failing the Null Hypothesis ($p < 0.05$), Welch’s One-Way ANOVA was used with the Games-Howell post hoc test. All analyses were performed with the Jamovi version 2.6.44 software [45]. Effect size (Cohen’s d , η^2) for the proteomic samples was calculated with an online calculator.

3. Results

3.1. Sensory Analysis

For both the 2020 and 2021 samples, the statistically significant inverse increase in total sensory score, from 3.6 on D0 to ≈ 10 on D3, was mainly due to changes in surface appearance (loss of skin brightness, bleeding from the operculum), eyes (cloudy cornea, dull pupil), and consistency (soft/elastic texture, soft abdomen) of the fish (Table 1, Figure S3). For the 2022 sample, the gills (colour and, to a lesser extent, smell), eyes, and consistency attributes, which also presented higher D0 values compared to the other two samples, were the main determinants of quality in the D3 fish (Table 1). Nevertheless, the total quality score in all three yearly samples was well below 18, which is considered the rejection point by this particular quality index, although it was developed and validated for a different anchovy species (*Engraulis anchoita*) [23].

Table 1. Quality assessment of freshly caught anchovies (D0) and of anchovies stored at sub-chilled conditions for 72 h (D3) in September samples from 2020, 2021, and 2022, according to the Quality Index Method proposed by Massa et al. [23]. Values represent the mean sum of all descriptors in each attribute, as scored by a panel of five evaluators. Single and double asterisks (*) indicate significant differences at $p < 0.05$ and $p < 0.001$, respectively.

Sample	Appearance	Eyes	Gills	Consistency	Total
2020 D0	0.8 ± 1.0	1.0 ± 1.1	0.8 ± 0.9	1.3 ± 1.1	3.6 ± 0.7
2020 D3	$3.5 \pm 0.8^*$	$2.8 \pm 1.2^*$	1.5 ± 1.0	$2.8 \pm 1.2^*$	$10.7 \pm 2.7^{**}$
2021 D0	1.0 ± 0.7	0.6 ± 0.5	0.8 ± 0.8	1.2 ± 0.8	3.6 ± 1.3
2021 D3	$3.0 \pm 0.7^*$	$2.4 \pm 1.3^*$	1.8 ± 0.8	$2.6 \pm 0.9^*$	$9.8 \pm 1.5^{**}$
2022 D0	1.6 ± 1.5	1.6 ± 1.3	0.8 ± 1.1	1.6 ± 0.5	4.8 ± 1.3
2022 D3	3.0 ± 0.7	3.2 ± 0.8	$3.2 \pm 1.3^*$	$3.0 \pm 0.7^*$	$11.6 \pm 1.1^{**}$

3.2. Proximate Composition

In the D0 fillets, the moisture content ranged from 76.4% to 77.3% (lowest value observed in the 2022 sample), the crude protein ranged from 19.5% to 21.0% (lowest in 2020, highest in 2022), the fat ranged from 1.8% to 2.1% (highest in 2021), and the ash from 1.6% to 1.8% (highest in 2022). The energy density of the D0 samples ranged from 4.5 to 5.0 kJ/g wet weight, with the lowest value in 2020, possibly resulting from the low crude protein content of this sample (Table 2).

Table 2. Proximate composition (g/100 g, wet weight) and energy density (kJ/g wet weight) of the fillet of freshly caught anchovies (D0) and anchovies stored at sub-chilled conditions for 72 h (D3) in September samples from 2020, 2021, and 2022. Values represent the mean $\pm$ standard deviation of independent experiments on fillets of three different fish ($n = 3$). Significant differences ($p < 0.05$) between days of storage are indicated by an asterisk (*).

Sample	Moisture	Protein	Fat	Ash	kJ/g
2020 D0	77.1 $\pm$ 0.7	19.5 $\pm$ 0.2	1.8 $\pm$ 0.2	1.7 $\pm$ 0.1	4.5 $\pm$ 0.04
2020 D3	78.7 $\pm$ 0.5 *	18.7 $\pm$ 0.1 *	1.8 $\pm$ 0.1	1.7 $\pm$ 0.2	4.3 $\pm$ 0.15
2021 D0	77.3 $\pm$ 1.4	20.1 $\pm$ 0.4	2.1 $\pm$ 0.4	1.6 $\pm$ 0.2	4.8 $\pm$ 0.30
2021 D3	77.5 $\pm$ 0.6	19.1 $\pm$ 0.6	1.9 $\pm$ 0.0	1.5 $\pm$ 0.1	4.6 $\pm$ 0.06 *
2022 D0	76.4 $\pm$ 0.4	21.0 $\pm$ 0.1	1.8 $\pm$ 0.3	1.8 $\pm$ 0.0	5.0 $\pm$ 0.15
2022 D3	77.3 $\pm$ 0.1 *	19.3 $\pm$ 0.7*	1.7 $\pm$ 0.1	1.8 $\pm$ 0.2	4.6 $\pm$ 0.40

Storage resulted in a significant moisture increase in both the 2020 and 2022 D3 anchovy fillets, resulting also in a significant decrease in protein content in these two samples. (Table 2). However, this was not accompanied by a significant decrease in energy density of the fillets in the corresponding samples. Furthermore, no significant differences in fat and ash content were observed in any of the yearly samples.

3.3. Microbiological Analysis

No significant differences ($p > 0.05$) were observed between storage days in either the TSA or the CFC growth media and in any of the yearly samples (Table 3). Specifically, on D0, the TSA bacterial counts ($\log_{10}$ CFU) were 3.65 ± 0.49 , 4.57 ± 0.76 , and 4.10 ± 0.58 for years 2020, 2021, and 2022, respectively, as compared to 4.31 ± 0.41 , 5.30 ± 0.29 , and 5.09 ± 0.39 on D3. The D0 CFC counts for years 2020, 2021, and 2022 were 2.75 ± 0.56 , 3.33 ± 0.19 , and $2.49 \pm 0.41 \log_{10}$ CFU, respectively, while on D3, the corresponding values were 3.64 ± 0.40 , 3.65 ± 0.36 , and 3.08 ± 0.23 . Consequently, between the yearly samples, the TSA counts presented no significant differences on either storage day, while those of CFU were significantly different on D0 only between the 2021 and 2022 samples ($p = 0.01$).

Table 3. Total aerobic plate counts (TSA agar) and *Pseudomonas* spp. counts (CFC agar) in freshly caught anchovies (D0) and anchovies stored at sub-chilled conditions for 72 h (D3) in September samples from 2020, 2021, and 2022. Values are presented as $\log_{10}$ cfu/g $\pm$ SD from three different replicates ($n = 3$).

	2020		2021		2022	
	TSA	CFC	TSA	CFC	TSA	CFC
D0	3.65 ± 0.49	2.75 ± 0.56	4.57 ± 0.76	3.33 ± 0.19	4.10 ± 0.58	2.49 ± 0.41
D3	4.31 ± 0.41	3.64 ± 0.40	5.30 ± 0.29	3.65 ± 0.36	5.09 ± 0.39	3.08 ± 0.23

3.4. Microbiome Analysis

Following quality filtering and ASV inference, a total of 812 bacterial ASVs were identified across all samples (Table S1). Among these, the 80 most abundant ASVs, spanning 8 phyla and 73 genera, accounted for 91.5% of the total bacterial relative abundance across the dataset (File S1). Alpha diversity analysis indicated some patterns of variation in bacterial diversity according to sample type, storage duration, and sampling year (Table S1). Head/viscera samples generally showed a tendency toward higher richness and diversity compared with fillet samples, while storage-related trends differed among sampling years. Rarefaction curves reached a plateau for all samples, indicating that the sequencing depth was sufficient to capture the majority of the bacterial community diversity (Figure S4). Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities showed no clear separation of bacterial communities, indicating high similarity in overall community composition during sub-chilled storage (Figure S5). Twenty-three of the above genera contain species with spoilage and/or pathogenic potential [46–69] and with a relative abundance >0.1% in either the fillet or the H/V in the three yearly samples (Figure 1).

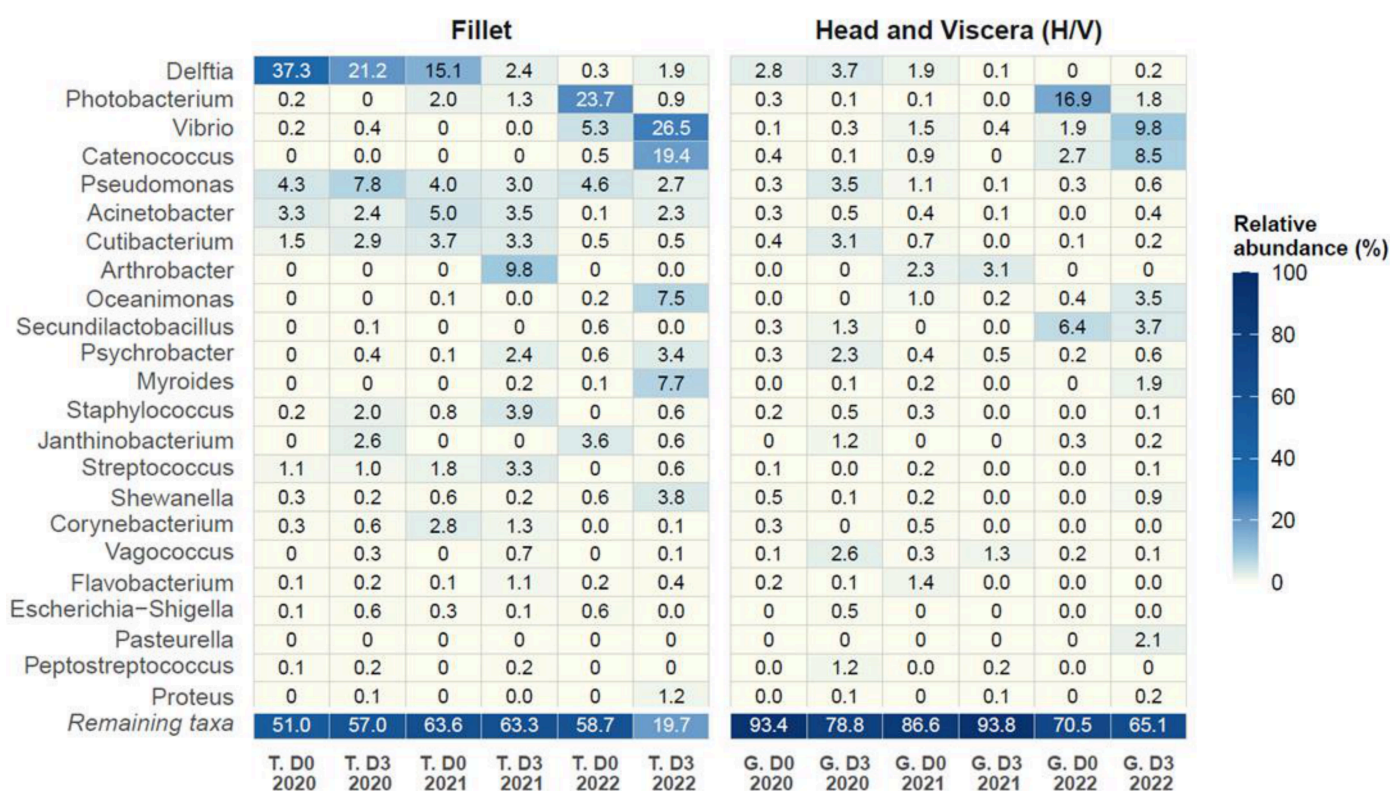


Figure 1. Heatmap of genus-level bacterial community composition in Fillet and Head and Viscera (H/V) samples at day 0 (D0) and day 3 (D3), over three consecutive years (T = Tissue/Fillet, G = Gut/Head-Viscera). Cell values indicate the relative abundance (%) of aggregated amplicon sequence variants (ASVs) assigned to each genus. Taxa with specific spoilage and/or pathogenic potential and a cumulative mean relative abundance > 0.1% across all samples are displayed; taxa below this threshold were grouped as “Remaining taxa”.

These genera represent from ≈50% to 80% of the total relative abundance in the fillet of the anchovy and from ≈7% to 35% in the H/V, illustrating important inter-annual shifts in dominant genera in all samples (Figure 1, File S1). Specifically, in the fillet, *Delftia* was the predominant genus in 2020 at both days of storage (37.3% at D0 declining to 21.2% at D3). *Delftia* also had a significant presence at D0 of 2021 (15.1%), but it declined considerably by D3 (2.4%). In contrast, in 2022, where *Delftia* was much less abundant, *Photobacterium*, a specific spoilage organism (SSO), was the predominant genus at D0 (23.7%). Its sharp

decline at D3 (0.9%) coincided with an important increase in relative abundance of *Vibrio* (5.3% at D0, 26.5% at D3), *Catenococcus* (0.5% at D0, 19.4% at D3), as well as in *Oceanimonas* (0.2% at D0, 7.5% at D3), and the SSO *Myroides* (0.1% at D0, 7.7% at D3). Notably, none of the above genera had a significant presence in any of the other samples. Regarding the other SSOs, *Pseudomonas* was consistently present across all three years, with its relative abundance increasing only at D3 of 2020 (4.3% at D0, 7.8% at D3); *Psychrobacter* exhibited an increasing trend in all three samples, while *Shewanella* and *Acinetobacter* increased in relative abundance only in 2022 (0.6% at D0, 3.8% at D3 and 0.6% at D0, 3.4% at D3, respectively). In terms of relative abundance, *Janthinobacterium* increased in 2020 but decreased in 2022, and *Flavobacterium* increased only in 2021 (Figure 1). With the exception of *Acinetobacter*, the mean relative abundance of the above SSOs across all samples and both storage days was $\leq 1\%$. Additionally, the spoilage-associated *Arthrobacter* showed an isolated spike at D3 of 2021 (9.8%) and was absent or at trace levels in all other samples. Also notable is the relative abundance of *Staphylococcus* across all samples, with increasing values at D3, as well as of *Escherichia-Shigella*, which, however, remained consistently low across all samples.

Compared to the fillet, the H/V microbiota, with the exception of the 2022 sample, was dominated by the non-spoilage, non-pathogenic *BD1-7_clade*, *Cyanobium_PCC-6307*, *Synechococcus_CC9902*, and *2013Ark19i* genera (File S1). In 2022, *Photobacterium*, along with *BD1-7_clade* and *Cyanobium_PCC-6307*, were the predominant genera at D0. As was the case in the fillet of the 2022 sample, the abundance of *Photobacterium* decreased sharply at D3 in H/V, concomitant with the increase in *Vibrio*, *Catenococcus*, *Oceanimonas*, and, to a lesser extent, *Myroides* (Figure 1). Regarding the other SSOs in the H/V, *Pseudomonas* was consistently detected in all samples, exhibiting the highest value at D3 of 2020 (3.5%), as was also the case in the fillet of that sample. *Acinetobacter* was also present in all samples, but with lower abundance compared to the fillet samples. Similarly, *Psychrobacter* was present in all three samples, exhibiting an increasing trend at D3, particularly obvious in 2020. Both *Janthinobacterium* and *Shewanella* mirrored the pattern observed for these two genera in the fillet, albeit at much lower abundance (Figure 1). Among the other genera with spoilage potential, *Secundilactobacillus* and *Vagococcus* were more prominent in the H/V than in the fillet. *Arthrobacter* was detected on both days of storage only in 2021, while *Pasteurella*, which was absent in all fillet samples, accounted for a 2.3% relative abundance at D3 of the 2022 H/V sample. Also noteworthy are the low levels of *Staphylococcus*, *Streptococcus* and *Escherichia-Shigella* in all H/V samples.

3.5. Biogenic Amines

The presence and quantities of putrescine, cadaverine, spermidine, spermine, and histamine were determined for both days of storage (D0 and D3) in the fillets of samples 2021 and 2022. In the 2021 sample, statistically significant changes during storage were observed in all amines, with the exception of histamine, which was not present in measurable quantities on either day (Table 4). As a result, the total biogenic amine concentration increased from ≈ 14 ppm (1.4 mg/100 g) in D0 to ≈ 29 ppm (2.9 mg/100 g) in D3. In contrast, in the 2022 sample, putrescine and cadaverine were the only amines exhibiting a statistically significant increase, although a higher mean value at D3 was observed for spermidine as well. As in the case of the 2021 sample, histamine was not present in measurable quantities on either storage day. Consequently, the total biogenic amine concentration in this sample increased from ≈ 8 ppm (0.8 mg/100 g) in D0 to ≈ 17 ppm (1.7 mg/100 g) in D3. The between-sample differences in total biogenic amines, at both days of storage, result from the higher spermidine and spermine values of the 2021 sample, as well as from the particularly low initial cadaverine concentration of the 2022 sample.

Table 4. Changes in biogenic amine concentrations in the fillet of freshly caught anchovies (D0) and anchovies stored at sub-chilled conditions for 72 h (D3) in September samples from 2021 and 2022. Values (ppm) represent the mean $\pm$ standard deviation of independent experiments on fillets of at least three different fish. Significant differences ($p < 0.05$) between days of storage are indicated by an asterisk (*).

Sample	Putrescine	Cadaverine	Spermidine	Spermine	Histamine	Total
2021 D0	1.62 $\pm$ 0.29	1.28 $\pm$ 0.34	5.51 $\pm$ 0.56	5.18 $\pm$ 0.81	ND	13.6 $\pm$ 2.0
2021 D3	3.79 $\pm$ 0.82 *	2.61 $\pm$ 0.19 *	11.28 $\pm$ 1.41 *	11.34 $\pm$ 0.35 *	Trace	29.02 $\pm$ 2.8 *
2022 D0	2.17 $\pm$ 0.29	0.16 $\pm$ 0.06	2.04 $\pm$ 1.44	3.98 $\pm$ 0.96	ND	8.35 $\pm$ 2.5
2022 D3	5.93 $\pm$ 1.68 *	2.54 $\pm$ 1.51 *	4.35 $\pm$ 1.05	3.74 $\pm$ 0.41	Trace	16.56 $\pm$ 4.7 *

3.6. Proteomic Analysis

The nano-LC-OrbiTrap-based bottom-up proteomic analysis of the anchovy's dorsal white muscle was performed on D0 and D3 fish from the 2021 sample. Collectively, from all D0 and D3 specimens examined, peptide sequences presenting homology to over 800 known proteins, mostly derived from the genome of herring (*Clupea harengus*), were recorded (File S2). However, most of these presented low scores and coverage, as well as small numbers of peptide spectrum matches (PSM), thus requiring further confirmation of their correct identification. Furthermore, only a limited number of the identified proteins were present in all three specimens examined on each of the days of storage, and an even smaller number was present in all specimens across both days (File S2). Given the above, a subset of 27 proteins was considered for the determination of potential degradation of the anchovy's muscle proteins during the storage conditions of the present study. These proteins were selected according to the score, coverage, and PMS criteria mentioned above, as well as by their presence in all specimens examined from both days of storage. This subset consisted of both sarcomeric and sarcoplasmic proteins, including five fast skeletal myosin heavy chain-like isoforms (mhc-fs-l), two myosin heavy chain isotypes (myh-4-1 and myh-7-1), three myosin light chain isotypes (mlc 1, 2, and 3), actin α 1a (act α 1a, the primary actin skeletal muscle isoform), two tropomyosin α isoforms (tpm1 and tpm α), troponin 1 (tpn1), two creatine kinase muscle isoforms (ckma and ckmb), calcium transporting ATPase 1 (atp2a1), pyruvate kinase (pk), aldolase A (aldoa), phosphoglycerate mutase (pgam), triosephosphate isomerase 1a (tp1a), glycogen phosphorylase muscle isotypes A and B (pygma and b), as well as of two isoforms of glyceraldehyde-3-phosphate dehydrogenase (gapdh and gapdh-l) and of β -enolase/enolase 3 (b-eno and eno3) (Table 5). Importantly, these proteins, despite being limited in number, represent >25% of the total peptide peak area of each sample, i.e., a significant portion of muscle's total protein content. According to our analysis (One-Way ANOVA), out of these 27 proteins, only act α 1a exhibited a statistically significant change in expression between D0 and D3 ($p = 0.04$, $\eta^2 = 0.712$), showing an important decrease in abundance. Interestingly, the gapdh-l protein also displayed an important decrease in abundance, although it failed to reach statistical significance ($p = 0.06$, $\eta^2 = 0.63$). However, the combination of the observed high inter-individual variability in protein abundance and the small replicate size ($n = 3$) limits the power of the statistical analysis, which can either mask or exaggerate the significance of the differences observed.

Table 5. Protein abundances in the dorsal muscle of freshly caught anchovies (D0) and anchovies stored at sub-chilled conditions for 72 h (D3) in the September 2021 sample. The mean (% $\pm$ SD) of normalized peak values of the nano-LC OrbiTrap analysis is presented, along with the probability (p) and effect size (Cohen's d , η^2). Protein abbreviations are as in the Text. The NCBI GenBank accession numbers against which the above proteins were identified are given below their abbreviations.

	Day	N	Mean	SD	η^2	p		Day	N	Mean	SD	η^2	p
mhc-fs-I (I)	D0	3	1.80	1.07	0.09	0.57	ckma	D0	3	0.33	0.18	0.11	0.51
A0A6P8G1S8	D3	3	2.23	0.61			A0A6P3VH03	D3	3	0.25	0.08		
mhc-fs-I (II)	D0	3	1.70	1.07	0.12	0.50	ckmb	D0	3	0.26	0.09	0.4	0.18
A0A6P8G2F8	D3	3	2.25	0.70			A0A6P3VVR3	D3	3	0.16	0.06		
mhc-fs-I (III)	D0	3	2.61	0.72	0.1	0.55	atp2a1	D0	3	0.23	0.05	0.07	0.63
A0A6P8F1H3	D3	3	2.24	0.70			A0A6P8F227	D3	3	0.19	0.12		
mhc-fs-I (IV)	D0	3	1.35	0.37	0.23	0.34	b-eno	D0	3	0.57	0.10	0.04	0.69
A0A6P3WCZ7	D3	3	1.87	0.73			A0A6P3W5E7	D3	3	0.49	0.33		
mhc-fs-I (V)	D0	3	2.44	0.89	0.00	0.96	eno3	D0	3	0.78	0.29	0.23	0.34
A0A6P8GBV3	D3	3	2.47	0.30			A0A6P3W3B1	D3	3	0.52	0.31		
myh_4-L	D0	3	1.08	0.36	0.01	0.88	pk	D0	3	0.35	0.19	0.31	0.25
A0A6P8GBN8	D3	3	1.13	0.46			A0A6P3VJW2	D3	3	0.19	0.07		
myh_7-L	D0	3	0.95	0.39	0.16	0.43	aldoa	D0	3	1.22	0.34	0.04	0.70
A0A6P8FLV3	D3	3	1.15	0.11			A0A6P3W9M1	D3	3	1.12	0.27		
mhc_1	D0	3	1.15	0.79	0.21	0.36	pgam	D0	3	0.13	0.02	0.15	0.45
Q9IB21	D3	3	0.65	0.29			A0A6P3VG48	D3	3	0.22	0.20		
mhc_2	D0	3	1.33	0.53	0.12	0.50	gapdh	D0	3	0.63	0.52	0.01	0.85
Q9IB20	D3	3	1.46	0.42			A0A6P3VZ23	D3	3	0.57	0.08		
mhc_3	D0	3	1.80	1.00	0.32	0.24	gapdh-like	D0	3	1.10	0.31	0.63	0.06
Q90W53	D3	3	0.98	0.25			A0A6P3W0T5	D3	3	0.61	0.10		
act α 1a	D0	3	3.21	0.84	0.71	0.04	tpi1a	D0	3	0.70	0.36	0.35	0.22
C9E3A5	D3	3	1.52	0.40			A0A6P3VJN0	D3	3	0.39	0.11		
tpm1	D0	3	0.55	0.46	0.08	0.60	pygma	D0	3	0.60	0.30	0.42	0.16
A0A6P8F8P4	D3	3	0.74	0.33			A0A6P8FPY8	D3	3	0.26	0.15		
tpm α	D0	3	1.11	0.63	0.01	0.87	pygmb	D0	3	0.60	0.30	0.41	0.17
A0A6P3WG81	D3	3	1.03	0.51			A0A6P3W0I6	D3	3	0.29	0.12		
trpn1	D0	3	0.04	0.01	0.47	0.13	Total peak area	D0	3	28.74	6.39	0.09	0.53
A0A6P8FDH3	D3	3	0.07	0.03				D3	3	25.96	4.34		

3.7. Fatty Acid Analysis

A total of 13 fatty acids were considered in this analysis and included the saturated fatty acids myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (PA, C16:0), heptadecanoic acid (C17:0) and stearic acid (SA, C18:0), collectively referred to herein as SAFA; the monounsaturated fatty acids palmitoleic acid (C16:1n7), oleic acid (OA, C18:1n9), vaccenic acid (VA, C18:1n7), and neuronc acid (C24:1), collectively referred to herein as MUFA; the n -6 series polyunsaturated fatty acids linoleic acid (LA, C18:2n6) and arachidonic acid (AA, C20:4n6), collectively referred to herein as n6; and the n -3 polyunsaturated fatty acids eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), collectively referred to herein as n3. The above fatty acids constituted >90% of the total peak area of the chromatograms and were consistently present in all specimens examined on both days of the yearly samples (Tables 6 and S3).

Accordingly, in the fillet, across all three yearly samples and on both days of storage, DHA was the dominant FA, followed by PA. Although decreased mean values were observed for DHA and n3 at D3, these were not statistically significant. Similarly, the increased mean values of PA and SAFA at D3 were also non-significant. In fact, the only significant difference observed concerned the n6 in the 2021 sample ($p = 0.001$). At

the individual fatty acid level, significant differences were not observed in any of the fatty acids between D0 and D3 in any of the three yearly samples, except for SA in 2021. Notably, the n3 content of the fillet ranged from 46% to 50% on D0 and from 43% to 46% on D3. Consequently, the mean polyene index, i.e., the EPA + DHA (n3) to SAFA ratio, an indicator of oxidative rancidity in fishery products [70], was >1 on both days of storage (Tables 6 and S3).

Table 6. SAFA, MUFA, n3, n6, PA (C16:0), and DHA contents in the total fat of the anchovy fillets from the three yearly samples. Values (%) represent the mean ($\pm$ SD) from at least three individuals ($n \geq 3$). In each group of fatty acids and lipid class, asterisk (*) denotes significant differences ($p \leq 0.05$) between days of storage in each yearly sample.

		SAFA	MUFA	n6	n3	C16:0	DHA	
TOTAL	2020	D0 ($n = 4$)	35.71 $\pm$ 4.94	11.39 $\pm$ 2.26	2.57 $\pm$ 0.52	50.33 $\pm$ 6.71	24.29 $\pm$ 2.95	44.50 $\pm$ 8.43
		D3 ($n = 4$)	38.04 $\pm$ 1.38	12.60 $\pm$ 0.53	2.93 $\pm$ 0.12	46.44 $\pm$ 0.85	25.76 $\pm$ 1.56	40.55 $\pm$ 0.62
	2021	D0 ($n = 3$)	37.26 $\pm$ 3.61	12.31 $\pm$ 1.42	3.27 $\pm$ 0.34	47.16 $\pm$ 4.64	26.42 $\pm$ 2.24	41.35 $\pm$ 4.02
		D3 ($n = 6$)	39.61 $\pm$ 1.32	13.65 $\pm$ 1.21	3.62 $\pm$ 0.06 *	43.24 $\pm$ 2.12	27.323 $\pm$ 0.82	37.23 $\pm$ 2.17
	2022	D0 ($n = 3$)	37.76 $\pm$ 1.81	12.50 $\pm$ 1.86	3.58 $\pm$ 0.73	46.17 $\pm$ 3.80	24.49 $\pm$ 2.56	39.67 $\pm$ 4.93
		D3 ($n = 6$)	39.71 $\pm$ 3.60	12.61 $\pm$ 1.25	3.30 $\pm$ 0.47	44.38 $\pm$ 4.23	26.75 $\pm$ 3.21	38.35 $\pm$ 4.09

3.7.1. The Fatty Acid Profiles of Lipid Classes

Neutral Lipids

In the neutral lipid fraction and on both days of storage, the major fatty acid was PA, followed by SA in the 2021 and 2022 samples, while in the 2020 sample, the second most abundant fatty acid was DHA (Table 7). Interestingly, DHA in both the 2020 and 2022 samples appeared to be increasing at D3. In contrast, it remained stable between storage days in 2021, where the lowest value for this fatty acid was recorded. Also notable was the significant decrease in PA at D3 of the 2020 sample, which contributed to the significant decrease in the SAFA group, as well as the significant increase in the LA content, which led to higher D3 values for the n6 group of the sample. In contrast, no significant differences were observed between days of storage in any fatty acid group of either the 2021 or the 2022 samples. However, it should be noted that in the 2021 sample, SA significantly increased while VA significantly decreased on D3. Between the yearly samples in this lipid class, significant differences were observed in the SAFA and n3 groups ($p = 0.004$ and $p = 0.005$, respectively) on D3 of the 2020 and 2021 samples. In the case of SAFA, this difference was due to higher values of all fatty acids within this group of the 2021 sample. In the case of the n3 group, it was due to the low DHA content in that same sample. (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

Triacylglycerols (TAG)

In the TAG class, the dominant fatty acid was PA on both days of storage, followed in content by DHA, with both OA and SA also contributing significantly. Importantly, the EPA content in this lipid class matched that of total fat and exceeded that of the neutral lipids on D0 across all yearly samples (Table S3). No significant differences were observed between storage days for any of the fatty acid groups in either year. At the individual fatty acid level, significant differences were limited to increased DHA content and decreased OA on D3 in the 2021 sample (Tables 7 and S3). Between the yearly samples, significant differences concerned the SAFA and n3 groups ($p = 0.023$ and $p = 0.048$, respectively) on D3 of 2020 and 2022. The differences in the SAFA group were due to higher PA and SA values in the 2022 sample, while those in the n3 group were due to both EPA and DHA values

significantly increasing in the 2020 sample compared to 2022. Furthermore, while the TAG DHA appeared to increase on D3 in both the 2020 and 2021 samples, mean values for this fatty acid decreased in 2022. However, it should be noted that the 2022 sample presented very high intra-specimen variations in the abundance of most fatty acids in this lipid class (Tables 7 and S3).

Table 7. SAFA, MUFA, n3, n6, PA (C16:0), and DHA contents in the total neutral lipids and in the TAG and FFA classes of the anchovy fillets from the three yearly samples. Values (%) represent the mean ($\pm$ SD) from at least three individuals ($n \geq 3$). In each group of fatty acids and lipid class, asterisk (*) denotes significant differences ($p \leq 0.05$) between days of storage in each yearly sample.

			SAFA	MUFA	n6	n3	C16:0	DHA
NEUTRAL	2020	D0 ($n = 3$)	53.82 $\pm$ 1.66	18.16 $\pm$ 3.49	3.04 $\pm$ 0.12	24.98 $\pm$ 4.67	30.38 $\pm$ 1.09	20.28 $\pm$ 3.85
		D3 ($n = 3$)	43.68 $\pm$ 3.05 *	18.86 $\pm$ 2.80	4.04 $\pm$ 0.43 *	33.42 $\pm$ 5.38	24.60 $\pm$ 1.95	25.15 $\pm$ 3.01
	2021	D0 ($n = 3$)	63.02 $\pm$ 2.10	17.36 $\pm$ 2.29	4.09 $\pm$ 0.61	15.52 $\pm$ 3.06	34.41 $\pm$ 1.04	12.74 $\pm$ 1.41
		D3 ($n = 3$)	67.63 $\pm$ 3.51	13.25 $\pm$ 1.57	4.32 $\pm$ 0.506	14.80 $\pm$ 3.59	35.42 $\pm$ 2.46	11.53 $\pm$ 2.20
	2022	D0 ($n = 3$)	59.58 $\pm$ 17.29	14.09 $\pm$ 6.53	5.29 $\pm$ 2.09	21.03 $\pm$ 9.20	31.72 $\pm$ 4.82	16.25 $\pm$ 3.93
		D3 ($n = 6$)	55.88 $\pm$ 8.12	13.71 $\pm$ 3.66	4.52 $\pm$ 0.91	25.89 $\pm$ 5.74	30.23 $\pm$ 1.71	20.48 $\pm$ 1.92
TAG	2020	D0 ($n = 3$)	48.00 $\pm$ 3.05	22.89 $\pm$ 4.52	4.29 $\pm$ 1.07	24.79 $\pm$ 2.48	27.03 $\pm$ 0.49	19.35 $\pm$ 2.06
		D3 ($n = 3$)	43.97 $\pm$ 2.17	19.05 $\pm$ 4.15	4.16 $\pm$ 0.37	32.90 $\pm$ 5.08	25.40 $\pm$ 0.98	24.35 $\pm$ 3.21
	2021	D0 ($n = 3$)	53.34 $\pm$ 0.53	22.57 $\pm$ 0.67	4.16 $\pm$ 1.06	19.93 $\pm$ 0.41	30.79 $\pm$ 1.58	14.47 $\pm$ 0.40
		D3 ($n = 4$)	50.25 $\pm$ 3.41	19.27 $\pm$ 2.17	4.78 $\pm$ 0.89	25.71 $\pm$ 1.67 *	28.86 $\pm$ 1.54	18.05 $\pm$ 1.48 *
	2022	D0 ($n = 3$)	53.29 $\pm$ 15.11	17.18 $\pm$ 3.51	4.28 $\pm$ 1.51	25.25 $\pm$ 10.66	30.66 $\pm$ 7.39	18.57 $\pm$ 7.62
		D3 ($n = 3$)	52.94 $\pm$ 5.58	20.70 $\pm$ 4.36	6.76 $\pm$ 2.98	19.61 $\pm$ 7.71	28.36 $\pm$ 3.15	14.22 $\pm$ 5.66
FFA	2020	D0 ($n = 3$)	59.31 $\pm$ 2.13	19.21 $\pm$ 4.92	2.59 $\pm$ 0.48	18.86 $\pm$ 2.85	31.68 $\pm$ 3.83	16.85 $\pm$ 1.98
		D3 ($n = 3$)	66.48 $\pm$ 0.44 *	14.10 $\pm$ 0.48	3.64 $\pm$ 0.15 *	15.77 $\pm$ 0.30	36.47 $\pm$ 2.53	12.38 $\pm$ 1.54 *
	2021	D0 ($n = 3$)	78.78 $\pm$ 2.52	12.11 $\pm$ 1.89	3.92 $\pm$ 0.30	5.19 $\pm$ 0.81	40.47 $\pm$ 1.76	3.70 $\pm$ 0.67
		D3 ($n = 6$)	74.49 $\pm$ 5.78	8.57 $\pm$ 1.76 *	6.12 $\pm$ 4.33	10.83 $\pm$ 2.65 *	39.71 $\pm$ 2.65	8.53 $\pm$ 2.15 *
	2022	D0 ($n = 3$)	79.78 $\pm$ 10.54	9.83 $\pm$ 6.26	5.40 $\pm$ 3.03	4.99 $\pm$ 2.22	39.44 $\pm$ 1.11	3.77 $\pm$ 1.69
		D3 ($n = 6$)	59.59 $\pm$ 6.19 *	13.42 $\pm$ 4.35	6.33 $\pm$ 2.09	20.66 $\pm$ 10.09 *	34.03 $\pm$ 3.00 *	16.26 $\pm$ 7.94 *

Free Fatty Acids (FFA)

In the FFA class, and as in the case of TAG, the dominant fatty acid was PA, which remained relatively stable between storage days (Table 7). High values were also recorded for SA, which on D0 of the 2022 sample matched those of PA, although it decreased significantly at D3. Consequently, the SAFA content was 60% on D0 in 2020 and on D3 in 2022, and 79% on D0 in both the 2021 and 2022 samples. In contrast, low DHA values were observed in this lipid class, especially on D0 of the 2021 and 2022 samples. Although the DHA content was significantly reduced on D3 of the 2020 sample, which had the highest content for this fatty acid at D0, it increased significantly in the other two samples. The EPA content of this class was significantly lower on both days of storage compared to that of either the total fat or the neutral lipids, exhibiting, however, an increasing trend on D3. These differences were reflected in the between-years comparison of the SAFA and n3 fatty acid groups (Table 7). (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

Polar Lipids

In the polar fraction of the fat, DHA was the major fatty acid across both days of storage. However, its content significantly decreased on D3 in both the 2020 and 2021

samples, while it also presented a lower mean value in 2022 (Table 8). This decrease was reflected in the total n3 fatty acid content, which was also affected by decreased EPA values on D3 in all three yearly samples (significant for 2020 and 2021). The decrease in n3 content was concomitant with an increase in SAFA, mostly due to the rise in SA in the 2020 and 2021 samples. Also notable was the increase in the MUFA group in the 2020 sample, which resulted from higher levels of both C16:1 and OA. Despite these changes, no significant differences were observed between any of the yearly samples in any of the fatty acid groups. (See Table S3 for levels of the aforementioned fatty acids on both storage days in each yearly sample).

Table 8. SAFA, MUFA, n3, n6, PA (C16:0), and DHA contents in the total polar lipids and in the PE, PI, PS and SM-PC classes of the anchovy fillets from the three yearly samples. Values represent the mean ($\pm$ SD) from at least three individuals ($n \geq 3$). In each group of fatty acids and lipid class, asterisk (*) denotes significant differences ($p \leq 0.05$) between days of storage in each yearly sample.

			SAFA	MUFA	n6	n3	C16:0	DHA
POLAR	2020	D0 ($n = 3$)	33.46 $\pm$ 2.99	10.73 $\pm$ 0.51	3.16 $\pm$ 0.29	52.66 $\pm$ 3.34	22.57 $\pm$ 2.50	49.00 $\pm$ 3.61
		D3 ($n = 3$)	48.32 $\pm$ 0.90 *	14.77 $\pm$ 1.26 *	2.74 $\pm$ 0.25	34.17 $\pm$ 1.58 *	26.17 $\pm$ 1.45	31.09 $\pm$ 1.74 **
	2021	D0 ($n = 3$)	41.94 $\pm$ 3.32	9.91 $\pm$ 2.73	3.33 $\pm$ 0.24	45.09 $\pm$ 5.51	27.22 $\pm$ 1.84	40.72 $\pm$ 5.14
		D3 ($n = 6$)	48.59 $\pm$ 3.33 *	12.49 $\pm$ 3.49	4.10 $\pm$ 0.92	34.82 $\pm$ 5.53 *	29.05 $\pm$ 0.92	31.62 $\pm$ 5.52 *
	2022	D0 ($n = 3$)	38.89 $\pm$ 4.28	12.28 $\pm$ 1.76	3.29 $\pm$ 0.70	45.54 $\pm$ 5.05	26.32 $\pm$ 4.16	39.56 $\pm$ 4.72
		D3 ($n = 6$)	43.77 $\pm$ 3.96	13.11 $\pm$ 2.49	3.46 $\pm$ 0.64	39.66 $\pm$ 5.54 *	28.07 $\pm$ 2.98	34.82 $\pm$ 5.59
PE	2020	D0 ($n = 3$)	30.22 $\pm$ 1.87	11.42 $\pm$ 1.02	3.47 $\pm$ 0.11	54.89 $\pm$ 2.99	18.94 $\pm$ 1.24	52.82 $\pm$ 3.10
		D3 ($n = 6$)	33.39 $\pm$ 1.57	10.68 $\pm$ 1.42	3.21 $\pm$ 0.37	52.71 $\pm$ 3.15	19.37 $\pm$ 2.39	50.36 $\pm$ 2.91 *
	2021	D0 ($n = 3$)	36.53 $\pm$ 0.80	13.11 $\pm$ 0.81	3.32 $\pm$ 0.23	47.04 $\pm$ 1.80	19.44 $\pm$ 2.26	44.59 $\pm$ 1.32
		D3 ($n = 5$)	42.30 $\pm$ 2.16 *	12.79 $\pm$ 3.17	3.69 $\pm$ 0.56	41.21 $\pm$ 3.22 *	23.81 $\pm$ 1.03 *	39.08 $\pm$ 3.34 *
	2022	D0 ($n = 3$)	40.00 $\pm$ 4.90	10.86 $\pm$ 2.05	3.51 $\pm$ 0.27	45.64 $\pm$ 6.36	22.64 $\pm$ 3.71	43.45 $\pm$ 5.84
		D3 ($n = 6$)	45.61 $\pm$ 5.73	12.20 $\pm$ 2.53	3.83 $\pm$ 0.79	38.35 $\pm$ 6.97	25.24 $\pm$ 3.00	36.27 $\pm$ 7.00
PI	2020	D0 ($n = 3$)	48.70 $\pm$ 4.09	12.60 $\pm$ 0.62	3.38 $\pm$ 0.72	35.32 $\pm$ 3.94	11.80 $\pm$ 0.14	33.57 $\pm$ 3.43
		D3 ($n = 3$)	54.93 $\pm$ 2.75	14.25 $\pm$ 0.11	3.45 $\pm$ 0.08	27.36 $\pm$ 2.71 *	19.94 $\pm$ 1.66 *	25.33 $\pm$ 3.43 *
	2021	D0 ($n = 3$)	56.83 $\pm$ 5.18	13.40 $\pm$ 1.39	4.47 $\pm$ 0.89	25.30 $\pm$ 4.85	30.76 $\pm$ 4.38	23.53 $\pm$ 4.48
		D3 ($n = 6$)	57.99 $\pm$ 6.02	17.37 $\pm$ 2.45 *	7.42 $\pm$ 3.84	17.22 $\pm$ 7.03	31.43 $\pm$ 2.15	15.33 $\pm$ 5.54
	2022	D0 ($n = 3$)	55.06 $\pm$ 6.48	12.40 $\pm$ 2.84	4.19 $\pm$ 0.43	28.35 $\pm$ 3.22	27.34 $\pm$ 2.11	26.92 $\pm$ 2.73
		D3 ($n = 6$)	63.09 $\pm$ 8.94	13.07 $\pm$ 6.19	5.41 $\pm$ 2.22	18.42 $\pm$ 6.45 *	29.13 $\pm$ 4.34	16.80 $\pm$ 6.29 *
PS	2020	D0 ($n = 3$)	45.77 $\pm$ 4.36	13.58 $\pm$ 2.07	1.60 $\pm$ 0.46	39.05 $\pm$ 3.91	18.90 $\pm$ 0.98	38.00 $\pm$ 3.81
		D3 ($n = 3$)	58.79 $\pm$ 8.00	17.60 $\pm$ 4.51	2.15 $\pm$ 0.38	21.46 $\pm$ 3.69 *	26.97 $\pm$ 5.01 *	19.31 $\pm$ 5.06 *
	2021	D0 ($n = 3$)	57.39 $\pm$ 4.20	13.00 $\pm$ 1.62	4.30 $\pm$ 0.79	25.30 $\pm$ 4.85	29.99 $\pm$ 5.02	23.53 $\pm$ 4.48
		D3 ($n = 6$)	57.84 $\pm$ 6.10	18.38 $\pm$ 3.37 *	7.38 $\pm$ 3.85	16.40 $\pm$ 8.38	31.23 $\pm$ 2.11	14.50 $\pm$ 6.98
	2022	D0 ($n = 3$)	67.30 $\pm$ 3.02	9.47 $\pm$ 1.29	3.69 $\pm$ 0.48	19.53 $\pm$ 3.11	30.44 $\pm$ 0.31	17.80 $\pm$ 2.68
		D3 ($n = 5$)	73.04 $\pm$ 10.76	8.68 $\pm$ 3.17	3.13 $\pm$ 0.73	15.15 $\pm$ 9.47	34.81 $\pm$ 4.31	13.36 $\pm$ 8.66
SM-PC	2020	D0 ($n = 3$)	35.87 $\pm$ 2.36	10.02 $\pm$ 0.60	2.88 $\pm$ 0.21	51.23 $\pm$ 2.81	29.92 $\pm$ 1.84	44.79 $\pm$ 2.65
		D3 ($n = 3$)	42.53 $\pm$ 3.34	9.85 $\pm$ 0.98	2.66 $\pm$ 0.03	44.96 $\pm$ 4.35	33.40 $\pm$ 2.43	39.31 $\pm$ 4.08
	2021	D0 ($n = 3$)	43.83 $\pm$ 5.47	11.66 $\pm$ 0.74	3.00 $\pm$ 0.59	41.51 $\pm$ 6.46	36.12 $\pm$ 4.65	35.35 $\pm$ 6.19
		D3 ($n = 5$)	46.34 $\pm$ 3.50	10.43 $\pm$ 1.77	3.76 $\pm$ 0.28 *	39.47 $\pm$ 2.43	33.37 $\pm$ 1.65	33.31 $\pm$ 1.84
	2022	D0 ($n = 3$)	44.81 $\pm$ 4.46	9.75 $\pm$ 0.90	2.97 $\pm$ 0.24	42.47 $\pm$ 3.43	32.36 $\pm$ 1.15	36.16 $\pm$ 2.55
		D3 ($n = 6$)	48.32 $\pm$ 3.50	11.22 $\pm$ 3.06	4.09 $\pm$ 1.69	36.38 $\pm$ 5.08	33.42 $\pm$ 2.86	30.76 $\pm$ 4.54

Phosphatidylethanolamine (PE)

In the PE class, the dominant fatty acid was DHA, exhibiting values that exceeded those recorded for the total fat, as well as those of the polar fraction in all three samples and on both days of storage. It remained stable between D0 and D3 in the 2020 and 2022 samples, but it decreased significantly in 2021 (Table 8). As in the case of the total fat and its polar fraction, the second most abundant fatty acid was PA, which also remained stable during storage in the 2020 and 2022 samples but, in contrast to DHA, significantly increased on D3 in 2021. Significantly higher values were also observed on D3 for the minor SAFA C14:0 and C15:0 in both the 2020 and 2021 samples. Notable in this lipid class was the low but stable EPA content across all samples, at values comparable to those observed in the FFA (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

Phosphatidylinositol (PI)

In this lipid class, DHA and SA exhibited comparable values on D0, while on D3 a decreasing trend was observed for DHA (significant in 2020 and 2022), with SA remaining stable (Tables 8 and S3). Low values for PA were recorded on D0 in the 2020 sample, but they increased significantly on D3. In the other two samples, the PA content remained stable during storage. A significant increase during storage was also observed for OA in both the 2020 and 2021 samples, which significantly affected the total MUFA content only in 2021. As in PE, the EPA content in this lipid class was low but stable between storage days, with values comparable to those observed in the PE and FFA classes. (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

Phosphatidylserine (PS)

High SA values were also recorded in this lipid class, with this fatty acid being the major SAFA on D0 in the 2020 and 2022 samples. In contrast to PA, which exhibited an increasing trend during storage, SA values remained relatively stable. Conversely, except for D0 in the 2020 sample, DHA exhibited values lower than SA or PA, as well as a decreasing trend during storage (Table 8). A significant increase in OA was also observed on D3 in the 2020 and 2021 samples, which significantly affected the total MUFA content in the latter (Table 8). Similar to PE and PI, EPA exhibited low but stable values during storage across the yearly samples. (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

Sphingomyelin-Phosphatidylcholine (SM-PC)

In this mixed class, DHA was the most abundant fatty acid in all three yearly samples and on both days of storage. However, a decreasing, albeit non-significant, trend in the values of this fatty acid was observed on D3 in all samples (Table 8). Conversely, the PA values remained stable during storage, as did the other fatty acids examined, with the notable exception of SA, for which a significant increase was observed in the 2020 and 2021 samples. In contrast to the other polar lipid classes, high EPA values were recorded in the SM-PC fraction of all three samples, comparable to those of the total fat. (See Table S3 for levels of aforementioned fatty acids on both storage days in each yearly sample).

4. Discussion

The proximate composition in the fillet of freshly caught anchovy (D0) across all yearly samples is in good agreement with the values reported for anchovies from other Mediterranean areas [71–76], as well as from European Atlantic Ocean waters [6,77]. Thus, in samples from these areas, the moisture content ranged from a low of 72% to a high of

79%, the Bay of Biscay and the Adriatic Sea, respectively [74,77], while the corresponding mean value of the three yearly samples of the present study was $\approx 77\%$. The protein content in the fillet of anchovy ranged from 18% in the Gulf of Cadiz [6] to 23% in the Gulf of Catania [72], as compared to the $\approx 20\%$ mean value of the three yearly samples presented herein. Anchovy fillet fat content has been reported to range from 1% in both the Gulfs of Catania and Lions to 4.3% in the Central Adriatic [71,72,74], compared with the present study's overall mean of $\approx 1.9\%$ for the three annual samples. However, at the local level, important variations in these values were observed when samples from different months or seasons were analyzed, as in the case of the Gulf of Cadiz, Bay of Biscay, and Central Adriatic [6,74,77], suggesting that the environment can significantly affect the quality characteristics of the fish. Relative to this, it is interesting to note that Black Sea anchovies, although of the same species, have a very different proximate composition, with moisture content $< 70\%$, protein content between 17% and 19%, and fat content ranging from 10% to 18% [76,78,79]. Whether these differences are due to the low salinity of the Black Sea (18–19 ppt), as compared to the Mediterranean Sea or the Atlantic Ocean (35–38 ppt) [80], or to other environmental (e.g., trophic conditions, diet composition) or genetic factors, remains to be established.

Regarding the energy density of the anchovy fillet, this parameter has been determined in four other studies, of which two concern fish from the Bay of Biscay [77,81], one from the Northwestern Mediterranean [82], and one from the Thracian Sea [83], with the latter conducted in an area very proximal to the sampling sites of the present study. The energy density of the Bay of Biscay anchovies ranged from 21 to 24 kJ/g dry weight (≈ 5 to 6 kJ/g wet weight), while for the Mediterranean anchovies it was ≈ 5 kJ/g wet weight, which is very similar to the values obtained in the present study.

The marginal differences in proximate composition and energy density that we observed between the D0 and D3 samples suggest that the fillet preserves most of its “crude” characteristics during storage, under the conditions used. This may appear to contrast the results of the sensory analysis, where the quality of the product dropped significantly at D3. However, the proximate composition analysis records the “crude” protein and fat content of the tissue, while during spoilage both protein and fat can be transformed or degraded by exogenous (microbial) or endogenous (autolytic) processes. Thus, as long as the transformation or degradation products remain within the tissue and given that the moisture content does not change dramatically, the proximate composition is not expected to differ significantly.

Microbial-caused spoilage is considered the most important factor in the spoilage of fish products, and microbiological assays are extensively used to assess fish and, more generally, seafood freshness and safety. Thus, total viable counts (TVC) ranging from 10^2 to 10^4 cfu/g, for either entire or filleted fish, are indicative of freshness, while counts exceeding 10^7 cfu/g indicate spoilage [84–86]. According to the results presented herein, the bacterial counts at both days of storage were well below the acceptable limits mentioned above. In addition, the initial TVC counts of all samples were comparable to those reported for fresh anchovies from the Northwestern and Central Mediterranean [19,87]. Furthermore, the TVC and *Pseudomonas* D0 and D3 counts reported herein were similar to those of fresh anchovies from the Adriatic Sea stored in ice in polystyrene boxes [16], although apparently higher than those of Central Mediterranean anchovies stored in ozonized slurry ice [87].

While microbiological analysis using general-purpose media is undeniably useful for evaluating the total bacterial load of the fish samples, it presents significant limitations in characterizing the microbiota composition, especially regarding genera with spoilage or pathogenic potential. To some extent, this also applies to the *Pseudomonas* CFC medium, on which other bacterial genera (e.g., *Shewanella*, *Enterobacteriaceae*, *Vibrio*) can grow [88].

Furthermore, only a small fraction of the total microbiota from environmental samples can be recovered using classical microbiological techniques, as many species either require specialized growth media or are presently unculturable. Culture-independent molecular applications overcome the above limitations and are presently widely used to evaluate the role of microbiome dynamics in the shelf life of foods, including seafood, under different storage conditions [25,64,65,89]. Our 16S rRNA gene analysis of the fillet microbiota indicates that the anchovies in the study region harbour a variety of bacterial genera with spoilage and/or pathogenic potential. However, with few exceptions, no obvious pattern was observed regarding either the presence of these genera in the three yearly samples, or their changes in relative abundance during the storage period. Specifically, from the SSOs identified, *Photobacterium*, *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Shewanella*, and *Flavobacterium* were consistently present in all samples, but the changes in their relative abundance during storage varied widely between years. Furthermore, apart from *Photobacterium*, *Pseudomonas*, and *Acinetobacter*, the relative abundance of the remaining SSOs was in most cases particularly low. However, it should also be noted that 16S rRNA amplicon sequencing provides information on the relative taxonomic composition of the bacterial community rather than on absolute bacterial abundance or metabolic activity; thus, an increase in the relative abundance of a given taxon might reflect either an increase in its absolute abundance or a decrease in other community members [90]. Hence, relative fluctuations in the abundance of specific SSOs should be interpreted with caution, as they do not necessarily reflect an increase in their absolute numbers or spoilage potential. In the present study, the microbiome shifts observed during storage likely represent changes in community structure rather than direct evidence of advanced spoilage, particularly considering the low culturable bacterial loads.

From the fillet samples, that of 2022 clearly stands out, as on D3 the relative abundance of the spoilage-associated genera represented 80% of the total. This was due to the sharp increase in the abundance of *Vibrio*, *Catenococcus*, *Oceanimonas*, and *Myroides*, while the SSOs *Psychrobacter* and *Shewanella* also exhibited the highest values among the fillet samples, though this, with the exception of sensory traits, did not obviously impact the qualitative traits of the fillets examined. The increased relative abundance of the above genera on D3 coincided with the drastic decline of *Photobacterium*, which was the predominant SSO genus on D0. Since *Photobacterium* is a cold-tolerant genus [91], it is unlikely that this decline was due to storage temperature alone. Notably, *Photobacterium*, *Vibrio* and *Catenococcus* belong to the Vibrionaceae family, share similar habitats in the marine environment and utilize the same resources [92]. Therefore, it would be of interest to examine whether the above observed effect results from antagonistic relationships between these genera, or if other factors -environmental or microbial- may also contribute the observed community dynamics.

The presence of *Staphylococcus* and of *Escherichia-Shigella* in all fillet samples, together with the increasing trend in relative abundance for *Staphylococcus* during storage, is of particular concern. Although members of these genera have frequently been associated with post-harvest handling and hygiene-related contamination in seafood, their detection by 16S rRNA amplicon sequencing should be interpreted with caution, as this method provides genus-level taxonomic assignments and does not distinguish between pathogenic and non-pathogenic species, nor between viable and non-viable cells. Nevertheless, their detection in fillet samples highlights the importance of hygienic handling practices throughout harvesting, transportation, and sample processing. Specifically, *Staphylococcus* is normally absent in wild fish populations [66,93], while humans are the primary reservoir of *Escherichia-Shigella* [67].

As expected, the microbiota of the H/V is largely dominated by genera that are normally associated with the gut and gills of the fish, such as *BD1-7_clade*, *Cyanobium_PCC-*

6307, *Synechococcus*_CC9902, and 2013Ark19i [68,69,94,95]. This results in a lower relative abundance of the spoilage- and pathogen-associated genera, although their presence in the H/V samples partially reflected patterns observed in the fillets. Thus, as in the fillet, the co-occurrence of *Photobacterium*, *Vibrio*, and *Catenococcus* in the 2022 H/V sample suggests comparable microbial dynamics in both sample types. In contrast to the fillet, *Staphylococcus* was detected at low relative abundance levels in the H/V samples, while *Escherichia-Shigella* was almost completely absent. The results support the notion that the presence of these genera in the fillet samples was due to contamination during fish handling.

Overall, the results of the 16S rRNA gene analysis indicate that sub-chilling prevented SSOs from significantly increasing in relative abundance within three days post-harvest, in agreement with the microbiological results. Additionally, as illustrated by the differences between the three yearly samples, the local environmental conditions and/or sampling period are important drivers of microbiota composition and may influence the overall quality of the fish.

Biogenic amines are naturally present in fish where they perform diverse biological functions [96]. However, they tend to accumulate during spoilage, because of proteolysis and decarboxylation of amino acids, either by endogenous enzymes or by bacteria possessing amino acid decarboxylase activity [97]. The occurrence of high concentrations of biogenic amines in fresh fish is associated with the risk of food poisoning. Histamine is the primary causative agent, although the presence of other amines, such as cadaverine and putrescine, can potentiate its action [96,98]. Currently, histamine is the only biogenic amine for which acceptable limits in fresh fish have been defined by various countries and international organizations, with 100 mg/kg being the maximum acceptable value in the European Union [99]. The absence of histamine in the anchovy fillets of the present study agrees with the results of other studies that have shown negligible formation of this biogenic amine in fish stored at 0 °C or below [97]. Specifically, in anchovies, it has been shown that histamine formation takes place after two days in fish that were iced prior to being stored at 4 °C [100]. Regarding the increased concentration of spermidine and spermine in the 2021 sample, it should be noted that these polyamines are of endogenous origin and are not synthesized by microorganisms. Therefore, their concentrations would be expected to decrease over time, if they change at all, rather than increase [101]. Thus, the spermine and spermidine 2021 concentration levels likely reflect an effect of the sample itself rather than of storage, as previously proposed for European hake stored in ice [102]. On the other hand, putrescine and cadaverine formation has been correlated with the presence of *Shewanella* [102] as well as with *Enterobacteriaceae* and *Pseudomonas* [98]. However, the data of the microbiome analysis do not point towards a correlation between the presence of *Enterobacteriaceae* in our samples (i.e., *Escherichia-Shigella* and *Proteus*) along with *Shewanella* and *Pseudomonas*, and the formation of putrescine and cadaverine, within this short-term storage period. This is because the relative abundance of these genera did not exhibit an increasing trend across all the yearly samples.

As mentioned above, sensory changes and texture properties are closely linked to fish freshness, with tissue tenderization being an important factor in determining the final quality attributes of the product. Because muscle tenderization results from the enzymatic degradation of proteins, proteomic analysis can help clarify the underlying biochemical processes and potentially contribute to improving the shelf life of fresh fish. However, muscle remains a challenging tissue for proteomic analysis, as it is dominated by the contractile proteins of the sarcomere, while several enzymes of energy metabolism are also highly abundant in the sarcoplasm [103]. As a result, the over-represented proteins render the detection of the low-abundance ones difficult, leading to incomplete proteome characterization [103,104].

Currently, the application of comparative proteomics to study the post-mortem changes in the muscle of fish species during cold storage is limited, and our study represents the first such attempt on the European anchovy. Relevant studies on other species employed mainly 2-dimensional electrophoresis (2-DE) to detect protein degradation in cold-stored fish muscle. Specifically, 2-DE and immunoblotting were used to study proteome changes in sea bass dorsal muscle after storage up to 96 h in ice [105]. According to the results of that study, the myofibrillar protein abundance remained stable during the storage period, with the major observed changes limited to two low-molecular-weight proteins, one of which was later identified as nucleoside diphosphate kinase [4]. The 2-DE analysis was also used to elucidate changes in the muscle proteins of cod stored on ice for up to eight days. Accordingly, several changes in protein spot intensities were observed, with the proteins involved not being identified [106]. In a more recent study, also on sea bass muscle stored at 1 °C for five days, 2-D Difference Gel Electrophoresis (DIGE) coupled to MS analysis revealed significant degradation of several proteins including myosin heavy chain, myosin light chain 2, GAPDH, and glyceraldehyde mutase [1]. The fact that no such differences were observed in the anchovy muscle in the present study could, among other factors, reflect species differences in muscle consistency, storage period and conditions, as well as the initial load of spoilage bacteria in the samples, which was not examined in any of the above studies. However, the possibility cannot be excluded that the small replicate size and high inter-individual variability may have obscured potentially important changes. Interestingly, and in contrast to the above studies, storage significantly affected the relative abundance of actin $\alpha 1a$. Actin is the second most abundant protein in muscle and is an indicator of post-mortem proteolytic degradation as it serves as a substrate of endogenous cathepsins (B, D, L) [4,107].

In our study, as well as in all the others mentioned above, the dorsal muscle of the fish was used, an obvious choice as it represents the major part of the fillet. Additionally, it is less likely to be contaminated by other tissues, as would have been the case if the hypaxial muscle were to be used. Therefore, the small or no changes observed in our study concern solely the dorsal part of the fillet. In the abdominal muscle, more important differences would be expected, as it is more exposed to the highly active endogenous digestive enzymes. Clearly, the fish muscle proteome analysis needs further refinement to overcome the challenges mentioned above. Further analytical challenges stem from the complexity of fish genomes, which have undergone three rounds of duplication in the Teleostei lineage [108]. Relevant to this is the identification of multiple fast skeletal myosin heavy chain-like proteins (mhc-fs-l) in the anchovy proteome. Specifically, 11 different isoforms were identified, although only five were present in all specimens examined (Table 5). Chen et al. [109] reported 14 different genes potentially encoding for fast skeletal mhc-fs-l proteins in the genome of the Chinese perch (*Siniperca chuatsi*), while in the herring genome (Genome assembly Ch_v2.02) 40 loci in eight different chromosomes were identified for this particular myosin heavy chain type. Similarly, a search in the recently made available European anchovy genome (Genome assembly IST-EnEncr_1.0) led to the identification of 31 mhc-fs-l loci in five different chromosomes. Importantly, as our results suggest, their expression pattern differs even among individuals from the same catch. However, even when the total mhc-fs-l content of the samples was considered, no significant differences were observed between the storage days (File S2).

Along with protein degradation, lipid hydrolysis is a major factor contributing to the deterioration of fresh fish quality during storage. Enzymatic lipid hydrolysis (lipolysis) occurs by the action of either endogenous or psychrotrophic bacteria-derived lipases and phospholipases and leads to the formation of free fatty acids. These, in turn, are highly susceptible to oxidation, a process that can alter flavour, texture, and colour of the fish [110].

According to the results presented here, the mean content of DHA and EPA in the fillet lipid of the fresh (D0) anchovies from the Thracian Sea was generally higher than that reported for anchovies from other Mediterranean areas [72,111–113] but comparable to Adriatic Sea anchovies [75,114]. Similarly, the Thracian and Adriatic Sea anchovies had higher SAFA content than those from the other regions but lower MUFA content. The above suggest that environmental conditions, and mainly diet composition, in different areas or seasons of sampling, can determine the fatty acid profile of fillet fat.

The results from the three yearly samples also suggest that the short-term storage of anchovies at sub-chilled conditions largely preserves the fatty acid profile in the total fat of the fillet, which is in line with the proximate analysis results regarding crude fat content. To our knowledge, only one other study has examined the changes in the fatty acid profile in the fillet of anchovy during cold storage [16]. As in the case of our samples, the authors observed small decreases in DHA and EPA with a concomitant increase in PA and, therefore, in SAFA, after storing the fish on ice for three days.

The total fat analysis provides useful information regarding the relative abundance of the different fatty acids therein but does not allow one to conclude whether the lipid classes that comprise the fat remain intact during storage. Our results regarding the fatty acid profile of the total neutral lipids, as well as that of the two major classes in this lipid fraction, i.e., TAG and FFA, demonstrated that there is an increasing trend in the abundances of DHA and EPA during storage, across the three yearly samples, in both the TAG and the FFA. Regarding the EPA, TAG appear to be the main depot site for this fatty acid in the neutral lipid. The rise in DHA and EPA in these two classes likely stems from the enzymatic hydrolysis of both neutral and polar lipids. In the case of TAG, the highly unsaturated EPA and DHA are apparently spared from the action of endogenous and/or exogenous lipases, due to the steric hindrance created by their rigid structures, which prevents the enzyme's active site from easily accessing the target ester bond [115–117]. In contrast, the more flexible structure of the esterified SAFAs and MUFAs renders them more susceptible to enzymatic hydrolysis, decreasing their relative abundance, while increasing that of EPA and DHA in this lipid class. On the other hand, phospholipases, and particularly those with high specificity for PUFA-containing phospholipids, such as PLA₂ [114,118], are likely to be responsible for the observed rise in abundance, especially of DHA and to a lesser extent of EPA, in the FFA class in two of the three yearly samples.

The fatty acid profile of the total neutral lipids in the muscle of fresh anchovies has also been studied on samples from the Gulf of Lions, Northwestern Mediterranean [71]. As compared with our D0 results, across all three yearly samples, the most notable differences are significantly lower contents of PA, SA, and OA, alongside higher EPA and DHA contents in the total neutral lipids of the anchovies from the Gulf of Lions. These differences highlight the effects of the local environment and diet composition in determining the anchovy fillet fatty acid profile.

According to the results presented here, the polar lipid fraction constitutes the main depot of DHA and EPA in the anchovy fillet fat, with PE and SM-PC being the main contributing classes. Especially for EPA, SM-PC appears to be the main storage site of this fatty acid in the polar lipid. However, the relative abundance of these *n*-3 PUFAs decreases significantly during storage, apparently due to the action of phospholipases, mainly on DHA, as discussed above. Interestingly, it appears that the PS and PI classes are more prone to the hydrolysis of DHA, despite their initial low content in this fatty acid. In contrast, in all the polar lipid classes, the relative abundance of both the SAFA and MUFA remains unchanged, if not increasing, during storage. Another interesting feature of the polar lipids is the high content of SA in all but the SM-PC classes, and especially in PI and PS. Regarding the latter, a previous study characterized the phospholipid molecular species

in the fillet of anchovies from the Adriatic Sea [114]. According to their results, in the PI and PS classes, the most abundant molecular species were 18:0/22:6, i.e., SA/DHA, and 16:0/22:6, i.e., PA/DHA, with the sum of these corresponding to 74% in the PI and to 64% in the PS. In contrast, in the PE class, the 18:0/22:6 species represented only 13.5% of the total, with the 16:0/22:6 amounting to 38.5%, while in the PC class, the 18:0/22:6 species represented only 1.4% of the total, and the 16:0/22:6 the 32%. These results are in good agreement with the abundances of these fatty acids in the polar lipid classes of our fillet samples (Table S2). It is noteworthy that the fatty acid profile of the total fillet fat exhibited high similarity across these two studies.

5. Conclusions

To our knowledge, the present study represents the first attempt to combine 16S rRNA gene sequencing, bottom-up proteomics, and lipid class analyses, along with standard sensory, proximate and microbiological analyses, to evaluate changes in the fillet of European anchovies stored under sub-chilled conditions. The results demonstrate that storage under such conditions effectively preserves the nutritional value of the fish, as evidenced by the stability of proximate composition, energy density, and overall fatty acid profiles of the fillet. Microbiological analyses, supported by 16S rRNA gene sequencing, showed that the relative abundance of spoilage-associated bacterial genera did not increase substantially during the storage period and, consequently, histamine was absent in all samples, while the concentrations of the other biogenic amines remained low. However, the notable interannual differences in microbiota composition point to the role that pre- and post-harvest environmental factors play in shaping the anchovy microbiome, which could potentially affect product quality. Furthermore, under the conditions examined, limited protein degradation was observed in the dorsal muscle proteome. In contrast, lipid class analyses indicated that selective hydrolysis processes affected the distribution of the health-beneficial fatty acids DHA and EPA among neutral and polar lipid fractions.

Future studies should investigate longer sub-chilling storage periods, while work focusing on abdominal muscle tissue, where endogenous enzymatic activity is expected to be greater, may provide further insights into post-mortem proteolytic processes. Integrating microbiome, proteome, lipidome, and sensory data under different environmental and seasonal conditions would further contribute to the development of more accurate shelf-life prediction models and optimized post-harvest handling strategies for the European anchovy as well as for other small pelagic fish species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse14151435/s1>, Figure S1: Location of sampling sites. The sampling sites of 8 September 2020 (circle, 40.693083 N, 24.459667 E), 13 September 2021 (Diamond, 40.725278 N, 24.485556 E), and 15 September 2022 (triangle, 40.657833 N, 24.480183 E) are indicated. The depth of the sea (m) at the sampling points is also given; Figure S2: Separation of the lipid classes from total anchovy fillet fat by a two-solvent TLC system. **I:** Unseparated Sphingomyelin and Phosphatidylcholine (SM-PC); **II:** Phosphatidylinositol (PI); **III:** Phosphatidylserine (PS); **IV:** Phosphatidylethanolamine (PE); **V:** Free Fatty Acids (FFA); **VI:** Triacylglycerol (TAG); **C:** Cholesterol; **CE:** Cholesterol esters; **SF:** Solvent front; **ORI:** Origin; Figure S3: Effects of storage in sub-chilled conditions for 72 hours on the appearance of anchovies. Panel A: Freshly caught anchovies (September 2020 sample). Panel B: The same batch of anchovies as in (A) after storage; Figure S4: Rarefaction curves of ASVs of replicates analyzed. G: head/viscera samples; T: fillet samples; Figure S5: Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis dissimilarities. Colors indicate the different yearly samples. G: head/viscera samples; T: fillet samples; Table S1: Alpha diversity (Shannon, Simpson, Inverse Simpson) and Good's coverage estimates across 16S rRNA gene sequencing samples; Table S2: Number of total read, DADA2 filtered reads, reads after taxonomy filtering, and final

observed reads for each sample; Table S3: The fatty acid profile of total fat and of the different neutral and polar lipid classes in the D0 and D3 anchovy fillets from the three yearly samples. Values (% of the sum of listed fatty acids) represent the mean ($\pm$ SD) from at least three individuals ($n \geq 3$). Significant differences ($p \leq 0.05$) between days of storage within each sample, are marked in red; File S1: Microbiome data; File S2: Proteomic data.

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References

1. Terova, G.; Addis, M.F.; Preziosa, E.; Pisanu, S.; Pagnozzi, D.; Biossa, G.; Gornati, R.; Bernardini, G.; Roggio, T.; Saroglia, M. Effects of postmortem storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: Analysis by 2-D DIGE and MS. *Proteomics* **2011**, *11*, 2901–2910. [[CrossRef](#)] [[PubMed](#)]
2. Boziaris, I.S.; Parlapani, F.F. Specific Spoilage Organisms (SSOs) in Fish. In *The Microbiological Quality of Food*; Bevilacqua, A., Corbo, M.R., Sinigaglia, M., Eds.; Woodhead Publishing Series in Food Science, Technology and Nutrition; Woodhead Publishing: Cambridge, UK, 2017; pp. 61–98. [[CrossRef](#)]
3. Wang, X.; Zheng, Z. Mechanistic Insights into Fish Spoilage and Integrated Preservation Technologies. *Appl. Sci.* **2025**, *15*, 7639. [[CrossRef](#)]
4. Delbarre-Ladrat, C.; Chéret, R.; Taylor, R.; Verrez-Bagnis, V. Trends in Postmortem Aging in Fish: Understanding of Proteolysis and Disorganization of the Myofibrillar Structure. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 409–421. [[CrossRef](#)] [[PubMed](#)]
5. Gram, L.; Huss, H.H. Microbiological Spoilage of Fish and Fish Products. *Int. J. Food Microbiol.* **1996**, *33*, 121–137. [[CrossRef](#)] [[PubMed](#)]
6. Careche, M.; García, R.; Borderías, A.J. Anchovy Shelf Life as Affected by Different Chilling Methods during Distribution. *J. Food Prot.* **2002**, *65*, 353–361. [[CrossRef](#)] [[PubMed](#)]
7. García-del-Hoyo, J.J.; Jiménez-Toribio, R.; Castilla-Espino, D. Price volatility transmission in the value chain of fresh anchovies in Spain. *Fish. Res.* **2023**, *260*, 106606. [[CrossRef](#)]
8. Raybaud, V.; Bacha, M.; Amara, R.; Beaugrand, G. Forecasting climate-driven changes in the geographical range of the European anchovy (*Engraulis encrasicolus*). *ICES J. Mar. Sci.* **2017**, *74*, 1288–1299. [[CrossRef](#)]
9. FAO. *The State of Mediterranean and Black Sea Fisheries 2025*; General Fisheries Commission for the Mediterranean; FAO: Rome, Italy, 2025. [[CrossRef](#)]
10. Giannoulaki, M.; Ibaibarriaga, L.; Antonakakis, K.; Uriarte, A.; Machias, A.; Somarakis, S.; Sanchez, S.; Roel, B.A. Applying a two-stage Bayesian dynamic model to a short lived species, the anchovy in the Aegean Sea (Eastern Mediterranean). Comparison with an Integrated Catch at Age stock assessment model. *Mediterr. Mar. Sci.* **2014**, *15*, 350–365. [[CrossRef](#)]
11. Viñas, J.; Sanz, N.; Peñarrubia, L.; Araguas, R.M.; García-Marín, J.L.; Roldán, M.I.; Pla, C. Genetic population structure of European anchovy in the Mediterranean Sea and the Northeast Atlantic Ocean using sequence analysis of the mitochondrial DNA control region. *ICES J. Mar. Sci.* **2014**, *71*, 391–397. [[CrossRef](#)]
12. Alexandridis, D.; Manousaki, T.; Antoniou, A.; Kristoffersen, J.; Apostolidis, C.; Cannas, R.; Spedicato, M.T.; Cariani, A.; Bellido, J.M.; Magoulas, A.; et al. Uncovering the Genetic Structure of European Anchovy Populations in Central and Western Mediterranean. *Ecol. Evol.* **2025**, *15*, e72441. [[CrossRef](#)] [[PubMed](#)]

13. Bouchenak-Khelladi, Y.; Durand, J.D.; Magoulas, A.; Borsa, P. Geographic structure of European anchovy: A nuclear-DNA study. *J. Sea Res.* **2008**, *59*, 269–278. [\[CrossRef\]](#)
14. Ruzicka, J.; Chiaverano, L.; Coll, M.; Garrido, S.; Tam, J.; Murase, H.; Robinson, K.; Romagnoni, G.; Shannon, L.; Silva, A.; et al. The role of small pelagic fish in diverse ecosystems: Knowledge gleaned from food-web models. *Mar. Ecol. Prog. Ser.* **2024**, *741*, 7–27. [\[CrossRef\]](#)
15. Innes, J.K.; Calder, P.C. Marine Omega-3 (N-3) Fatty Acids for Cardiovascular Health: An Update for 2020. *Int. J. Mol. Sci.* **2020**, *21*, 1362. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Dambrosio, A.; Quaglia, N.C.; Colonna, M.A.; Capuozzo, F.; Giannico, F.; Tarricone, S.; Caputi Jambrenghi, A.; Ragni, M. Shelf-Life and Quality of Anchovies (*Engraulis encrasicolus*) Refrigerated Using Different Packaging Materials. *Fishes* **2023**, *8*, 268. [\[CrossRef\]](#)
17. Available online: <https://www.interregeurope.eu/good-practices/responsible-fishing#evidence-of-success> (accessed on 23 July 2026).
18. Chaouqy, N.E.; Gallardo, J.M.; El Marrakchi, A.; Aubourg, S.P. Lipid damage development in anchovy (*Engraulis encrasicolus*) muscle during storage under refrigerated conditions. *Grasas Aceites* **2008**, *59*, 309–315. [\[CrossRef\]](#)
19. Pons-Sánchez-Cascado, S.; Vidal-Carou, M.C.; Nunes, M.L.; Veciana-Nogués, M.T. Sensory analysis to assess the freshness of Mediterranean anchovies (*Engraulis encrasicolus*) stored in ice. *Food Control* **2006**, *17*, 564–569. [\[CrossRef\]](#)
20. Ferreri, R.; Genovese, S.; Barra, M.; Biagiotti, I.; Bourdeix, J.; De Felice, A.; Gašparević, D.; Hattab, T.; Iglesias, M.; Juretić, T.; et al. Variability in size at maturity of the European anchovy (*Engraulis encrasicolus*) in the Mediterranean Sea. *Mediterr. Mar. Sci.* **2021**, *22*, 858–870. [\[CrossRef\]](#)
21. Albo-Puigserver, M.; Pennino, M.G.; Bellido, J.M.; Colmenero, A.I.; Giráldez, A.; Hidalgo, M.; Gabriel Ramírez, J.; Steenbeek, J.; Torres, P.; Cousido-Rocha, M.; et al. Changes in Life History Traits of Small Pelagic Fish in the Western Mediterranean Sea. *Front. Mar. Sci.* **2021**, *8*, 570354. [\[CrossRef\]](#)
22. Kutsyn, D. Ecological patterns of variability in the life history of the European anchovy (*Engraulis encrasicolus*). *J. Fish Biol.* **2025**, *108*, 1284–1299. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Massa, A.E.; Manca, E.; Yeannes, M.I. Development of quality index method for anchovy (*Engraulis anchoita*) stored in ice: Assessment of its shelf-life by chemical and sensory methods. *Food Sci. Technol. Int.* **2012**, *18*, 339–351. [\[CrossRef\]](#) [\[PubMed\]](#)
24. AOAC International. *Official Methods of Analysis of AOAC International*, 18th ed.; Methods 981.10, 922.06, 923.03, and 925.10; Association of Official Analytical Chemists: Washington, DC, USA, 2005.
25. Parlapani, F.F.; Michailidou, S.; Pasentsis, K.; Argiriou, A.; Krey, G.; Boziaris, I.S. A meta-barcoding approach to assess and compare the storage temperature-dependent bacterial diversity of gilt-head sea bream (*Sparus aurata*) originating from fish farms from two geographically distinct areas of Greece. *Int. J. Food Microbiol.* **2018**, *278*, 36–43. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Kyritsi, M.; Tsourekis, A.; Koukaras, K.; Kamidis, N.; Krey, G.; Michailidou, S.; Argiriou, A. Seasonal Dynamics of Marine Bacterial Communities in Aquaculture Farms: The Case of the Northern Ionian Coastal Ecosystem (Mediterranean Sea). *J. Mar. Sci. Eng.* **2023**, *11*, 1332. [\[CrossRef\]](#)
27. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Bokulich, N.A.; Kaehler, B.D.; Rideout, J.R.; Dillon, M.; Bolyen, E.; Knight, R.; Huttley, G.; Gregory Caporaso, J. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **2018**, *6*, 90. [\[CrossRef\]](#) [\[PubMed\]](#)
30. R Core Team. *R: A Language and Environment for Statistical Computing*, Version 4.4; R Core Team: Vienna, Austria, 2024. Available online: <https://cran.r-project.org>. (accessed on 7 August 2024).
31. McMurdie, P.J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Wickham, H. *ggplot2 Elegant Graphics for Data Analysis*, 2nd ed.; Springer: New York, NY, USA, 2009.
33. Andersen, K.S.; Kirkegaard, R.H.; Karst, S.M.; Albertsen, M. ampvis2: An R package to analyse and visualise 16S rRNA amplicon data. *bioRxiv* **2018**. [\[CrossRef\]](#)
34. Kandlikar, G.S.; Gold, Z.J.; Cowen, M.C.; Meyer, R.S.; Frise, A.C.; Kraft, N.J.B.; Moberg-Parker, J.; Sprague, J.; Kushner, D.J.; Curd, E.E. ranacapa: An R package and Shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. *F1000Research* **2018**, *7*, 1734. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Özogul, F.; Taylor, K.D.A.; Quantick, P.; Özogul, Y. Biogenic amines formation in Atlantic herring (*Clupea harengus*) stored under modified atmosphere packaging using a rapid HPLC method. *Int. J. Food Sci. Technol.* **2002**, *37*, 515–522. [\[CrossRef\]](#)
36. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [\[CrossRef\]](#) [\[PubMed\]](#)

37. Folch, J.; Lees, M.; Sloane-Stanley, G.M. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [\[CrossRef\]](#)
38. Falch, E.; Rustad, T.; Jonsdottir, R.; Shaw, N.B.; Dumay, J.; Berge, J.P.; Arason, S.; Kerry, J.P.; Sandbakk, M.; Aursand, M. Geographical and seasonal differences in lipid composition and relative weight of by-products from gadiform species. *J. Food Compos. Anal.* **2006**, *19*, 727–736. [\[CrossRef\]](#)
39. Boukouvala, E.; Cariani, A.; Maes, G.E.; Sevilla, R.E.; Verrez-Bagnis, V.; Jérôme, M.; Guarniero, I.; Monios, G.; Tinti, F.; Volckaert, F.A.M.; et al. Restriction Fragment Length Analysis of the Cytochrome b Gene and Muscle Fatty Acid Composition Differentiate the Cryptic Flatfish Species *Solea solea* and *Solea aegyptiaca*. *J. Agric. Food Chem.* **2012**, *60*, 7941–7948. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Junaidi, A. Lipid Homeostasis in Farmed Fish: Role of Peroxisome-Proliferator Activated Receptor-Gamma (PPAR γ). Ph.D. Thesis, University of Stirling, Stirling, UK, 2019.
41. Laillet, B.; Viau, M.; Ta, H.P. Analysis of Neutral Lipids, Phospholipids, Glycolipids, and Total Lipids by High-Performance Thin Layer Chromatography. In *Multidimensional Characterization of Dietary Lipids*; Lopez, C., Genot, C., Riaublanc, A., Eds.; Methods and Protocols in Food Science; Humana: New York, NY, USA, 2024. [\[CrossRef\]](#)
42. Laggai, S.; Simon, Y.; Ransweiler, T.; Kiemer, A.K.; Kessler, S.M. Rapid chromatographic method to decipher distinct alterations in lipid classes in NAFLD/NASH. *World J. Hepatol.* **2013**, *5*, 558–567. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Deranieh, R.M.; Joshi, A.S.; Greenberg, M.L. Thin-layer chromatography of phospholipids. In *Membrane Biogenesis*; Rapaport, D., Herrmann, J., Eds.; Methods in Molecular Biology; Humana Press: Totowa, NJ, USA, 2013; Volume 1033. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Heden, T.D.; Neufer, P.D.; Funai, K. Looking Beyond Structure: Membrane Phospholipids of Skeletal Muscle Mitochondria. *Trends Endocrinol. Metab.* **2016**, *27*, 553–562. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Jamovi, Version 2.6. The Jamovi Project. Jamovi: Sydney, Australia, 2024. Available online: <https://www.jamovi.org> (accessed on 23 July 2026).
46. Anagnostopoulos, D.A.; Parlapani, F.F.; Boziaris, I.S. The Evolution of Knowledge on Seafood Spoilage Microbiota from the 20th to the 21st Century: Have We Finished or Just Begun? *Trends Food Sci. Technol.* **2022**, *120*, 236–247. [\[CrossRef\]](#)
47. Joffraud, J.-J.; Cardinal, M.; Cornet, J.; Chasles, J.-S.; Léon, S.; Gigout, F.; Leroi, F. Effect of Bacterial Interactions on the Spoilage of Cold-Smoked Salmon. *Int. J. Food Microbiol.* **2006**, *112*, 51–61. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Antunes-Rohling, A.; Calero, S.; Halaihel, N.; Marquina, P.; Raso, J.; Calanche, J.; Beltrán, J.A.; Álvarez, I.; Cebrián, G. Characterization of the Spoilage Microbiota of Hake Fillets Packaged Under a Modified Atmosphere (MAP) Rich in CO₂ (50% CO₂/50% N₂) and Stored at Different Temperatures. *Foods* **2019**, *8*, 489. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Gram, L.; Dalgaard, P. Fish Spoilage Bacteria—Problems and Solutions. *Curr. Opin. Biotechnol.* **2002**, *13*, 262–266. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Jammal, A.; Bariche, M.; Zu Dohna, H.; Kambris, Z. Characterization of the Cultivable Gut Microflora in Wild-Caught Mediterranean Fish Species. *Curr. Nutr. Food Sci.* **2017**, *13*, 147–154. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Toldrá, F.; Nollet, L.M.L. (Eds.) *Handbook of Seafood and Seafood Products Analysis*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2024. [\[CrossRef\]](#)
52. Wang, M.; Kong, Q.; Yu, H.; Jiang, M.; Han, Z. Identification of Dominant Spoilage Bacteria, Bacterial Diversity Analysis, and Antibacterial Exploration of Chilled Monkfish. *Food Biosci.* **2023**, *56*, 103121. [\[CrossRef\]](#)
53. Odeyemi, O.A.; Burke, C.M.; Bolch, C.C.J.; Stanley, R. Seafood Spoilage Microbiota and Associated Volatile Organic Compounds at Different Storage Temperatures and Packaging Conditions. *Int. J. Food Microbiol.* **2018**, *280*, 87–99. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Rosado, D.; Canada, P.; Silva, S.M.; Ribeiro, N.; Diniz, P.; Xavier, R. Disruption of the Skin, Gill, and Gut Mucosae Microbiome of Gilthead Seabream Fingerlings after Bacterial Infection and Antibiotic Treatment. *FEMS Microbes* **2023**, *4*, xtad011. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Agnew, W.; Barnes, A.C. *Streptococcus iniae*: An Aquatic Pathogen of Global Veterinary Significance and a Challenging Candidate for Reliable Vaccination. *Vet. Microbiol.* **2007**, *122*, 1–15. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Gadoin, E.; Desnues, C.; Bouvier, T.; Roque D'orbecastel, E.; Auguet, J.-C.; Crochemore, S.; Adingra, A.; Bettarel, Y. Tracking Spoilage Bacteria in the Tuna Microbiome. *FEMS Microbiol. Ecol.* **2022**, *98*, fiac110. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Zhong, H.; Wei, S.; Kang, M.; Sun, Q.; Xia, Q.; Wang, Z.; Han, Z.; Liu, Y.; Liu, M.; Liu, S. Effects of Different Storage Conditions on Microbial Community and Quality Changes of Greater Amberjack (*Seriola dumerili*) Fillets. *LWT* **2023**, *179*, 114640. [\[CrossRef\]](#)
58. Wu, J.; Wei, G.; Jin, R.; Lin, R.; Weng, W.; Liang, D.; Qiu, X.; Yang, S. Assessment of *Bacillus cereus* Spoilage Potential and the Antibacterial Mechanism of *Larimichthys crocea*-Derived Peptide SDH73 against *Bacillus cereus*. *Int. J. Food Microbiol.* **2025**, *439*, 111235. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Corral-Jara, K.F.; Skírnisdóttir, S.; Knobloch, S.; Briem, H.; Cobo-Díaz, J.F.; Carlino, N.; Bergsten, P.; Armanini, F.; Asnicar, F.; Pinto, F.; et al. Inter-Facility Characterization of Bacteria in Seafood Processing Plants: Exploring Potential Reservoirs of Spoilage Organisms and the Resistome. *Heliyon* **2024**, *10*, e33866. [\[CrossRef\]](#) [\[PubMed\]](#)
60. Feldhusen, F. The Role of Seafood in Bacterial Foodborne Diseases. *Microbes Infect.* **2000**, *2*, 1651–1660. [\[CrossRef\]](#) [\[PubMed\]](#)

61. Jia, S.; Li, Y.; Zhuang, S.; Sun, X.; Zhang, L.; Shi, J.; Hong, H.; Luo, Y. Biochemical Changes Induced by Dominant Bacteria in Chill-Stored Silver Carp (*Hypophthalmichthys molitrix*) and GC-IMS Identification of Volatile Organic Compounds. *Food Microbiol.* **2019**, *84*, 103248. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Parlapani, F.F.; Meziti, A.; Kormas, K.A.; Bozariis, I.S. Indigenous and Spoilage Microbiota of Farmed Sea Bream Stored in Ice Identified by Phenotypic and 16S rRNA Gene Analysis. *Food Microbiol.* **2013**, *33*, 85–89. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Waters, C.M.; Bassler, B.L. The *Vibrio harveyi* Quorum-Sensing System Uses Shared Regulatory Components to Discriminate between Multiple Autoinducers. *Genes Dev.* **2006**, *20*, 2754–2767. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Syropoulou, F.; Parlapani, F.F.; Kakasis, S.; Nychas, G.-J.E.; Bozariis, I.S. Primary Processing and Storage Affect the Dominant Microbiota of Fresh and Chill-Stored Sea Bass Products. *Foods* **2021**, *10*, 671. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Sequino, G.; Valentino, V.; Esposito, A.; Volpe, S.; Torrieri, E.; De Filippis, F.; Ercolini, D. Microbiome dynamics, antibiotic resistance, and spoilage-associated genomic potential in fresh anchovies stored in different conditions. *Food Res. Int.* **2024**, *175*, 113788. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Matuszewska, M.; Dabrowska, A.; Murray, G.G.R.; Kett, S.M.; Vick, A.J.A.; Banister, S.C.; Pantoja Munoz, L.; Cunningham, P.; Welch, J.J.; Holmes, M.A.; et al. Absence of *Staphylococcus aureus* in Wild Populations of Fish Supports a Spillover Hypothesis. *Microbiol. Spectr.* **2023**, *11*, e04858-22. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Iwamoto, M.; Ayers, T.; Mahon, B.E.; Swerdlow, D.L. Epidemiology of Seafood-Associated Infections in the United States. *Clin. Microbiol. Rev.* **2010**, *23*, 399–411. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Wang, T.; Li, J.; Jing, H.; Qin, S. Picocyanobacterial *Synechococcus* in marine ecosystem: Insights from genetic diversity, global distribution, and potential function. *Mar. Env. Res.* **2022**, *177*, 105622. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Hu, X.; Su, H.; Zhang, P.; Chen, Z.; Xu, Y.; Xu, W.; Li, J.; Wen, G.; Cao, Y. Microbial Community Structure and Metabolic Characteristics of Intestine and Gills of Dwarf-Form Populations of *Sthenoteuthis oualaniensis* in South China Sea. *Fishes* **2022**, *7*, 191. [\[CrossRef\]](#)
70. Lubis, Z.; Buckle, K.A. Rancidity and lipid oxidation of dried-salted sardines. *Intern. J. Food Sci. Technol.* **1990**, *25*, 295–303. [\[CrossRef\]](#)
71. Pethybridge, H.; Bodin, N.; Arsenaault-Pernet, E.J.; Bourdeix, J.H.; Brisset, B.; Bigot, J.L.; Roos, D.; Peter, M. Temporal and inter-specific variations in forage fish feeding conditions in the NW Mediterranean: Lipid content and fatty acid compositional changes. *Mar. Ecol. Prog. Ser.* **2014**, *512*, 39–54. [\[CrossRef\]](#)
72. Sanfilippo, M.; Reale, A.; Ziino, M.; Romeo, V.; Lembo, E.; Manganaro, A. Chemical Composition and Nutritional Value of *Engraulis encrasicolus* (Linnaeus, 1758) Caught by Driftnet “Menaide” along Sicilian Coast: A Natural Food for Mediterranean Diet. *World J. Fish Mar. Sci.* **2011**, *3*, 44–50.
73. Roncarati, A.; Brambilla, G.; Meluzzi, A.; Iamiceli, A.L.; Fanelli, R.; Moret, I.; Ubaldi, A.; Miniero, R.; Sirri, F.; Melotti, P.; et al. Fatty acid profile and proximate composition of fillets from *Engraulis encrasicolus*, *Mullus barbatus*, *Merluccius merluccius* and *Sarda sarda* caught in Tyrrhenian, Adriatic and Ionian seas. *J. Appl. Ichthyol.* **2012**, *28*, 545–552. [\[CrossRef\]](#)
74. Šimat, V.; Bogdanović, T. Seasonal changes in proximate composition of anchovy (*Engraulis encrasicolus*, L.) from the central Adriatic. *Acta Adriat.* **2012**, *53*, 125–132.
75. Polak, T.; Žlender, B.; Glavič, A.; Skvarča, M.; Lušnic Polak, M.; Demšar, L. Physico-chemical Composition and Sensory Quality of Anchovies (*Engraulis encrasicolus*) from the Northern Adriatic. In Proceedings of the 59th International Congress of Meat Science and Technology, Izmir, Turkey, 18–23 August 2013.
76. Kocatepe, D.; Mehmet, E.; İrfan, K.; Bayram, K.; Yalçın, K. Differences on lipid quality index and amino acid profiles of European Anchovy caught from different area in Turkey. *Ukr. J. Food Sci.* **2019**, *7*, 6–15. [\[CrossRef\]](#)
77. Spitz, J.; Mouroucq, E.; Schoen, V.; Ridoux, V. Proximate composition and energy content of forage species from the Bay of Biscay: High- or low-quality food? *ICES J. Mar. Sci.* **2010**, *67*, 909–915. [\[CrossRef\]](#)
78. Kaya, Y.; Turan, H. Comparison of Protein, Lipid and Fatty Acids Composition of Anchovy (*Engraulis encrasicolus* L. 1758) During the Commercial Catching Season. *J. Muscle Foods* **2010**, *21*, 474–483. [\[CrossRef\]](#)
79. Karşı, B.; Öğretmen, Ö.Y.; Emanet, M. Proximate composition, fatty acid characteristics, and amino acid profile of European anchovy (*Engraulis encrasicolus* L. 1758): Monthly and seasonal influences. *Front. Nutr.* **2025**, *12*, 1684958. [\[CrossRef\]](#) [\[PubMed\]](#)
80. Poulos, S.E. Water Masses of the Mediterranean Sea and Black Sea: An Overview. *Water* **2023**, *15*, 3194. [\[CrossRef\]](#)
81. Gatti, P.; Cominassi, L.; Duhamel, E.; Grellier, P.; Le Delliou, H.; Le Mestre, S.; Petitgas, P.; Rabiller, M.; Spitz, J.; Huret, M. Bioenergetic Condition of Anchovy and Sardine in the Bay of Biscay and English Channel. *Prog. Oceanogr.* **2018**, *166*, 129–138. [\[CrossRef\]](#)
82. Campanini, C.; Albo-Puigserver, M.; Gérez, S.; Lloret-Lloret, E.; Giménez, J.; Pennino, M.G.; Bellido, J.M.; Colmenero, A.I.; Coll, M. Energy content of anchovy and sardine using surrogate calorimetry methods. *Mar. Environ. Res.* **2021**, *172*, 105510. [\[CrossRef\]](#) [\[PubMed\]](#)

83. Schismenou, E.; Chatzifotis, S.; Tsiaras, K.; Somarakis, S. Anchovy and sardine condition and energy content in the North Aegean Sea (eastern Mediterranean) in relation to their contrasting reproductive strategies. *J. Fish Biol.* **2024**, *105*, 1178–1188. [[CrossRef](#)] [[PubMed](#)]
84. International Commission on Microbiological Specifications for Foods (ICMSF). *Microorganisms in Foods 2: Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd ed.; Blackwell Scientific: Oxford, UK, 1986; p. 190.
85. Duarte, A.M.; Silva, F.; Pinto, F.R.; Barroso, S.; Gil, M.M. Quality Assessment of Chilled and Frozen Fish—Mini Review. *Foods* **2020**, *9*, 1739. [[CrossRef](#)] [[PubMed](#)]
86. Esteves, E.; Anibal, J. Sensory evaluation of seafood freshness using the quality index method: A meta-analysis. *Int. J. Food Microbiol.* **2021**, *337*, 108934. [[CrossRef](#)] [[PubMed](#)]
87. Bono, G.; Okpala, C.O.R.; Vitale, S.; Ferranteli, V.; Di Noto, A.; Costa, A.; Di Bella, C.; Lo Monaco, D. Effects of different ozonized slurry-ice treatments and superchilling storage (−1 °C) on microbial spoilage of two important pelagic fish species. *Food Sci. Nutr.* **2017**, *5*, 1049–1056. [[CrossRef](#)] [[PubMed](#)]
88. Tryphinou, P.; Drosinos, E.H.; Nychas, G.J.E. Performance of Pseudomonas CFC-selective medium in the fish storage ecosystems. *J. Microbiol. Methods* **2001**, *47*, 243–247. [[CrossRef](#)] [[PubMed](#)]
89. Broekaert, K.; Heyndrickx, M.; Herman, L.; Devlieghere, F.; Vlaemynck, G. Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several general growth media. *Food Microbiol.* **2011**, *28*, 1162–1169. [[CrossRef](#)] [[PubMed](#)]
90. Gloor, G.B.; Macklaim, J.M.; Pawlowsky-Glahn, V.; Egozcue, J.J. Microbiome Datasets Are Compositional: And This Is Not Optional. *Front Microbiol.* **2017**, *8*, 2224. [[CrossRef](#)] [[PubMed](#)]
91. Moi, I.M.; Roslan, N.N.; Leow, A.T.C.; Ali, M.S.M.; Rahman, R.N.Z.R.A.; Rahimpur, A.; Sabri, S. The biology and the importance of Photobacterium species. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 4371–4385. [[CrossRef](#)] [[PubMed](#)]
92. Farmer, J.J.; Hickman-Brenner, F.W. The Genera Vibrio and Photobacterium. In *The Prokaryotes*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E., Eds.; Springer: New York, NY, USA, 2006; pp. 508–563. [[CrossRef](#)] [[PubMed](#)]
93. Saito, E.; Yoshida, N.; Kawano, J.; Shimizu, A.; Igimi, S. Isolation of Staphylococcus aureus from raw fish in relation to culture methods. *J. Vet. Med. Sci.* **2011**, *73*, 287–292. [[CrossRef](#)] [[PubMed](#)]
94. Friedland, K.D.; Ahrenholz, D.W.; Haas, L.W. Viable gut passage of cyanobacteria through the filter-feeding fish Atlantic menhaden, *Brevoortia tyrannus*. *J. Plankton Res.* **2005**, *27*, 715–718. [[CrossRef](#)]
95. Toxqui-Rodríguez, S.; Riera-Ferrer, E.; Del Pozo, R.; Palenzuela, O.; Sitjà-Bobadilla, A.; Estensoro, I.; Piazzon, M.C. Molecular interactions in an holobiont-pathogen model: Integromics in gilthead seabream infected with *Sparicotyle chrysophrii*. *Aquaculture* **2024**, *581*, 740365. [[CrossRef](#)]
96. Ding, T.; Li, Y. Biogenic amines are important indices for characterizing the freshness and hygienic quality of aquatic products: A review. *LWT* **2024**, *194*, 115793. [[CrossRef](#)]
97. Visciano, P.; Schirone, M.; Tofalo, R.; Suzzi, G. Biogenic Amines in Raw and Processed Seafood. *Front. Microbiol.* **2012**, *3*, 188. [[CrossRef](#)] [[PubMed](#)]
98. Arulkumar, A.; Paramithiotis, S.; Paramasivam, S. Biogenic amines in fresh fish and fishery products and emerging control. *Aquac. Fish* **2023**, *8*, 431–450. [[CrossRef](#)]
99. The European Commission. Regulation 1019/2013/EU, Commission Regulation EU No 1019/2013. Amending annex I to regulation EC No 2073/2005 as regards histamine in fishery products. *Off. J. Eur.* **2013**, *282*, 46–47.
100. Rossano, R.; Mastrangelo, L.; Ungaro, N.; Riccio, P. Influence of storage temperature and freezing time on histamine level in the European anchovy *Engraulis encrasicolus* (L., 1758): A study by capillary electrophoresis. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2006**, *830*, 161–164. [[CrossRef](#)] [[PubMed](#)]
101. Mietz, J.L.; Karmas, E. Chemical Quality Index of Canned Tuna as Determined by High-Pressure Liquid Chromatography. *J. Food Sci.* **1977**, *42*, 155–158. [[CrossRef](#)]
102. Baixas-Nogueras, S.; Bover-Cid, S.; Veciana-Nogués, M.T.; Mariné-Font, A.; Vidal-Carou, M.C. Biogenic Amine Index for Freshness Evaluation in Iced Mediterranean Hake (*Merluccius merluccius*). *J. Food Prot.* **2005**, *68*, 2433–2438. [[CrossRef](#)] [[PubMed](#)]
103. Jiao, X.; Li, X.; Zhang, N.; Zhang, W.; Yan, B.; Huang, J.; Zhao, J.; Zhang, H.; Chen, W.; Fan, D. Postmortem Muscle Proteome Characteristics of Silver Carp (*Hypophthalmichthys molitrix*): Insights from Full-Length Transcriptome and Deep 4D Label-Free Proteomic. *J. Agric. Food Chem.* **2024**, *72*, 1376–1390. [[CrossRef](#)] [[PubMed](#)]
104. Geary, B.; Young, I.S.; Cash, P.; Whitfield, P.D.; Doherty, M.K. Comparing Simplification Strategies for the Skeletal Muscle Proteome. *Proteomes* **2016**, *4*, 10. [[CrossRef](#)] [[PubMed](#)]
105. Verrez-Bagnis, V.; Ladrat, C.; Morzel, M.; Noël, J.; Fleurence, J. Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. *Electrophoresis* **2001**, *22*, 1539–1544.
106. Kjærsgård, I.V.H.; Flemming, J. Proteome Analysis Elucidating Post-mortem Changes in Cod (*Gadus morhua*) Muscle Proteins. *J. Agric. Food Chem.* **2003**, *51*, 3985–3991. [[CrossRef](#)] [[PubMed](#)]
107. Ahmed, Z.; Donkor, O.; Street, W.A.; Vasiljevic, T. Calpains- and cathepsins-induced myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive peptides. *Trends Food Sci. Technol.* **2015**, *45*, 130–146. [[CrossRef](#)]

108. Taylor, J.S.; Braasch, I.; Frickey, T.; Meyer, A.; Van de Peer, Y. Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome Res.* **2003**, *13*, 382–390. [[CrossRef](#)] [[PubMed](#)]
109. Chen, L.; Pan, Y.; Cheng, J.; Zhu, X.; Chu, W.; Meng, Y.Y.; Bin, S.; Zhang, J. Characterization of myosin heavy chain (MYH) genes and their differential expression in white and red muscles of Chinese perch, *Siniperca chuatsi*. *Int. J. Biol. Macromol.* **2023**, *250*, 125907. [[CrossRef](#)] [[PubMed](#)]
110. Tavares, J.; Martins, A.; Fidalgo, L.G.; Lima, V.; Amaral, R.A.; Pinto, C.A.; Silva, A.M.; Saraiva, J.A. Fresh Fish Degradation and Advances in Preservation Using Physical Emerging Technologies. *Foods* **2021**, *10*, 780. [[CrossRef](#)] [[PubMed](#)]
111. Soriguer, F.; Serna, S.; Valverde, E.; Hernando, J.; Martín-Reyes, A.; Soriguer, M.; Pareja, A.; Tinahones, F.; Esteve, I. Lipid, protein, and calorie content of different Atlantic and Mediterranean fish, shellfish, and molluscs commonly eaten in the south of Spain. *Eur. J. Epidemiol.* **1997**, *13*, 451–463. [[CrossRef](#)] [[PubMed](#)]
112. Zlatanov, S.; Laskaridis, K. Seasonal variation in the fatty acid composition of three Mediterranean fish—Sardine (*Sardina pilchardus*), anchovy (*Engraulis encrasicolus*) and picarel (*Spicara smaris*). *Food Chem.* **2007**, *103*, 725–728. [[CrossRef](#)]
113. Tsopelakos, A.; Miliou, H. Lipid and Fatty Acid Composition of Low-Value Mediterranean Fish in Winter and Spring for Discard Valorization. *Fishes* **2025**, *10*, 454. [[CrossRef](#)]
114. Boselli, E.; Pacetti, D.; Lucci, P.; Frega, N. Characterization of Phospholipid Molecular Species in the Edible Parts of Bony Fish and Shellfish. *J. Agric. Food Chem.* **2012**, *60*, 3234–3245. [[CrossRef](#)] [[PubMed](#)]
115. Cho, Y.; Kim, B.H.; Kim, Y.; Kim, I.H. Concentration of docosahexaenoic acid from tuna oil via a two lipase-catalyzed reaction. *Food Chem.* **2024**, *458*, 140253. [[CrossRef](#)] [[PubMed](#)]
116. Martin, D.; Nieto-Fuentes, J.A.; Señoráns, F.J.; Reglero, G.; Soler-Rivas, C. Intestinal digestion of fish oils and ω -3 concentrates under in vitro conditions. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 1315–1322. [[CrossRef](#)]
117. Lyberg, A.-M.; Adlercreutz, P. Lipase specificity towards eicosapentaenoic acid and docosahexaenoic acid depends on substrate structure. *Biochim. Biophys. Acta—Proteins Proteom.* **2008**, *1784*, 343–350. [[CrossRef](#)] [[PubMed](#)]
118. Murawska, G.M.; Armando, A.M.; Dennis, E.A. Lipidomics of phospholipase A2 reveals exquisite specificity in macrophages. *J. Lipid Res.* **2024**, *65*, 100571. [[CrossRef](#)] [[PubMed](#)]

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