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Seasonal Pattern of the Effect of Slurry Ice during Catching and Transportation on Quality and Shelf Life of Gilthead Sea Bream

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Abstract: The objective of the present study was the evaluation of the effect of slurry ice, as an alternative cooling medium during harvesting and transportation, on the quality parameters (e.g., microbiological stability, sensory attributes, physicochemical changes) and shelf life of fish. The effect of seasonal variability of seawater temperature on fish preservation using the tested cooling media was also investigated. Gilthead sea bream (*Sparus aurata*) was slaughtered and transported in different mixtures of conventional flake ice and slurry ice for 24 h. Three mixtures of ice were tested as T: slaughtered in flake ice and transported in flake ice (control), TC: slaughtered in slurry ice and transported in flake ice, T50: slaughtered and transported in slurry ice 50%–flake ice 50%. Samples were subsequently stored isothermally at 0 °C for shelf-life evaluation. Three independent experiments were performed at three different periods, i.e., January, April, and September, referring to a sea water temperature range of 13.3–26.8 °C. Higher sea water temperatures at catching led to lower microbial growth rates and proteolytic enzyme activities and longer shelf life of refrigerated whole fish. The partial replacement of conventional flake ice with slurry ice improved the quality and extended the shelf life of fish at 0 °C by 2–7 days. The results of the study support that the use of slurry ice may enable better quality maintenance and significant shelf-life extension of whole gilthead sea bream.

Keywords: gilthead sea bream; slurry ice; slaughtering; transportation; flesh quality; proteolytic enzymes; shelf life

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1. Introduction

Aquatic products deteriorate very quickly after slaughtering as a result of complex microbiological, enzymatic, and biochemical mechanisms. Quality and freshness loss postmortem is attributed to structural and biochemical changes (activation of fish proteases) accompanied by microbial spoilage [1]. Enzymatic cleavage of fish proteins in the extracellular matrix connecting the myofibrils leads to postmortem reduction in the connective collagen tissue of the muscle [2] and muscle fibrillar proteins, causing muscle softening and gaping in the myocommata [3]. The integrity of these proteins is of great importance for fish flesh quality, as texture deterioration begins with the destruction of the intramuscular and Z-discs and the consequent collapse of the muscle fibril [4]. Proteases responsible for the deterioration of the myotomia can derive from both the muscle tissue and the digestive system if the latter is not removed prior to storage [2]. Key roles are held by collagenases,

which hydrolyze the collagen of the connective tissue, and cathepsins and calpains, which proteolyze muscle fibril proteins.

Spoilage begins immediately postmortem depending on both preservation methods and environmental parameters [5]. Spoilage can be a result of enzymatic, oxidative and microbial processes. Onboard storage conditions affect fish quality and product acceptability and shelf life [6]. Fish should be cooled immediately after catch and kept on ice to prevent quality loss, ensure safety, and decrease postharvest losses [7].

An alternative methodology for catching, transportation, and storage of aquatic products is ice slurry cooling. Slurry ice is a mixture of a carrier liquid, either pure freshwater or a binary solution of water and a freezing point depressant (the most commonly used in industry are sodium chloride, propylene glycol, and ethylene glycol), and small ice particles [8]. It presents certain advantages compared with conventional flake ice, such as better flow ability, faster rates of chilling, limited damage on the surface of fish, the prevention of dehydration via higher exchange of heat, microscopic ice crystals, and full surface coverage [9]. Additionally, the use of slurry ice can lead to an increase in the contact area, up to full coverage of the surface, leading to the surface's protection from oxygen [10]. Slurry ice is effective because ice crystals of micron size are combined with the large heat capacity of ice, and therefore, slurry ice maintains a biphasic system for a longer period than conventional flake ice [11–13]. Studies have reported the beneficial effects of slurry ice application on quality retainment of fish products (e.g., European sea bass, hake, herring, sardine, shrimp) [14–18]. Annamalai et al. concluded that both the biochemical and microbial activity of yellow croaker fish was delayed when slurry ice was used, and therefore, shelf life was extended. Researchers have previously declared that slurry ice presents approximately three times faster precooling speed than flake ice [19]. Additionally, slurry ice can maintain fish at subzero temperatures, from −0.5 to −1.5 °C, whereas flake ice can only reduce storage temperature to around to 0 °C. Therefore, slurry ice has a stronger cooling capacity than flake ice [19,20]. Although the use of slurry ice has remarkable advantages, there are limitations of slurry ice, such as the tendency of the ice to float to the container surface and form hard ice crusts. That said, slurry ice is gel-like in texture and therefore less abrasive. Finally, it is created directly from salt water, thereby negating the need for fishermen to buy their ice. Water temperature at catch is an important factor that may affect the effectiveness of the tested cooling media used for transportation of perishable foods, as well as the microbial flora composition of the fish, which develops spoilage and quality degradation during subsequent refrigerated storage. The seasonal pattern of different fish quality indicators has been reported in the literature [21].

Gilthead sea bream (*Sparus aurata*) is one of the most popular aquatic products in the Mediterranean [18]. Its high commercial value is due to its desirable sensory properties (e.g., appearance, taste, aroma). However, fresh gilthead sea bream is highly susceptible to spoilage during refrigerated storage due to enzymatic and chemical reactions, which are usually responsible for the initial loss of freshness, and microbial activity, with the latter leading to the obvious spoilage and gilthead sea bream's limited shelf life of 14–15 days [22,23]. Therefore, the main concern is increasing its shelf life by novel, minimal processing technologies or packaging systems [24]. The consumption of fresh gilthead sea bream is common in Greece and the European region; 199,476 tn of gilthead sea bream was globally produced in 2019 [25]. However, microbial development, filleting, and sensory degradation during storage must be effectively addressed to allow the exploitation of its full commercial potential. These undesired alterations may be minimized by appropriate temperature control and compliance to storage conditions or by alternative postharvest processes.

The objective of the present study was the evaluation of the environmental temperature (seasonality) and the cooling medium of fish (slurry ice vs. conventional flake ice), during catching and transportation, on fish quality. Key parameters (microbiological/physicochemical indices and sensory characteristics) of fish that determine the shelf life were studied. The impact of ice slurry during catching and transportation on gilthead sea

bream quality, considering different combinations of slurry ice and flake ice, has not yet been evaluated. Huidobro et al. [13] compared the effects of slaughtering in liquid ice and commercial slaughtering in ice on water gilthead sea bream quality. Quality evaluation was based on sensory parameters and physicochemical indices of fish during iced storage, and microbial spoilage was not considered. The preservative effect of slurry ice as an alternative cooling medium for whole gilthead sea bream at catching and transportation might show a seasonal pattern because of the deviations of environmental temperature between the different periods of the year, which need to be systematically investigated. Additionally, it is important that the final temperature of the products cooled with flake ice reaches near 0 °C and that those cooled with slurry ice reach −0.5 to −1.0 °C [13]. Therefore, the present study was focused on fish quality deterioration during subsequent storage at 0 °C. Limited work has been conducted on slurry ice's effects on the quality and shelf life of Mediterranean fish [13,17]. The systematic study and mathematical modeling of the parameters (such as water temperature, catching, processing and transportation conditions) may provide technological solutions for fish handling to enhance the quality and increase the shelf life of fresh fish and reduce food waste.

2. Materials and Methods

2.1. Experimental Design

Gilthead sea bream (200–400 g weight: total of 120 samples) was slaughtered in either ice water or 50% or 100% slurry ice (Philosofish S.A., Larymna Fthiotida, Greece, FAO 37.3.1) and transported to the laboratory in polystyrene containers within 24 h. Slurry ice (of −3.2 ± 0.2 °C temperature) was produced from filtered seawater (35 g/kg salinity) using a semi-industrial scale slurry ice machine (ZIEGRA ice maker, Germany). Three mixtures of slurry ice and conventional flake ice were tested: T—flake ice slaughtered and transported (control samples), TC—slurry ice slaughtered and flake ice transported, T50—in slurry ice 50% (*w*/*w*)–flake ice 50% (*w*/*w*) slaughtered and transported. The weight ratio (*w*/*w*) of fish to ice (slurry or flake) was 1:1. Sampling was performed in three different periods, in September 2020 (26.8 ± 5.2 °C sea water temperature), January 2021 (13.9 ± 4.5 °C), and April 2021 (13.3 ± 4.4 °C), in the same fish farm located in Larymna (Fthiotida, Greece). Whole fish samples (T, T50, TC), immediately after receipt, were aerobically packaged in polyamide/polyethylene side sealed bags unprinted (one whole fish per package) and stored isothermally in controlled incubators at 0 (±0.2) °C for 28 days. The storage temperature was measured with the use of computer-downloadable miniature temperatures loggers (COX TRACER, Belmont, NC, USA). Sampling was conducted to allow quality evaluation of fish and shelf life determination during refrigerated storage. Quality evaluation was based on microbial growth (total microbial count, yeasts and molds, *Brochothrix thermosphacta*, *Pseudomonas* spp., *Enterobacteriaceae* spp., H_2S-producing bacteria), pH, color, total volatile basic nitrogen, proteolytic enzymes, and sensory evaluation. Microbiological and chemical analysis was carried out on fish fillets. Sensory evaluation was carried out on whole fish.

2.2. Measurement Methods

2.2.1. Microbiological Parameters

Total microbial count (TVCs), yeasts and molds, *Brochothrix thermosphacta*, *Pseudomonas* spp., H_2S-producing bacteria, and *Enterobacteriaceae* spp. (expressed as log CFU/g) were enumerated in fish fillet (including skin) according to the spread plate technique detailed in Ntzimani et al. (2021) (n = 2) [17].

2.2.2. Physicochemical Quality Parameters

The pH value of all fish samples (dilution ratio of fish fillet samples to Ringer's solution, 1:10) was measured during storage (pH-meter AMEL 338, Italy) (n = 3).

The analysis of total volatile basic nitrogen (TVB-N) was conducted in a Kjeldahl rapid distillation unit after extraction with trichloacetic acid (30 g of fish flesh in 60 mL TCA 6% v/w) (Büchi 321, Switzerland) [26].

Quantification of color of tested samples was based on measurement on the dorsal part of the body with a color meter (Minolta CR-200, Japan) using the CIE L*, a*, b* color scale (CIE 1978) (n = 3). Color measurement of fish skin was carried out on 5 specimens.

2.2.3. Fatty Acids Analysis

For the determination of fatty acids (FA) in the fish flesh, the raw material was freeze-dried for 48 h and then subjected to fatty acid extraction (n = 2). Three consecutive extraction cycles were carried out, using hexane as solvent (1:20 sample to solvent ratio), in order to recover the total oil content of samples. Then, direct transesterification of FA was conducted based on the method described by Lepage and Roy (1984) with modifications [27]. Ten milligrams of lipids were mixed with 5 mL of methanol–toluene 3:2 (v/v) and 5 mL of acetyl chloride–methanol mixture 1:20 (v/v). After heating at 100 °C for 1 h, the samples were mixed with 5 mL of distilled water and 5 mL of n-hexane, and two different phases appeared after centrifugation at 1006× g (3000 rpm) for 5 min (Thermo Scientific Heraeus Megafuge 16R Thermo Fisher Scientific, Waltham, MA, USA). The methyl esters of fatty acids (FAMEs) were collected from the upper (organic) phase after solvent evaporation in a rotary evaporator (Heidolph G1, Schwabach, Germany) with a vacuum pump and diluted in 2,2,4-trimethylpentane (Sigma–Aldrich, Steinheim, Germany). FA analysis was carried out on an HP 7890 GC system (plus +) coupled to an HP 5975 mass selective detector. The temperature of the HP-5 MS column (30 m × 250 µm, 0.25 µm, Hewlett Packard, Palo Alto, CA, USA) was increased from 125 °C to 240 °C at a constant rate (5 °C min^{-1}) and was then held at the highest temperature for 12 min. The split ratio was set at 50:1. The flow rate of the used carrier gas, which was helium, was set at 1 mL min^{-1}; the inlet temperature was set at 220 °C and split at 20:1. FA identification was based on comparison of retention times and mass spectra (mass range from 40 to 400 m/z) with the NIST and Wiley mass spectral libraries and quantified using a standard mixture of 37 FAMEs (Supelco 37 Component fatty acids methyl esters mix, Sigma–Aldrich, Steinheim, Germany). The results for each FA were calculated as percentages (%) of the total FA.

2.2.4. Proteolytic Enzymes Activity

Calpain, collagenase, cathepsin B, and cathepsin L activities were evaluated in gilthead sea bream collected in September 2020, January 2021, and April 2021 (data not shown). To that end, samples of white muscle, just above the lateral line and close to the skin (~200 mg), were obtained from the fish fillet on catch day (day 0) and days 1, 2, 4, 8, and 15 postharvest. In total, 288 samples were sacrificed (8 samples per day per method per temperature). Samples were snap frozen in liquid nitrogen and kept at a temperature of −80 °C for further examination. Briefly, the sample was minced using a tissue homogenizer (IKA® ULTRA-TURRAX® T25) in appropriate buffer as previously described [17]. Cathepsin L and B, collagenase, and calpain activity (expressed as fluorescence unit (FLU) change per minute per mg protein (ΔF)) were determined using the Barrett and Kirschke method with minor modifications, using Z-phenylalanine-arginine-7-amido-4-methylcoumarin hydrochloride, Suc-Gly-Pro-Leu-Gly-Pro-AMC, and L-methionine-AMC trifluoroacetic salts as substrates. The fluorescence of 7-amino-4-methylcoumarin (AMC) produced by all the aforementioned enzymes was detected using a spectrofluorometer (VarioskanTM LUX multimode microplate reader, Thermofisher) (Em = 460 nm, Ex = 360 nm). The crude extracts' protein content was determined using the Bradford method and a standard of bovine serum albumin [28]. Samples were measured in duplicates.

2.2.5. Sensory Evaluation

The sensory evaluation of raw and cooked fish was conducted by eight trained assessors (ISO 8586-1, 1993) [29,30]. All fish samples were wrapped individually in aluminum

foil, cooked for 40 min at 180 °C, and served after cooling for 1–2 min, as described by Tsironi et al. (2015) [31]. The sensory parameters (appearance, texture and odor of raw and cooked samples, and taste of cooked samples) were evaluated, and sensory scores were recorded in appropriate forms, reflecting the organoleptic evolution of quality deterioration. Additionally, panelists were asked to score the overall impression and acceptability. Rating was assigned separately for each parameter on a 1–9 scale (9 = high quality and 1 = poor quality). A sensory score of 5 was taken as the average score for minimum acceptability [31,32].

2.3. Data Analysis

In order to evaluate the quality changes of chilled fish during storage, the "apparent kinetics" methodological approach was applied. Based on this procedure, a primary kinetic model was used, where values obtained from the different measured quality indices were plotted vs. time. The apparent order of quality loss was estimated based on the least square statistical fit. The bacterial growth was modelled by the Baranyi model, and the experimental data were fitted to curves using the DMfit program (IFR, UK) [33]. The rate (k) and lag phase (λ) of the bacterial growth of all tested microorganisms were calculated.

Enzymatic activity's statistical analysis was conducted with the use of R packages in RStudio. Since our data deviated significantly from the normal distribution (p-value of the Shapiro–Wilk test < 0.05), nonparametric tests were carried out, including the Kruskal–Wallis H Test, the Dunn Test, and a calculation of the Spearman correlation coefficient. Permutational multivariate analysis of variance (PERMANOVA) and principal coordinate analysis (PCoA) were also conducted.

3. Results and Discussion

3.1. Microbial Growth

TVCs, H_2S-producing bacterium, and *Pseudomonas* spp. loads increased during storage, whereas *Brochothrix thermosphacta* and yeasts/molds (<2.00 log CFU g^{-1}) were not detected the first 22 days (data not shown). *Enterobacteriaceae* were initially not detected (<1.00 log CFU g^{-1}) and reached 3.25 log CFU g^{-1} at the end of storage (data not shown), indicating good hygiene of the marine environment and good fishing practices, including farming, catching, and handling. Similar counts of TVCs, H_2S-producing bacteria, *Pseudomonas* spp., and *Enterobacteriaceae* spp. have been reported in the literature [21,34–36].

The microbial loads of TVCs in the different periods (September, January, April) on gilthead sea bream (whole) are depicted in Figure 1a–c. Initial TVCs were approximately 2 log CFU/g for all tested samples. As shown in Figure 1a, samples slaughtered in September had similar TVCs growth curves for the T and T50 samples, whereas total viable counts for the TC samples were lower. For the T/T50 and TC gilthead sea bream samples, TVCs reached 8.74 and 7.32 log CFU/g, respectively (26 days of storage at 0 °C). The inhibitory effect of ice slurry application on TVCs was significant ($p < 0.05$) because of an evident lag phase (λ) in 100% slurry ice (for example for TC samples, the lag phase wa s 4 days, while that for T samples was 1 day, 0 °C). Lower bacterial growth rates of TC (0.24 d^{-1}) samples were also calculated compared with those of T (0.33 d^{-1}) samples.

Counts of *Pseudomonas* spp. remained at the detection limit (2.0 log CFU/g) for approximately 4–5 days for TC samples, while counts increased throughout storage for T and T50 samples, reaching 6 log $CFUg^{-1}$ after 14, 16, and 21 d for the T, T50, and TC samples, respectively (Figure 2a). Similar results concerning the growth of *Pseudomonas* spp. were reported in other studies, showing a similar effect of ice slurry as compared with conventional ice [35]. The *Pseudomonas* spp. growth rates had significantly lower values for TC (0.24 d^{-1}) samples than for T (0.36 d^{-1}) and T50 (0.33 d^{-1}) samples ($p < 0.05$), showing that slurry ice was effective in controlling and in fact slowing the growth of *Pseudomonas* spp. as compared with conventional ice when the sea water temperature was 27 °C.

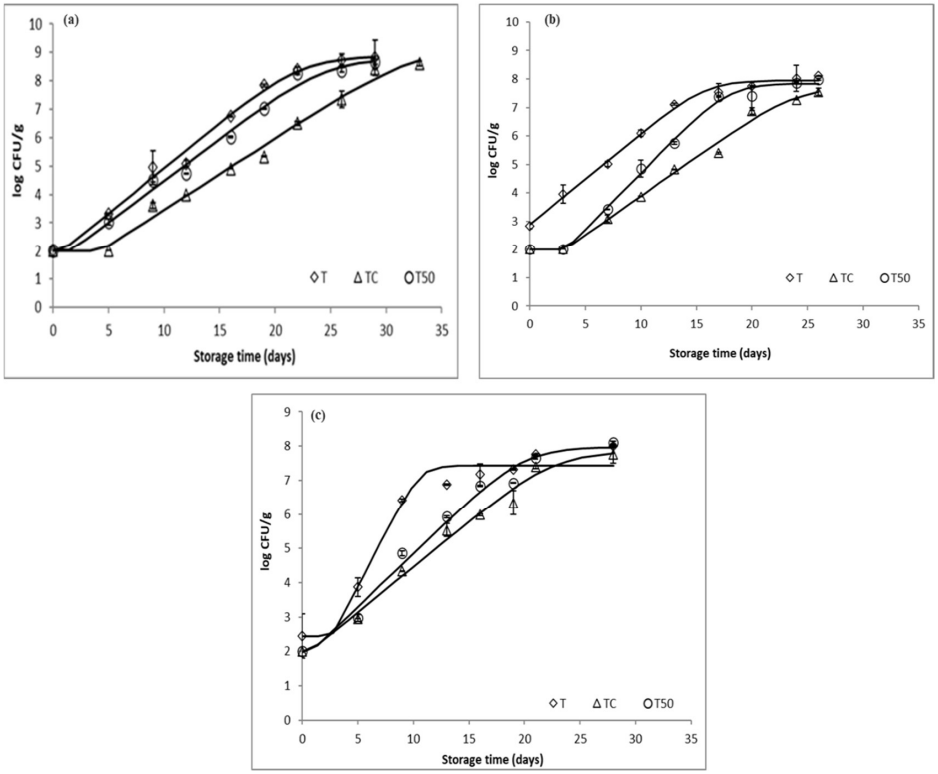

Figure 1. Growth of total viable counts (TVCs) in whole gilthead sea bream slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50) ($p < 0.05$).

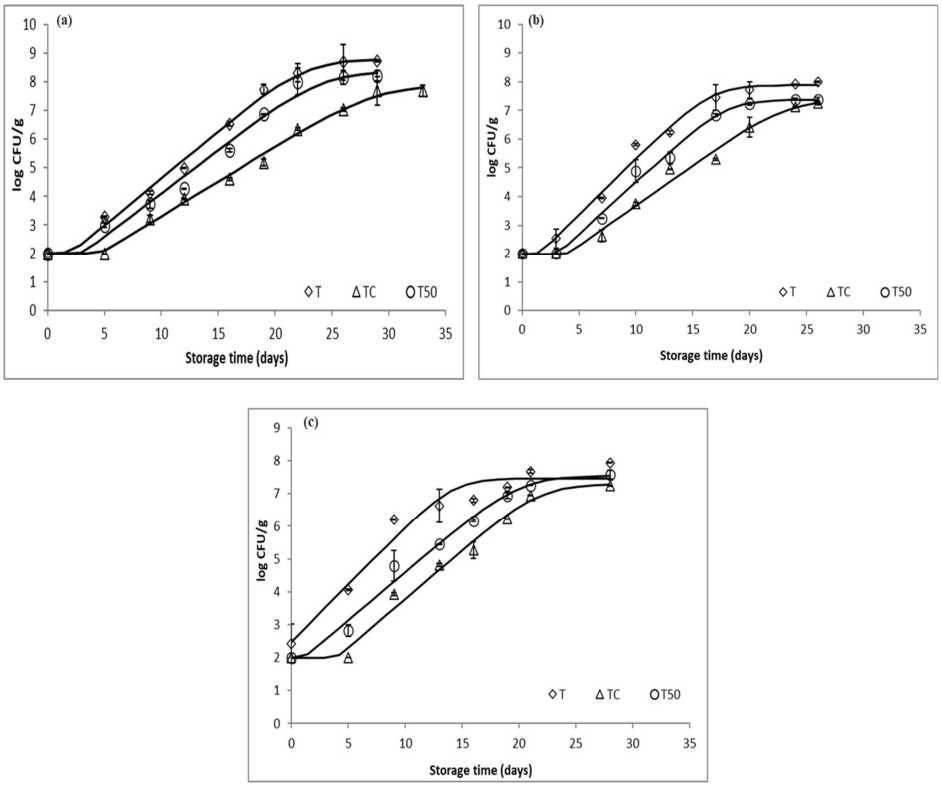

Figure 2. Growth of *Pseudomonas* spp. in gilthead sea bream samples slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50) ($p < 0.05$).

H_2S-producing bacteria (e.g., *Shewanella putrefaciens*) showed similar behavior as *Pseudomonas* spp. (Figure 3a). Lower rates were noted for TC and T50 samples in comparison with T samples. Application of 100% slurry ice lowered the H_2S-producing bacteria growth rate; therefore, replacing the conventional method of slaughtering resulted in greater control of bacteria growth.

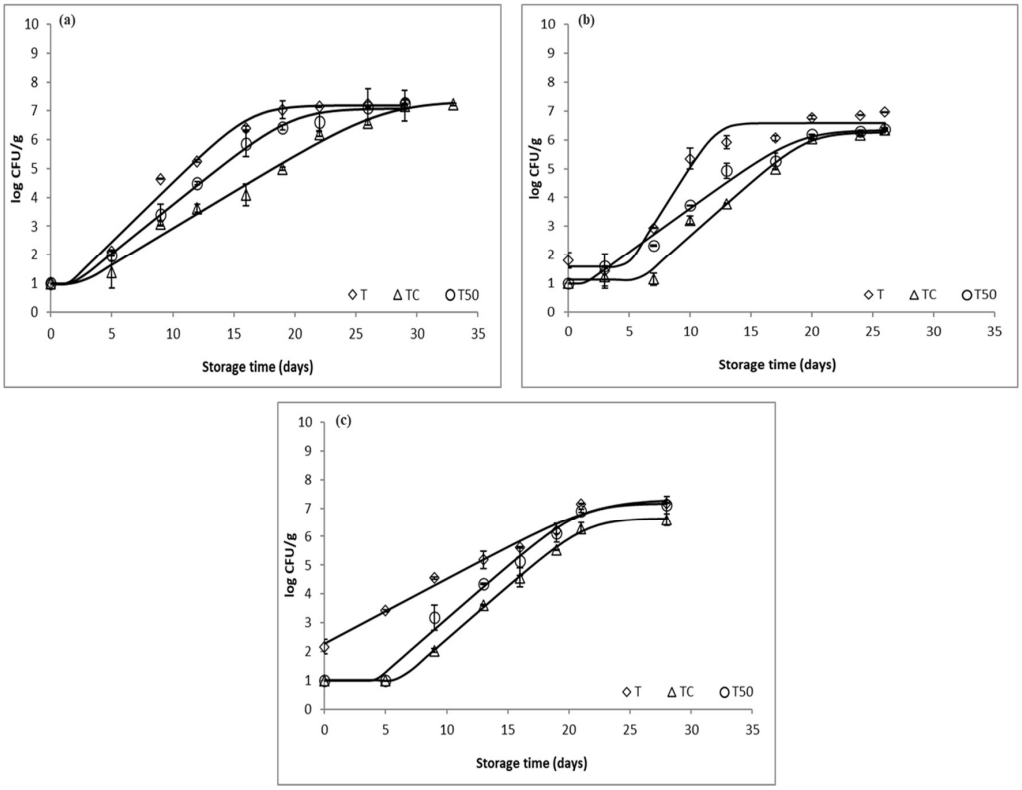

Figure 3. Growth of H_2S-producing bacteria in gilthead sea bream samples slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50) ($p < 0.05$).

The results were in agreement with the reported data in the literature, with similar initial H_2S-producing bacteria and *Pseudomonas* bacteria counts (2.4–2.5 log CFU g^{-1} for both) [10,37,38]. Zhao et al. (2022) observed that H_2S-producing and *Pseudomonas* spp. counts in ice slurry were lower (significant differences, $p < 0.05$) in comparison with those in flake ice on day 3 [10]. Therefore, it was found that the growth of the aforementioned bacteria was effectively restrained by ice slurry during storage, which was found in the present study as well.

Data from the experiments during the different periods showed that when the sea water temperature was higher (September), the growth rates of the microorganisms tested were lower, and the shelf life longer, than during colder periods, showing that microorganisms adapted more quickly to lower preservation temperatures when the samples were slaughtered during colder periods. Therefore, it was shown that the ambient water temperature might shape the dominant microflora, microbial counts, and shelf life. When ice is used, the fish surface microflora in summertime (which is mainly responsible for microbial spoilage) may receive shock, which could explain the higher growth rates in winter [21]. Based on the obtained microbiological data, the preservative effect of the slurry ice-based cooling media did not show any seasonal pattern.

3.2. Physicochemical Analysis

The pH of TC samples remained almost constant during the first 9 days. The pH values of control and T50 slurry ice-treated samples were higher (data not shown). Initial

values of pH were 6.517, 6.485, and 6.479 for T, T50, and TC samples, respectively (January). The pH of fish flesh increased for all tested conditions until the end of storage time at 0 °C, reaching the values of 6.537, 6.711, and 6.601 for T, T50, and TC samples, respectively (January) Similar results were obtained for sea bream during April and September. pH increase indicates agglomeration of alkaline compounds (e.g., trimethylamine-, ammonia compounds) as a result of microbial activity [39]. The pH of live fish is near 7.0, whereas postmortem, pH is between 6.0 and 7.1 depending on factors such as species and season [39]. The above measured pH values for control fish samples were similar to those of sea bream fillet samples under storage in ice observed by Tejada and Huidobro (2002) [40].

Color parameters of fish surface, such as L*, a*, b*, of all tested samples were determined. Researchers have noted that changes in myoglobin leads to the formation of brown color during chilled storage as a result of biochemical reactions. Brightness of the samples is also affected [41]. Data showed that the average L* (lightness) values of the samples tested in September were higher than those of the samples tested in January and April, during the storage period. It was observed that L* values for TC samples were not changed until the end of storage at 0 °C (a change was observed on the last day of storage). TC samples showed the highest L*values when fish samples were caught during September. Therefore, the combination of slurry and flake ice led to color (brightness) retention. At the beginning of the storage period, average L* values were 54.52 ± 2.52, 54.20 ± 3.13, and 55.36 ± 2.62 for T, T50, and TC samples, respectively, caught in September. For sea bream caught in January, average L* values were determined as 58.17 ± 1.87, 46.95 ± 3.74, and 47.91 ± 2.84 for T, T50, and TC samples, respectively. Lower L* values were noted for the samples caught in April (45.19 ± 4.2, 44.77 ± 2.87, and 42.98 ± 4.31 for T, T50, and TC, respectively). At the end of the storage period, L* values for TC samples than those for T and T50 samples, regardless the period the fish was caught. Final values of L* color parameter for TC samples were ca. 71.10 ± 3.25 in September, 50.90 ± 4.15 in January, and 51.43 ± 2.57 in April. Color changes in fish during storage may be due to autolytic and microbial activity, and they are expressed as yellow flesh color or brown discoloration [42]. The better retainment of fish color in TC samples is in agreement with the observations from the enzymatic activity and pH of fish flesh, as TC samples exhibited stable pH and small fluctuations of calpain and collagenase activities. Additionally, TC treatment resulted in significant inhibition of microbial spoilage, as indicated by lower microbial growth rates, compared with the alternative tested treatments.

3.3. Fatty Acid Analysis

Fatty acid composition, as well as total lipid content, vary with species and whether fish are cultured or wild. Even among the same fish species, variations might be observed due to differences in sex, age, seasonality, diet, the presence of various contaminants, and water temperature and salinity [43].

Gilthead sea bream samples were rich in both unsaturated (mono- and poly-) and saturated fatty acids. The major FAs of all tested sea bream samples were oleic acid (C18:1), which represented up to 34.5% of the total amount of FA depending on the seasonality (32.8–34.5%); palmitic acid (C16:0), which represented 15.7–16.6%; linoleic acid (C18:2n-6), which represented 14.1–15.5%; docosahexaenoic acid (DHA/C22:6n-3), which represented 8.3–9.7%; eicosapentaenoic acid (EPA/C20:5n-3), which represented 4.3–4.6%; and palmitoleic acid (C16:1), which represented 4.3–4.9%. These FAs have also been reported as the main fatty acids in fillets of cultured gilthead sea bream, as well as in farmed seabass flesh [44,45].

Different seasons of catching (January, April, and September) did not result significant differences in the fatty acid profile, except for total MUFA. More precisely, total MUFA concentration in sea bream flesh was significantly higher in January (42.9 ± 1.68%) and April (45.1 ± 1.62%) samples than in September (40.5 ± 1.21%) samples ($p < 0.05$) (Figure 4). Similar results were previously reported for rainbow trout, as well as for gilthead sea bream [46,47].

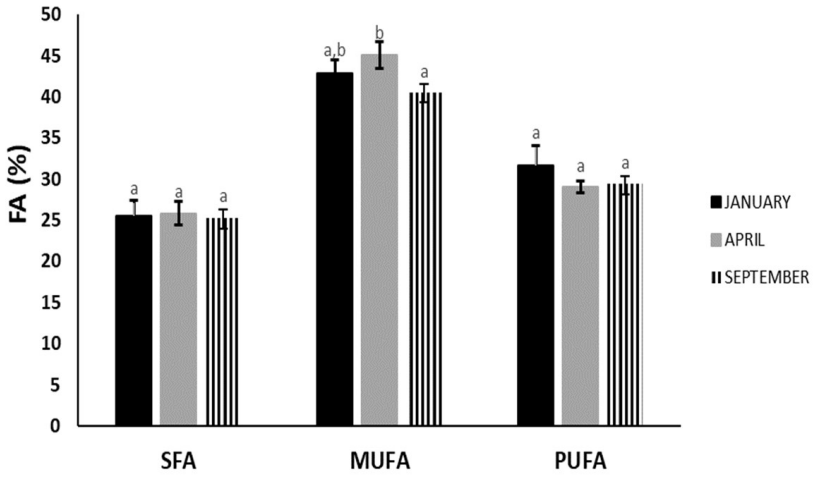

Figure 4. Fatty acid composition of sea bream samples caught in September 2020, January 2021, and April 2021 (SFA: Saturated Fatty Acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids). Different letters within the same set of columns indicate statistically significant difference.

The partial or total replacement of flake ice with slurry ice resulted in similar FA profiles in sea bream samples at all the examined catching periods. It could be concluded that lipid damage (e.g., lipid hydrolysis or oxidation) did not occur in the fish flesh during slaughtering or transportation in slurry ice instead of conventional flake ice.

3.4. Enzymatic Activity

Calpain showed the highest activity levels of all proteolytic enzymes regardless of the ambient temperature. The pattern of calpain activity up to day 15 postharvest differed with the temperature and the catching method. Overall, calpain activity was significantly lower at high ambient temperature (Figure 5). The use of slurry ice (TC, T50) delayed the activation of calpain by one and two days at high and low ambient water temperatures, respectively (Figure 5). However, a second phase of calpain activation was recorded at high ambient temperature in T50 samples. In both periods, calpain activities showed early activation postmortem, in accordance with previous studies [17,48].

Collagenase was activated as early as calpain (Figure 6), and the pattern of the activation differentiated with ambient water temperatures. Higher activities on average were recorded at low ambient temperature, and delayed activation was recorded in the TC and T50 groups. At low ambient temperature, collagenase activity declined gradually from day 2 to day 15 postharvest, in contrast with the findings of Hernandez-Herrero et al. (2003), which showed a gradient increase until day 9 postharvest in Atlantic cod's fillet [49]. Still, the lowest activities at low ambient temperature were observed in the T50 and TC groups at high ambient temperature (Figure 6).

The enzymatic activity of cathepsins also differed with ambient temperature, showing higher activities at low ambient temperature (Figures 7 and 8). In addition, a correlated activation pattern was observed between cathepsin B and cathepsin L regardless of the ambient temperature and the catching method, with correlation coefficients ranging between 0.76 (September 2020) and 0.79 (January 2021). The use of slurry ice did not delay the activation of cathepsins in either ambient temperature. At high ambient temperature, TC exhibited the highest on average cathepsin B and L activities (Figures 7 and 8).

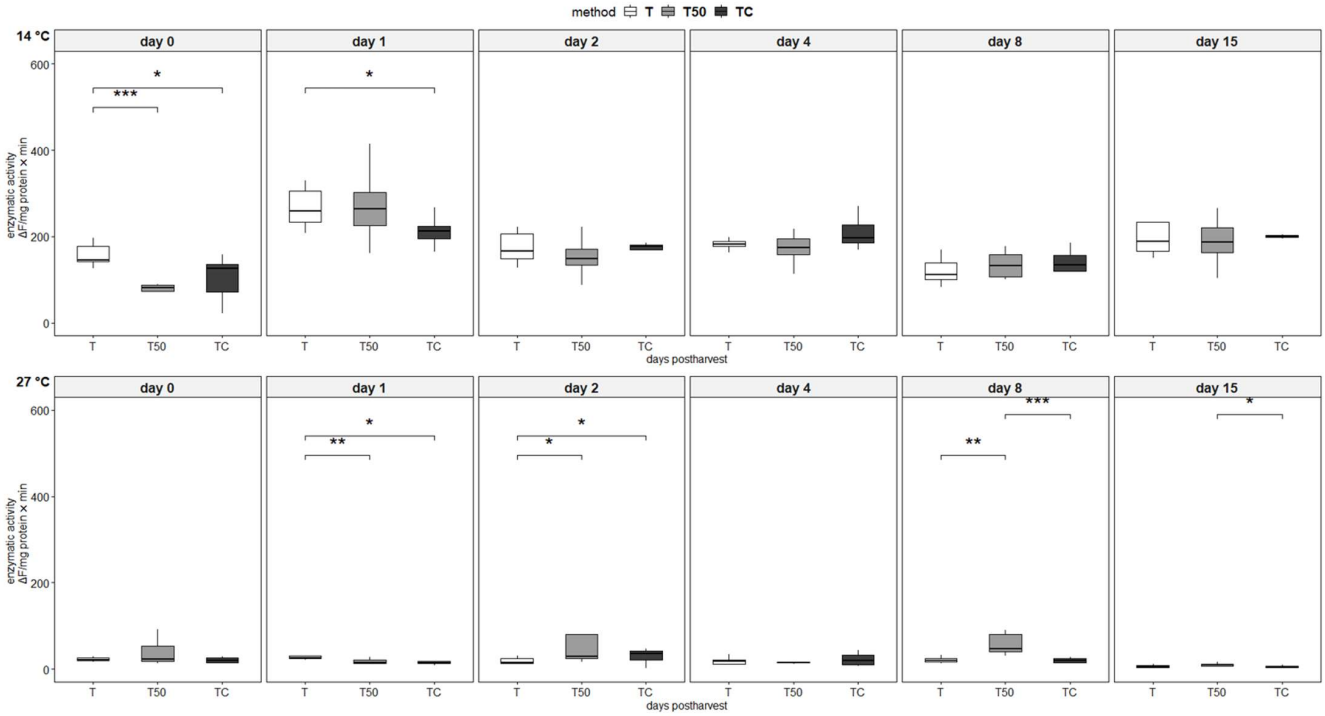

Figure 5. Calpain activity in all tested slaughter and storage conditions. T: control group, slaughter and storage in ice flakes; T50: slaughter and storage in 1:1 slurry ice to ice flakes; TC: slaughter in slurry ice, storage in ice flakes. Enzymatic activity is expressed as fluorescence unit (FLU) change per minute per mg protein (ΔF). Superscripts: statistically significant differences between treatments on each day (***: $p < 0.001$, ** $p < 0.01$, *: $p < 0.05$).

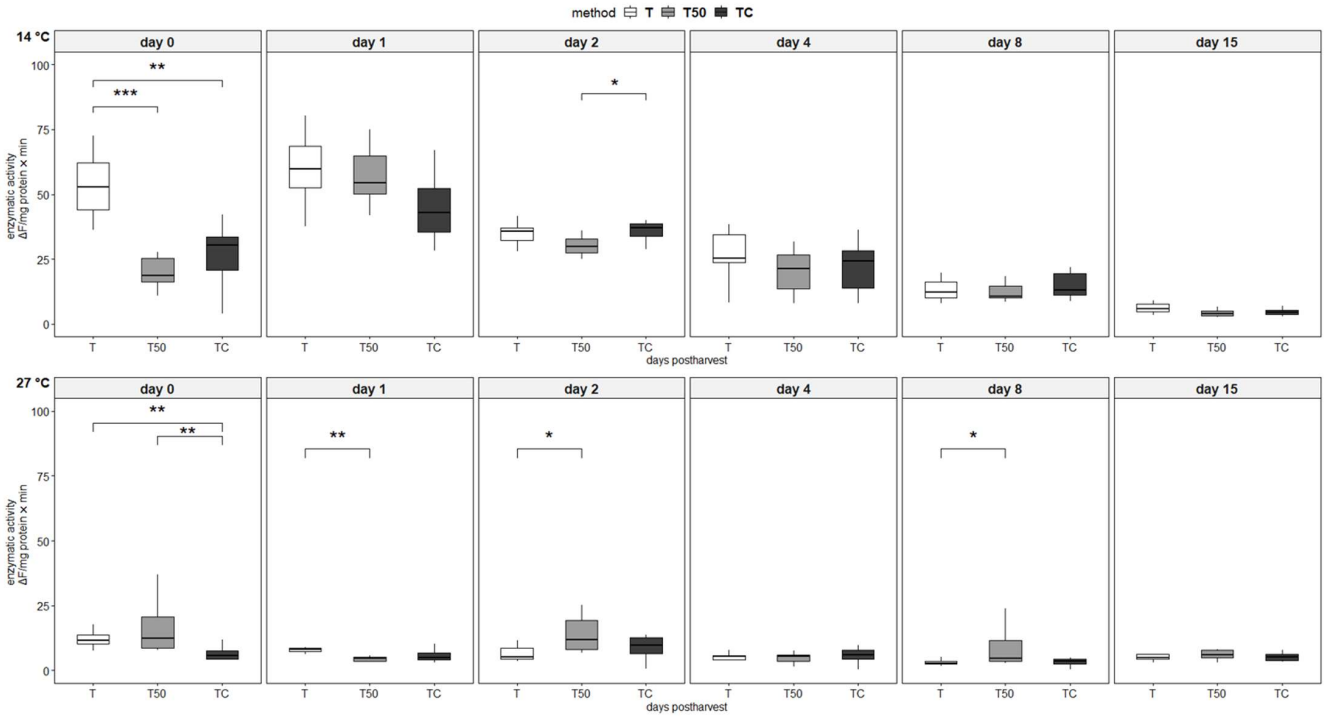

Figure 6. Collagenase activity in all tested slaughter and storage conditions. T: control group, slaughter and storage in ice flakes; T50: slaughter and storage in 1:1 slurry ice to ice flakes; TC: slaughter in slurry ice, storage in ice flakes. Enzymatic activity was expressed as fluorescence unit (FLU) change per minute per mg protein (ΔF). Superscripts: statistically significant differences between treatments on each sampling day (***: $p < 0.001$, ** $p < 0.01$, *: $p < 0.05$).

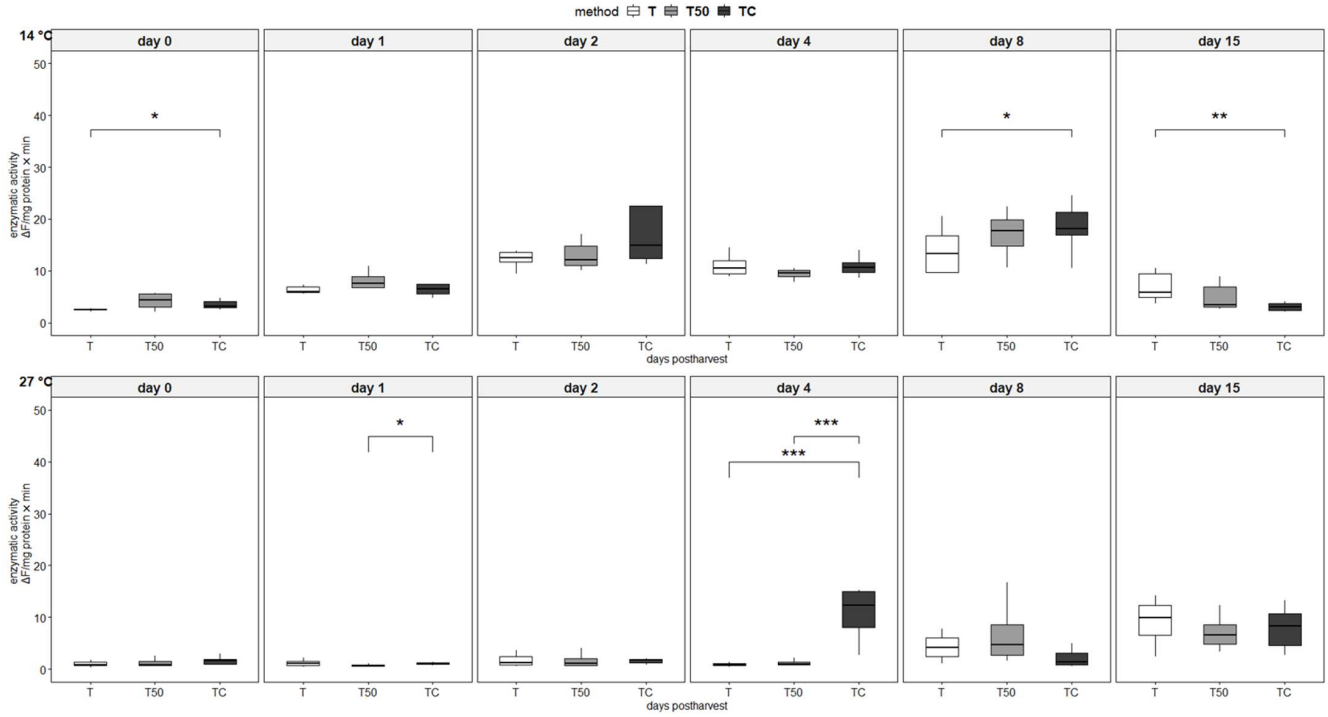

Figure 7. Cathepsin L activity in all tested slaughter and storage conditions. T: control group, slaughter and storage in ice flakes; T50: slaughter and storage in 1:1 slurry ice to ice flakes; TC: slaughter in slurry ice, storage in ice flakes. Enzymatic activity was expressed as fluorescence unit (FLU) change per minute per mg protein (ΔF). Superscripts: statistically significant differences between treatments on each sampling day ((***: $p < 0.001$, ** $p < 0.01$, *: $p < 0.05$).

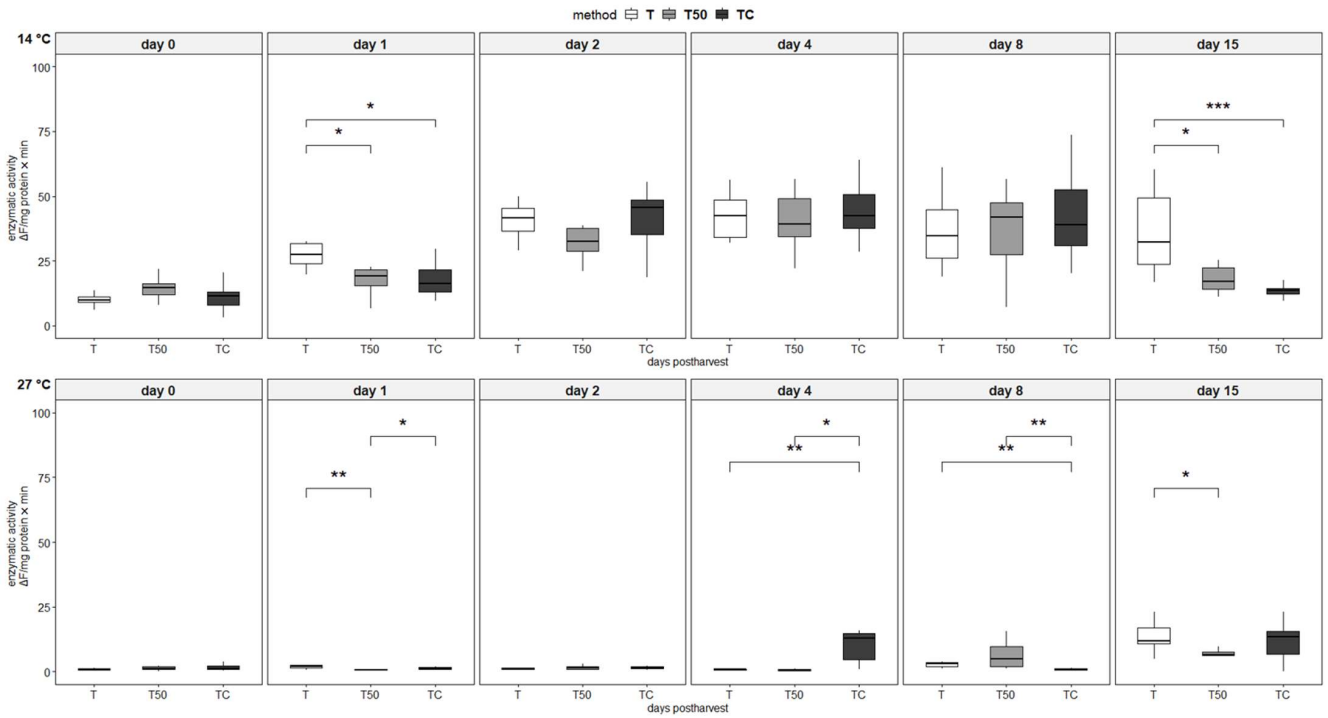

Figure 8. Cathepsin B activity in all tested slaughter and storage conditions. T: control group, slaughter and storage in ice flakes; T50: slaughter and storage in 1:1 slurry ice to ice flakes; TC: slaughter in slurry ice, storage in ice flakes. Enzymatic activity was expressed as fluorescence unit (FLU) change per minute per mg protein (ΔF). Superscripts: statistically significant differences between treatments on each sampling day (***: $p < 0.001$, ** $p < 0.01$, *: $p < 0.05$).

The multivariant analysis revealed two distinct groups that differed only by sea water temperature and not by catching method. Despite the high dispersion of values, multivariant analysis supported a high impact of ambient temperature on the enzyme activation (Figure 9). Fish being poikilothermic, i.e., with varied body temperature, their physiology and biochemistry are directly affected by water temperature changes. Differing calpain activity with water temperature was also observed in stickleback (*G. aculeatus*) [50]. Calpain is a family of cytosolic proteases (in contrast with collagenases, which act extracellularly), and cathepsins that are located in the lysosomes [51–53]. Changes in pH postmortem are responsible for the release and activation of those enzymes [54]. In the present study, TC resulted in stable muscle pH up to day 9 postharvest, which might have contributed to smaller fluctuations in calpain and collagenase activities (Figures 5 and 6), though it did not influence the activities of cathepsins (Figures 7 and 8).

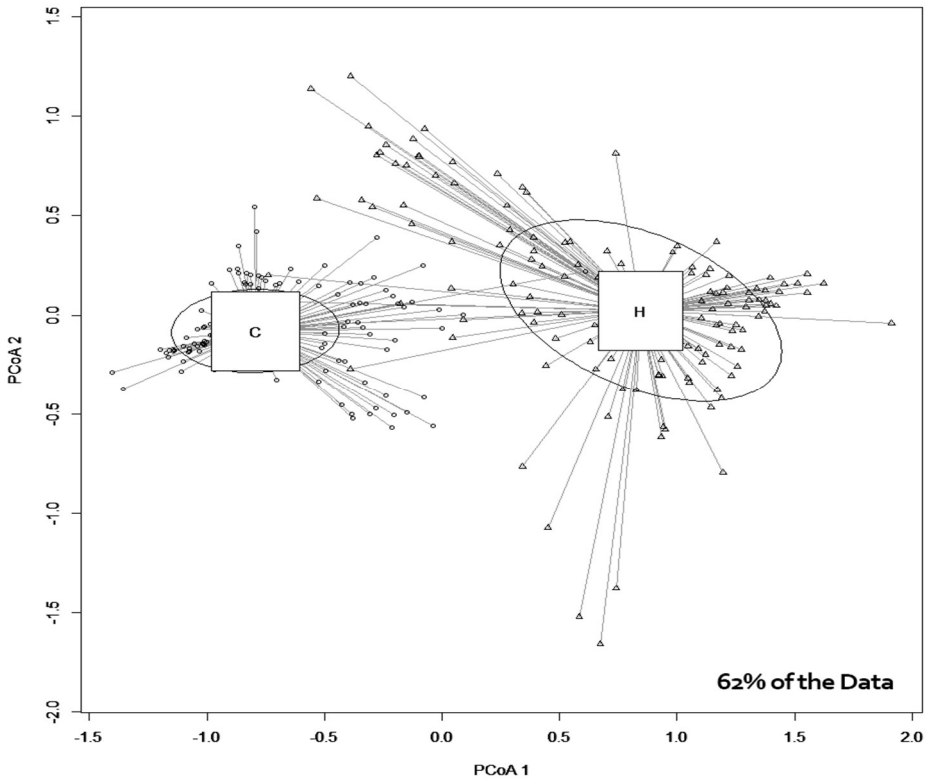

Figure 9. Principal coordinate analysis (PCoA, =multidimensional scaling, MDS); 62% of the data showed dissimilarities indicating an effect of water temperature on enzymatic activities. C: 14 °C H: 27 °C. △: represents the values of enzymatic activity in 27 °C. ○: represents the values of enzymatic acitivity in 14 °C.

These enzymes were expected to be affected by temperature changes, possibly resulting in different quantities and activation patterns. It is well established that environmental factors (such as water temperature, etc.) influence fish growth by affecting the metabolism of skeletal muscles, which account for the majority of the fish's body mass and accumulate structural proteins. In turn, skeletal muscle proteins provide amino acids that are employed physiologically as plastic and energetic substrates. Thus, the rate of protein degradation in skeletal muscles, which is regulated by calpain, collagenase, and lysosomal cathepsins [4,55], is recognized as a proxy for fish growth, protein metabolism, and the development of adaptive responses to environmental changes that regulate these processes throughout ontogenesis [56,57]. Fish calpains, in particular, are also involved in the establishment of compensatory or pathogenic alterations in response to abiotic environmental factors such as temperature, salinity, and so on [58]. Another feature of lower water temperature is a profound effect on mitochondria proliferation in tissues with high

demand for oxygen, resulting in an increase in aerobic capacities [59]. This kind of rapid increase in mitochondria numbers can have a domino effect, activating various processes. Such processes may provoke alterations in lysosomal membrane permeabilization and membrane structures, leading to the release of cathepsins [60,61]. The findings of this study contribute to the body of knowledge about the significant effect of ambient temperature on fish physiology and support the scope of exploring how slaughter methods interplay with ambient temperature to shape the shelf life of fish.

3.5. Sensory Evaluation

Based on preliminary experiments (data not shown), the use of slurry ice (100% replacement) (at temperature approximately -3.2 ± 0.2 °C) resulted in significant detrimental changes to fish appearance, mainly fish clouding, and in partial freezing of samples. The purpose was to investigate the effect of ice slurry application with the concept of superchilling. For this reason, the catching and transportation methods were based on slurry ice–flaked ice mixtures within the temperature range -1.0 to -2.0 °C.

Sensory evaluation results of appearance, odor and overall acceptability of seabream samples are depicted in Figures 10–12, respectively. At day 0, sensory assessors observed typical attributes of the raw and cooked fish in all testing conditions, indicating maximum freshness (odor, appearance, taste) and quality (sensory score = 9). The sensory scores decreased with storage time. The sensory scores for overall acceptability of all tested samples remained high for 4–7 days of storage. The scores for TC samples were higher than those for T and T50 samples (10–15 days of storage). The maximum differences between T/T50 and TC samples were shown for the seasons September 2020 and January 2021. On day 15, T and T50 received the score of 5 (approximately). On day 20, TC samples were slightly acceptable. Fresh fish had a sharp, sea weed smell, with bright red and odorless gills, whereas the sensory spoilage characteristics were sourness, a putrid "off" flavor, and grey-yellowish color and intense ammonia odor in the gills. The main detrimental effect of slurry ice on gilthead sea bream quality corresponded to the appearance of the eyes and the gills (which became slightly dull and opaque). This was probably due to the precipitation of an eye protein at subzero temperature levels, at which most of the current ice slurry systems are operated [62]. Slurry ice applied in TC samples led to significantly lower degradation rates of sensory attributes compared with the control samples (T) ($p < 0.05$), as also reported in the literature [13,63].

Ice slurry delayed H_2S-producing bacteria and *Pseudomonas spp.* growth, resulting in better control of bacterial growth as compared with flake ice, regardless the period of slaughtering. The slurry ice application (combined with flake ice) led to a 2- to 7-day shelf life increase for gilthead sea bream (at 0 °C). Under all treatments, sensory rejection coincided with a 10^6 CFU/g *Pseudomonas spp*. load. Microbial loads of 10^6–10^7 CFU/g of *Pseudomonas* spp. have been used as the rejection limit in aerobically stored fish [30,33]. The shelf lives of T, T50, and TC samples were calculated as 14, 16, and 21 days, respectively, when the temperature of sea water was 27 °C (approximately), whereas the shelf lives of T samples were 12 and 10 days; for T50, 14 and 15 days; and for TC, 18 and 17 days during January and April, respectively. Rodríguez et al. (2005) reported a 10-day shelf life increase for horse mackerel stored in brine-based ice slurry (from 5 days, for samples stored in flake ice, to 15 days) [64]. The results of the present study were in agreement with those of Campos et al. (2005), who observed a 7-day extension in sardine shelf life when slurry ice was applied (from 8 days, for samples stored in flake ice, to 15 days) [65], as well as with those of Navarro-Segura et al. (2019), who calculated a 3-day shelf life extension for slurry ice-treated mackerel compared with control samples, and with those of Annamalai et al. (2018) [19]. This effect of slurry ice application may be due to the surface wash together with the subzero temperature achieved with this advanced storage system, the latter of which resulted in delayed activation of the proteases [56]. The results of the present study did not show a significant effect of slurry ice during transportation, in agreement with Cakli et al. (2006) [66].

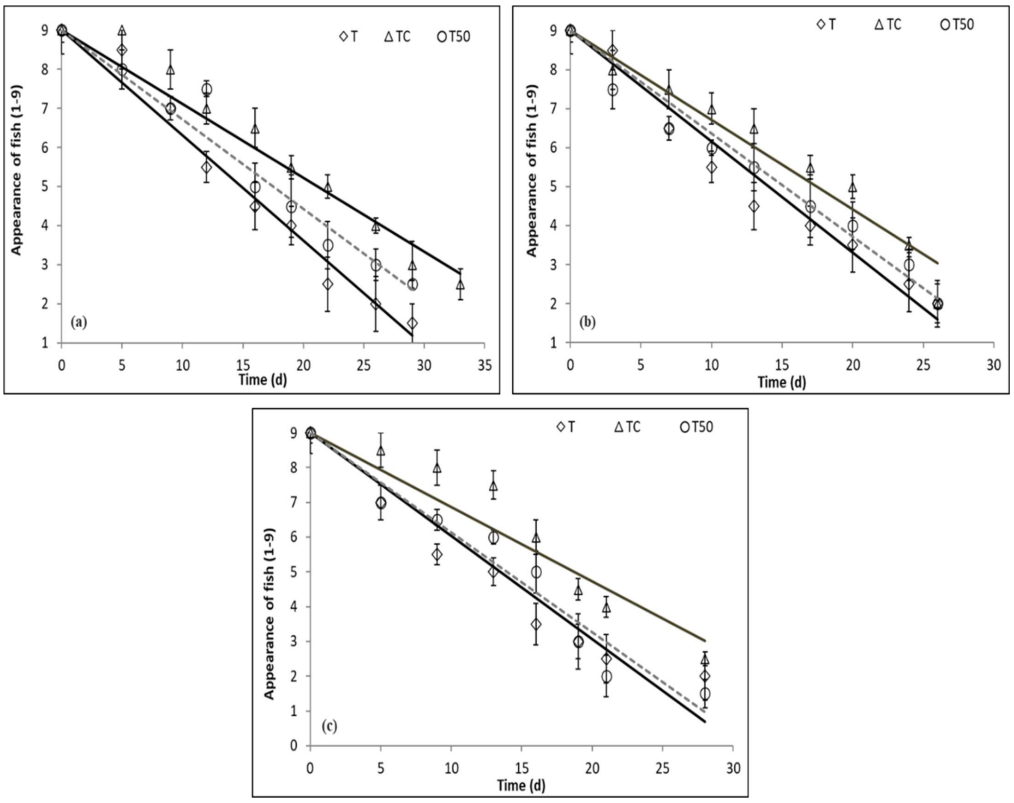

Figure 10. Scores of appearance of sea bream samples slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50).

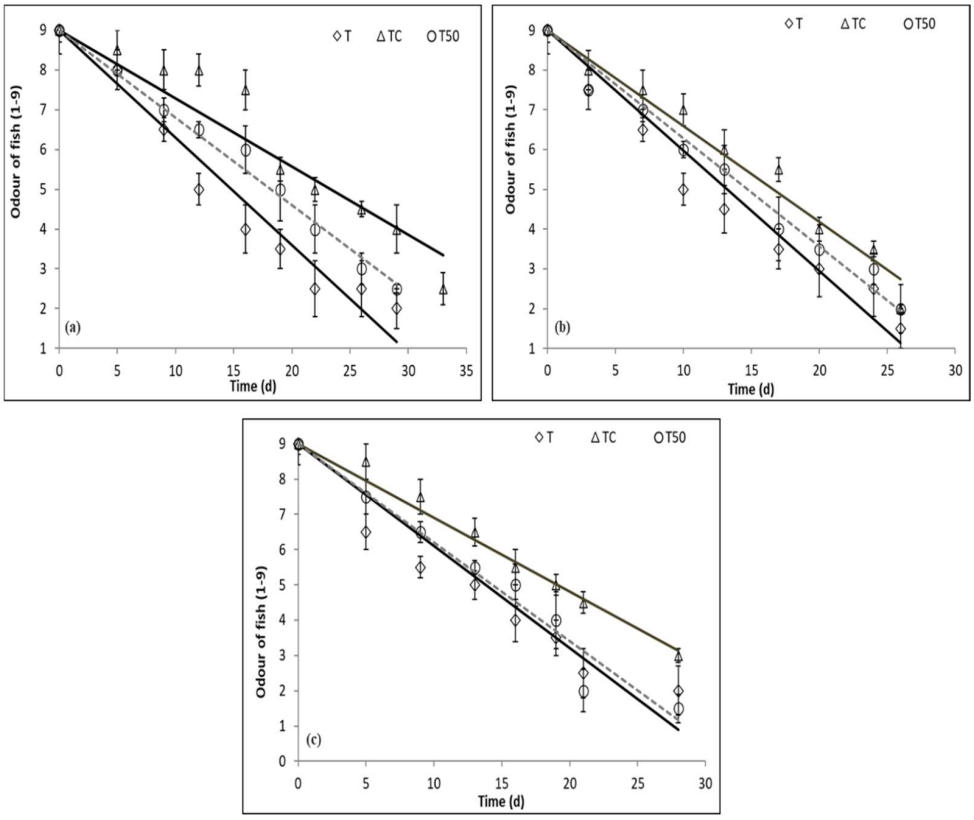

Figure 11. Scores of odor of sea bream samples slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50).

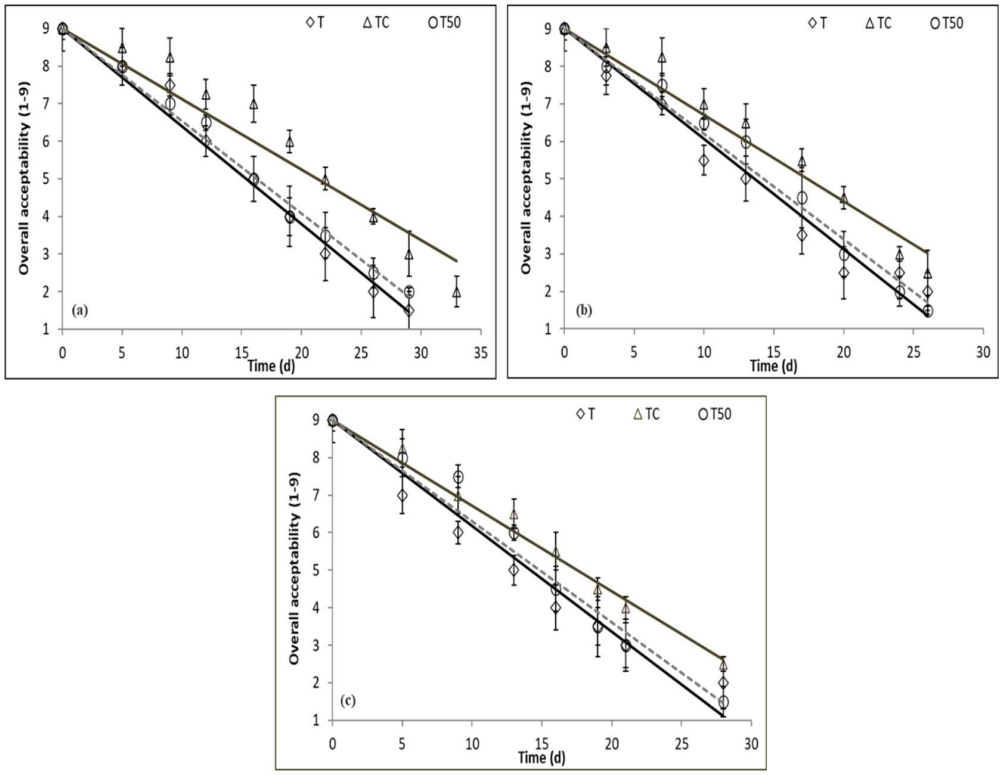

Figure 12. Scores of overall acceptability of sea bream samples slaughtered in (**a**) September 2020, (**b**) January 2021, and (**c**) April 2021 during storage at 0 °C (◇T, △TC, ○T50).

4. Conclusions

Slurry ice may lead to improved quality, in terms of microbial stability and physico-chemical indices, and increased shelf life of chilled aquatic products. In the present study, the use of slurry ice with flake ice for slaughtering purposes led to a 2–7-day shelf life increase at 0 °C based on both bacterial growth and organoleptic characteristics. Water temperature appeared to shape the action of proteases that determine postslaughter quality.

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