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The addition of ascorbic acid improves the microbiological quality and shelf life of Atlantic mackerel (*Scomber scombrus*) fillets stored in ice

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Abstract
Ascorbic acid is one of the most promising additives for improving fish shelf life, but studies on its antimicrobial activity during product storage are limited. In this experiment, we assessed the effect of ascorbic acid on the preservability of Atlantic mackerel (*Scomber scombrus*) fillets during storage in ice. Fillets were treated to reach an ascorbic acid concentration of 0.30 mg/g (T1) and 0.15 mg/g (T2). Samples were stored in ice at 0°C and analyzed at 0, 2, 7, 9, and 14 days for total viable count, specific spoilage organisms (SSOs), *Pseudomonas* spp., potentially histamine-producing bacteria (HPB), and *Enterobacteriaceae*. Growth curves were constructed using the DMfit tool of Combase, and the shelf life was estimated in relation to microbiological limits reported in the literature. Sensory evaluation was performed using a quality index method scheme. At 7 and 9 days of storage, treated samples exhibited bacterial counts from 0.5 to 1.7 Log CFU/g lower than controls, with a logarithmic reduction proportional to the additive concentration. The antimicrobial action appeared to diminish after 14 days. A consistent effect was observed for potentially HPB, with counts of 1.7 Log CFU/g lower in T1 samples compared to controls at 9 days. The moderate effect on SSOs limited the estimated shelf life, as the critical limit was reached after 7 days and 8.4 days (T1 and T2). Ascorbic acid positively impacted the microbiological characteristics of mackerel fillets. Further investigation into the decay dynamics of the additive in fish products during storage is advisable.

Introduction
Fish and seafood products are characterized by a very short shelf life due to the rapid deterioration of their organoleptic properties. Autolytic degradation, microbial growth, and lipid oxidation are the main mechanisms responsible for fish changes after death; the new compounds formed during these processes cause alteration in texture, flavor, and odor, thus making seafood products unfit for human consumption (Ghaly *et al.*, 2010; Nie *et al*., 2022). Endogenous and exogenous factors affect fish perishability, including tissue composition (high quantity of water and low amount of glycogen in muscle, poor presence of connective tissue), health status, variety of enzymes, aquatic habitat, and fishing methods, which can cause lesions that facilitate bacterial contamination (Colavita, 2012).
To slow down fish deterioration and extend its shelf life, several strategies have been considered, such as refrigeration, freezing, new packaging techniques, hyperbaric storage, and other emerging preservation methods, like edible films and coatings (Ghaly *et al.*, 2010). Additives are commonly used to maintain the sensorial and nutritional properties of fish. Currently, over 200 additives can be used in processed seafood products, but only 36 are authorized for unprocessed fish and shellfish (20 employable only in frozen products), and even fewer for fresh seafood (European Parliament and Council of the European Union, 2008).
Among these, additives based on ascorbic acid (AA) are commonly used in the food industry for their strong antioxidant activity. In accordance with Regulation No. 1333/2008 and amendments (European Parliament and Council of the European Union, 2008), the authorized molecules in Europe are AA (E300), sodium ascorbate (E301), calcium ascorbate (E302), and fatty acid esters of AA (ascorbyl palmitate and ascorbyl stearate, E304). In the USA, New Zealand, and Australia, the use of potassium ascorbate (E303) is also approved. The employment of additives based on AA hinders enzymatic browning in fruits and vegetables, works as a leavening agent in bakery products, prevents product oxidation and discoloration during meat storage, and maintains organoleptic properties in fish (Varvara *et al.*, 2016). AA, particularly its fatty acid esters (E304), is often used to avoid rancidity in fatty fish species since it removes oxygen and reduces the first step in the chain reaction of lipid oxidation (Rostamzad *et al*., 2011; Kilic and Oztan, 2016). AA also exerts an antimicrobial effect in fish, but there is still limited research about it, and many studies have assessed its action only on the total viable count (TVC) (Lee *et al*., 2019; Sáez *et al.*, 2020). Concerning its degradation, AA is stable in dry environments but deteriorates in aqueous ones and when added to foods. Temperature, pH, light, ion concentration, matrix composition, and thermal treatments are the main factors affecting the rate of this process. In the organism, AA is absorbed from the intestine and then reversibly oxidated into dehydroascorbic acid, which is further transformed into 2,3-diketogulonic acid by an

irreversible hydrolysis reaction. Other minor metabolites such as L-threonic acid, oxalic acid, glyceric acid, and glyoxylic acid are formed (EFSA ANS Panel, 2015). Regarding toxicity, the EFSA ANS Panel evaluated the combined exposure of the population to AA, calcium ascorbate (E301), and sodium ascorbate (E302) added to food, considering loss factors too. They calculated that the amount of total exposure, both from added and natural sources, would arrive at 1 g/person per day, representing a percentage of approximately 50-65%. After analyzing short-term studies on animals, since there were no available data for humans, the Panel concluded that acute toxicity is very low; long-term tests led to the same conclusions about chronic toxicity. Additionally, no adverse reactions were observed in prenatal development studies. So, the examined additives have neither genotoxic nor carcinogenic effects and can be safely used in foods without the necessity of setting a numerical acceptable daily intake limit (EFSA ANS Panel, 2015). Considering the potential of AA as an additive for fish preservation, the aim of this study was to investigate the effect of this compound on the shelf life and safety of Atlantic mackerel fillets (*Scomber scombrus*) stored in ice. Special emphasis was placed on the overall antimicrobial potential of the additive, considering not only the TVC but also monitoring other microbial populations responsible for fish spoilage.

Materials and Methods

Sample collection, processing, and storage

A total of 60 fresh, whole Atlantic mackerels (*Scomber scombrus*), each weighing 300-400 g and caught in FAO area 27.7.d, were collected from the wholesale fish market of Turin (Piedmont, Italy). The products were transported within 30 minutes inside a polystyrene box with an appropriate ice cover to our laboratory and immediately processed. After beheading and gutting the fish, fillets of epaxial muscle weighing approximately 20-25 g each were obtained. During filleting, sterile conditions were maintained by working near a Bunsen burner and using sterile knives, tweezers, and scissors. AA powder (Ascorbic acid, E-300, Ph. Eur., Food Grade; ACEF Spa, Fiorenzuola d'Arda, Italy) was diluted in sterile deionized water to obtain solutions with known concentrations. Samples were treated with 6 mg/mL and 3 mg/mL AA stock solutions. An adequate amount (1 mL) was injected into the flesh of fillets to reach concentrations of AA in the product equal to 0.30 mg/g (samples T1) and 0.15 mg/g (samples T2). To achieve a homogeneous distribution of the solution within the fillet, each was divided into ten equal parts, and 0.1 mL of the additive solution was injected into each of these parts.

All treated samples and controls (C), in which 1 mL of sterile deionized water was injected, were placed individually in sterile bags and stored in ice at 0°C. Storage temperature values were monitored through a data logger (Datalogger Wi-Fi, Saveris 2 T-3; Testo Spa, Germany).

Microbiological analysis

From each fillet, 10 g of muscle tissue were sterilely removed using sterile knives, tweezers, and scissors, diluted in 90 mL of peptone saline solution (0.85% NaCl + 0.1% bacteriological peptone), and homogenized for 30 s at 230 rpm (Stomacher 400 Circulator; Seward, London, UK). Subsequently, serial dilutions were prepared and the following microbiological enumerations were carried out: i) TVC on Plate Count Agar (Oxoid, Basingstoke, UK), incubated at 30°C for 72 hours; ii) specific spoilage organisms (SSOs) on Lyngby Iron Agar (Condalab, Madrid, Spain), incubated at 20°C for 72 hours; iii) potentially histamine-producing bacteria (HPB) on modified Niven Agar (Mavromatis and Quantick 2002), incubated at 30°C for 48 hours; iv) *Pseudomonas* spp. on *Pseudomonas* Agar + CFC Supplement (Oxoid), incubated at 25°C for 48 hours; v) *Enterobacteriaceae* on Violet Red Bile Glucose Agar (Oxoid), incubated at 37°C for 24 hours. All analyses were performed in triplicate. Enumerations were carried out immediately after sample processing (day 0) and after 2, 7, 9, and 14 days.

Identification of potentially histamine-producing bacteria
Suspect colonies grown on modified Niven medium, which exhibited a distinctive purple halo, were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Individual colonies were placed on a target plate (MSP 96 target; Bruker Daltonics, Bremen, Germany). An aliquot (1 µL) of a 70% formic acid solution was subsequently added, and, after evaporation of the latter, 1 µL of a matrix solution (α-cyano-4-hydroxycinnamic acid; Bruker Daltonics) was used to cover the spots. The spectra were obtained (mass range 2000-20,000 Da) with the Microflex LT MALDI-TOF (Bruker Daltonics) associated with FlexControl software 4.1.9 (Bruker Daltonics) and MALDI BioTyper database (Bruker Daltonics).

Determination of pH and water activity
At each sampling point, the pH of samples was measured using a pH meter (Crison 507; Crison Instruments, Barcelona, Spain) after homogenizing 5 g of sample in 5 mL of deionized water. The water activity (a_w) was determined with an a_w meter (Aqualab Series 3TE; Decagon Devices, Pullman, Washington, DC, USA).

Curve fitting and shelf life estimate
The DMfit tool of Combase (Combase, https://www.combase.cc/index.php/en/), based on the Baranyi and Roberts (1994) primary model, was used to construct the complete growth curves for each microbial group, starting from the data points collected. This approach was applied to identify the moment in which each microbial group reached a level considered a critical limit of spoilage or safety, to estimate the shelf life of samples belonging to the three groups.

Sensory evaluation
A quality index method (QIM) scheme was specifically constructed for Atlantic mackerel fillets (Table 1), starting from two schemes available in the literature (Pons-Sánchez-Cascado *et al*., 2006; Cardenas Bonilla *et al*., 2007). The developed QIM included the following parameters: general appearance, color, odor, blood, and texture. Sensory evaluation was performed by six untrained panelists from our department on days 7 and 14 of storage.

Statistical analysis
The results of microbiological enumerations and product characteristics were subjected to the Shapiro-Wilk and Kolmogorov-Smirnov tests to assess the normality of data. A two-way analysis of variance, followed by a Tukey's multiple comparison test ($p<0.05$; GraphPad Prism version 9.0.0, GraphPad Software, San Diego, CA, USA), was applied to identify significant differences between control and treated samples.

Results
Microbiological analysis
The outcomes of the microbiological analyses carried out during the 14-day storage period are reported in Table 2. The obtained results highlighted significant differences ($p<0.05$) in terms of microbial loads and growth dynamics between samples C, T1, and T2. After 7 and 9 days of storage, observed microbial loads were from 0.5 to 1.7 Log CFU/g lower in treated samples compared to the controls, with logarithmic reductions proportional to the additive concentration. The highest effect was observed for potentially HPB, with loads of 1.7 Log CFU/g lower in T1 samples compared to controls after 9 days. After 14 days, the differences between the treated and control samples were generally not significant.

Identification of potentially histamine-producing bacteria
The results related to the MALDI-TOF identification of suspect colonies grown on modified Niven medium are presented in Figure 1. Potentially HPB belonged to the genera *Pseudomonas* (66.7%),

Brochothrix (2.1%), *Erwinia* (2.1%), *Pseudoescherichia* (2.1%), *Acinetobacter* (2.1%), *Rahnella* (2.1%), and others (22.9%).

Determination of pH and water activity

The pH of the product was 6.3 at the beginning and gradually increased to values of 6.9-7.0 on day 14. The value of a_w remained between 0.984 and 0.987 for the entire duration of our experiment. Neither of the two parameters showed significant differences among the three groups.

Curve fitting and shelf life estimate

Figure 2 represents the fitted growth curves of individual microbial populations predicted in the three groups of samples based on observed data points. By comparing the data points predicted with the critical limits identified for each microbial population, it was possible to estimate the shelf life of the product. The boundaries used were obtained from the Centre for Research and Documentation on Food Safety of the Piedmont region (https://www.ceirsa.org/matrice_alim.php#inizio) and are related to the TVC, *Pseudomonas* spp. and *Enterobacteriaceae*, having critical limits of 6, 7, and 4 Log CFU/g, respectively. For SSOs, we used the threshold proposed by Giarratana *et al*. (2022) for Atlantic mackerel, with a critical value of 6 Log CFU/g. To the best of our knowledge, no critical limits related to potentially HPB have been reported. At the end of our investigation, no group of samples had reached the critical limit for *Enterobacteriaceae*. The critical limit for the TVC was reached at 7 days by the control samples and at 9.5 days by both treated groups. At 7 days, the controls also reached the critical limit relating to SSOs, while the treated ones reached the threshold at 8.4 days.

Sensory evaluation

At 7 days, the total demerit scores were inversely proportional to AA concentration. Specifically, the scores for C, T2, and T1 samples were 13, 15, and 22, respectively. At 14 days, both treated samples obtained the same score (15), while the control group scored 27.

Discussion

The effects of AA on fish have been extensively investigated in the past years, but most studies have focused on its antioxidant action, especially in frozen products (Aubourg *et al*., 2004; Rostamzad *et al.,* 2011; Taheri *et al*., 2012; Monirul *et al*., 2019). Surveys that consider antimicrobial action are often limited to quantifying only the TVC (Lee *et al*., 2019; Sáez *et al.,* 2020). In assessing the performance of additives, it would be desirable to monitor the behavior of other microbial groups, such as SSOs, which are major contributors to spoilage phenomena in fish (Gram and Dalgaard, 2002; Zhuang *et al.,* 2021). To monitor the evolution of spoilage-associated bacteria, it is advisable to employ various types of media and diverse culture conditions (Saelens and Houf, 2022). Thus, we designed an experimental protocol to assess the impact of AA on fish products during storage in ice, studying its antimicrobial action against the specific microbial populations responsible for fish spoilage. Atlantic mackerel (*Scomber scombrus*) was selected considering the availability of fresh products in the local market, the existing knowledge about the specific spoilage flora of this species (Svanevik and Lunestad, 2011; Giarratana *et al*., 2022), and the fact that mackerel belongs to a histamine risk family according to Regulation No. 2073/2005 (European Commission, 2005). It was in fact our interest to evaluate the effect of AA addition also against potentially HPB. We selected two additive concentrations based on legal limits and industrial standards, aiming to replicate real commercial conditions. The higher AA level corresponds to the maximum allowable dose in tuna (300 mg/kg or 0.30 mg/g), as imposed by the recent Regulation 2022/1923 (European Commission, 2022) amending Annex II of Regulation No. 1333/2008 (European Parliament and Council of the European Union, 2008). This value was considered a reference because it represents the sole legal limit set for AA in fishery products. The lower dosage (150 mg/kg or 0.15 mg/g), on the other hand,

was close to the concentration generally used by the industry for fish products (100 mg/kg), as reported to EFSA by the food industry itself (EFSA ANS Panel, 2015).
The results of this study revealed significant differences between the control group and the fillets treated with AA, indicating the effectiveness of the additive. The effects were observed on all bacterial populations, with some differences in relation to the additive concentration and the considered microbial group. For the TVC, as highlighted in Table 2 and Figure 2, the greatest differences between controls and treated samples were observed between the fifth and twelfth days of storage. However, at the end of the experiment, all groups showed similar microbial loads. The maximum difference was quantifiable at 1.3 Log CFU/g on the day 9 of storage. It is important to note that, considering the TVC, there are no substantial differences between the two groups treated with different concentrations of the additive. Regarding *Pseudomonas* spp., the slight effects of AA were observed quite early, already at 40-45 hours of storage (Table 2 and Figure 2). Towards the end of the observation period, the effect appeared to diminish in the group treated with 0.15 mg/g of AA, while it persisted in the group treated with the higher dosage. The effect of AA on total microbial count and *Pseudomonas* spp. could be boosted by using the additive in combination with other compounds. Zambuchini *et al.* (2008), as an example, showed that ellagic acid (0.03%) in combination with L-AA (1.71%) and sodium ascorbate (1.98%) retarded the proliferation of aerobic bacteria, psychrotrophic bacteria, and *Pseudomonas,* extending the shelf life of *Solea solea* up to 10 days (8 days for controls). On SSOs, AA had a less significant effect that appeared later; we found a difference of less than 1 Log CFU/g only between days 9 and 11 of storage. The positive aspect is that the effect, albeit minimal, persisted until the end of the observation period, but only in the group treated with the higher dosage (Table 2 and Figure 2). *Enterobacteriaceae* were poorly represented in the product, and no proliferation was highlighted in any group during the entire observation period (Table 2 and Figure 2). However, AA could also exert action against this microbial group. In fact, a recent study showed that dip treatments with AA solutions of hot-smoked mackerel fillets resulted in low *Enterobacteriaceae* loads in samples treated with the additive (Ude *et al.,* 2024). The observed effect on potentially HPB deserves special attention. Despite the low initial presence and the maintenance of low levels in the early days of storage, potentially HPB started to increase in all groups from the fourth day of storage. In the group treated with the higher concentration of AA, the extent of this growth was less evident, while no substantial differences were observed between the control group and the group treated with the lower dosage (except in the last days of observation) (Table 2 and Figure 2). AA at 0.30 mg/g reduced the loads of potentially HPB up to a maximum of 1.7 Log CFU/g, with an effect that was noticeable from the fifth day of storage and persisted for the entire duration of the experiment. Regarding the identification of potentially HPB, we observed a prevalence of bacteria belonging to the genus *Pseudomonas* (Figure 1). Microorganisms belonging to this genus are indeed reported as HPB (Visciano *et al.,* 2020). On the other hand, no information could be obtained regarding the presence of histaminogenic enterobacteria, since no psychrotrophic enterobacteria were quantified or identified during this experiment.
By comparing the growth dynamics of the various microbial populations and the identified critical spoilage levels, we can estimate the shelf life of fillets belonging to the three groups. In general, we observed that critical levels were reached later in treated fillets. This observation is in line with the different effectiveness of AA against the various considered microbial groups, as discussed above. The limiting parameter for the shelf life of treated fillets was represented by the SSOs, whose critical limit was reached at 7 days for the control group and at 8.4 days for the treated groups. These, therefore, are the reference times for determining the shelf life of treated and untreated mackerel fillets stored in ice. The identified shelf life does not consider the growth of HPB, as, to the best of our knowledge, an unambiguous limit of safety is not indicated in the literature. Anyway, the storage temperature of our experimental test, carried out at an average of -0.9°C (as recorded by the data logger used), did not realistically expose samples to the risk of developing histamine. Analysis of product characteristics revealed that there were no significant differences in pH and a_w among the three groups, demonstrating that the observed effects on microbial populations were mainly due to

the direct action of AA. The results of the sensory evaluation, although only indicative and carried out by untrained panelists, corroborated the outcomes of the microbiological analysis.

Conclusions

In this study, AA exerted a positive effect on the microbiological and organoleptic features of mackerel fillets. The effects on microbial loads were proportional to the additive concentration, both in terms of magnitude and duration. The efficacy was particularly evident in TVC, *Pseudomonas* spp., and potentially HPB, mainly belonging to the genus *Pseudomonas*. The action of AA on the latter microbial group undoubtedly deserves further investigation; if this effect is observed at higher storage temperatures as well, the additive could help prevent histamine formation in cases of thermal abuse. The lower efficacy observed in some cases with increasing time was probably related to the progressive degradation of the additive. The decay dynamics of AA in fishery products during storage should be further studied to optimize the employment of this additive at the industrial level.

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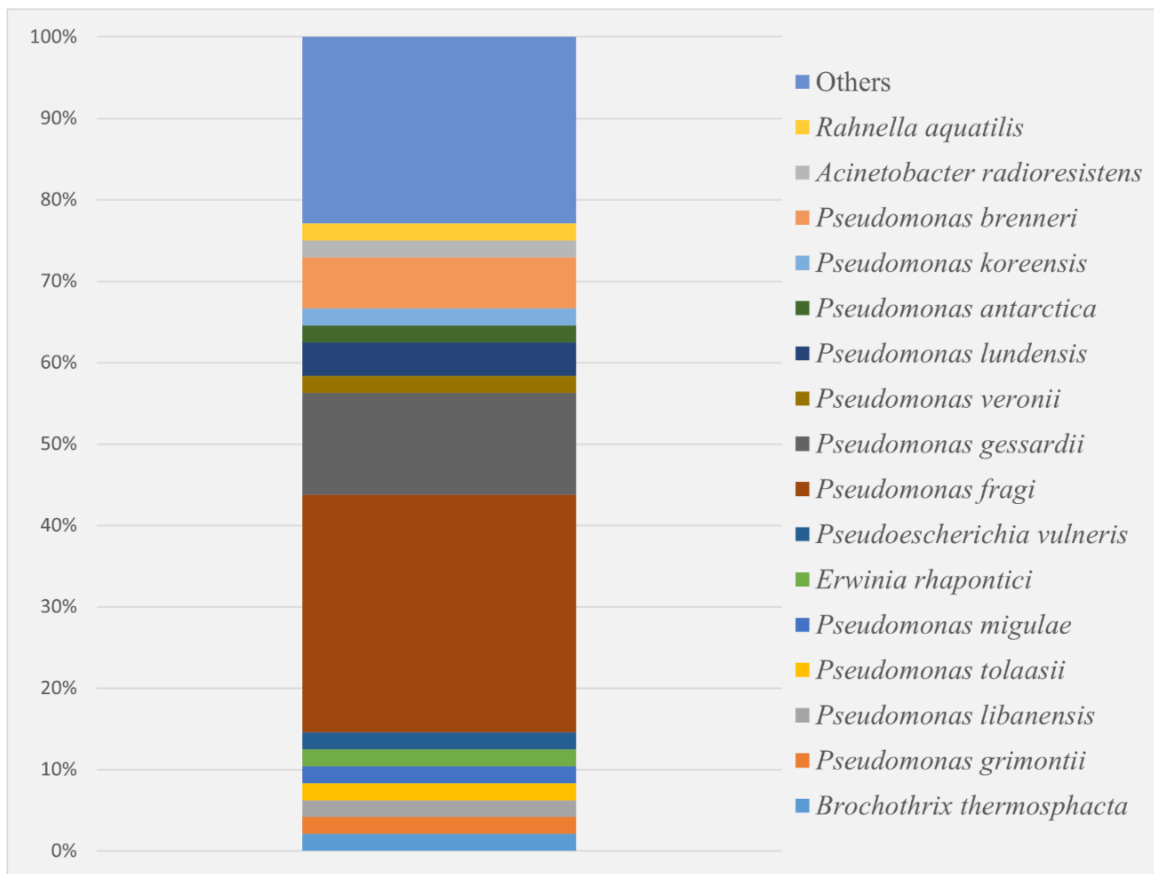

Figure 1. Potentially histamine-producing bacteria identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Figure 2. Fitted growth curves of different microbial populations in treated (T1 and T2) and control samples. Growth curves were obtained by using the DMfit tool of Combase (https://www.combase.cc/index.php/en/), based on the Baranyi and Roberts (1994) primary model.

Table 1. Quality index method scheme developed for Atlantic mackerel fillets.

Quality parameter	Description	Score
General appearance	Bright	0
	Dull	1
	Milky	2
Color	Greyish/pinkish and translucent	0
	Waxy pink slightly opaque	1
	Dark red extremely opaque	2
Odor	Fresh, neutral	0
	Seaweedy, marine	1
	Slightly acrid	2
	Acetic, ammonia	3
Blood	Bright red, not present	0
	Dull red	1
	Shadowy, brown	2
Texture	Firm	0
	Rather soft	1
	Very soft	2

Table 2. Outcomes of microbiological analyses of treated and control mackerel fillets during storage in ice at 0°C.

Samples	Microbiological parameters (Log CFU/g)				
	Total viable count				
	0 h	**45 h**	**168 h**	**216 h**	**335 h**
C	3.0±0.0	3.6±0.3	6.1±0.1 a	7.1±0.1 a	7.8 ±0.5 a
T1	3.0±0.0	3.4±0.3	5.3±0.2 b	5.8±0.3 b	7.4 ±0.5 ab
T2	3.3±0.0	3.5±0.1	5.0±0.0 b	5.9±0.2 b	7.9 ±0.1 ac
	Specific spoilage organisms				
	0 h	**45 h**	**168 h**	**216 h**	**335 h**
C	2.7±0.3	3.6±0.3	5.7±0.2 a	7.3±0.1 a	8.0±0.4 a
T1	2.4±0.3	3.5±0.1	5.2±0.2 b	6.5±0.2 b	7.7±0.2 ab
T2	2.4±0.1	3.7±0.1	5.2±0.1 b	6.5±0.1 b	8.3±0.2 ac
	***Pseudomonas* spp..**				
	0 h	**45 h**	**168 h**	**216 h**	**335 h**
C	2.8±0.4	3.5±0.1	6.0±0.4 a	7.1±0.2	8.5±0.4 a
T1	2.7±0.1	3.4±0.1	5.6±0.2 a	6.7±0.4	7.8±0.3 b
T2	2.9±0.1	3.3±0.1	5.2±0.2 b	6.9±0.1	8.4±0.2 a
	Potentially histamine-producing bacteria				
	0 h	**45 h**	**168 h**	**216 h**	**335 h**
C	1.0±0.0	1.1±0.2	4.3±0.0 a	5.4±0.5 a	5.8±0.2 a
T1	1.0±0.0	1.2±0.4	3.0±0.0 b	3.7±0.3 b	5.0±0.0 b
T2	1.1±0.2	1.0±0.0	4.0±0.0 a	5.0±0.4 a	5.2±0.4 b
	Enterobacteriaceae				
	0 h	**45 h**	**168 h**	**216 h**	**335 h**
C	1.8±0.2	1.1±0.2	1.8±0.4 a	1.7±0.3 a	1.4±0.4 a
T1	1.7±0.2	1.1±0.2	1.2±0.4 b	1.0±0.0 b	<1.0±0.0 b
T2	1.7±0.2	1.2±0.3	1.2±0.3 b	1.3±0.2 ab	1.0±0.0 a

T1 and T2, microbial populations in treated samples; C, control sample. Data followed by different letters in the same column are significantly different ($p<0.05$) according to Tukey's multiple comparison test.