AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

Deliverable WP5

D 5.2.2 New types of packaging Control sheet/Control Document

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CONTENT OF THE DELIVERABLE

This document is divided in:

- **PART 1**

  A short presentation of the deliverable. It is a control document and a demonstrative review of the deliverables that are listed in Application Form in different WP that consists of the producing an object (like feeds, model, protocols, set of tools for market analysis, ec), buying equipment/Services, in this case new types of packaging.

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- **PART 2**

  The second part provides the final results and a collection of data from the WP and project in relation to the General objectives at the Programme level.

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PART 1

1. *Introduction (objective and purpose of the deliverable)*

Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are the main marine fish species farmed in Europe and, in particular, in Mediterranean countries. Their white flesh, low fat and high content of polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), make them popular and the most important economically cultured fish among aqua-cultured species [1–5]. Indeed, the growing interest of consumers in nutritional aspects and the parallel attention to food quality issues have contributed to their consideration as a value-added seafood product, with increasing consumption and demand in the international seafood market [1,3]. For this reason, great interest has been given to rearing systems and feeding regimes because they may affect flesh quality, especially in terms of fat concentration, nutritional compounds, and hygienic quality [6,7]. Both fish are very perishable because of indigenous and microbial enzymes, which determine deterioration and shelf life [8] immediately postmortem [9]. The deterioration processes, which lead to an important, sequential, and progressive modification of the initial state of freshness, are fast and depend on rearing, harvesting, slaughtering, handling, and storage conditions [9–11].

Microbial spoilage is attributed to specific spoilage organisms (SSOs) that prevail over the rest of the microbiota, reach high concentrations (>8 log CFU/cm^2 or g), are favoured by different parameters (such as atmosphere, temperature, and flesh chemical composition; processing, transportation, and storage in the market) and produce various metabolites responsible for off-flavours/odours [8,9,12,13].

Aerobic and facultative anaerobic Gram-negative (*Pseudomonas*, *Moraxella/Acinetobacter*, *Photobacterium*, *Flavobacterium/Cytophaga*, *Xanthomonas*, *Vibrio*, *Shewanella*, *Proteus*, *Aeromonas*, *Serratia*, *Hafnia*) or Gram-positive (*Bacillus*, *Corynebacterium*, *Micrococcus*, *Carnobacterium,* and other cocci and lactobacilli) microorganisms can grow during storage and are the main bacteria isolated in spoiled fish that are stored in air or modified atmosphere packaging (MAP) [14,15]. *Photobacterium phosphoreum* is a spoiler of cold water fish, but it is also present in Mediterranean species [15–19].

The spoilage of fish products includes the production of short-chain peptides, amino acids and other nonprotein nitrogen molecules [20–23], trimethylamine (TMA), total volatile nitrogen (TVB-N), sulfuric compounds, aldehydes, ketones and esters [2–4,8], lactic acid, acetic acid, ethanol, hydrogen sulfide, thiols, mercaptans, dimethyl sulfides, and indole, which produce urinary odours in fish meat [24–31]. When aerobically stored, fresh fish are particularly spoiled by *Pseudomonas* spp., producing TVB-N [25,31], which increases at the end of storage. Conversely, when stored in MAP, TVB-N development is slower [2,3] because *Pseudomonas* growth is suppressed, and lactic acid bacteria (LAB) and *Brochothrix thermosphacta* predominate and produce organic acid and volatile compounds [32]. Finally, *Hafnia alvei*, *Proteus* spp., *Pseudomonas* spp., *Shewanella putrefaciens* and *Morganella morganii* [17,33,34] decarboxylate amino acids to biogenic amines, which represents a risk for consumers [35–37]. An increase in the amount of these compounds is suitable only as an acceptance/rejection criterion and is not suitable as a freshness index [24,25].

Different and current technologies, including refrigeration of the products after air or MAP or adding natural preservatives (e.g., essential oils), have been used in order to increase the shelf-life of sea bass and sea bream fish. Unfortunately, these strategies do not permit the entire control of spoilage bacteria. Therefore, new technologies are needed to increase the shelf life of fresh fish [20]. Recently, a new potential approach to

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prolong the shelf life of fresh products was developed using biopreservation systems [1,23], which consist of the use of natural or controlled microbiota or natural antimicrobials as a way of preserving food and extending its shelf life [1,38]. In particular, the use of lactic acid bacteria (LAB), which have antagonistic properties against spoilage and pathogenic microorganisms and are considered generally recognized as safe (GRAS), has been suggested. Indeed, LAB compete for nutrients and produce metabolites with antimicrobial activities such as lactic and acetic acid, hydrogen peroxide, and peptide bacteriocins [1,20]. Different LAB strains are normally used directly or in combination with other preservative techniques (antimicrobials, sodium alginate) because they can inhibit the activities of a wide spectrum of microorganisms, including spoilers and pathogens in food [39,40].

The aim of this work was to improve the shelf life of farmed sea bass and sea bream using different methods, including vacuum packaging (VP), MAP, and bioprotective culture consisting of *Latilactobacillus sakei*. Microbiological, physicochemical, and sensory quality indices were monitored to confirm the effectiveness of biopreservation on product quality during proper (4 ± 2 °C) or abuse (6 ± 2 °C) temperature storage periods and to define a new way of packaging that can be used by the farmed fish production.

2. Methodology

Samples consisted of 3 different lots of gutted sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). The sea bass weighed approximately 474–578 g and were 35 cm long, and the sea bream weighed 404–440 g and were 25 cm long. Both fish were bred in sea cages by Orada Adriatic d.o.o. in Cres (Split), Croatia, collected, and slaughtered after a bath in water and ice. The postmortem period lasted 24 h on ice, and then the fish were vacuum or modified atmosphere Packaged and stored at different temperatures and times before the analysis. VP (−1.0 bar) and MAP (70% N_2, <1% O_2, 30% CO_2) technologies were chosen according to our experience in the field (data not shown), and applied by an Orved VM53 vacuum machine (Italy). The packaging was a multilayer film consisting of polyethylene terephthalate (PET) with a thickness 12 my, aluminium with a layer thickness 9 my, nylon with a thickness 15 my, and polyethylene (PE) NEUTRO with a thickness 75 my. On different days, three samples were collected and subjected to microbial and physicochemical analysis (moisture, pH, TVB-N, thiobarbituric acid reactive substance **(**TBARS) assay, rancidity value). Additional samples were subjected to sensorial analysis.

(a) To compare VP and MAP samples stored at 4 ± 2 °C, analyses were performed on days 0, 6, and 12. At each time point and for each packaging condition and fish species, three samples were collected and analysed. Each lot and each type of packaging included 9 sea bass and 9 sea bream samples.

(b) To study the microbial and physicochemical development of both fish stored in VP at 6 ± 2 °C (simulating abuse temperatures), analyses were performed on days 0, 3, 6, 9, and 12. At each time point and for each fish species, three samples were collected and analysed. Each lot included 15 sea bass and 15 sea bream samples.

(c) To prolong the shelf life of both fish samples stored at 6 ± 2 °C (simulating abuse temperatures) with or without supplementation, a bioprotective starter consisting of *Latilactobacillus sakei* (LAK-23, Sacco s.r.l., Via Alessandro Manzoni 29/A, 22071 Cadorago, CO, Italy) was used. The strain was selected and isolated from meat products and tested for its genetic and phenotypic characteristics. Then, its use was proposed as a starter for meat fermentation and as a bioprotective agent, being a bacteriocin producer, versus *L. monocytogenes* and spoilage microorganisms in meat and fish products.

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Three samples were collected and analysed on days 0, 7, and 14.

2.1. Bioprotective Starter Suspension

The chosen starter was sold freeze-dried in a foil pouch. At the time of use, the starter was thawed, homogenized, and diluted in sterile peptone water (NaCl 0.6%; Peptone, Oxoid, 0.1%, distilled water 1 L). To evaluate its load, dilutions were performed in sterile peptone water, and 0.1 mL of each dilution was inoculated in Petri dishes, to which de Man, Rogosa, and Sharpe (MRS) medium (Oxoid, Italy) was subsequently added by the double layer method. The plates were incubated at 37 °C for 48–72 h, and the grown colonies were counted. Each suspension contained on average approximately 11 log CFU/g. Then, the starter culture was diluted in natural water used to wash the fish before packaging at a level of approximately 7 log CFU/mL.

2.2. Bioprotective Starter Inoculum

The three sequential lots of both gutted sea bass and sea bream samples were dipped in three different washing waters and left for 10 min. Each lot included 27 samples for each fish species. The washing waters included the following:

(a) Natural water (control), 9 each samples/fish/lot;
(b) Natural water supplemented with a bioprotective starter (7 log CFU/mL), 9 samples/fish/lot;
(c) Natural water supplemented with a bioprotective starter (7 log CFU/mL) and 0.1% glucose, 9 samples/fish/lot.

Briefly: the chosen *L. sakei* was further examined as a starter to preserve both sea bass and sea bream in VP. *L. sakei* was added to distilled water, which was then used to wash the fish before packaging. In particular, one group of both fish (St) was washed with water supplemented with the starter; another group (StG) was washed with starter supplemented with 0.1% glucose (Oxoid, Italy), and another group (C) was washed with distilled water as a control. After washing and VP, the level of the starter in both fish was approximately 5 log CFU/g. Then, the washed samples were vacuum packed using the abovementioned methodology and stored at 6 ± 2 °C for up to 14 days. On days 0, 7, and 14, three samples were collected and subjected to microbial and physicochemical analysis. Additional washed samples were used at 14 days for sensorial analysis.

2.3. Microbiological Analysis

Total viable microbial counts (TVCs) were evaluated on plate count agar (Oxoid, Milan, Italy) incubated at 30 °C for 48–72 h. LAB were counted in MRS medium (Oxoid, Milan, Italy) after incubation at 30 °C for 48 h; total coliforms were counted on violet red bile lactose agar (VRBLA, Oxoid, Milan, Italy) incubated at 37 °C for 24–48 h; *Enterobacteriaceae* were counted in violet bile glucose agar (VRBGA, Oxoid, Milan, Italy) incubated at 37 °C for 48 h; *E. coli* were counted in Coli-Id (bioMèrieux, Marcy-l'Étoile, France) incubated at 37 °C for 48 h; *Pseudomonas* spp. were counted on *Pseudomonas* agar base (Oxoid, Milan, Italy) supplemented with CFC (Oxoid, Milan, Italy) and incubated at 30 °C for 48 h; Enterococci were counted in kanamycin aesculin agar (Oxoid, Milan, Italy) incubated at 37 °C for 48 h; sulfite-reducing Clostridia were quantified in differential reinforced clostridial medium (DRCM, VWR, Radnor, PA, USA) after incubation at 37 °C for 24–48 h in an anaerobic jar, using an anaerobic kit (gas pack anaerobic system, BBL, Becton Dickinson, Franklin Lakes, NJ, USA). *Listeria monocytogenes* and *Salmonella* spp. were investigated according to ISO methods 11290/1 [41] and 6579–1 [42], respectively.

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2.4. Physico-Chemical Analysis

The pH value was measured using a pH metre (Basic 20, Crison Instruments, Spain), by inserting the probe into 3 different points on each sample. The moisture content was measured by the A.O.A.C. [43] method. The Pearson method [44] was used to evaluate the TVB-N concentration (expressed in milligrams of N/100 g product), and the method described by Ke et al. [45] was used to evaluate the oxidation stability during storage (TBARS; expressed in nanomoles of malonaldehyde/g).

Sensorial Analysis

Sensorial analyses were performed by 20 nonprofessional and non-trained assessors (10 women and 10 men, representing food technology students aged between 22 and 24 years of age). Nonprofessional assessors were chosen because they represent typical consumers. Each tested sample was packaged in aluminized paper and cooked at 180 °C for 30 min in an oven before the sensorial analysis, which was used to evaluate the following:

(a) Comparison between VP and MAP of three sequential lots of gutted sea bass and sea bream samples stored at 4 ± 2 °C at 12 days. Nine samples of each fish species and of each type of packaging were tested;

(b) Comparison between VP sea bass and sea bream samples of three sequential lots stored at 6 ± 2 °C for 14 days. The fish samples were labelled as controls if they were not supplemented with a bioprotective starter (samples a), St if they were supplemented with a bioprotective starter (samples b), and StG if they were supplemented with a bioprotective starter and glucose (0.1%) (samples c). Nine samples of each treatment for each lot were tested.

For both tests, sensory analysis was performed using the triangle test methodology, ISO 4120:2004 [46]. After cooking, the products were cooled at 65 °C and used for sensory evaluation.

For test a, the nonprofessional assessors were presented with three products, two of which were identical. The assessors were asked to state which product they believed was the odd one out ($p < 0.05$). The assessors who identified the different samples were asked to indicate their preference. The scoring system used was (VP samples versus MAP samples): 1 (excellent), 2 (good), 3 (sufficient), and 4 (scarce).

For test b, the nonprofessional trained assessors were asked to state which product they believed was a unique sample. The assessors who indicated the presence of two distinct samples were asked to identify the best sample, considering the following parameters: flavour, odour, colour, texture (appearance, surface moisture, and colour), and overall acceptance of the product. The scoring system used was (samples versus samples): 1 (excellent), 2 (good), 3 (sufficient), and 4 (scarce).

2.6. Statistical Analysis

Data were analysed using Statistica 7.0 vers. 8 software (Statsoft Inc., Tulsa, OK, USA, 2008). The values of the different parameters were compared by a one-way analysis of variance and the means were then compared using the Tukey's honest significance test. Differences were considered significant at $p < 0.05$.

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3. Results and Discussion

3.1. Microbial and Physicochemical Evaluation of VP and MAP Gutted Sea Bass and Sea Bream Samples Stored at 4 ± 2 °C

The microbial loads of fresh-gutted, vacuum-packaged, and modified atmosphere-packaged sea bass and sea bream are reported in Tables 1 and 2. Initially, fish freshness was excellent, and as microbial and TVB-N levels increased, the freshness characteristics gradually diminished with time in both VP and MAP samples. VP seemed to maintain the freshness quality better than MAP. More specifically, the freshness characteristics remained of excellent quality for up to 6 days; however, both VP and MAP fish can also be accepted, considering the level of microbial load reached at 12 days, which was equal or less than 8–9 log CFU/g, representing the microbial concentration required to spoil chilled fish [2–4,8]. Conversely, considering the TVB-N value, only the VP samples should be accepted. Indeed, in these VP samples, the TVB-N was always at a level of 35 mg N/100 g, which is the limit of acceptability of fish [47], while the TVB-N of MAP fish exceeded this limit.

Indeed, by consulting the literature, it was determined that the shelf life of fresh fish is based on the storage temperature and atmosphere, the level of initial microbial contamination, and the handling techniques, such as gutting, filleting, and packaging [2,3]. Consequently, in each work, different shelf-life durations are demonstrated. Usually, for fresh whole or gutted sea bass in air or MAP and stored in ice or at 4 °C, the shelf life varies from 8 to 19 days [7,48–50]. Our results are not in agreement with those of various authors [2,3,51,52], who found that the shelf life is reduced to 8 days when the fish are commercialized as fillets stored at 2–4 °C under air, or to 12 days in MAP.

Table 1. Development of microorganisms, TVB-N and TBARS in gutted sea bass packaged under vacuum or in MAP and stored at 4 ± 2 °C.

Microorganisms	**Time (Days)**					
	T0		**T6**		**T12**	
	VP	**MAP**	**VP**	**MAP**	**VP**	**MAP**
Total viable count	2.0 ± 0.2 a	2.0 ± 0.2 a	5.3 ± 0.2 a	7.9 ± 0.5 b	7.9 ± 0.5 a	8.7 ± 0.9 b
Enterobacteriaceae	2.1 ± 0.2 a	2.1 ± 0.2 a	3.4 ± 0.4 a	3.6 ± 0.1 a	4.0± 0.7 a	4.5 ± 0.8 a
Pseudomonas	2.3 ± 0.2 a	2.3 ± 0.2 a	2.3 ± 1.2 a	2.6 ± 0.6 a	2.1 ± 0.6 a	2.4 ± 0.5 a
E. coli	<10 a	<10 a	2.0 ± 0.3 a	2.0 ± 0.2 a	2.0 ± 0.5 a	2.0 ± 0.6 a
Total coliforms	<10 a	<10 a	2.5 ± 0.4 a	2.3 ± 0.3 a	4.0 ± 0.3 a	4.8 ± 0.1 b
Clostridium H_2S+	<10	<10	<10	<10	<10	<10
Lactic acid bacteria	<10 a	<10 a	2.2 ± 0.4 a	2.2 ± 0.3 a	5.7 ± 0.2 a	5.0 ± 0.3 b
Enterococci	<10 a	<10 a	2.0 ± 0.1 a	2.0 ± 0.2 a	2.6 ± 0.5 a	2.3 ± 0.3 a

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TVB-N	13.0 ± 0.2 a	13.5 ± 0.5 a	19.9 ± 0.5 a	20.1 ± 0.3 a	35.2 ± 0.1 a	43.4 ± 0.2 b
TBARS	1.5 ± 0.2 a	1.5 ± 0.1 a	2.7 ± 0.3 a	2.4 ± 0.3 a	2.5 ± 0.3 a	2.6 ± 0.2 a

Legend: *Data represent the means ± standard deviations of the total samples; Mean with the same letters within each line (following the values), regardless of packaging method and storage time are not significantly differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; <10 CFU/g; TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g.*

Table 2. Development of microorganisms, TVB-N and TBARS in sea bream packaged under vacuum or in MAP and stored at 4 ± 2 °C.

Microorganisms	**Time (Days)**					
	T0		**T6**		**T12**	
	VP	**MAP**	**VP**	**MAP**	**VP**	**MAP**
Total viable count	2.3 ± 0.1 a	2.3 ± 0.2 a	4.5 ± 1.5 a	5.4 ± 0.2 a	5.3 ± 0.3 a	5.9 ± 0.2 b
Enterobacteriaceae	2.1 ± 0.3 a	2.0 ± 0.1 a	2.6 ± 0.3 a	2.3 ± 0.1 a	3.9 ± 0.4 a	4.7 ± 0.3 b
Pseudomonas	2.4 ± 0.1 a	2.3 ± 0.2 a	2.8 ± 1.6 a	2.7 ± 0.3 a	2.0 ± 0.2 a	2.4 ± 0.6 a
E. coli	<10 a	<10 a	2.1 ± 0.1 a	2.2 ± 0.3 a	2.1 ± 0.3 a	2.4 ± 0.2 a
Total coliforms	<10 a	<10 a	1.9 ± 0.8 a	2.0 ± 0.7 a	3.5 ± 0.4 a	4.7 ± 0.5 b
Clostridium H_2S+	<10	<10	<10	<10	<10	<10
Lactic acid bacteria	<10 a	<10 a	2.4 ± 0.7 a	2.0 ± 0.1 a	5.5 ± 0.4 a	4.7 ± 0.2 b
Enterococci	<10 a	<10 a	2.0 ± 0.1 a	2.0 ± 0.2 a	2.0 ± 0.1 a	2.9 ± 0.9 a
TVB-N	12.9 ± 0.5 a	12.7 ± 0.3 a	21.9 ± 0.1 a	23.5 ± 1.2 a	35.0 ± 0.2 a	42.1± 0.3 b
TBARS	1.6 ± 0.2 a	1.5 ± 0.2 a	2.5 ± 0.2 a	2.6 ± 0.3 a	2.5 ± 0.3 a	2.6 ± 0.2 a

Legend: *Data represent the means ± standard deviations of the total samples; Mean with the same letters within each line (following the values), regardless of packaging method and storage time are not significantly differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; <10 CFU/g; TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g.*

The initial concentration of TVC, *Pseudomonas* and *Enterobacteriaceae* (Day 0) was approximately 2 log CFU/g in both the packaging of the fish samples, while other investigated microorganisms (such as *E. coli*, total coliforms, *Clostridium* H_2S-producing LAB and Enterococci) were not detected (less than the threshold limit of the methods).

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L. monocytogenes and *Salmonella* spp. were not found in any of the tested samples.

Additionally, TVB-N and TBARS levels were initially acceptable (Table 1).

Over 12 days of storage, different microorganisms, except for *Pseudomonas* and *Clostridium* H_2S producers, grew. In particular, in vacuum- or modified atmosphere-packaged MAP sea bass, the TVC level exceeded 7 and 8 log CFU/g, respectively. For sea bream, the TVC level was 5.3 log CFU/g in VP and 5.9 log CFU/g in MAP. The main spoilage bacteria, such as Enterobacteriaceae and total coliforms, grew less than in TVC, but both microbial groups exceeded concentrations of 3 and 4 log CFU/g, respectively. VP and MAP and the low level of oxygen (<0.5%) promoted LAB growth; consequently, they reached values between 4.7 and 5.7 log CFU/g (Tables 1 and 2). In VP for both fish species, the LAB concentration was higher than that for MAP, while the total coliforms were lower. VP and MAP affect not only the growth rate but also the final populations of spoilage bacteria [2,3].

Again, the increase in CO_2 and the reduction in O_2, mostly suppressing Gram-negative and favoring Gram-positive microorganisms, increase the shelf life of fresh fish, as Gram-negative microorganisms are the main contributors to spoilage, represented by TVB-N production [2,3,8]. Indeed, the main spoilage bacteria, such as *Pseudomona*s spp. and H_2S-producing bacteria, grow fast in air, where they become dominant; however, in reduced oxygen environments, their growth is blocked or limited, as demonstrated by our results, and consequently, the shelf life of fresh fish increases. Most likely, the lower presence of spoilage bacteria justifies the lower TVB-N ($p < 0.05$) concentration and demonstrates the acceptability at 12 days of VP in both fish species. As shown, the TVB-N concentration was 35 mg N/100 g at 12 days in VP for both sea bass and sea bream; this value is considerably acceptable according to Directive 95/149/EEC [47]. Conversely, the TVB-N concentration in MAP fish exceeded the limit, reaching 43.4 mg N/100 g in sea bass and 42.1 mg N/100 g in sea bream samples.

In addition, TBARS values increased (Tables 1 and 2) and remained at a maximum level of 2.5–2.6 nmol/g at the end of storage (12 days). At 0 days, the TBARS values were 1.5 and 1.6 nmol/g for both fish species, and then they slightly increased ($p > 0.05$), reaching acceptable levels (2.5–2.6 nmol/g); consequently, these values must be accepted. According to several authors [45,53], food products are not rancid when TBARS values are <8 nmol/g of the sample, slightly rancid when TBARS is between 9–20 nmol/g, and rancid and unacceptable when the TBARS is >21 nmol/g.

Indeed, the sensory acceptability of the VP or MAP samples was determined by the triangular test. The jury was composed of 20 nonprofessionally trained evaluators. Fifteen out twenty evaluators perceived only light differences between samples in VP and MAP. Before cooking the sea bass and sea bream samples, the jury agreed in affirming that all samples did not show any white or viscous patinas, slime, discolouration, or browning, or off-flavours or off-odours after cooking; 15 out of 20 evaluators believed the sea bass and sea bream samples in VP maintained the typical odours and flavours of fresh fish better. The scoring system used was (VP versus MAP) one (excellent), two (good), three (sufficient) and four (scarce). Based on this scoring, all samples were acceptable by the 20 evaluators. However, the 15 tasters, who found differences between the two types of packaging, preferred the VP samples with respect to the MAP ones, and the final value score was, respectively, two (good) and three (sufficient). Consequently, they preferred samples of sea bass or sea bream in VP. In any case, the evaluators did not perceive any ammoniac odour in samples in MAP, although they showed a higher TVB-N concentration than products in VP.

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Despite the light differences in the microbial loads and the TVB-N values of MAP samples exceeding 35 mg N/100 g, as proposed by the Directive 95/149/EEC [47], it was concluded that the shelf-life of VP and MAP samples of both the fish was approximatively of about 12 days.

Considering that the best sensorial, physicochemical, and microbial results were obtained for gutted sea bass and sea bream samples in VP stored at 4 ± 2 °C, the next phase of the work was to study the shelf life of gutted sea bass and sea bream in VP stored at 6 ± 2 °C, which simulates the normal abuse temperature of supermarkets or consumer fridges.

3.2. Microbial and Physico-Chemical Evaluation of Vacuum-Packaged Sea Bass and Sea Bream Samples Stored at 6 ± 2 °C

The changes in the physicochemical parameters and microbial population of aquaculture farmed gutted sea bream and sea bass in VP during storage at 6 ± 2 °C are shown in Tables 3–6.

For the sea bass samples, all microorganisms, except *Pseudomonas* spp. and *Clostridium* H_2S producers, grew during storage. The TVC level of gutted sea bass was 3.7 log CFU/g (Table 3), which was slightly higher than the initial value obtained previously (Table 1). This confirms that the initial contamination depends on different parameters, such as breeding, farming, slaughtering, gutting, filleting, and packaging [2,3,50,51]. Then, TVC grew at 6 days to a level of 6 log CFU/g and after 12 days of storage to over 8.0 log CFU/g. The obtained data agreed with Cakly et al. [9,13], who measured the same concentration in gutted and ungutted sea bass after 14 days of storage at 4 °C. TVC, reaching 8 log CFU/g, exceeded the value of 7 log CFU/g considered to be the maximum level of acceptability for gutted and ungutted freshwater and marine fish [54], and in our experiment, this was reached 2 days before the TVC counts observed by other authors investigating European whole sea bass stored in ice [7,9,13,48,55]. In our experiment, this highest TVC level depended on the abuse temperature of storage (6 ± 2 °C), which is 2–4 °C higher than the storage temperatures (2–4 °C) used by the abovementioned cited authors. Enterobacteriaceae strains and total coliforms were initially measured at levels of 1.4 and 1.6 log CFU/g, respectively, but after 12 days, the counts increased to values higher than 5 log CFU/g.
E. coli seemed to have grown, but considering it is a mesophilic strain, it is doubtful that this was real growth but rather growth that depended on the sample, which changed at any analytical time.

Additionally, LAB and Enterococci grew, stimulated by the vacuum (Table 3). In contrast, *Pseudomonas* spp. did not increase solely due to the vacuum, considering that they are closely aerobic. Indeed, *Pseudomonas* spp. are the dominant spoilage microorganisms of chilled stored fish, either air caught or farmed from the warm, temperate waters of the Mediterranean Sea [2,3,7,49,56–58], but fish in VP or MAP are dominated by LAB, which are strictly microaerophiles [2,3,59]. In this group of analyses, neither *L. monocytogenes* nor *Salmonella* spp. were found, which demonstrated good hygienic quality and good manufacturing practice applied during the processing of fresh gutted sea bass.

Table 3. Development of microorganisms in gutted sea bass packaged under vacuum and stored at 6 ± 2 °C.

Microorganisms	Time (Days)				
	0	**3**	**6**	**9**	**12**
Total viable count	3.7 ± 1.2 a	5.7 ± 0.4 b	6.0 ± 0.2 b	7.4 ± 0.1 c	8.0 ± 0.4 d

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Enterobacteriaceae	1.4 ± 0.1 a	3.5 ± 0.3 b	3.8 ± 0.3 b	4.3 ± 0.6 b	5.8 ± 0.1 c
Pseudomonas spp.	2.4 ± 0.7 a	2.0 ± 0.2 a	2.0 ± 0.3 a	2.1 ± 0.1 a	2.2 ± 0.1 a
E. coli	<10 a	2.7 ± 0.2 b	2.9 ± 0.1 b	<10 a	3.6 ± 0.6 c
Total Coliforms	1.6 ± 0.1 a	3.5 ± 0.1 b	3.3 ± 0.2 b	3.5 ± 0.1 b	5.1 ± 0.2 c
Clostridium H_2S+	<10	<10	<10	<10	<10
Lactic acid bacteria	<10 a	3.7 ± 0.4 b	4.7 ± 0.2 c	6.0 ± 0.3 d	6.1 ± 0.7 d
Enterococci	<10 a	<10 a	<10 a	2.9 ± 0.3 b	3.4 ± 0.7 b

Legend: *Data represent the means ± standard deviations of the total samples; Mean with the same letters within each line (following the values), regardless of packaging method and storage time are not significantly differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; <10 CFU/g.*

Table 4 shows the physicochemical parameters of the tested gutted sea bass. As shown, the moisture, pH and TBARS values did not change significantly. During storage, the means of the abovementioned parameters seemed to change, but considering the large standard deviation, the change was not significant ($p > 0.05$). In addition, the different means and standard deviations observed could be due to the three analysed samples, which changed at each analytical time. Conversely, the TVB-N concentration changed during storage ($p < 0.05$), which confirmed the effects of microbial growth, as suggested by Hebard et al. [60]. At the beginning of storage, the TVB-N value of the tested gutted sea bass was 12.9 ± 0.3 mg N/100 g and then increased and reached a value of approximately 39 ± 1.3 mg N/100 g (Table 4). This value indicates that spoilage had just started at 12 days. Indeed, at 6 days of storage, the TVB-N value was approximately 31.5 ± 1.3 mg N/100 g; this value is considered acceptable according to the limit proposed by EC/1995 [52], which is 35 mg N/100 g. The initial TVB-N concentration is typically between 5 and 20 mg N/100 g [9,13], but at the end of storage, it was over 30–35 N/100 g, which is the concentration that is generally regarded as the limit of acceptability for ice-stored cold water fish [10,61].

Table 4. Physicochemical values of gutted sea bass packaged under vacuum and stored at 6 ± 2 °C.

Parameter	**Time (Days)**				
	0	**3**	**6**	**9**	**12**
Moisture	79.5 ± 0.3 a	77.6 ± 0.9 b	76.3 ± 0.9 b	77.2 ± 2.0 b	76.6 ± 0.8 b
pH	6.16 ± 0.03 a	6.03 ± 0.09 a	6.06 ± 0.07 a	5.91 ± 0.01 a	6.03 ± 0.04 a
TVB-N	12.9 ± 0.3 a	11.0 ± 3.5 a	21.0 ± 0.9 b	31.5 ± 1.3 c	39.0 ± 1.2 d
TBARS	1.6 ± 1.2 a	2.4 ± 1.2 a	2.8 ± 0.5 a	2.4 ± 0.6 a	2.6 ± 0.3 a

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***Legend**: Moisture %, TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g. Data represent the means ± standard deviations of the total samples; Mean with the same letters within a lanes (following the values), considering each single parameter regardless of the times, are not significantly differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point.*

Table 5. Development of microorganisms in sea bream packaged under vacuum and stored at 6 ± 2 °C.

Microorganisms	**Time (Days)**				
	0	**3**	**6**	**9**	**12**
Total viable count	2.3 ± 0.1 a	2.3 ± 0.2 a	4.5 ± 1.5 b	5.4 ± 1.2 b	5.5 ± 1.9 b
Enterobacteriaceae	2.1 ± 0.3 a	2.0 ± 0.1 a	2.6 ± 0.3 b	2.3 ± 0.1 b	4.9 ± 0.4 c
Pseudomonas spp.	2.2 ± 0.3 a	2.0 ± 0.4 a	2.0 ± 0.5 a	2.1 ± 0.2 a	2.5 ± 0.3 a
E. coli	<10 a	<10 a	2.1 ± 0.1 b	2.2 ± 0.3 b	2.1 ± 1.1 b
Total coliforms	<10 a	<10 a	1.9 ± 0.8 b	2.0 ± 0.9 b	4.5 ± 0.8 c
Clostridium H_2S+	<10	<10	<10	<10	<10
Lactic acid bacteria	<10 a	<10 a	2.4 ± 0.7 b	2.0 ± 0.1 b	5.5 ± 0.4 c
Enterococci	2.0 ± 0.1 a	2.0 ± 0.2 a	2.0 ± 0.2 a	2.1 ± 0.2 a	2.0 ± 0.1 a

***Legend:** Data represent the means ± standard deviations of the total samples; Mean with the same letters within lanes (following the values), considering each single parameter regardless of the times, are not significantly differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; <10 CFU/g.*

Table 6. Physicochemical values of gutted sea bream packaged under vacuum and stored at 6 ± 2 °C.

Parameter	**Time (Days)**				
	0	**3**	**6**	**9**	**12**
Moisture	75.3 ± 0.1 a	75.6 ± 0.3 a	76.1 ± 0.2 b	76.2 ± 0.3 b	76.0 ± 0.2 b
pH	6.1 ± 0.1 a	6.0 ± 0.1 a	6.1 ± 0.1 a	5.9 ± 0.1 a	6.0 ± 0.1 a
TVB-N	12.3 ± 0.2 a	11.3 ± 1.5 a	22.0 ± 0.3 b	33.2 ± 0.3 c	35.0 ± 1.2 d
TBARS	1.2 ± 0.8 a	2.2 ± 0.9 a	2.4 ± 0.3 a	2.6 ± 0.3 a	2.7 ± 0.2 a

***Legend**: Moisture %, TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g. Data represent the means ± standard deviations of the total samples; Mean with the same letters within lanes (following the values), considering each single parameter regardless of the times, are not significantly*

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differently ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point.

However, despite the microbial level (8 log CFU/g) and TVB-N values (39 mg N/100 g), the gutted sea bass samples must be accepted, considering that there was no unacceptable odour and that Cakly et al. [9,13] suggested the acceptability of aquacultured sea bass stored in ice, which presented a TVC value above 8 log CFU/g and TVB-N and TBARS values of approximately 50.13 ± 0.25 mg N/100 g, and 2.66 ± 0.06 mg malonaldehyde/kg, respectively.

During the storage of sea bream samples, all the microorganism groups, except *Clostridium* H_2S and Enterococci, grew. At the beginning the TVC concentration was approximately 2.3 log CFU/g (Table 5). Then, it grew, and at 12 days reached 5.5 ± 1.9 log CFU/g. This concentration was similar to data of Cakly et al. [9,13], who measured the same values in gutted and ungutted sea bream after 7 days of storage at 4 °C. Consequently, the level of TVC indicates the fish can be largely acceptable, considering that the final TVC did not exceed 7 log CFU/g, as requested for gutted and ungutted freshwater and marine fish [54].

This adequate TVC value confirms the application of excellent production processes [2,3,50,51], and although it was obtained at abuse temperatures (6 ± 2 °C), it was lower than that obtained by different authors for European whole sea bream stored in ice [2,3,9,13,48,50,51]. Additionally, the concentrations of Enterobacteriaceae and total coliforms were initially low, 2.1 ± 0.3 log CFU/g and less than 10 CFU/g, respectively. During 12 days of storage, both microbial groups grew to 4.9 ± 0.4 log CFU/g and 4.5 ± 0.8 log CFU/g, respectively. *E. coli* did not grow, and at each analysis time, the difference was not significant ($p > 0.05$). Additionally, LAB grew and reached 5.5 ± 0.4 log CFU/g, considering that they are microaerophilic (Table 3). Conversely, *Pseudomonas* spp. demonstrated soft growth, dependent on the residual oxygen in the VP, because *Pseudomonas* spp. are strictly aerobic, but the averages at any time were not significantly different ($p > 0.05$). In sea bream, a higher microbial concentration was represented by LAB, and are strictly microaerophiles [2,3,59]. Additionally, neither *L. monocytogenes* nor *Salmonella* spp. were found in sea bream samples.

The physicochemical parameters of the tested gutted sea bream are shown in Table 6. The moisture, pH, and TBARS values did not significantly change ($p > 0.05$). Only the TVB-N changed at any time during the analysis ($p < 0.05$), confirming the effects of microbial growth suggested by different authors [2,3,9,13,55,60]. At the beginning of storage, the TVB-N values of the tested gutted sea bream were similar to those of sea bass and were determined to be 12.3 ± 0.2 mg N/100 g. Then, the TVB-N value increased according to the time of storage and reached a value of approximately 35.0 ± 1.2 mg N/100 g (Table 6) at 12 days of storage. This value must be considered largely acceptable according to the limit proposed by EEC/1995 [52] and for ice-stored cold water fish [10,61], which is 35 mg N/100 g. The final TVB-N concentration in sea bream samples was lower than that in sea bass samples because the former contained a lower concentration of spoilage microorganisms. The levels of TVC and Enterobacteriaceae in sea bream were 2.5 and 1 log CFU/g lower, respectively, than those in sea bass. Therefore, it could be demonstrated that the presence of lower spoilage microorganism concentrations corresponds to lower TVB-N concentrations. Microbial and physicochemical data demonstrated that the sea bream tested must be accepted, considering that there was no unacceptable odour and that they presented a TVB-N of less than 8 log CFU/g, a TVB-N of less than 50.13 ± 0.25 mg N/100 g and a TBARS of less than 2.66 ± 0.06 mg malonaldehyde/kg [9,13].

Finally, considering the TVC, TVB-N, and TBARS values, it seems that both the VP gutted fish can be accepted until 12 days of storage at 6 ± 2 °C and, consequently, this time can represent the limit of their shelf-life.

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Considering their economic value and the growing interest of consumers in their nutritional aspects, the next aim was to prolong their shelf life until 14 days.

Fresh fish are rapidly susceptible to spoilage due to microbiological and biochemical degradation [1,17], and, to extend their shelf life, different preservative technologies are used, such as heat processing, chemical preservatives, MAP, and refrigeration [1]. These technologies are extensively used, but they do not completely control spoilage bacteria. In particular, some technologies, such as heat processing and antimicrobial compounds, cannot be used to preserve fresh fish. Heat processing changes the texture of fish, which becomes processed food, and synthetic preservatives are not acceptable by consumers, who increasingly demand high-quality, but minimally processed, seafood [62]. Therefore, the abovementioned technologies cannot be used to preserve fish.

The use of bioprotective methods is a new, modern, and promising method largely used in other food fields to obtain good results against spoilage and pathogenic microorganisms without changing the texture, flavour, or odour of the product [23,63,64]. Among LAB, *Latilactobacillus sakei* is frequently used in bioprotective technology [65,66]. In particular, LAK-23, a commercialized bioprotective starter culture based on *L. sakei*, was chosen to try to achieve our objective, considering that LAB originally isolated from certain food products are the best starter cultures for these same products, because they would be more competitive than LAB from other sources [23,67]. Starter cultures and LAB, in particular, are considered as GRAS by the Food and Drug Administration [68]. This status may be based either on a history of safe use in food prior to 1958 or on scientific procedures, which require the same quantity and quality of evidence as would be required to obtain food additive regulations. In Europe, starter cultures are granted Qualified Presumption of Safety (QPS) status if reasonable evidence is provided. A safety assessment can be made based on four pillars: taxonomic identification, body of knowledge, possible pathogenicity ('safety concerns'), and end use [69]. The body of knowledge is one of the pillars of the QPS evaluation and is investigated based on the scientific literature [70]. QPS provides a safety status for microorganisms intentionally used in the food and feed chain, certifying that they do not pose a risk to human and animal health [69,70]. Consequently, *L. sakei* is traditionally and largely used as a starter to promote food ripening, and as a bioprotective agent against pathogenic and spoilage microorganisms.

The data regarding the different washing treatments are shown in Tables 7 and 8. During storage at 6 ± 2 °C, the starter consisting of *Latilactobacillus sakei* grew until the end of the experiment (14 days) and reached values over 6 log CFU/g; conversely, in the control samples, the level of autochthonous LAB was always less than 5.5 log CFU/g (Tables 7 and 8). LAB growth inhibited spoilage microorganisms such as total coliforms and *Enterobacteriaceae*, considering that *Pseudomonas* growth was blocked by LAB and, above all, by VP. Indeed, at the end of the storage, the *Enterobacteriaceae* concentrations in the sea bass samples washed with starter (St) and starter added with sugar (StG) were lower than in the samples washed with water (C), and were 4.4 ± 0.1 and 3.3 ± 0.2 CFU/g ($p < 0.05$), respectively. Conversely, in the C samples, they were 4.9 ± 0.3 CFU/g. Different concentrations were also present at level of total coliforms ($p < 0.05$).
Indeed, in C., St, and StG samples the total coliforms reached values of 5.0 ± 0.3, 4.1 ± 0.3, and 3.1 ± 0.2 CFU/g, respectively. Similar behaviour could be observed in sea bream samples. Indeed, the *Enterobacteriaceae* concentrations in samples washed with starter (St) and starter added with sugar (StG) were lower than in the samples washed with water (C), and were 4.3 ± 0.1 and 3.4 ± 0.2 CFU/g ($p < 0.05$), respectively. Conversely, in the C samples, they were 4.9 ± 0.3 CFU/g. The different concentrations were also present at the level of total coliforms ($p < 0.05$).
Indeed, in C., St, and StG samples the total coliforms reached values of 4.4 ± 0.3, 4.0 ± 0.3, and 3.0 ± 0.2 CFU/g, respectively. The reduced growth of both the total coliforms and *Enterobacteriaceae* depended on

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the added LAB starters, which grew over 6 log CFU/g. Indeed, in sea bass and sea bream C samples, the LAB reached 5.3 ± 0.2 and 5.3 ± 0.1 CFU/g, while in St they were 6.5 ± 0.2 and 6.0 ± 0.1 CFU/g and in StG they were 7.2 ± 0.2, and 6.9 ± 0.5 CFU/g, respectively.

Table 7. Development of microorganisms, TVB-N, and TBARS in VP packaged sea bass added with or without bioprotective cultures and glucose (0.1%) and stored at 6 ± 2 °C.

Microorganisms	**Time (Days)**								
	T0			**T7**			**T14**		
	C	**St**	**StG**	**C**	**St**	**StG**	**C**	**St**	**StG**
Total viable count	2.0 ± 0.2 a	2.0 ± 0.1 a	2.1 ± 0.3 a	3.3 ± 0.2 b	3.1 ± 0.3 b	4.1 ± 0.1 c	6.0 ± 0.1 d	6.4 ± 0.3 d	6.3 ± 0.2 d
Pseudomonas spp.	2.3 ± 0.2 a	2.3 ± 0.1 a	2.0 ± 0.2 a	2.3 ± 0.5 a	2.3 ± 0.5 a	2.4 ± 0.1 a	2.1 ± 0.3 a	2.1 ± 0.2 a	2.2 ± 0.1 a
Lactic acid bacteria	2.0 ± 0.1 a	5.0 ± 0.3 b	5.0 ± 0.3 b	2.8 ± 0.4 a	6.2 ± 0.2 c	7.0 ± 0.5 d	5.3 ± 0.2 b	6.5 ± 0.2 c	7.2 ± 0.5 d
Enterococci	$<10^2$ a	$<10^2$ a	$<10^2$ a	2.0 ± 0.1 a	2.0 ± 0.1 a	2.0 ± 0.1 a	2.6 ± 0.5 a	2.6 ± 0.5 a	2.3 ± 0.3 a
Total coliforms	<10 a	<10 a	<10 a	3.0 ± 0.2 a	2.5 ± 0.4 a	2.2 ± 0.5 b	5.0 ± 0.3 c	4.1 ± 0.3 d	3.1 ± 0.2 b
E. coli	<10 a	<10 a	<10 a	2.3 ± 0.3 b	2.1 ± 0.3 b	2.2 ± 0.1 b	2.5 ± 0.3 b	2.4 ± 0.3 b	2.3 ± 0.2 b
Enterobacteriaceae	2.1 ± 0.2 a	2.3 ± 0.1 a	2.2 ± 0.2 a	3.4 ± 0.4 a	2.9 ± 0.1 a	2.5 ± 0.3 a	4.9 ± 0.3 b	4.4 ± 0.1 b	3.3 ± 0.2 a
Clostridium H_2S+	<10	<10	<10	<10	<10	<10	<10	<10	<10
pH	6.0± 0.1 a	6.0± 0.1 a	6.0± 0.1 a	6.0± 0.2 a	6.0 ± 0.1 a	6.0± 0.3 a	6.1± 0.2 a	6.0± 0.2 a	6.0 ± 0.1 a
TVB-N	12.7 ± 0.1 a	12.9 ± 0.1 a	12.9 ± 0.3 a	22.5 ± 1.5 b	21.5 ± 1.5 b	19.0 ± 1.2 b	42.2 ± 0.2 c	37.2 ± 1.2 d	30.2 ± 0.3 e
TBARS	1.7 ± 0.2 a	1.5 ± 0.1 a	1.7 ± 0.3 a	2.0 ± 0.3 a	2.0 ± 0.1 a	2.1 ± 0.2 a	2.2 ± 0.3 a	2.3 ± 0.1 a	2.2 ± 0.2 a

Legend: *C: Control: without bioprotective starter; St: with bioprotective starter, and StG: with bioprotective starter and glucose (0.1%) added. Data represent the means ± standard deviations of the total samples; Mean with the same letters within lanes (following the values) are not significantly different ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; <10–10^2 CFU/g; TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g.*

Table 8. Development of microorganisms and TVB-N, and TBARS in VP packaged sea bream added with or without bioprotective cultures and glucose (0.1%) and stored at 6 ± 2 °C.

Microorganisms	**Time (Days)**								
	T0			**T7**			**T14**		
	C	**St**	**StG**	**C**	**St**	**StG**	**C**	**St**	**StG**
Total viable count	2.3 ± 0.2 a	2.3 ± 0.1 a	2.3 ± 0.3 a	4.3 ± 0.2 b	4.1 ± 0.3 b	4.0 ± 0.1 b	6.9 ± 0.3 c	6.2 ± 0.5 c	6.1 ± 0.4 c
Pseudomonas spp.	2.3 ± 0.2 a	2.3 ± 0.1 a	2.0 ± 0.2 a	2.8 ± 1.2 a	2.3 ± 0.3 a	2.0 ± 0.2 a	2.3 ± 0.5 a	2.1 ± 0.2 a	2.1 ± 0.1 a
Lactic acid bacteria	2.0 ± 0.1 a	5.0 ± 0.3 b	5.0 ± 0.5 b	2.8 ± 0.6 a	5.9 ± 0.2 b	6.8. ± 0.3 c	5.3± 0.1 d	6.0 ± 0.1 b	6.9 ± 0.5 c

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Enterococci	$<10^2$ a	$<10^2$ a	$<10^2$ a	2.0 ± 0.2 a	2.0 ± 0.3 a	2.0 ± 0.1 a	2.5 ± 0.3 b	2.6 ± 0.3 b	2.0 ± 0.5 ab
Total coliforms	<10 a	<10 a	<10 a	2.2 ± 0.4 b	2.3 ± 0.3 b	2.1 ± 0.3 b	4.4 ± 0.3 c	4.0. ± 0.3 c	3.0 ± 0.2 d
E. coli	<10 a	<10 a	<10 a	2.3 ± 0.3 b	2.1 ± 0.3 b	2.2 ± 0.1 b	2.1 ± 0.3 b	2.1 ± 0.1 b	2.1 ± 0.2 b
Enterobacteriaceae	2.2 ± 0.3 a	2.2 ± 0.1 a	2.2 ± 0.1 a	2.5 ± 0.2 a	2.3 ± 0.6 a	2.2 ± 0.3 a	4.9 ± 0.3 b	4.3 ± 0.1 c	3.4 ± 0.2 d
Clostridium H_2S+	<10	<10	<10	<10	<10	<10	<10	<10	<10
pH	6.0 ± 0.1 a	6.0 ± 0.1 a	6.0 ± 0.1 a	6.0 ± 0.1 a	6.0 ± 0.3 a	6.0 ± 0.3 a	6.1± 0.1 a	6.0 ± 0.3 a	6.0 ± 0.2 a
TVB-N	12.3 ± 0.2 a	12.2 ± 0.3 a	12.2 ± 0.1 a	22.9 ± 0.5 b	21.9 ± 0.8 b	19.0 ± 0.2 c	42.5 ± 1.2 d	38.2 ± 0.8 e	31.2 ± 0.2 f
TBARS	1.5 ± 0.2 a	1.5 ± 0.1 a	1.6 ± 0.2 a	2.0 ± 0.1 b	2.0 ± 0.2 b	2.1 ± 0.1 b	2.2 ± 0.1 b	2.1 ± 0.2 b	2.2 ± 0.1 b

Legend: C: Control: without bioprotective starter; St: with bioprotective starter, and StG: with bioprotective starter and glucose (0.1%) added. Data represent the means ± standard deviations of the total samples; Mean with the same letters within a lanes (following the values). are not significantly different ($p < 0.05$). Analyses were conducted in triplicate on three different samples per each sampling point. Data log CFU/g; $<10–10^2$ CFU/g; TVB-N—Total volatile basic nitrogen mg N/100 g; TBARS: nmol malonaldehyde/g.

In addition, the TVC level was similar in all samples independent of the starter, and no significant differences were observed among the samples ($p > 0.05$), as found by Bassi et al. [23]. Finally, in these groups of fish, neither *L. monocytogenes* nor *Salmonella* spp. was ever found. The activity of the starters was confirmed by the change in pH and TVB-N level. Indeed, the final pH was approximately 6.08 (St) and 6.04 (StG) in sea bass-inoculated samples and 6.04 (St) and 6.02 (StG) in sea bream-inoculated samples, while in the controls, the final pH was 6.11 in both fish species. These data do not agree with those of other authors, who found that in vacuum-packed sea bass, the pH decreased to 5.6 units [23]. In StG samples, a higher pH decrease was expected because of the added sugar. This can be explained by the limited final LAB loads (less than 7.5 CFU/g). In each case, its value was less than that in the control samples, where the pH decrease was very limited, given the small level of glucose initially present in the fish flesh [8].

Again, the TVB-N value increased in all samples. At 14 days of storage, the TVB-N concentration of the StG samples was approximately 30.2 and 31.2 mg N/100 g and that of the St samples was approximately 37.2 and 38.3 mg N/100 g in sea bass and sea bream, respectively. Conversely, in the C samples for both fish species, the level of TVB-N was always greater than 40 mg N/100 g (Tables 7 and 8).

This lower TVB-N value in the StG and St samples depends on the reduced activity of *Enterobacteriaceae*, as previously demonstrated by Gram and Huss [8]. Indeed, *Enterobacteriaceae* and, consequently, total coliforms, are recognized to be responsible for TVB-N and trimethylamine production [8,23]. Therefore, the starter LAB suppressed the spoiling bacteria, yielding a reduction in the TVB-N concentration. This is in agreement with data on the LAB inoculation effect [8,23,65,71]. The abovementioned authors noticed that the use of starter cultures with antimicrobial properties against *Listeria* sp. and psychotropic bacteria could reduce the risk of biogenic amine and, consequently, TVB-N formation, whose production in vacuum-packed fishes depends on psychotropic bacteria that proliferate slowly and dominate the mesophilic bacterial load, because low temperatures favour their growth [8,30,59,72–75]. Finally, the TBARS levels of all the tested samples (C, St, StG) always remained less than or equal to 2.2 nmol/g, demonstrating that VP protects both

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fish from rancidity (Tables 7 and 8). Additionally, in this case, all the samples can be acceptable given the TBARS values, as suggested by Cakly et al. [9,13].
Based on the physicochemical results, it can be concluded that the use of starter culture can prolong the shelf life of sea bass and sea bream in VP until 14 days of storage at 6 ± 2 °C, a temperature that is considered typical of supermarkets and consumer fridges. The TVC, Enterobacteriaceae, total coliforms, and TVB-N concentrations of the StG-inoculated samples met the limit proposed by the ICMSF [54] and EEC/1995 [52], and consequently, they must be largely acceptable until 14 days at 6 ± 2 °C. Additionally, the samples treated with only the starter can be accepted, despite the level of TVB-N exceeding the limit proposed by EEC/1995 [52].

3.3. Sensorial Analysis

In addition to food preservation, sensory characteristics cannot be neglected as the main factors responsible for product acceptance [1]. Indeed, the samples developed in this study were also judged by nontrained nonprofessional evaluators; therefore, it was decided to show only the overall quality attributes, because the other sensory descriptors demonstrated a similar trend. The sensory assessment was performed on day 14 of the storage period, because, on this day, the products could be accepted, considering the microbial and TVB-N level, and the results obtained by a triangular test are shown in Table 9. The obtained results demonstrated that all nonprofessional evaluators identified the samples based on the three treatments (Table 9) and that there were no great differences in the samples.

Table 9. Sensorial evaluation by not professional trained panelists.

Fishes Samples		Difference	Final Values Score *
Sea bass	C versus St	+20/20	3/1
	C versus StG	+20/20	3/1
	St versus StG	+20/20	2/1
Sea bream	C versus St	+20/20	3/1
	C versus StG	+20/20	3/1
	St versus StG	+20/20	2/1

Legend: + n. positive assessments/total assessments; C not inoculated samples with. bioprotective starter; St samples inoculated with bioprotective starter; StG samples inoculated with bioprotective starter and added with dextrose (0.1%). * Scores (samples versus samples) 1 (excellent). 2 (good). 3 (sufficient).

The small difference in pH between samples washed with St, STG, and C was not valued significantly by the panelists. Indeed, the panel identified a slightly acidic, nondisturbing taste in the St and StG samples.

Finally, the three treatments were differentiated by the score attributed to the investigated parameters, such as flavour, odour, colour, texture (appearance, surface moisture, and colour), and overall acceptance of the product. The scoring system used was (samples versus samples) one (excellent), two (good), three (sufficient), and four (scarce). Based on this scoring, all samples were acceptable; in particular, the 20

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evaluators preferred the following in descending order of acceptability: StG, St, and C. Therefore, it was proposed to use bioprotective starters diluted in water supplemented with glucose to prolong the shelf life until 14 days at 6 °C for either fresh sea bass or fresh sea bream.

4. Conclusions

Fish meat is very perishable because of indigenous and microbial enzymes, which determine spoilage and shelf life. The deterioration processes depend on different parameters and, in particular, by the type of packaging and by the storage conditions.

In this paper, different technologies have been used in order to prolong the shelf-life of fresh sea bass and sea bream:

Vacuum and modified atmosphere packaging (MAP) were first compared in order to choose the best packaging for both the fish. Independently of the type of packaging, data showed light differences between the fish samples during proper refrigeration (4 ± 2 °C) and a similar shelf-life of about 12 days. However, either for TVB-N values or for sensorial analysis, the VP was considered the best packaging.

Indeed, based on the applied scoring, the 15 out 20 tasters, who found difference between the two type of packaging, preferred the VP samples with respect to MAP ones, and the final value score was respectively two (good) and three (sufficient).

The shelf-life of 12 days was also confirmed by the evaluation of microbiological, physicochemical (TVB-N), and sensory quality indices in VP fish stored at abuse temperature (6 ± 2 °C, simulating supermarkets and consumer fridges) during the storage period.

However, to prolong the shelf life of both the fish, different methods occurred. In particular, a method was employed that washed the gutted sea bass and sea bream in water added with or without dextrose (0.1%), and inoculated them with bioprotective starter (7 log CFU/mL). After washing the samples, they were subjected to VP and stored at 6 ± 2 °C.
The bioprotective starter permitted a reduction in growth of spoilage microorganisms and the increasing of the TVB-N concentration, which in both fish was less than 35 mg N/100 g product. Consequently, the shelf-life of both fish was about 14 days.
In addition, nonprofessional and untrained evaluators confirmed the acceptability of the inoculated samples by sensorial analysis. Indeed, they considered the fish treated with StG excellent, and the ones treated with St good.

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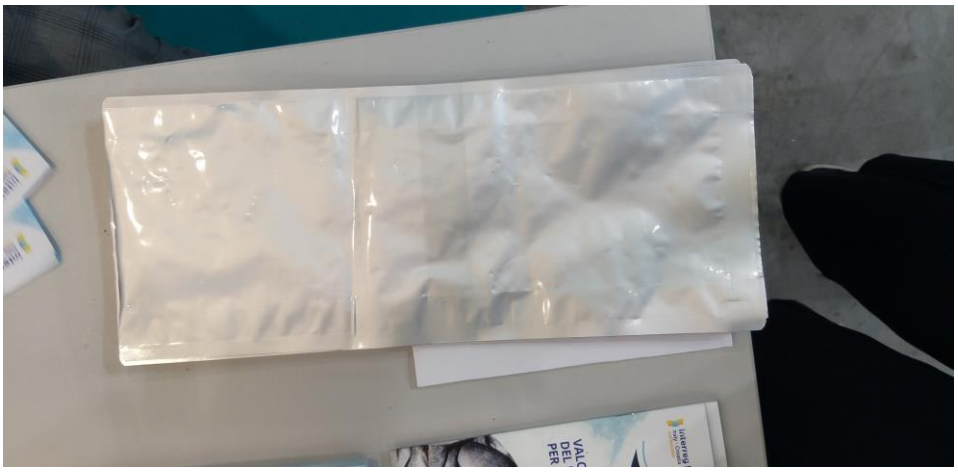
Figure 1: vacccum packed eviscerated sea bass tested for extended shelf life, UNIUD, 2021.

Figure 2: New packeging, presented at the training in Pordenone, May 2022



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List of equipment used during project:

Ref.	Image (photo with the project label on)	Description (name of the equipment and short description of the object)	Station Town and PP where is places
1		Incubator – To maintain standard temperature for bacteria growth	Di4a
2		Conventional PCR thermocycler To test genes and identify microorganisms	Di4a
3		Minicentrifuge Used for centrifugation of small quantities of ingredients used in molecular techniques	Di4a
4		Orved VM53 vacuum machine Used to package fish and fish products	Di4a
5		Autoclave Used to sterilize broths and agars	Di4a
6		Microscope Used to observe the morphology of the microorganisms.	Di4a

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- **PART 2**

  The second part provides the final results and a collection of data from the WP and project in relation to the General objectives at the Programme level that we will need to add to the final report.

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A. CONTRIBUTION TO EUSAIR

Please provide a description of the project contribution to the EUSAIR in terms of synergy with the Strategy's pillars and alignment of implemented project's activities with the Action Plans and labelled projects.

Project contributes to the EUSAIR Strategy's pillar "blue growth" and through innovation and development of the sustainability of aquaculture in the Adriatic Sea establishes a basis for the development of aquaculture in the whole EUSAIR region. In particular, a network of academia and industry worked together in the enhancing profitable, high-quality and sustainable aquaculture production which is capable to contributes to job creation and economic growth of rural and outlying island communities as well as to supply of healthy food products, respecting the EU and international rules. The results of task 5.2 will increase the shell-life of fresh fish and consequently the economic value of marine aquaculture sector and can be easily transferred to other territories of the EUSAIR especially those missing specialised research centres as well as other Mediterranean areas.

B. CONTRIBUTION TO HORIZONTAL PRINCIPLES

Please provide a description of the project contribution to the horizontal principles of equality between men and women, non-discrimination and sustainable development.

The project gathered different experts based on the skills regardless of race, nationality, ethnic origin, religion, disability, age or sexual orientation. In particular, it provides a description of the project contribution to the horizontal principles of equality between men and women, non-discrimination and sustainable development. The focus was the promotion of a healthy and sustainable product from the Adriatic regions, bringing together farmers, scientists, consumers, veterinarians and experts in the field. In particular, task 5.2.2 contributes to the sustainable aquaculture thanks to a sustainable use of new packaging and bioprotective cultures in order to improve the safety and the shelf-life of fish.

C. COMMUNICATION ACTIVITIES

Please refer to the Final Communication Report template and provide a summary on the main achievements trying also to identify which were the most successful communication tools in reaching general public/decision makers/other target groups.

All activities were disseminated through different media channels (social media such as Facebook, Twitter, LinkedIn), project website, international and national journals and portal sand through different virtual conferences and face to face conferences. Many experts were reached through virtual and online workshops organised to disseminate the project results. However, the most important events were press conference organised in Rijeka that presented the project outputs in 2019, as well as the press conference immediately before the final conference in Zadar on 3rd June 2022, in Udine (June, 21rst, 2022), in Padua (November, 19th, 2021), in Pordenone (Aquafarm, May 25th, 2022), in Ostuni (May 7th, 2022).

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These press conferences raised a huge interest of journalists and reached huge number of general public.

D. NATURA 2000

Please describe, if it is the case, measures foreseen and implemented by the project:

a) In case the project involved Natura 2000 sites, describe what measure the project envisaged and implemented to avoid any negative impact: No Natura 2000 sites are involved
b) In case the project had a positive effect on Natura 2000 sites, please describe which measure the project has foreseen and implemented in order to reach a direct or indirect positive impact: No Natura 2000 sites are involved

E. TYPES OF ACTIONS ADDRESSED (as defined in the Cooperation Programme)

These are our primary objective's types of actions, that we addressed by the Project:

Specific Objectives	***Types of action***	***the most relevant one within the SO addressed by your project***
1.1 Enhance the framework conditions for innovation in the relevant sectors of the blue economy within the cooperation area	*Joint projects and actions aimed at creating platforms, networks and at supporting exchange of good practices in order to enhance the knowledge transfer and capitalization of achieved results in the field of blue economy*	***X***
	Actions aimed at cluster cooperation, joint pilot initiatives in order to boost the creation of marketable innovative processes and products, in the field of blue economy	***X***

Our project is based on *output indicators*

CO01 – 24 enterprises received support,

CO02 – 4 enterprises received grants,

CO04 – 20 enterprises received non-financial support,

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CO42 – 7 research institutions participated in cross-border, transnational or interregional research projects,
CO44 – 578 participants involved in joint local employment initiatives and

F. TYPES OF OUTPUTS PRODUCED

Specify the types of outputs generated by your activity that are reported here and provide a brief description

Output typology	Description
Trainings	Different course either in Italy or in Croatia regarding the safety of the fish have been performed during the project.
Monitoring systems	N.A.
SMEs clusters	Potential collaboration and exchange of work and resources among enterprises involved in the aquaculture business chain, such as fish farms and industries for fish end fish products aquafeeds producing and waste recycling were established. The innovative techniques and protocols implemented during the project within the task 5.2.2 can be applied in other Italian and/or Croatian fish farms and facilities. The cross border production chain that involves Italian hatcheries, which grow sea bass and sea bream fingerlings and juveniles, and Croatian on-growing sea cages-based farms, which than exported the fish to the Italian market, was implemented thanks to the project training courses and events.
New networks	New collaborations among project partners and researchers of Udine University were developed during the project in order to achieve the task 5.2.2 objectives. Moreover, an active cooperation among researchers of LP and fish farmers was developed so as to improve the interest of entrepreneurs for R&D and innovation as well as allow the project to respond to their needs.
Platforms	N.A.
Adaptation plan	N.A.
Building renovation	N.A.
Others (please specify)	N.A.

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G. TYPOLOGY OF IMPACTS

Please indicate what type of impact(s) your project has had. You can choose more than one answer. For each tangible impact selected, please provide a concrete example from your project, where possible supported by quantitative information.

TANGIBLE IMPACTS

Tangible impacts	**Example/ quantitative information**
Improved access to services	N.A.
Cost savings	The new packaging can reduce the costs of fish production
Time savings	The new packaging can reduce the time of fish production
Reduced energy consumption	To Reduce the waste
Reduced environmental impact	The application permits indirectly to reduce energy in fish production, less waste.
(Man-made, natural) risk reduction	N.A.
Business development	In sea bass/bream intensive farms in the Adriatic area will ensure a better productivity and more eco-compatible productions that will be more appreciated by the consumers, increasing the profitability of the mariculture sector.
Job creation	New and permanent employment opportunities to costal populations of both sides of the Adriatic Sea can increase thanks to the knowledge transfer and skills.
Improved competitiveness	New packaging and new products of farmed sea bass/bream on intensive farms in the Adriatic area can improve and ensure an increased competitiveness of SMEs on regional and international markets.
Other tangible impacts (specify)	N.A.

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INTANGIBLE IMPACTS

Intangible impacts	**Example/quantitative information**
Building institutional capacity	N.A.
Raising awareness	The project has stimulated the attention of fish farmers and fish product producers in particular the topics related to the production improvement, with less waste and the use of new marketing techniques.
Changing attitudes and behaviour	New trend of production (new products) and new marketing materials can change the attitudes and behaviour of the consumers and improve the nutritional habits of the population.
Influencing policies	N.A.
Improving social cohesion	N.A.
Leveraging synergies	The project lead to the strengthening of relations between Italian and Croatian research groups, as well as between universities or centres of excellence and fish farmers. The project provides to fish farmers new techniques and protocols for the safe and healthy fish production that can be applied in hatcheries and sea plants, so to improve the sustainability of Mediterranean aquaculture and consequently the competitiveness of sector.

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