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ORIGINAL PAPER

Tuna Burgers Preserved by the Selected *Lactobacillus paracasei* IMPC 4.1 Strain

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Abstract

The use of protective microbial strains in combination with modified atmosphere packaging (MAP) and refrigerated storage on the shelf life of tuna burgers was investigated. Preliminary, the protective ability of three lactic acid bacteria (LAB) cultures (*Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus plantarum*) have been assessed on ready-to-cook tuna burgers. Among them, *L. paracasei* showed the best preserving performance and significantly controlled both aerobic mesophilic bacteria and *Pseudomonas* spp. growth. Subsequently, the efficacy of the selected LAB culture under MAP conditions (5% O_2 and 95% CO_2) was assessed evaluating microbial and sensory quality, as well as volatile aldehyde content. Results indicated that the shelf life of burgers containing *L. paracasei* and packaged under MAP was 4 days longer than the control (shelf life about 6 days) and that the applied procedure represents an effective approach for the mild preservation of fish products.

Keywords Bio-preservation · Mild treatment · Ready-to-cook fish burger · Fish shelf life · MAP · Processed seafood

Introduction

Fish is perceived by consumers as beneficial for health due to its content of high biological value proteins, vitamins, mineral salts, and omega-3 polyunsaturated fatty acids. However, some factors, particularly the high perishability and its time-consuming preparation, represent a barrier to fish consumption (Trondsen et al. 2003; Costa et al. 2014). Poli et al. (2006) have suggested that ready-to-cook headed, gutted, and filleted fish products can promote fish consumption even though the high perishable nature of processed fish strongly limits shelf life. Public awareness on healthy and food safety has increased consumers demand for foods free from pathogens, minimally processed to guarantee an unimpaired sensory quality and preserved without chemical additives (Castellano et al. 2008). An interesting approach to improve microbial food safety is the use of natural microbiota and/or their antimicrobial products as a bio-preservation method (Stiles 1996). Selected LAB strains could be used as protective cultures as they exert an antagonistic effect against potential pathogens and other undesired microorganisms. Their preserving action is mainly related to the competition for nutrients, to the pH lowering and to the production of inhibitory compounds as a result of their metabolism, principally organics acids, such as lactic acid, diacetyl, fatty acids, CO_2, peroxide, and bacteriocins (Gibbs 1987; Klaenhammer 1988; Daeschel 1989; De Vuyst and Vandamme 1994; Stiles 1996; Caplice and Fitzgerald 1999). As reported by Stiles (1996), the protective strains that grow rapidly and produce antagonistic substances can be added directly to food, even though their addition could compromise the sensory properties (Schillinger et al. 1996). Otherwise, bio-preservation could be obtained by adding purified antagonistic substances or fermentation products. Many studies on bio-preservation have focused on the in vitro identification and characterization of bacteriocin-producing LAB strains, as well as on purified bacteriocins and their mode of action in different food model systems. Tomé et al. (2008) isolated bacteriocin-producing LAB from vacuum-packaged cold-smoked salmon with an in vitro anti-listerial activity. Chahad et al. (2012) have demonstrated the ability of *Enterococcus* spp. strains, isolated from farmed sea bream and sea bas, to inhibit the growth of pathogenic and spoiling

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bacterial species due to the production of several enterocins. However, the effectiveness of LAB protective cultures and/or their metabolites needs to be assessed for each specific food. Particularly for fishery products, considering the legal restrictions imposed for the use of purified bacteriocins and the cost-effectiveness of this preservation method, the use of live cells of LAB strains may represent an alternative solution to extend their shelf life, combining this approach with packaging in modified atmosphere and low storage temperature. Modified atmosphere packaging (MAP) is a technology designed to prolong the shelf life of foods by slowing down or blocking the chemical and biological reactions responsible of food matrix decay. The shelf life extension depends on several factors, such as fish species, fat content, initial microbial population, gas mixture, and most importantly, storage temperature. In literature, different combinations of oxygen, carbon dioxide and nitrogen were used to package fish products with different effects on the shelf life (Arvanitoyannis et al. 2005; Corbo et al. 2005; Torrieri et al. 2006; Goulas and Kontominas 2007; Sivertsvik 2007). However, preservation provided by MAP and low temperatures may be improved applying other preservation techniques. With this aim, bio-protective LAB strains with antimicrobial properties were tested to preserve tuna burgers packed under MAP. In particular, three potentially bio-preservative LAB strains were screened based on their protective behavior on ready-to-cook tuna burgers (tuna burgers); furthermore, the bio-preserving efficacy of the selected *L. paracasei* culture was verified under MAP conditions (5% O_2–95% CO_2) at refrigerated temperature.

Materials and Methods

Bacterial Strains and Culture Conditions

Three bacterial strains, *L. casei* P1, *L. paracasei* IMPC 4.1 (LMG S-27068; ITEM15479), and *L. plantarum* 5BG (ITEM17403) were obtained from the CNR-ISPA collection and stocked at − 80 °C in de-Man-Rogosa and Sharpe (MRS) broth containing 50% glycerol. Working cultures of each strain were prepared by inoculating them (2%, *v*/*v*) in MRS broth (37 °C for 24 h); before use in experiments, working cultures were sub-cultured twice in the same media.

Tuna Burgers Preparation

The experimental design was organized in two subsequent trials. Frozen yellow-fin tuna fillets (*Thunnus albacares*) (around 10 kg) were kindly provided by a local fishing company (Caggianelli, Manfredonia, Italy), delivered to the laboratory and kept in a freezer at − 18 °C. Before use, fish has been thawed at 5 °C for 24 h in a refrigerated chamber and sliced.

In the STEP 1, for the fish-burger preparation, tuna slices were minced by an industrial food processor (Everest, Rimini, Italy) and ingredients were added as follows (g per kg of raw material): minced fish (765), extra-virgin olive oil (100), sodium chloride (5), parsley (5), rosemary (5), curry powder (5), potato starch (50), and potato flakes (65). All ingredients were homogenized in a bowl mixer (Multichef, Ariete, Firenze, Italy) equipped with a spiral hook for 5 min. Finally, tuna burgers were prepared by hand (40 g, 50 mm diameter, thickness 10 mm). This first batch represented the control samples. In addition, three different LAB strains (*L. casei*, *L. paracasei*, and *L. plantarum*) were tested in order to screen their bio-preservation properties on tuna burgers. Therefore, fish dough was obtained as previously described and separately inoculated with microbial strains. A total of 96 tuna burgers were prepared, considering samples with and without LAB. For experimental use, bacterial cultures grown in MRS Broth (37 °C for 24 h) were centrifuged in a SL 40-R centrifuge (Thermo Fisher scientific, Waltham, USA) at 10,000×*g* for 15 min at 4 °C. The pellets were resuspended in sterile water at 4 °C, and the resulting cell suspensions (9 log cfu/mL for each microorganism) were used as inoculum and added to fishery dough prior to burger forming operation. Two tuna burgers per treatment were packaged in air using commercially available bags (Nylon/Polyethylene) with thickness of 150 μm, provided by Biochemia (Bari, Italy) and kept under refrigeration (4 ± 1 °C) for 12 days.

During the experimental STEP 2, only *L. paracasei* strain was added to fish dough. Uninoculated burgers were used as control. The samples were realized as reported for the STEP 1 and packaged in air and under MAP (5% O_2 and 95% CO_2) (Danza et al. 2017). A total of 96 tuna burgers were prepared and stored at 4 ± 1 °C for 13 days.

Samples were labeled as follows: Ctrl-Air and Ctrl-MAP (tuna burgers without microbial addition and packaged in air and under MAP, respectively), *L. paracasei*-Air and *L. paracasei*-MAP (tuna burgers inoculated with the selected protective culture and packaged in air and under MAP, respectively).

Microbiological Analyses, Headspace Gas Composition, and pH Determination

From each tuna burger, samples (20 g) were aseptically transferred to sterile plastic pouches and homogenized with a Stomacher LAB Blender 400 (Pbi International, Milan, Italy) for 90 s with 220 mL of peptone water. Appropriate decimal dilutions of the samples were made using the same diluents, and aliquots of each dilution were inoculated in duplicate in different growth media by spread (0.1 mL) or pour (1 mL) plating to estimate microbial counts; the results are reported as colony-forming units per gram (cfu/g). The media and the conditions used were as follows: Gelatin Peptone

Agar incubated at 30 °C for 48–72 h for total aerobic mesophilic bacterial count and used for excluding the LAB strains inoculated for technological use; De Man, Rogosa, and Sharpe Agar anaerobically incubated at 37 °C for 48 h for LAB strains; Plate Count Agar incubated at 30 °C for 24–48 h and 7 °C for 10 days for mesophilic and psychrotrophic bacteria, respectively; Pseudomonas Agar Base, added with cetrimide-fucidin-cephaloridine selective supplement, incubated at 25 °C for 48 h for *Pseudomonas* spp.; Iron Agar, pour plated incubated at 25 °C for 3 days, for hydrogen sulfide-producing bacteria (HSPB); spread plated chilled IA, supplemented with 5 g/l NaCl and incubated at 15 °C for 7 days, for psychrotolerant and heat labile aerobic bacteria (PHAB); Violet Red Bile Glucose Agar incubated at 37 °C for 24 h for *Enterobacteriaceae*; and Violet Red Bile Agar incubated at 37 °C for 24 h for total coliforms. The conditions used during the counts of HSPB and PHAB were those suggested by the Nordic Committee on Food Analyses, with regard to fish and fishery products (NCFA 2006). All media were supplied from Oxoid (Milan, Italy). The microbiological analyses were carried out twice on two different batches, and microbiological data were transformed into logarithms of the number of colony-forming units (cfu/g). All plates were examined visually for typical colony types and morphology characteristics associated with each growth medium. The microbial threshold was set to 7 log/g or log/cm^2 at 30 °C for total viable count (MAL^{mesophylic}) as recommended by the ICMSF (1986) for freshwater and marine species.

The measurement of pH, conducted in duplicate, was performed on the first homogenized dilution of the fish samples with a pH meter (Crison, Barcelona, Spain). O_2 and CO_2 concentrations were monitored by a PBI Dansensor analyzer (Checkmate 3, Milan, Italy). To avoid modifications in the headspace gas composition due to gas sampling, each package was used only for a single measurement. Two bags were used for each measurement.

Strain Monitoring

To monitor the survival of the inoculated strains on tuna burgers during storage, at each sampling time, the 20% of total LAB colonies, randomly picked from countable MRSA plates containing from 50 to 100 colonies, were isolated and checked for purity. Bacterial DNA was extracted from overnight cultures grown in MRS broth at 37 °C using a Clonsaver Card Kit (Whatman, Maidstone, UK) and analyzed by repetitive element sequence-based PCR (rep-PCR) as previously described in De Bellis et al. (2010). The amplification fragments were resolved by microfluidic Lab-on-a-Chip (LoaC) electrophoresis with an Agilent 2100 Bioanalyzer and the DNA LabChip Kit 7500 (Agilent Technologies, Waldbronn, Germany). Sample preparation and chip loading was performed according to manufacturer's instructions and data, elaborated by 2100 Expert Software provided by the same company, were visualized as peaks in an electropherogram and bands on a gel. To estimate the dimension of each PCR product, a sizing ladder containing 10 reference fragments ranging from 50 to 7.000 bp flanked by an upper (10.380 bp) and lower (50 bp) marker, was resolved in a separate well. Genetic identification of the inoculated strains was based on the comparison of the REP-PCR profile of each isolate with the specific pattern obtained from the pure cultures of the inoculated strains. Therefore, the concentration of each strain (cfu/g) was calculated on the basis of the number of identified colonies.

Sensory Analyses

Sensory properties of uncooked tuna burgers were evaluated by a panel of eight experienced judges using nine-point scale: odor, color, appearance, texture, and overall quality were evaluated. The texture was judged considering the force required for cutting the product by using a knife. In the scale, score of 9 corresponded to "very good quality," score of 1–4 corresponded to "unacceptable quality," and 5 represented the acceptability threshold (Paulus et al. 1979). All the burgers were coded by a letter and presented to each panelist simultaneously in random order.

Volatile Aldehydes Analysis

A 2.0 g aliquot of burger was transferred to a 20-mL glass headspace sample vial, and 50 μL of internal standard solution (3-octanol) were added to obtain a final concentration of 20 ppm. The mixture was carefully shaken and then left to equilibrate 1 h in the dark, at room temperature before the analysis. For the extraction, the method described and optimized by Parlapani et al. (2017) was used. To the aim, a SPME fiber coating 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 23 gauge was used. The fiber was purchased from Supelco and thermally conditioned in accordance with the manufacturer's recommendations before first use. The samples of burger were warmed to 40 °C for 15 min before exposing the SPME fiber to the headspace of the sample. Headspace sampling/extraction times of 30 min at 40 °C were evaluated with continuous stirring (250 rpm). Analyses were performed in duplicate on two different samples, and blank runs were made with empty glass vial before and after each analysis. A Gerstel MPS autosampler (Gerstel, Baltimore, MD, USA) mounted to an Agilent 6890N gas chromatograph (Little Falls, DE, USA) paired with an Agilent 5975 mass selective detector constituted the analytical system. The software used was MSD ChemStation (Agilent). SPME injections were in splitless mode using a SPME injection sleeve (0.75 mm ID) at 250 °C for 350 s during which the thermal desorption of analytes from the fiber occurred in a HP-INNOWax column

(60 m × 0.25 mm, 0.25-μm film thickness, J&W. Scientific, Folsom, USA). Helium carrier gas was used with a total flow of 1.0 mL min^{-1}. The oven parameters were as follows: initial temperature was 40 °C held for 15 min, followed by an increase to 150 °C at a rate of 3 °C min^{-1} and held for 5 min, then increased to 200 °C at a rate of 15 °C min^{-1} and held at this temperature for 10 min before returning to the initial temperature. The total cycle time was 70 min. The MS detector was operated in scan mode (scan range 35–500 *m*/*z*), and the transfer line to the MS system was maintained at 250 °C. Peak identification was carried out by comparison of mass spectra with those obtained from NIST library (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 05, software version 2.0d) and with the Kovats indices (KI) of authentic standards (Sigma-Aldrich) calculated by running a paraffin series (from C_5 to C_{25}) under the same working conditions. The Kovats indices (KI) were compared with those reported, when present, in Flavornet (www.flavornet.org), LRI & odor database (www. odour.org.uk), Pherobase (www.pherobase.com), and Pubchem (https://pubchem.ncbi.nlm.nih.gov).

Shelf Life Calculation

In order to determine the shelf life of tuna burgers, in each step, the microbial acceptability limit (MAL) (i.e., the storage time at which the viable cell concentration reaches the threshold) and the sensory acceptability limit (SAL) (i.e., the storage time at which the overall quality reaches the threshold) were calculated by means of a reparameterized Gompertz equation, according to previous studies also reported in the literature (Conte et al. 2009).

Statistical Analysis

Microbiological data, presented as mean values ± standard deviation (SD) ($n = 2$) and expressed as $\log_{10}$ cfu/g of burger, were compared by using Student's *t* test or the one-way factor analysis of variance (ANOVA). Significant differences ($p < 0.05$) among groups were determined by using the post hoc LSD Fisher test. Duncan's test was performed to find out the source of significant differences among $MAL^{mesophiles}$ and $SAL^{overall\ quality}$ data. All statistical analyses were performed using the Statistica 7.1 for Windows (StatSoft Inc., Tulsa, OK, USA).

Results and Discussion

As reported beforehand, the work was organized into two subsequent experimental steps: during experimental STEP 1, the bio-preservation capacity and cell viability of three selected LAB strains on tuna burgers were tested monitoring microbial loads during refrigerated storage. In experimental STEP 2, the selected *L. paracasei* strain was used in combination with MAP to evaluate the combined effect on headspace gas concentration, volatile compound composition, sensory quality, and microbial acceptability of packaged fish products.

Bio-Preserving Efficacy of Microbial Strains

Figure 1a reports the viable cell load of aerobic mesophilic bacteria recorded in the tuna burgers during the storage time. The best fit of the reparameterized Gompertz equation to the experimental data is represented by the curves reported in the same figure; the parallel line indicates the threshold imposed for mesophiles. As can be observed, control sample and *L. plantarum* tuna burger exceeded the value of 7 $\log_{10}$ cfu/g, considered as the upper acceptability limit for marine species (ICMSF 1986), at the same day of storage (9 days). On the other hand, some differences between *L. casei* and *L. paracasei* were recorded. In particular, in *L. casei* sample, the aerobic viable cell concentration increased up to reach 6.82 $\log_{10}$ cfu/g, at the end of day 12. While, at the same storage time, the presence of *L. paracasei* was effective in inhibiting the mesophilic bacterial growth reducing the microbial proliferation by about 2 and 3 logarithmic cycles, with respect to *L. casei* and *L. plantarum* samples, respectively. This positive result may be related to the competition for nutrients and to the production of organic acids, hydrogen peroxide, bacteriocins, and protein or proteinaceous compounds (Stiles 1996; Lindgren and Dobrogosz 1990; Ström et al. 2002; Dalié et al. 2009). The mechanisms of action of different antimicrobial compounds produced by LAB are not fully understood, and it can be hypothesized that several mechanisms can take place at the same time. As a matter of fact, it is known that the lowering of the product pH, determined by organic acid production, is responsible for the inhibitory activities of LAB, and concomitantly may favor bacteriocin production (Muhialdin et al. 2011; Yang and Ray 1994). During storage, all tuna burger samples tested in STEP 1 showed a decrease of pH respect to the initial value (ranging 6.15–6.28). In particular, tuna burgers both inoculated with *L. casei* and *L. paracasei* reached at day 12 a pH value of 5.34 and 5.03, respectively, while, at the same storage time, a minor variation of pH was observed for control and *L. plantarum* samples (6.19 and 6.02, respectively).

Figure 1b shows the evolution of *Pseudomonas* spp. plotted as a function of storage time. A similarity with the evolution of aerobic mesophilic bacteria can be observed. In particular, *Pseudomonas* spp. counts were initially quantified at low

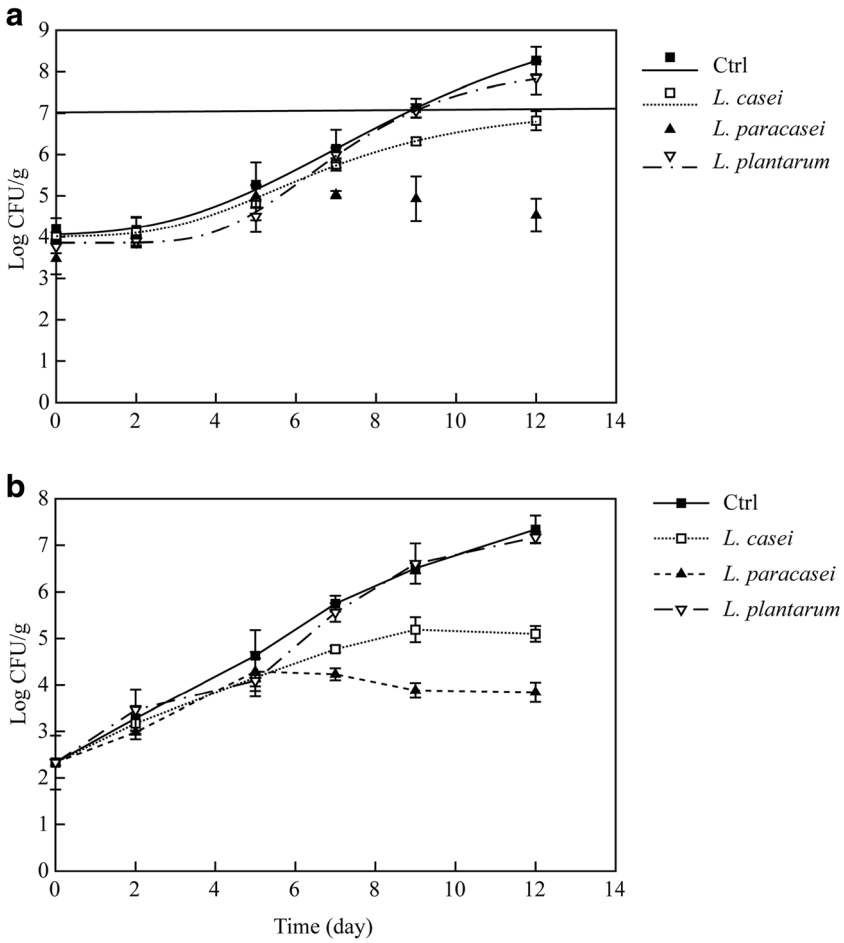

Fig. 1 Evolution of mesophiles (**a**) (the curves are best fit of a reparameterized Gompertz equation to experimental data) and *Pseudomonas* spp. (**b**) cell load ($\log_{10}$ cfu/g) for control, tuna burgers inoculated with *L. casei*, *L. paracasei*, *L. plantarum* strains, and packed in air under refrigeration conditions (STEP 1)

levels (2.33 $\log_{10}$ cfu/g) in all the investigated samples, thus indicating a good fish quality. During refrigerated storage for control and *L. plantarum* samples, an increase in *Pseudomonas* viable cells (up to 7 $\log_{10}$ cfu/g) was detected with no significant differences ($p < 0.05$) observed between them, while a significant inhibition of *Pseudomonas* spp. was observed when samples were inoculated with *L. paracasei* (3.84 $\log_{10}$ cfu/g) and *L. casei* (5.10 $\log_{10}$ cfu/g) probably due to the competitive action against the spoiler. Similarly, Katikou et al. (2007) have observed a significant reduction of *Pseudomonas* spp. counts and of other spoilage indicators (*Enterobacteriaceae*, H_2S-producing bacteria, yeasts, and molds) and a significant improvement of chemical parameters and off-odors in vacuum-packed rainbow trout fillets inoculated with *Lactobacillus sakei* CECT 4808 and *L. curvatus* CECT 904T protective cultures. A very low proliferation was recorded for the other specific spoilage organisms (SSOs). In particular, HSPB, PHAB, *Enterobacteriaceae* and coliform cell loads were always below the level of detection in all the investigated samples and for the entire observation period, indicating good hygiene of marine environment, as well as good fishing practices and handling (data not shown).

To monitor the presence of the inoculated strains during storage of tuna burgers in both experiments, a total of 192 isolates from inoculated burgers and relevant controls were analyzed by REP-PCR and Loac analyses comparing the resulting profiles with the three distinct and specific electrophoretic patterns obtained from pure cultures of the biopreserving strains (Fig. 2). In the experimental STEP 1, at each sampling time (0, 5, and 7 days), the 100% of LAB colonies (counts ranging from 8.04 ± 0.27 to 7.81 ± 0.44 $\log_{10}$ cfu/g) deriving from burgers inoculated with the *L. paracasei* strain was identified as *L. paracasei* IMPC 4.1 as the comparison of profiles of all isolates analyzed delineated a single pattern corresponding to the profile of the inoculated strain. With regard to burgers inoculated with *L. casei* (LAB counts ranging from 8.02 ± 0.30 to 7.66 ± 0.05 $\log_{10}$ cfu/g) and *L. plantarum* (LAB counts ranging from 7.69 ± 0.19 to 3.50 ± 0.54 $\log_{10}$ cfu/g), the REP profiles of the analyzed LAB colonies revealed different banding patterns: a survival rate ranging from 91.7% (time zero) to 33.3% (after 5 days) was obtained for *L. casei*, while for *L. plantarum*, the 100% (time zero) and the 44.4% (after 9 days) of analyzed colonies was identified as the inoculated strain. In the control burgers, none of the REP-PCR profiles of the analyzed colonies was

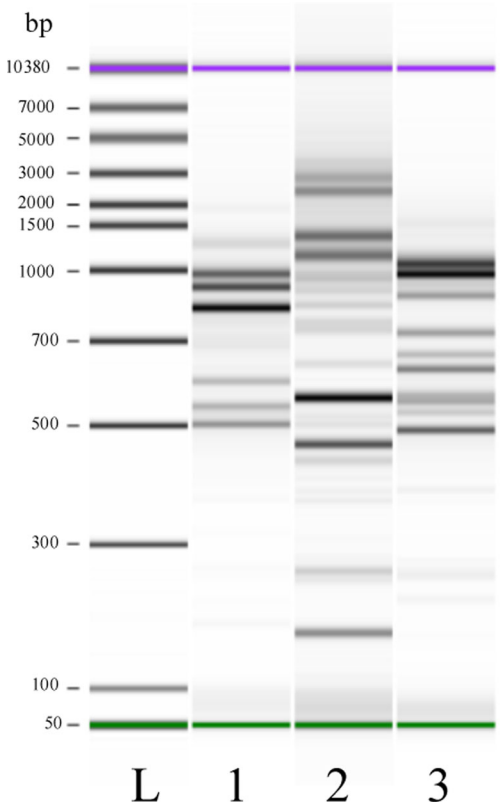

Fig. 2 Gel-like image of the specific REP-PCR patterns of the bio-preserving strains generated by the Bioanalyzer Agilent2100 using DNA7500 Labchip kit: sizing ladder (L), *L. casei* P1 (lane 1), *L. plantarum* 5BG (lane 2), *L. paracasei* IMPC 4.1 (lane 3)

comparable to the specific patterns of bio-preserving strains (data not shown).

Effects of *L. paracasei* Strain and MAP on Tuna Burger Shelf Life

As the *L. paracasei* population remained steady ($p > 0.05$) in experimental STEP 1 during the entire storage period (> 7.8 $\log_{10}$ cfu/g), it was selected for experimental STEP 2 in combination with MAP. Strain monitoring was performed, and the Loac analysis allowed to differentiate three different REP-PCR patterns (LAB1, LAB2, and LAB3) among the analyzed colonies with LAB1 pattern corresponding to the inoculated *L. paracasei* strain (Fig. 3, lane 1). At each sampling time, the presence of the bio-preserving culture was confirmed on burgers stored both in MAP or air with populations always exceeding 8.0 $\log_{10}$ cfu/g (Table 1). The inoculated strain resulted well adapted in the fish matrix representing at initial time the almost totality (90%) of LAB population (Fig. 3, lane 2). The remaining colonies, accounting for the 10% of total LAB population, were characterized by a single profile (LAB2 pattern; Fig. 3, lane 3). At the 1st day, the bio-preserving strain was recovered on burgers stored both in MAP or air at similar values ($p > 0.05$); in both samples, the 77.8% of analyzed colonies showed the *L. paracasei*-specific REP profile (Fig. 3, lanes 4 and 10) and the remaining 22.2% presenting the LAB2 profile (Fig. 3, lanes 5 and 11). At the successive experimental times (6 and 8 days for *L. paracasei*-Air; 8 and 13 days for *L. paracasei*-MAP), the 100% of analyzed colonies was identified as *L. paracasei* IMPC 4.1 (Fig. 3, lanes 6, 7, 12, and 13). During the entire experimental period, in control burgers stored both in air and MAP, LAB counts were always below the detection limit (2 $\log_{10}$ cfu/g) except for 6th day sampling in Ctrl-MAP in which two REP-PCR profiles (LAB2 and LAB3 pattern) were found (Fig. 3, lanes 8 and 9). As observed previously in swordfish fillets bio-preserved by a probiotic *L. paracasei* strain (Valerio et al. 2015), our results indicate that the bio-preserving strain replaced LAB populations hampering the growth of LAB strains that can be responsible for defects in products (Alvarez and Moreno-Arribas 2014; Lyhs and Björkroth 2008). When the bio-protective culture was combined with MAP (5% O_2 and 95% CO_2), a reduction of the mesophilic population occurred, compared to the control. In Fig. 4a, the evolution of aerobic mesophilic bacteria plotted as a function of storage time is reported. The initial mesophilic population was about 2 $\log_{10}$ cfu/g, and in Ctrl-Air samples, values rapidly overlap the imposed microbial threshold of 7 $\log_{10}$ cfu/g while in Ctrl-MAP values were lower (6.04 $\log_{10}$ cfu/g) at the end of storage time. When the protective culture was used, values of 5.82 $\log_{10}$ cfu/g were found for *L. paracasei*-air sample and 4.11 $\log_{10}$ cfu/g for *L. paracasei*-MAP. Hence, a better preservation of microbial quality seems to be assured by using *L. paracasei* and MAP condition. Concerning the packaging system, results obtained in this study are in agreement with other studies reported in literature since packaging with a low oxygen concentration is generally effective for fish products (Del Nobile et al. 2009; Costa et al. 2014). The headspace gas concentration (5% O_2 and 95% CO_2) of samples remained quite constant during the entire observation period.

Figure 4b shows the *Pseudomonas* spp. viable cell load plotted as a function of storage time. The highest contamination level was observed in Ctrl-Air sample after 8 days (7 $\log_{10}$ cfu/g) while at the same time, the application of MAP to not inoculated tuna burgers inhibited this microbial group (5.70 $\log_{10}$ cfu/g). As reported by Velu et al. (2013), the presence of *Pseudomonas* can be drastically reduced by the oxygen restriction of modified atmospheres, while CO_2-tolerant organisms may be favored. Under these conditions, the dominating microorganisms are LAB, mainly *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* (Jones 2004; Fontana et al. 2006). The protective culture under investigation has limited *Pseudomonas* spp. proliferation in both packaging conditions. Hence, *L. paracasei*-Air and *L. paracasei*-MAP samples have registered low *Pseudomonas* counts until day 11 of

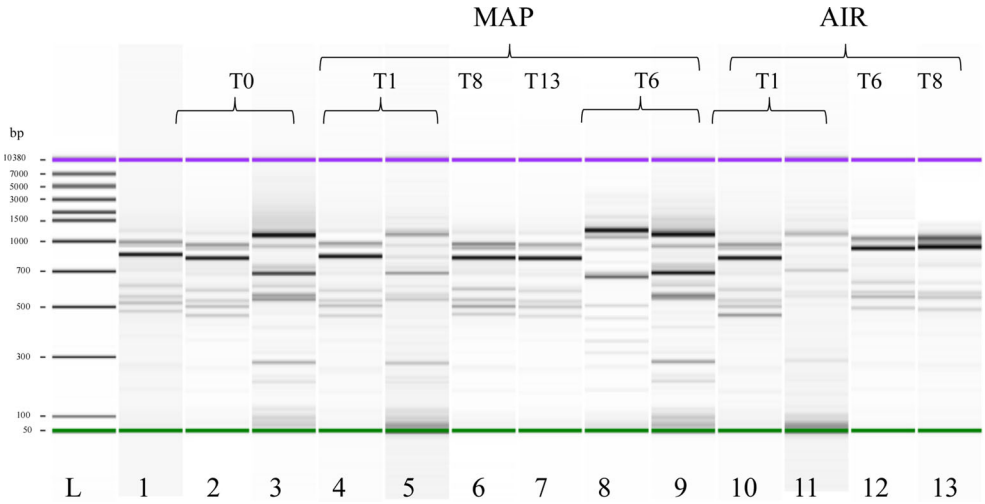

Fig. 3 Gel-like image, generated by the Bioanalyzer Agilent2100 using DNA7500 Labchip kit, of representative REP-PCR patterns of LAB isolates from tuna burgers stored in MAP (lanes 4–9) or air (lanes 10–13) atmosphere in experimental STEP 2: sizing ladder, L; *L. paracasei* IMPC 4.1, lane 1; *L. paracasei* inoculated burgers T0, lanes 2 and 3; *L. paracasei*-MAP, lanes 4–7; Ctrl-MAP, lanes 8 and 9; *L. paracasei*-Air, lanes 10–13

storage (4.18 $\log_{10}$ cfu/g and 3.40 $\log_{10}$ cfu/g, respectively). These results are in agreement with a previous study on the bio-preservation of foods through the use of live cells (Jinlan et al. 2010). In our study, similarly to *Pseudomonas* spp., the psychrotrophic and PHAB bacteria resulted inhibited, while cell loads for the other investigated spoilage microorganisms were always below the level of detection for the entire observation period (data not shown).

Regarding the pH of the tuna burgers, significant differences were observed among samples during storage. For the control samples (Ctrl-Air and Ctrl-MAP), initial and final pH values of 5.79 and 5.90 were observed, respectively. A significant pH decrease was observed in both samples inoculated with *L. paracasei* strain with respect to control samples: final values were 5.01 (*L. paracasei*-Air) and 4.95 (*L. paracasei*-MAP) lower than the initial values (5.79), probably due to the acidic compounds produced from the metabolic pathway of the protective culture.

Curves shown in Fig. 5 result from the fitting procedure of *overall quality* data, whereas the $SAL^{overall\ quality}$ values obtained are listed in Table 2. The selected MAP applied to burgers inoculated with *L. paracasei* strain has promoted a good sensory preservation and limited the odor changes until 11 days of storage, while a more rapid decay was observed for the other samples. The positive effect of bio-protective cultures on preventing off-odors in cold-smoked salmon and rainbow trout fillets has been already reported by Leroi et al. (2015) and Katikou et al. (2007). In particular, the *overall quality* of Ctrl-Air rapidly decreased below the threshold value within 6 days of storage, due to a general spoiling of all the sensory attributes (color, odor, appearance, and consistence). The presence of oxygen in the samples packed in air was responsible for the color changes linked to the browning of spices and meat; moreover, changes of odor, appearance, and consistence were due to the great microbial proliferation (Abbas et al. 2009; Gram and Dalgaard 2002). Ctrl-MAP and *L. paracasei*-Air samples were refused from panel test after 8 days. Therefore, as indicated in Table 2, sensory properties limited shelf life of tuna burgers even though the product preservation can be promoted by the use of the protective *L. paracasei* strain in combination with MAP (5% O_2, 95% CO_2).

Effect of *L. paracasei* Strain and MAP on Aldehydes Development

Among the aroma substances of fish, special attention was paid to volatile aldehydes, including saturated, α,β-monounsaturated, and polyunsaturated. These compounds are formed principally as secondary products from the lipid oxidation of polyunsaturated fatty acid but also by other chemical or enzymatic

Table 1 LAB and *Lactobacillus paracasei* 4.1 counts ($\log_{10}$ cfu/g) recorded in bio-preserved and control tuna burgers stored in MAP or air in experimental STEP 2 ($n = 2$)

Label								
	L. paracasei	*L. paracasei*-MAP			*L. paracasei*-Air			Ctrl-MAP
Days	0	1	8	13	1	6	8	6
LAB	8.34 ± 0.68	7.98 ± 0.03	9.19 ± 0.04	9.12 ± 0.24	8.20 ± 0.17	8.36 ± 0.06	8.98 ± 0.42	4.22 ± 0.53
L. paracasei 4.1[a]	7.93 ± 0.11	7.97 ± 0.04	9.19 ± 0.04	9.12 ± 0.24	8.11 ± 0.06	8.36 ± 0.06	8.98 ± 0.42	nd

ND not detected

[a] Counts were confirmed by REP-PCR

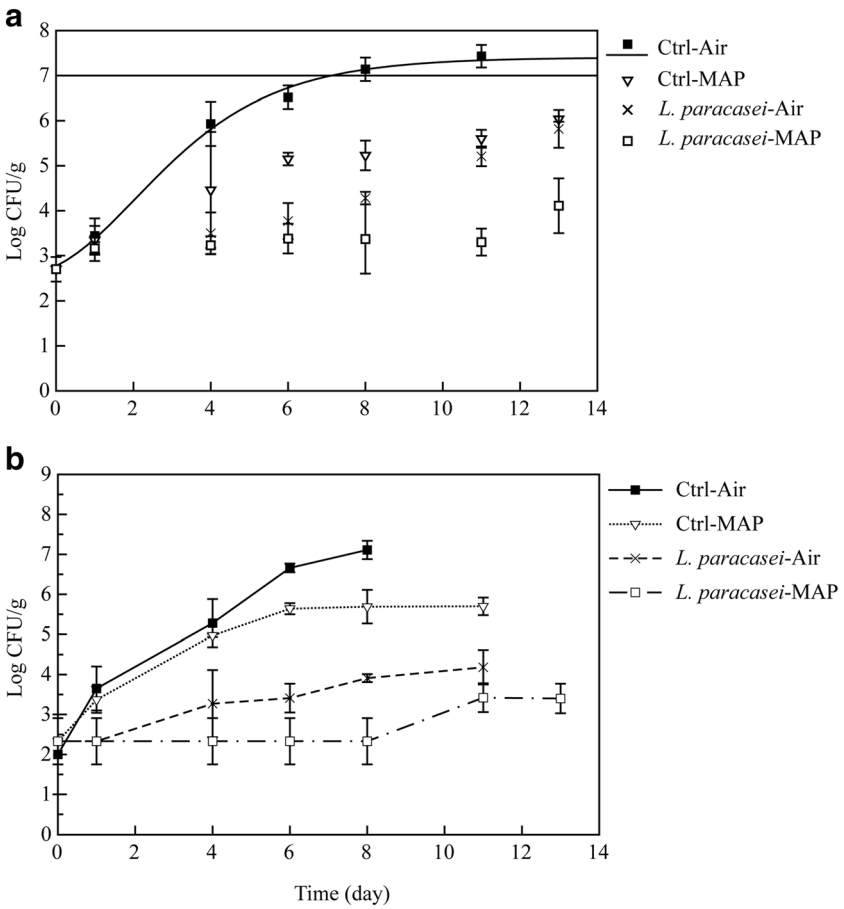

Fig. 4 Evolution of mesophiles (**a**) (the curves are best fit of a reparameterized Gompertz equation to experimental data) and *Pseudomonas* spp. (**b**) cell load (log_{10} cfu/g) for control and tuna burgers inoculated with *L. paracasei* strains packed in air and in MAP under refrigeration conditions (STEP 2)

processes that take place during the storage period in the fish product. In particular, saturated aldehydes, with a very low odor threshold, greatly contribute to food rancidity, thus provoking loss of nutritional quality (Veloso et al. 2001). As consequence, aldehydes are also responsible for alteration in odor, color, and texture. The profile of volatile aldehydes of tuna burgers studied in the current study is reported in Fig. 6, in terms of mean peak area recorded in each sample during the storage time. The results of analysis emphasize that, in general, aldehydes were lower in the *L. paracasei* samples than in the Ctrl samples and especially in *L. paracasei*-MAP. Among the various volatile compounds, saturated aldehydes deriving from the lipid oxidation of linoleic acid, i.e., hexanal, heptanal, and pentanal (Lirong et al. 2017), exerted similar trends. Specifically, at the beginning of the storage (t0), in the Ctrl samples, the hexanal, a compound with grassy or mushroom-like green odors (Zhou et al. 2016), was not significantly different ($p > 0.05$) with respect to *L. paracasei* samples, but during the storage period, it remained quite constant in the control burgers and disappeared in *L. paracasei* samples. Similarly,

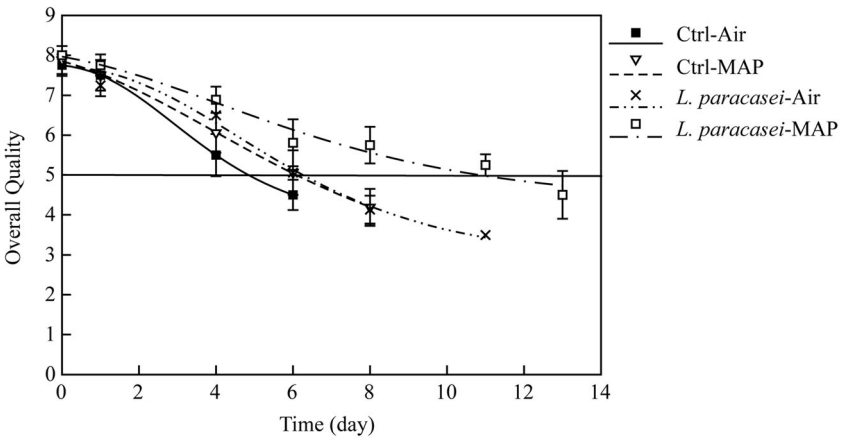

Fig. 5 Overall quality decay of tuna burger samples. The curves are the best fit of a reparameterized Gompertz equation to the experimental data. Control and tuna burgers inoculated with *L. paracasei* strains packed in air and in MAP under refrigeration conditions (STEP 2)

Table 2 Shelf life (day; mean ± SD) of tuna burger samples, assumed as the lowest value between microbial (MAL) and sensory (SAL) acceptability limit

Sample	$MAL^{mesophiles}$ (day)	$SAL^{overall\ quality}$ (day)	Shelf life (day)
Ctrl-Air	7.11 ± 0.44	4.84 ± 0.00a	4.84 ± 0.00a
Ctrl-MAP	> 13	6.08 ± 0.04b	6.08 ± 0.04b
L. paracasei-Air	> 13	6.14 ± 0.12b	6.14 ± 0.12b
L. paracasei-MAP	> 13	10.84 ± 1.00c	10.84 ± 1.00c

[a-c] Means in the same column followed by different letter are significantly different ($P < 0.05$)

heptanal with a fatty and pungent odor was only found in the Ctrl samples at comparable concentrations during the entire observation period. With regard to pentanal, characterized by almond, malt, pungent odor, at t0, it was not significantly different ($p > 0.05$) in Ctrl samples and *L. paracasei* samples, but with time, it only increased in the Ctrl-MAP while was decreasing in all the *L. paracasei* samples (both in air and under MAP). Concerning the Strecker aldehydes, 2-methylbutanal (pungent and caprylic odor) arising from leucine degradation, and benzaldehyde (almond odor), originated from the degradation of phenylglycine, two different evolutions were found in control and treated samples. In particular, while the 2-methylbutanal significantly decreased ($p < 0.05$) during time in the *L. paracasei* samples with respect to the Ctrl samples, the benzaldehyde remained constant in all the samples.

In the same figure, the cuminaldehyde, a volatile essential oil, was also reported, probably deriving from spices used in the preparation of tuna burgers. As the most aldehydes, it was also affected by the bio-preservative microorganism; in fact, a more evident reduction less evident in the Ctrl samples was recorded during storage, compared to *L. paracasei* samples.

To sum up, recorded findings of the current study confirmed that bio-preservation can affect food spoilage, and consequently, the volatile aldehyde profile. Our results also allow concluding that *L. paracasei*, above all when combined with MAP, affected the aldehydes evolution, probably due to the microbial origin of these compounds. The contribution of aldehydes to the total aroma of burgers was in general confirmed by the sensory evaluation. To give evidence, in the same Fig. 6, the results of odor recorded from the panel test were also reported. The picture shows a strict correlation between the volatile aldehyde evolution and the acceptability of samples in terms of odor. It is worth noting that sample *L. paracasei*-MAP was very different from the other three samples: in *L. paracasei* tuna burgers under MAP, the less amounts of aldehydes were recorded and the odor parameter remained acceptable for almost 2 weeks, compared to samples that were refused within the first week.

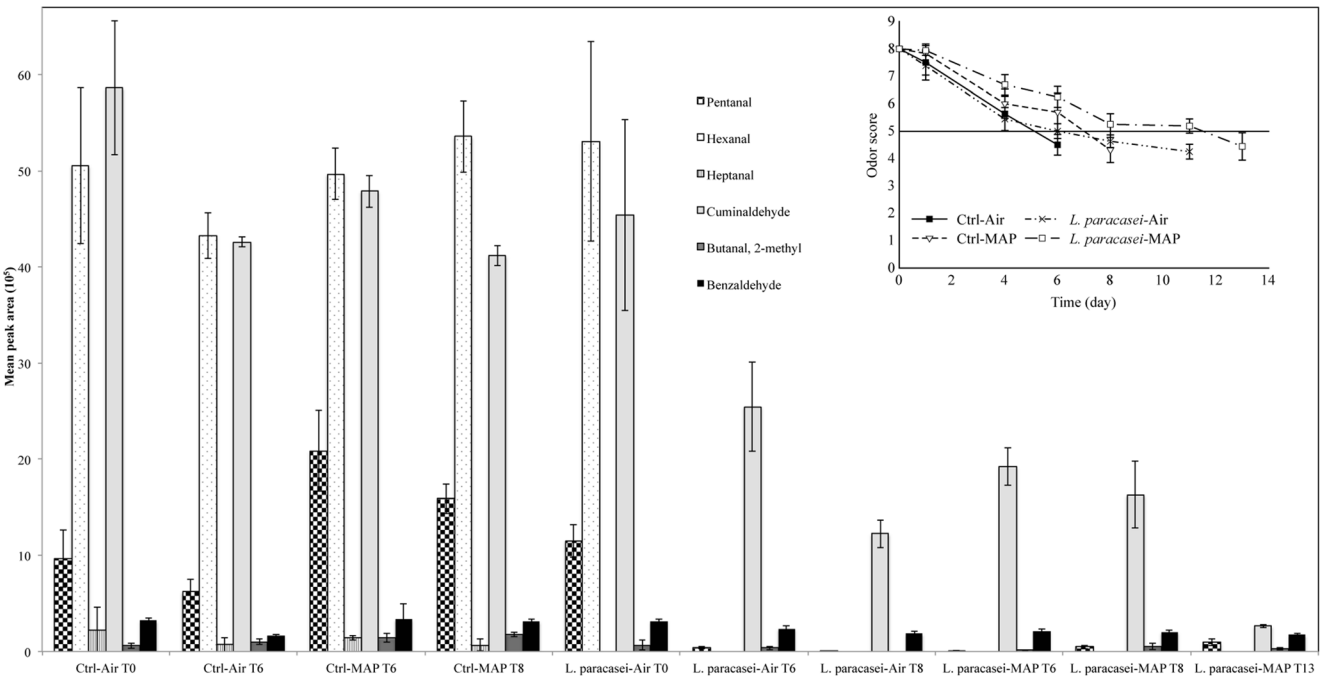

Fig. 6 Volatile aldehydes of tuna burgers during the storage. Control and tuna burgers inoculated with *L. paracasei* strains packed in air and in MAP under refrigeration conditions (STEP 2). In the same figure, a picture of the evolution of the odor perceived by the panelists was also reported

Conclusions

Results highlight that the combined use of the selected protective culture of *L. paracasei* and packaging system characterized by high CO_2 headspace concentration was able to improve the shelf life of tuna burgers (about 11 days) with respect to control sample packed in air (about 5 days) or in MAP (about 6 days). The efficacy of the tested strategy was proved against total mesophilic and psychrotophic bacteria, *Pseudomonas* spp., and PHAB. The aldehyde profile confirmed the trend of acceptability recorded during sensory section, in particular in terms of odor. As compared to several other mild preservation procedures like addition of bacteriocins or spray-dried microbial cultures, the addition of live bacterial cells used in combination with MAP is an inexpensive and easy method to extend shelf life of fresh fishery products. Under the normative force, the selection of LAB possessing the GRAS (generally recognized as safe) status as protective cultures is generally agreed as beneficial for extending seafood shelf life.

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