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Antimicrobial Performance of Two Different Packaging Materials on the Microbiological Quality of Fresh Salmon

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Abstract: In the present research the antimicrobial activity of two active packaging materials on the spoilage microbiota of fresh salmon fillets was tested. A PET-coated film (PET: Polyethylene Terephthalate) containing lysozyme and lactoferrin was tested in parallel with a carvacrol-coextruded multilayer film. Salmon fillet samples were stored up to four days at 0 and 5 °C, comparatively. The carvacrol multilayer film was found effective in preventing mesophiles and psychrotrophs at shorter storage time and at lower temperature (4.0 compared to 5.0 log CFU/g in the control sample—CFU: Colony Forming Units). Lysozyme/lactoferrin-coated PET was instead efficient in decreasing H_2S-producing bacteria at longer storage time and higher temperature (2.7 instead of 4.7 log CFU/g in the control sample). Even if is not intended as a way to "clean" a contaminated food product, an active package solution can indeed contribute to reducing the microbial population in food items, thus lowering the risk of food-related diseases.

Keywords: active packaging; carvacrol; coextrusion; lysozyme; lactoferrin; coating; salmon

1. Introduction

Salmon is an important product of aquaculture: 1,400,000 ton were produced in 2010 with a value of more than seven billion US dollars. In 2009, the main producers of Atlantic salmon were Norway, Chile, the EU and Canada [1]. In the EU the main farmed species is Atlantic salmon, accounting for 93% of the total aquaculture production. The EU is very dependent on the rest of the world for salmon since it imports 80% of its supply from other countries, and 80% of that from Norway.

Fresh seafood is characterized by a relatively short shelf-life and is typically spoiled by aerobic Gram-negative bacteria. In fish, the spoilage process is well documented and consists of autolytic degradation by fish enzymes and the production of unpleasant odors and flavors as a result of microbial action [2]. Typically, in the chilled seafood supply chain, microbial-mediated changes dominate the spoilage process [3]. The bacteria responsible for spoilage in marine fish vary according to the harvest environment, the degree of cross-contamination and the preservation methods applied post-harvest. The primary spoilage bacteria in aerobically packed fish are Gram-negatives from the genera *Pseudomonas* and *Shewanella* while in modified atmospheres, they are *Photobacterium* as well as lactic acid bacteria (LAB), such as *Lactobacillus* and *Carnobacterium* [3,4]. *Shewanella putrefaciens* and *Pseudomonas* spp. become the main producers of the volatile compounds associated with spoilage,

such as trimethylamine (TMA), ammonia and sulphides. TMA is particularly responsible for the unpleasant odor of spoiled fish, and is a common index of seafood quality. However, the changes in sensory attributes often occur before products are hygienically spoiled [5].

During storage, spoilage bacteria are selected primarily as a result of the physical and chemical condition in the products; however, seafood spoilage obviously involves growth of the microorganisms to a high amount (>10^6–10^7 CFU/g) and the interaction between groups of microorganisms may influence their growth and metabolism [6]. In particular the high iron-binding capacity of the *Pseudomonas* siderophores may cause this bacterial group to be positively selected, as well as LAB inhibit the growth of other bacteria due to the formation of lactic acid and/or bacteriocins or by competition for nutrients [7].

A significant support to the fight against microbial spoilage may derive from food packaging, which not only acts as a barrier against moisture, water vapor, gases and solutes, but may also serve as a carrier of active substances, such as antimicrobials, in active packaging. Active packaging is defined as an integrated system in which the package, product and environment interact to prolong shelf-life or to enhance safety and/or quality of food products [8].

The antimicrobial features of food packaging materials can be achieved by different strategies: among others, the incorporation in the bulky polymer of migrating compounds, grafting of antimicrobial moieties, and immobilization of antimicrobial agents on the surface of the material in direct contact with the food are the most widely adopted routes. However, direct incorporation in the plastic polymer matrix is not a feasible approach when dealing with antimicrobial agents that are highly sensitive to package production conditions; indeed, high processing pressure and high temperature, or incompatibility with the packaging material, can inactivate the active agents [9]. Alternative production methods have recently been considered. In particular, coating technology has gained increasing attention due to its promising potential as a valid route to generate antimicrobial packaging materials [10].

The aim of the present research was to evaluate the antimicrobial effectiveness of two active packaging materials on the spoilage microbiota of fresh salmon fillets. In particular, a PET-coated film containing lysozyme and lactoferrin was tested in parallel with a carvacrol-coextruded multilayer film. These natural compounds have been selected for their interesting antimicrobial performance evidenced in the frame of the European funded project NAFISPACK—Natural Antimicrobials for Innovative and Safe Packaging (EU212544). Salmon samples after packaging were stored up to four days at 0 and 5 °C, comparatively, to show any significant effect of the developed materials on spoilage microbiota. These two temperatures were chosen as the first represents the one applied by the company to deliver salmon samples to the market, while the second is commonly used by consumers for home storage.

2. Experimental Section

2.1. Antimicrobial Films

Lysozyme-Lactoferrin (LZ-LF) water solutions were coated together onto PET: the formulation included gelatin as the main biopolymer network, glycerol as plasticizer, and a lipid phase (a monoglyceride acetylate) as slipping agent, in order to avoid blocking of the film coils during the unwinding operations on the reels. The total dry matter was 19.1 wt %, where LZ and LF accounted for 3 wt % (on the total weight). The procedure has been up-scaled in a pilot plant coating machine at a speed of 10 m/min. The removal of the solvent (water) was achieved by the combined effect of IR lamps and a flux of mild air. Right before the coating deposition, the plastic web underwent an in-line corona treatment to improve surface wettability.

Carvacrol (4.8%) was instead incorporated in a coextruded multilayer film 73 µm thick, made up of two external layers of polypropylene, two tie layers, and an internal layer of ethylene–vinyl alcohol copolymer with a 29% ethylene molar content (EVOH-29), prepared as reported elsewhere [11]. Briefly, films were obtained by extrusion processes using a flat sheet die 500 mm wide and three single screw

extruders (Dr. Collin GmbH, Edersberg, Germany) with 30 mm of screw diameter and L/D ratio of 30. Die, feeding block, and transfer lines were maintained at 250 °C. Temperature of chill roll was set at 45 °C and an additional airknife was used. Line speed was 9.5 m/min.

2.2. Sample Preparation

Fresh salmon fillets cut to cubes (*ca.* 120 g) were packaged in laminated material made up of polyethylene/polyamide/polyethylene in Bremerhaven (Germany) and then transported on ice to the University of Milan, where they arrived the next day (Figure 1). Fillets were repackaged into pouches (20 cm × 12 cm) made from a high barrier multilayer film after being wrapped in sheets of the antimicrobial films. The transfer of the samples from the original package to the experimental packages was made in sterile conditions under a safety cabinet. Samples were all packaged under vacuum. Reference samples were also prepared in an identical way, without wrapping in antimicrobial films.

Figure 1. Sample of salmon fillets, cut into cubes, employed in shelf-life trials.

After packaging, samples were all stored at 0 °C and analyses were performed at days 0, 1 and 4. At day 1, some samples were moved to 5 °C, where they were left until the microbiological analysis.

2.3. Microbiological Analysis

Microbial analyses were performed at days 0, 1 and 4 after packaging. Samples were taken from the surface of the samples (2–3 mm) and transferred aseptically and weighed in a sterile Stomacher bag, diluted with peptone water (PW) (Scharlab, Barcelona, Spain) and blended in Stomacher (IUL S.L., Barcelona, Spain) for 6 min. Ten-fold dilution series in PW of the obtained suspensions were made and plated on selective solid media: TSA (Merck, Germany) for mesophiles and psychrotrophs, MEA (Sigma Aldrich, St. Louis, MO, USA) for yeasts and fungi, *Pseudomonas* agar base (Himedia, India) for *Pseudomonas* spp., VRBLA (Violet Red Bile Agar, Merck, Germany) for coliforms, MRS agar (Scharlab, Barcelona, Spain) for lactic acid bacteria, and Lyngby Iron agar (Oxoid, UK) for H_2S-producing bacteria. Colonies were counted after incubation at 30 °C for 24 h for mesophiles, 10 °C for 10 days for psychrotrophs, 30 °C for five days for yeasts and fungi, 25 °C for 24 h for *Pseudomonas*, 37 °C for 24 h for coliforms, 25 °C for five days for lactic acid bacteria and 20 °C for three days for H_2S-producing bacteria. Counts were performed in triplicate and reported as logarithms of the number of colony forming units (log CFU/g salmon), and means and standard deviations were calculated.

3. Results and Discussion

Aerobic mesophiles and psychrotrophs as well as H_2S-producing bacteria were the prevalent population in the salmon at time of packaging (3.3–3.6 log CFU/g), followed by LAB (2.2 log CFU/g and *Enterobacteriaceae* (1.5 log CFU/g) (Table 1). According to the International Commission on Microbiological Specifications for Foods, most aquatic animals at the time of harvest have microbial counts in the range of 2 to 5 log CFU/g [12]. In the present study the initial value of microbial load was in the same range, as also reported by other authors [13]. After one day of storage at 0 °C,

the psychrotroph population increased up to 5.5 log CFU/g in control samples. Salmon packed in carvacrol active films was characterized by a reduced psychrotroph population (3.9 log CFU/g), while LZ-LF–coated films were ineffective (5.8 log CFU/g) (Table 2). Gram-negative psychrotrophic bacteria are the major group of microorganisms responsible for the spoilage of fresh fish at chilled temperatures [2]. In this study, mesophiles in the first 24 h remained in the range 3.9–4.1 log CFU/g, without significant difference between the control sample and those stored in the active packages.

Table 1. Microbial population (log CFU/g) present in salmon at time of packaging.

Microorganisms	Microbial Count (log CFU/g)
Aerobic mesophiles	3.6 ± 0.2
Aerobic psychrotrophs	3.5 ± 0.1
Enterobacteriacae	1.5 ± 0.4
Lactic acid bacteria	2.2 ± 0.2
Pseudomonas	<2
H_2S-producing bacteria	3.3 ± 0.2

Table 2. Microbial population (log CFU/g) in salmon packed in two different antimicrobial materials and in one reference material (control) after storage for one day at 0 °C.

Microorganisms	Control	Carvacrol	LZ LF
Aerobic mesophiles	3.9 ± 0.7	4.1 ± 0.6	4.0 ± 0.5
Aerobic psychrotrophs	5.5 ± 0.4	3.8 ± 0.5	5.8 ± 0.2
Enterobacteriaceae	1.6 ± 0.2	1.6 ± 0.5	1.6 ± 0.2
Lactic acid bacteria	2.2 ± 0.3	2.2 ± 0.4	3.1 ± 0.1
Pseudomonas	<2	<2	<2
H_2S-prod. Bacteria	2.7 ± 0.6	3.6 ± 0.4	2.1 ± 0.2

At day 1 some samples were moved to 5 °C while, in parallel, other samples were kept at 0 °C. All samples were then stored for up to four days, one day more than the normal shelf-life suggested by the company.

After four days at 0 °C, salmon packed in carvacrol films maintained psychrotroph and mesophile populations at low levels, *i.e.*, around 4.0 log CFU/g, compared to 5.0 log CFU/g in control samples (Table 3). Also, LZ-LF–coated films showed an interesting performance, reducing the populations up to 3.0 and 3.7 log CFU/g, respectively. Note also, at this storage temperature, the efficacy of the two active films in reducing the *Pseudomonas* population of approximately 1 log cycle compared to control, from 3.2 to 2.2–2.3 log CFU/g.

Table 3. Microbial population (log CFU/g) in salmon packed in two different antimicrobial materials and in one reference material (control) after storage for four days at 0 °C and 5 °C, comparatively.

Temperature	Microorganisms	Control	Carvacrol	LZ LF
0 °C	Aerobic mesophiles	5.1 ± 0.2	3.9 ± 0.2	3.7 ±0.3
	Aerobic psychrotrophs	5.0 ± 0.3	4.0 ± 0.1	3.0 ± 0.1
	Enterobacteriaceae	2.0 ± 0.0	1.9 ± 0.3	2.2 ± 0.2
	Lactic acid bacteria	2.9 ± 0.2	2.6 ± 0.1	2.0 ± 0.1
	Pseudomonas	3.2 ± 0.5	2.2 ± 0.2	2.3 ± 0.6
	H_2S-prod. bacteria	3.3 ± 0.2	3.4 ± 0.4	3.4 ± 0.4
5 °C	Aerobic mesophiles	5.3 ± 0.6	4.6 ± 0.6	4.5 ± 0.3
	Aerobic psychrotrophs	5.3 ± 0.6	6.9 ± 0.4	3.8 ± 0.2
	Enterobacteriaceae	1.9 ± 0.0	2.5 ± 0.5	2.2 ± 0.2
	Lactic acid bacteria	2.8 ± 0.5	3.2 ± 0.4	3.1 ± 0.2
	Pseudomonas	2.5 ± 0.1	2.8 ± 0.3	2.2 ± 0.2
	H_2S-prod. bacteria	4.7 ± 0.6	4.0 ± 0.7	2.7 ± 0.5

Salmon samples stored for four days at 5 °C without active packaging were characterized by mesophile and psychrotroph population of 5.3 log CFU/g. Carvacrol active films were effective in maintaining only the mesophile population at a low level of 4.6 log CFU/g, while LZ-LF–coated PET reduced both the populations down to 4.5 and 3.8 log CFU/g, respectively. It must be noted, also, that in this last case there was a reduction of H_2S-producing bacteria to 2.7 instead of 4.7 log CFU/g as seen in the control samples. This positive observation that LZ/LF–coated PET films could prevent growth of H_2S-producing bacteria is of actual relevance since this type of microorganism has been identified as the most potent in causing rejection of fresh salmon fillets, due to the production of off-odors during growth [4].

Enterobacteriaceae were also found to be members of the microbial association implicated in the spoilage of fresh sliced salmon during refrigerated storage. This finding is in agreement with results reported for different fish species, including fresh Atlantic salmon [14] as well as rainbow trout [15], in which *Enterobacteriaceae* were determined as a part of the microbial population at the end of the product shelf-life under refrigerated storage. In this study, *Enterobacteriaceae* were always found in low levels, less than 3 log CFU/g; although *Enterobacteriaceae* can grow at low temperatures, their proliferation was slow during refrigerated storage, possibly because their growth rate is lower than that of other Gram-negative psychrotrophic spoilers [16].

The LAB population was always found lower than 3.2 log CFU/g, and was not influenced by the active packaging employed. The low LAB count in this study was expected since they tend to grow slowly at refrigeration temperatures [13].

The obtained data satisfied the recommended microbiological limits for fresh and frozen fish reported in [12], which are defined only for aerobic plate counts performed at 20–25 °C (5.5 to 7 log CFU/g) and for *E. coli* (1 to 2.7 log CFU/g). The first data reflect handling practices in the fish industry, from shipboard to market delivery, while the second parameter is considered an indicator of contamination and, when present in high numbers, suggests temperature abuse in product handling.

The present research was aimed at investigating the efficacy of two antimicrobial food packaging materials on microbial spoilage of fresh salmon fillets. Incorporation of natural antimicrobials into food packaging materials to control the growth of spoilage and pathogenic organisms has been researched for the last decades. As regards fish products and shelf-life, the vast majority of data relates to the possibility of applying antimicrobial edible films in contact with the fish surface. Jasour *et al.* [17] evaluated the effect of an edible coating based on chitosan coated with the lactoperoxidase system (LPS) on the quality and shelf-life extension of rainbow trout during refrigerated storage at 4 °C. Results indicated that antimicrobial coating was found efficient in reducing *Shewanella putrefaciens*, *Pseudomonas fluorescens* as well as psychrotrophic and mesophilic bacterial populations compared to the control sample.

Few authors have investigated the performance of an antimicrobial package prepared by applying a coating procedure. Gomez Estaca *et al.* [18] produced a complex gelatin-chitosan film incorporating clove essential oil which was applied to fish during chilled storage: the growth of microorganisms was drastically reduced in Gram-negative bacteria, especially *Enterobacteriaceae*, while LAB remained constant for much of the storage period. Neetoo and Mahomoodally [19] compared the antimicrobial efficacy against *Listeria monocytogenes* in smoked salmon fillets of films or direct coatings incorporating nisin (Nis) and sodiumlactate (SL), sodiumdiacetate (SD), potassiumsorbate (PS), and/or sodium benzoate (SB) in binary or ternary combinations on cold smoked salmon. Surface treatments incorporating Nis (25,000 IU/mL) in combination with PS (0.3%) and SB (0.1%) had the highest inhibitory activity, reducing the population of *L. monocytogenes* by a maximum of 3.3 log CFU/cm^2 (films) and 2.9 log CFU/cm^2 (coatings) relative to control samples after 10 days of storage at 21 °C. During refrigerated storage, coatings were more effective in inhibiting growth of *L. monocytogenes* than their film counterparts. Cellulose-based coatings incorporating Nis, PS, and SB reduced the population of *L. monocytogenes*, and anaerobic and aerobic spoilage microbiota by a maximum of 4.2, 4.8, and 4.9 log CFU/cm^2, respectively, after four weeks of refrigerated storage.

In the present study the carvacrol-incorporated multilayer film was found effective in preventing mesophiles and psychrotrophs at shorter storage time and at lower temperature, while lysozyme-lactoferrin–coated PET was mostly efficient in decreasing H_2S-producing bacteria at longer storage time and higher temperature. Even if it is not intended as a way to "clean" a contaminated food product, an antimicrobial package solution can indeed contribute to reducing the microbial population in food items, thus lowering the decay of organoleptic features and increasing shelf life.

4. Conclusions

This work provides examples of active food packages, in which the antimicrobial compounds, *i.e.*, the volatile carvacrol and the soluble association lysozyme-lactoferrin, were applied, the first incorporated in a coextruded multilayer film while the second association was coated onto PET. Application of these materials on actual salmon samples under conditions similar to those foreseeable for a future practical use gave positive results, in particular for the reduction of H_2S-producing bacteria in coated films. Future work will indicate whether the antimicrobial-loaded films used here may find other practical uses (e.g., liners or wraps) and whether they will be effective to improve the safety and to extend the shelf-life of other food products.

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Conflicts of Interest: The authors declare no conflict of interest.

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