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Strategies to increase the hygienic and economic value of fresh fish: Biopreservation using lactic acid bacteria of marine origin

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ABSTRACT

In this work we describe the development of a biopreservation strategy for fresh fish based on the use of bacteriocinogenic LAB of marine origin. For this purpose, two multibacteriocinogenic LAB strains, *Lactobacillus curvatus* BCS35 and *Enterococcus faecium* BNM58, previously isolated from fish and fish products were selected owing to their capability to inhibit the growth of several fish-spoilage and food-borne pathogenic bacteria. Two commercially important fish species were chosen, young hake (*Merluccius merluccius*) and megrim (*Lepidorhombus boscii*), and the specimens were acquired at the Marín (Pontevedra, Spain) retail fish market, after one night in the chilled hold of a near-shore fishing vessel. The biopreservation potential and the application strategies of these two LAB strains were first tested at a laboratory scale, where several batches of fresh fish were inoculated with: (i) the multibacteriocinogenic LAB culture(s) as protective culture(s); and/or (ii) their cell-free culture supernatant(s) as food ingredient(s), and (iii) the lyophilized bacteriocin preparation(s) as lyophilized food ingredient(s). All batches were stored in polystyrene boxes, permanently filled with ice at 0–2 °C, for 14 days. Microbiological analyses, as well as sensorial analyses, were carried out during the biopreservation trials. Subsequently, *Lb. curvatus* BCS35 was selected to up-scale the trials, and combinations of the three application methods were assayed. For this purpose, this strain was grown in a semi-industrial scale fermentor (150 l) in modified MRS broth, and three batches of fresh fish were inoculated with the protective culture and/or food ingredient, and stored on ice in a chilled chamber at 0–2 °C at the Marín retail fish market for 14 days. Microbiological analyses were carried out during the storage period, showing that when *Lb. curvatus* BCS35 culture or the corresponding cell-free culture supernatant was used as protective culture or food ingredient, respectively, bacterial counts were significantly lower than those of the untreated control batches, both for young hake and megrim. In addition, the presence of *Listeria* spp. in megrim was inhibited in both analyses. The effect of protective culture or food ingredient on the sensory characteristics of fish was evaluated by an official fish appraiser from the Marín retail fish market, who concluded that all the biopreserved batches were worth a higher price in the fish market than the respective control batches, demonstrating that the multibacteriocinogenic strain of marine origin *Lb. curvatus* BCS35 may be considered as a suitable candidate for its application as fresh fish biopreservative.

1. Introduction

Fresh fish is an extremely perishable food compared to other food commodities, partly because of the considerable distance existing between harvesting areas and consumers. Moreover, the time elapsing between catching and landing fish is often much longer than that between landing and selling in the shop, and consequently fishermen bear much of the responsibility for the state of freshness in which the fish reaches the consumer. This issue is of special importance in fisheries distant from the fleet base port where the long periods of time during fish transportation provide abundant opportunities for microbial growth and cross-contamination from different sources, making it more difficult to maintain the hygienic quality required for fish and seafood (Gram and Huss, 1996). The loss of quality of fresh fish and therefore the reduction of its market value is strongly correlated with the decrease of its hygienic–sanitary conditions. The fish species selected for this study, young hake (*Merluccius merluccius*, Merlucciidae) and megrim (*Lepidorhombus boscii*, Scophthalmidae),

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are caught by various types of bottom-trawling gear in multispecific fisheries (Sánchez et al., 1998) in the Northern Stock (ICES Divisions VIIIa, b, d, and VII) and Southern Stock (ICES Divisions VIIIc and IXa), and have a particularly important commercial value (Vázquez-Rowe et al., 2011a, 2011b). The time passed since the fish from the first haul is refrigerated and stored in the hold of the vessel until it is sold in the retail fish market is about 12–14 days. Consequently, the capture obtained in the first few days of each tide reaches considerably lower prices than individuals caught in the last days of the tide (Vázquez-Rowe et al., 2011a). The preservation of fish during this period of time is a major concern to fishermen, since products brought to market in well preserved conditions will generally achieve higher prices, and thus give better returns to the fish industry.

Fish freshness is an important factor when determining whether the fish meat is edible or not, or in considering other possible uses. The sensory evaluation of raw fish in markets and landing sites is carried out by assessing the appearance, texture and odor (Gram and Huss, 1996). Traditionally, several factors have been used to determine fish freshness, including visual inspection of the gills' color and shine, skin color and texture, slime color and thickness, and smell and flesh texture. Freshness can also be assessed by chemical and microbiological analyses. The attempts for controlling the growth of microbiota causing fish spoilage have been fundamentally centered, apart from the action of cold, on the use of modified atmospheres. Nevertheless, this technology has not been widely implemented in this field as it involves a significant and costly transformation of the ship holds that may restrict the uses that outfitters had foreseen for their ships. Furthermore, biopreservation technology, which refers to the shelf-life extension and improvement of food safety by using microorganisms and/or their metabolites, raises as an interesting and cost-effective alternative; however, biopreservation technology must be combined with different hurdles (refrigeration, vacuum-packing, salting, etc.) in order to become a viable alternative. In this respect, lactic acid bacteria (LAB) may be considered as biopreservative agents since they produce a wide range of antimicrobial metabolites including the production of bacteriocins (Cintas et al., 2001; Deegan et al., 2006). For these reasons, during the last years, extensive research has been devoted to investigate the potential application of LAB and/or their antimicrobial compounds in food preservation of a variety of foods including dairy and dairy products, fermented vegetables, meat and meat products, and fish products (Calo-Mata et al., 2008; El Bassi et al., 2009; Leroi et al., 2015; Ndaw et al., 2008). In this context, the aim of this work was to evaluate a biopreservation strategy for fresh fish by the use of bacteriocinogenic LAB of marine origin and/or their bacteriocin-containing supernatants. In recent years, several studies have reported the utilization of bacteriocinogenic- and non-bacteriocinogenic-LAB strains to improve the quality and shelf-life of fish products (El Bassi et al., 2009; Leroi et al., 2015; Ndaw et al., 2008; Nilsson et al., 2004; Tahiri et al., 2009; Tomé et al., 2008). Furthermore, for the development of biopreservation strategies for fish and fish products it is preferable to employ suitable LAB strains isolated from marine species, since the strains already acclimatized to a seafood habitat would be advantageous in terms of biopreservation (El Bassi et al., 2009). The majority of LAB species have a long history of safe human exposure, and therefore have been proposed by the European Food Safety Authority (EFSA) for the Qualified Presumption of Safety (QPS) status (EFSA, 2015), geared to ensure the safety of microorganisms used in food and feed. There are four main general strategies to use bacteriocins as food biopreservatives: (i) inoculation of a culture to produce the bacteriocin in situ as a protective and/or starter culture; (ii) use of a substrate previously fermented by a bacteriocin-producing strain as a food ingredient; (iii) addition of a purified/semi-purified bacteriocin preparation as a food additive, and/or (iv) incorporation or immobilization of the bacteriocin in or onto packaging materials for development of bioactive food packaging (Deegan et al., 2006; Leroi et al., 2015). In a previous study (Gómez-Sala et al., 2015) we described the antimicrobial spectrum of *Lactobacillus curvatus* BCS35 and *Enterococcus faecium* BNM58, isolated from dry-salted cod (*Gadus morhua*, Gadidae) and albacore (*Thunnus alalunga*, Scombridae), respectively, and selected them because of their broad and strong antimicrobial spectrum against spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes*, *Clostridium* spp., *Brochothrix thermosphacta*, *Shewanella putrefaciens* and *Pseudomonas fluorescens*. The aim of the present study was to assess the biopreservative effect of two multibacteriocinogenic LAB strains and/or their antimicrobial metabolites (i.e., bacteriocins) by using several application strategies to improve the microbiological and sensory quality of fresh fish of high economical relevance, namely young hake and megrim.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The LAB strains of marine origin *Lb. curvatus* BCS35 and *E. faecium* BNM58, previously isolated from dry-salted cod and albacore muscle, respectively (Gómez-Sala et al., 2015), were selected for the biopreservation studies of fresh fish. *E. faecium* BNM58 harbors the genetic determinants encoding enterocin L50, enterocin P and enterocin Q, while *Lb. curvatus* BCS35 contains sakacin P, sakacin X and sakacin Q (Gómez-Sala et al., 2015). The LAB strains were stored at −80 and −20 °C with 15% (*v*/*v*) of sterile glycerol as cryopreservative.

For the first part of the study, which was carried out at a laboratory scale (lab-scale), *Lb. curvatus* BCS35 and *E. faecium* BNM58 were grown in MRS broth (Scharlau, Barcelona, Spain) at 30 °C for 16 h with moderate agitation (120 rpm) in an orbital shaker (Thermo Fisher Scientific Inc., USA). Cultures (approx., 1×10^9 cfu/ml) were then divided into three fractions: (i) culture fractions were taken, supplemented with 3% (*w*/*v*) NaCl, and refrigerated until use as protective cultures; (ii) cell-free culture supernatants were obtained by culture centrifugation (12,000 rpm, 4 °C, 10 min), supplemented with 3% (*w*/*v*) NaCl, and kept at −20 °C until use as food ingredients, and (iii) aliquots of cell-free culture supernatants (CFS) were frozen at −40 °C, lyophilized with a Dura Dry II MP apparatus (Kinetics Group Inc., USA), supplemented with 3% (*w*/*w*) NaCl, and stored at room temperature until use as lyophilized food ingredients.

In the second part of the study, which was carried out at a semi-industrial scale, *Lb. curvatus* BCS35 was grown in home-made modified MRS broth (150 l) at 30 °C for 16–18 h in a 180 l fermentor (Innaves S.A., Spain), with pH control (between 6.0 and 6.2) and moderate agitation (120 rpm). The protective culture, food ingredient and lyophilized food ingredient were obtained as described above, except that centrifugation was performed in a Westfalia separator Mod. OTC 3-02-1337 (GEA Westfalia Separator Iberica S.A., Spain).

In both studies, cultures were analyzed for total viable counts (TVCs), and food ingredients and lyophilized food ingredients were assessed for bacteriocin activity. TVCs were performed by the standard dilution method on MRS agar (Scharlau) after incubation at 37 °C for 24–48 h. The bacteriocin activity was measured by an agar-well diffusion test (ADT) as previously described (Cintas et al., 1995) using *L. monocytogenes* CECT4032 as indicator microorganism.

2.2. Fresh fish, batch description and storage conditions

Fresh gutted young hake and ungutted megrim were acquired at the Marín (Pontevedra, Spain) retail fish market. After being caught, the fish was placed in polystyrene boxes, in two layers separated by a plastic sheet and a layer of crushed ice, and maintained overnight at 0–2 °C in the chilled hold of a near-shore fishing vessel. The study was designed to reproduce a fishing trip of 14 days and it was carried out twice.

For the lab-scale study, the fish-containing polystyrene boxes were transported to the laboratory of Innaves S.A. (Pontevedra, Spain) in chilled conditions using crushed ice supplied by the Marin retail fish