In aquaculture, several criteria should be considered to select an appropriate probiotic, including the aquatic origin and safety of the strain and its ability to modulate the host immune response. The properties and effects of probiotics are strain-specific and some factors such as viability, dose and duration of diet supplementation may regulate their immunomodulatory activities. In this study, we assessed the in vitro effect of eight heat-inactivated and viable lactic acid bacteria (LAB) of aquatic origin belonging to the genera Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Weissella on the viability and innate immune response of turbot (Scophthalmus maximus L.) leucocytes. Head-kidney leucocytes were incubated with viable and heat-inactivated LAB at different concentrations. After incubation, the viability of leucocytes was evaluated using colorimetric assays (MTT and LDH) and flow cytometry (annexin V/propidium iodide). Heat-inactivated LAB showed no cytotoxic effect while viable LAB exerted variable influence on apoptosis of turbot phagocytes and lymphocytes. Leucocyte respiratory burst activity and phagocytosis were also differentially activated, as viable LAB stimulated leucocytes more efficiently than the heat-inactivated LAB. Our results suggest diverse strain-specific mechanisms of interaction between the evaluated LAB and turbot leucocytes. Furthermore, our work sets up in vitro systems to evaluate the effect of LAB as potential probiotics, which will be useful to develop efficient screening.

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In fish farming, probiotics are defined as live microorganisms which have a beneficial effect on the host by modifying the host-associated microbiota, improving nutrient digestion and enhancing the host response towards disease [3–10]. Several studies have demonstrated the beneficial effects of probiotics in different fish species [7,8], being the lactic acid bacteria (LAB) and Bacillus spp. the best documented groups [5,9,11,12]. The effect of bacterial viability on the ability of probiotics to stimulate these beneficial effects in fish should be considered before their in vivo routine use. One of the most important effects of probiotics is their ability to modulate immunity [8]. However, the mechanisms by which probiotics interact with the host immune system are poorly known [13–15]. Nevertheless, the properties and effects of probiotics are considered strain-specific [16] and some factors such as viability, dose and duration of supplementation may regulate their immunomodulatory activities [17,18]. Recent molecular, genomic and in vitro studies are improving our knowledge about the host intestinal epithelial and immune response to probiotic strains [14,19–21]. Moreover, the results obtained using in vitro systems have revealed correspondence to those found using in vivo fish assays [15,22]. In this respect, in vitro assays are being developed as tools to: (i) supply a comprehensive understanding of the mechanisms involved in the direct interaction between probiotic bacteria and fish cells; (ii) reduce time and number of experimental animals and, consequently, economic costs and ethic problems; (iii) optimize feeding doses and viability of bacteria; (iv) guide the research on the immune modulatory effect; and (v) identify the most effective probiotic strain to be used for in vivo treatments [15,23].

In a previous work, we characterized 99 LAB from fish, seafood and fish products intended for human consumption [24], and according to their antimicrobial activity against fish pathogens, in vitro safety and promising functional probiotic properties, eight LAB strains were selected as potential turbot probiotics [25,26]. The aim of the present work was to evaluate the effect of these eight LAB on the viability and innate immune response parameters of leucocytes of a relevant marine aquaculture species such as turbot. These assays were performed using heat-inactivated and viable bacteria in order to determine if viability could be essential for the immunomodulatory effect of these LAB on this flatfish species.

2. Materials and methods

2.1. Fish

Healthy turbot specimens with an average body weight of 700 g were reared at the facilities of the Spanish Institute of Oceanography in Vigo (Galicia, Spain). They were maintained in seawater tanks at 18 °C and fed daily with commercial diet. For sampling, specimens were euthanized by an overdose of tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd., Vancouver, Canada).

2.2. Bacterial strains and culture conditions

A total of eight LAB (Enterococcus faecium CV1, E. faecium LPP29, Lactobacillus curvatus subsp. curvatus BCS35, Lactococcus lactis subsp. cremoris SMF110, Leuconostoc mesenteroides subsp. cremoris SMM69, Pediococcus pentosaceus SMM73, P. pentosaceus TPP3 and Weissella cibaria P71) isolated from fish, seafood and fish products intended for human consumption [24] were used in this study. LAB were grown aerobically in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Ltd., Basingstoke, United Kingdom) at 30 °C for 16 h and, when needed, cultures were centrifuged at 6000 × g for 5 min. Bacterial pellets were washed twice with sterile phosphate saline buffer (PBS; pH 7.2) and resuspended in the same buffer. The concentrations of the bacterial suspensions were determined by measuring their optical density, assuming that an OD500 of 1.0 corresponds to 1.2 × 109 cells/ml, according to the McFarland standard (BioMerieux, Marcy L’Étoile, France). Heat-inactivation of the LAB was conducted by exposing the bacterial suspensions to 80 °C for 30 min. To verify the effectiveness of the heat-inactivation process, 10 μl of heat-inactivated suspensions were spread onto MRS agar (1.5%, w/v) (30 °C, 24 h) to check for bacterial growth.

2.3. Isolation of head-kidney leucocytes

Head-kidneys (HK) were isolated from adult turbots under sterile conditions and passed through a 100 μm nylon mesh with heparinized Leibovitz’s L-15 medium (Gibco, Paisley, Scotland, UK) containing 5% (v/v) of heat-inactivated fetal calf serum (FCS) (PAA, Linz, Austria), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco). In the case of cells treated with viable LAB, no antibiotics were added to the culture medium. Turbot HK leucocytes were obtained by density-gradient centrifugation (1800 × g, 25 min) over Ficoll–Paque solution (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The interface was collected, washed and resuspended in the culture medium described above. The cells were counted, at least, four times using a Neubauer chamber and their viability was determined immediately by trypan blue exclusion test (1% in PBS, v/v) (Sigma–Aldrich, St. Louis, Missouri, USA). Finally, the number of cells required for the different assays was adjusted by dilution in the culture medium described above.

2.4. MTT assay

Cellular viability and proliferative effect of LAB was assessed by MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assays performed according to the protocol described by Mosmann [27], with modifications. Briefly, 2 × 106 HK leucocytes in 200 μl of medium supplemented with FCS (5%, v/v) were dispensed in 96-well microtiter plates and incubated in presence and absence of heat-inactivated and viable LAB (109 cfu/ml) at 18 °C for 24 h. After incubation, 100 μl of medium were removed from each well and 10 μl of the MTT solution (5 mg/ml; Sigma–Aldrich Corporation, Missouri, USA) were added. Plates were incubated at room temperature in dark for 4 h and finally the formazan precipitates were solubilized with 100 μl of 0.07 N HCl-isopropanol (Panreac, France). Absorbance (A) was read at 450 nm using a microplate spectrophotometer system (EnVision® Multilabel Reader, PerkinElmer, Massachusetts, USA). HK leucocytes without bacteria or HK leucocytes incubated with Triton X-100 (1%, v/v) were used as negative and positive controls of cytotoxicity, respectively. Wells without HK leucocytes and wells without HK leucocytes but containing LAB were set as background controls. In this assay, the decrease of absorbance was considered as a sign of cell viability loss. Viability (%) was calculated using the following formula after subtracting the respective background values:

\[ \% \text{Viability} = \frac{A_{\text{sample}}}{A_{\text{negative control}}} \times 100 \]

2.5. LDH (lactate dehydrogenase) assay

Cellular cytotoxicity was analyzed using the LDH Cytotoxicity Assay Kit (BioVision Research Products, Mountain View, California, USA) following the manufacturer’s instructions. After 24 h of incubation at 18 °C of 2 × 106 HK leucocytes (200 μl) in the presence and absence of heat-inactivated and viable LAB (109 cfu/ml), microtiter plates were centrifuged (1000 × g, 1 min) and 100 μl of supernatant were transferred to a new 96-well microtiter plate.
Finally, 100 μl of the reaction solution were added and the absorbance was measured after 30 min at 490 nm with 655 nm as reference wavelength in a microplate spectrophotometer system (EnVision® Multilabel Reader, PerkinElmer). The controls were used as the same as described for the MTT assay. In this assay, an increase of absorbance was considered as a sign of cytotoxicity. Cytotoxicity (%) was obtained using the following formula after subtracting the respective background values:

\[
\% \text{Cytotoxicity} = \left( \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \right) \times 100
\]

2.6. Evaluation of apoptosis by flow cytometry

Evaluation of apoptosis in HK leucocytes in the presence or absence of heat-inactivated and viable LAB was performed using the annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining assay described by Saha et al. [28]. Turbot HK leucocytes (2 × 10⁸ cells) were treated and incubated (18 °C, 24 h) in 96-well microtiter plates under the same experimental conditions cited above for colorimetric assays and using the same concentration of LAB. After treatment, HK leucocytes were washed with ice-cold culture medium and stained with 3 μl of annexin V-FITC (Immunotools, Friesoythe, Germany) and 2.5 μl PI (1 mg/ml; Sigma–Aldrich Corporation). Cells untreated and treated with staurosporine (0.1 μg/ml) were used as negative and positive apoptosis controls, respectively. After incubation at room temperature for 10 min in the dark, cells were analyzed using the Coulter XL-MCL Flow Cytometer (Coulter Electronics, Florida, USA). Annexin V-FITC and PI were detected using green (FL-1) and red fluorescence (FL-3) detectors, respectively. Approximately 20,000 counts were made for each sample. Flow cytometry results were expressed as percentages of viable (annexin-V⁻/PI⁻), early apoptotic (annexin-V⁺/PI⁻), and necrotic (or late apoptotic) (annexin-V⁻/PI⁺) leucocytes.

2.7. Respiratory burst activity

The respiratory burst activity of HK leucocytes (1 × 10⁶ cells; 200 μl) in the presence of heat-inactivated and viable LAB (10⁶, 10⁷ and 10⁸ cfu/ml) was determined by the reduction of nitroblue tetrazolium (NBT) assay described by Boesen et al. [29]. After 2 h of incubation at 18 °C, microtiter plates were centrifuged (1000 × g, 1 min) and the medium was aspirated out. Leucocyte monolayers were incubated with 100 μl of NBT solution (1 mg/ml; Sigma–Aldrich Corporation) at room temperature for 2 h in the dark and then plates were centrifuged (1000 × g, 1 min) to remove NBT solution. Cells were fixed with methanol (70%; v/v) for 10 min and air dried, and intracellular formazan blue crystals were solubilized in 120 and 140 μl of potassium hydroxide (2 M; KOH; Merck KGaA, Darmstadt, Germany) and dimethyl sulphoxide (DMSO; Merck KGaA), respectively. Absorbance was measured at 655 nm in a microplate spectrophotometer system (EnVision® Multilabel Reader, PerkinElmer). HK leucocytes without bacteria and HK leucocytes incubated in presence of phorbol myristate acetate (PMA, 1 μg/ml; Sigma–Aldrich) were used as negative and positive controls of respiratory burst activity, respectively. Wells without HK leucocytes and wells without HK leucocytes but containing LAB were set as background controls. In this assay, an increase of absorbance was considered as a sign of an increase of respiratory burst activity.

2.8. Phagocytosis assay

The effect of heat-inactivated and viable LAB on ingestion of zymosan A (Saccharomyces cerevisiae) BioParticles® labeled with FITC (Invitrogen, Madrid, Spain) by turbot phagocytes was evaluated by flow cytometry. After isolation by density gradient centrifugation, HK leucocyte suspensions were enriched with macrophages by their adherent properties onto plastic surfaces as previously described by Sugamata et al. [30]. Briefly, the cell suspension was incubated for 2 h in four Petri dishes pretreated with heat-inactivated turbot serum (45 °C, 1 h) [31]. Following incubation, the supernatant enriched with non-adherent cells was carefully collected. Then, Petri dishes were washed thoroughly once with Leibovitz's L-15 medium to collect adherent cells from the plastic surface. Next, 1 × 10⁶ cells in culture medium (1 ml) supplemented with FCS (5%, v/v) and turbort decomplemented serum (2%, v/v) were incubated in 24-well plates at 18 °C for 24 h. Finally, wells were treated with sonicated particles of FITC-conjugated zymosan at a cell:zymosan ratio of 1:10 in the presence of heat-inactivated or viable LAB at a cell:bacteria ratio of 1:1 at 18 °C for 2 h. Cells plus particles of FITC-conjugated zymosan in the absence LAB were incubated at 18 and 4 °C as positive control of active phagocytosis and to exclude that the ingestion of zymosan was a passive process, respectively. After incubation, the medium was removed and an accutase solution (Life Technologies, California, USA) was added according to the manufacturer’s instructions to detach and collect cells. Finally, cells were washed with culture medium and placed on ice to stop phagocytosis. Trypan blue (0.04%, p/v) was used as a quenching agent to differentiate between membrane attachment and ingestion of zymosan. The samples were mixed gently and analyzed in a Coulter XL-MCL Flow Cytometer. Approximately 20,000 counts were made for each sample. Phagocytes and lymphocytes populations were identified using forward (FS) and size scatter (SS) in a dot plot and the detection of green fluorescence of the respective gated events was calculated from histograms analysis (FL-1). The cytometer was set to analyze the phagocytic cells, showing highest SSC and FSC values. Results were expressed both as phagocytic activity, considered as the percentage of cells with internalized particles of FITC-conjugated zymosan within the phagocytic cell population, and as the phagocytosis index (Phi), which reflects the relative number of ingested zymosan per cell. Phi was calculated according to the following formula: Phi = (% of phagocytosing cells × mean green fluorescence intensity in the gated leucocytes)/100.

2.9. Statistical analysis

Student’s t test was used to determine statistically significant differences between treated cells and negative controls in MTT and LDH assays. Data from annexin V/PI, NBT reduction and phagocytic activity assays were analyzed by one-way ANOVA test with Dunnett’s post hoc comparison to determine the significant differences between the treatments. Statistical analyses were performed using Statgraphics Plus v.5.1 program and the significant level was accepted at p < 0.05.

3. Results

3.1. Evaluation of turbot leucocytes viability by colorimetric assays

The effect of heat-inactivated LAB (10⁸ cfu/ml) on turbot HK leucocytes viability determined by the MTT assay is shown in Fig. 1A. After 24 h of incubation, no significant changes in HK leucocytes viability were observed. Nevertheless, the results obtained with heat-inactivated E. faecium CV1 and P. pentosaceus SMM73
indicate a slight decrease of viability compared to cells in the absence of LAB (p > 0.05). On the other hand, none of the probiotic strains stimulated HK leucocytes proliferation. To confirm these results we performed the LDH test in parallel. As measured by MTT assay, none of the heat-inactivated LAB reduced significantly the viability of turbot HK leucocytes compared to untreated cells (p > 0.05) (Fig. 1B). However, the LDH release was slightly increased in turbot HK leucocytes exposed to heat-inactivated E. faecium CV1 (p > 0.05), suggesting a decreased viability as seen in the MTT test. The effect of live LAB on HK leucocytes viability could not be determined using these tests since their metabolism interfered with the enzymatic activities involved in colorimetric assays (data not shown).

3.2. Leucocyte apoptosis: annexin V and PI staining assay

To evaluate the effect not only of the heat-inactivated LAB but also the viable LAB on the leucocyte viability, we carried out a double staining flow cytometric assay using annexin V and PI. According to the results obtained in the MTT and LDH assays, the percentages of apoptotic/necrotic turbot HK leucocytes (annexin V+/PI- and annexin V+/PI+ cells) after 24 h of incubation with heat-inactivated LAB (10^8 cfu/ml) and control cells were not statistically different (p > 0.05) (Table 1). We also looked for differential effects of LAB strains on leucocytes from the lymphocyte and phagocyte gates (Supplementary Fig. 1). Untreated turbot HK lymphocytes and phagocytes typically show different viabilities after 24 h incubation ex vivo, with higher proportion of apoptotic/necrotic in lymphocytes (16.00 ± 2.81) compared to phagocytes (5.78 ± 1.66). These percentages were not significantly modified after 24 h of incubation in the presence of heat-inactivated LAB, except for phagocytes treated with Lc. cremoris SMM69 which showed a significant increase of apoptosis/necrosis (p < 0.05) (Table 1). With respect to the viable LAB, it is worthy to note that the different strains exerted variable effects on the induction of turbot HK leucocyte apoptosis. As shown in Table 1, when all leucocytes were considered, a significant reduction of apoptotic/necrotic cells was detected after 24 h of incubation with E. faecium CV1 (10^8 cfu/ml) compared to untreated cells (p < 0.05), which was due to a significant decrease of apoptotic/necrotic lymphocytes (p < 0.05). This significant reduction of apoptotic/necrotic lymphocytes was also observed with E. faecium LPP29, L. cremoris SMF110 and Lc. cremoris SMM69 (p < 0.05). Moreover, these three strains had an opposed effect on phagocytes, with a significant increase of apoptosis of phagocytes treated with Lc. cremoris SMM69 (p < 0.05). Also, a significantly higher percentage of annexin-V−/PI− leucocytes was observed with W. cibaria P71 (p < 0.05), while no effect was observed when Lb. curvatus BCS35, P. pentosaceus SMM73 or P. pentosaceus TFP3 were used (p > 0.05) (Table 1). These strains showed the same results when lymphocyte and phagocyte gates were analyzed separately. In addition, when viable (annexin-V+/PI−), early apoptotic (annexin-V+/PI−) and necrotic (or late apoptotic) (annexin-V+/PI−)
leucocytes were analyzed independently (Table 2), we detected a significant increase of viable lymphocytes after 24 h of incubation with viable *E. faecium* CV1, *E. faecium* LPP29 and *Lc. mesenteroides* SMF110. On the other hand, a significant increase of annexin-V/PI− lymphocytes (p < 0.05) were observed with viable *Lc. mesenteroides* SMF669, *W. cibaria* P71, *E. faecium* LPP29 and *Lc. mesenteroides* SMF110. These two last viable LAB strains also increased significantly the annexin-V−/PI+ lymphocytes (p < 0.05).

### 3.3. Respiratory burst activity

After 2 h of incubation with 10⁶ cfu/ml of heat-inactivated and viable LAB, the stimulation of respiratory burst activity of turbot HK leucocytes was variable with bacterial strains (Fig. 2A). With *E. faecium* CV1 and *E. faecium* LPP29, only heat-inactivated bacteria stimulated significantly the respiratory burst activity (p < 0.05), whereas the incubation with live *Lc. mesenteroides* SMF110 or *P. pentosaceus* SMM73 stimulated the respiratory burst activity (p < 0.05). Both live and heat-inactivated *Lb. curvatus* BCS35, *Lc. mesenteroides* SMF669 and *W. cibaria* P71 increased significantly the respiratory burst activity (p < 0.05), while heat-inactivated or live *P. pentosaceus* TPP no longer had stimulatory effects at this concentration (p > 0.05). However, when 10⁵ or 10⁶ cfu/ml were used, a significant stimulatory effect was observed for all LAB compared to controls (p < 0.05) regardless of their viability. In most cases the viable LAB showed a higher respiratory burst induction ability (Fig. 2B and C).

### 3.4. Phagocytic activity

To gain further insight into the effect of heat-inactivated or viable LAB on innate immune response, flow cytometry was used to evaluate their impact on zymosan phagocytosis by turbot HK leucocytes. As shown in Fig. 3, none of the heat-inactivated LAB caused a significant increase of the phagocytic activity or PhI compared to control cells (p > 0.05), except *E. faecium* CV1, that showed a significant increase of PhI (p < 0.05). Importantly, the exposure to viable LAB increased significantly the phagocytic activity and PhI compared to untreated cells (p < 0.05) in all cases excepted for *Lb. curvatus* BCS35, for which the increase in phagocytic activity only was significant (p < 0.05).

### 4. Discussion

The use of probiotics in aquaculture can be an effective strategy to reduce the use of antibiotics and other treatments, and the LAB are the most widely proposed candidates for this purpose [4,9,12,32]. Amongst the potential beneficial effects of probiotics,
the stimulation of innate immune response is a key aspect to help fighting infectious diseases in production systems. However, the probiotic mechanisms which modulate this immune response are still not well elucidated. We hypothesize that results obtained from in vitro assays might be predictive of those observed in vivo [15,22]. However, it should be noted that, although in vitro characteristics suggest the potential usefulness of bacteria as fish probiotics, in vitro results not always correspond to in vivo outcomes [33–35].

**Fig. 2.** Effect of heat-inactivated (●) and viable LAB (▲) on the respiratory burst activity in turbot HK leucocytes determined by NBT reduction assay. Untreated cells were used as control (■). Different LAB concentrations were tested: $10^6$ (A), $10^7$ (B) and $10^8$ cfu/ml (C). Results are expressed as optical density (O.D.) (mean ± S.D.). All experiments were performed threefold in triplicate. Means that share the same letter indicate no significant difference amongst groups of each treatment ($p > 0.05$).
A variety of parameters such as dose, treatment duration, route of administration and frequency of delivery may explain the differences observed. The in vitro assays are useful to evaluate the effect after direct contact between the immunocompetent cell and LAB but, after LAB administration in the diet or by bath, we need to be sure that the candidate probiotic strain will be able to get the localization where its effect is required and, in addition, they have to proliferate efficiently to reach enough concentration to exert their immunostimulant properties detected by in vitro studies. In this sense, we undertook the development of such in vitro systems to evaluate the immune modulatory properties of candidate LAB probiotics for aquaculture use. When validated, such tools would be instrumental to select the most appropriate LAB strain before starting expensive and time consuming in vivo assays.

Ideal probiotics should stimulate the immune response without causing cytotoxicity to the host cells [36]. Two colorimetric assays, MTT and LDH, which rely on the metabolic activity of cells, were used to evaluate if the eight LAB of aquatic origin are cytotoxic for turbot HK leucocytes. Both assays allowed to determine that there is no influence of heat-inactivated LAB on turbot HK leucocytes viability. However, they were not useful to evaluate the effect of viable LAB, since their metabolism interferes with the enzymatic activities involved in these colorimetric assays. Therefore, a double staining flow cytometric assay using annexin V and PI were carried out in order to evaluate the effect not only of the heat-inactivated LAB but also the viable LAB on the leucocyte viability due to the limitation of these colorimetric assays. This test confirmed the results obtained with heat-inactivated LAB strains in MTT and LDH assays. Furthermore, flow cytometry allowed to distinguish the LAB effect on lymphocytes and phagocytes subpopulations. As observed with all HK leucocytes, no cytotoxic effect was observed for the heat-inactivated bacterial forms on any of the two subpopulations, except for Lc. cremoris SMM69, which increased phagocyte apoptosis. On the contrary, viable LAB showed five different effects on the viability of turbot HK leucocytes: (i) Lb. curvatus BCS35, P. pentosaceus SMM73 and P. pentosaceus TPP3 did not affect phagocyte or lymphocyte apoptosis/necrosis; (ii) E. faecium CV1 decreased lymphocyte apoptosis; (iii) Lc. cremoris SMM69 decreased lymphocyte apoptosis and increased phagocyte late apoptosis/necrosis; (iv) E. faecium LPP29 and L. cremoris SMF110

Fig. 3. Effect of heat-inactivated (a) and viable LAB (b) on the phagocytosis of turbot HK leucocytes determined by flow cytometry. Untreated cells were used as control (g). Results are expressed as phagocytic activity (A) and phagocytosis index (B) (mean ± S.D.). Data represent three independent experiments. Means that share the same letter indicate no significant difference amongst groups of each treatment (p > 0.05).
increased lymphocyte and phagocyte late apoptosis/necrosis but the net effect over the viability of lymphocytes was positive; and (v) *W. cibaria* P71 caused the death of lymphocytes and phagocytes. Altogether, these data indicate that the effect of LAB on leucocytes is strain-specific and they suggest that lymphocytes and phagocytes might be differentially affected. Therefore, measuring the LAB influence on the viability of immunocompetent cells, in combination with *in vitro* assays to determine their immunostimulatory effect, would be an appropriated method of screening to evaluate how diverse LAB strains and forms influence the status of immune cells before performing the *in vivo* studies. In this sense, we could also expect different modulator effects on leucocytes from other teleost species, as previously described in HK leucocytes isolated from gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) treated with the probiotic *Vagococcus fluvialis* [23]. In addition, complete genome sequences are now available for many species belonging to a great number of fish families. Their analysis reveals particular features, such as the presence of duplicated genes [37] that provides a large resource for sub-functionalization, and likely favors the diversity of defense mechanisms among teleost fish species. Hence, different responses could be expected against the same agent.

Apoptosis cells have been considered as a contributor to the pathology of many diseases, including disorders of the immune function [38]. Some studies have reported the direct effect of probiotics on apoptosis of fish non-immune cells [39,40] but little is known with respect to their effect on apoptosis in fish immune cells. Different results have been described depending on the tested bacterial strain, tissue or even among different fish species. In this respect, Salinas et al. [40] evaluated the effect of the cytoplasmic extracts obtained from two probiotic strains on cell proliferation and apoptosis of two fish cell lines: SAF-1, a fibroblast cell line from gilt head sea bream dorsal fin, and EPC, a cell line from epidermillo papulosum cyprini derived from a skin tumor of carp (*Cyprinus carpio*). The extracts elicited different effects, being that obtained from *Lactobacillus delbrueckii* subsp. *lactic CECT 287* the only one showing a clear antiproliferative and apoptotic effect on both cell lines. Other studies showed a dual effect of probiotics on apoptosis in diverse cell lines [39,41]. Lazado and Caipang [39] observed no significant changes on caspase-3 activity in primary epidermal cells of Atlantic cod (*Gadus morhua*) exposed to probiotics (*Pseudomonas* sp. GP21 and *Psychrobacter* sp. GP12), which indicates the absence of any effect on apoptosis. In the same study, these probiotics significantly reduced the apoptosis induced by *Vibrio anguillarum* 2133 in dorsal primary epidermal cells at 24 h post incubation, but not in ventral primary epidermal cells at the same incubation time, showing a possible duality of the protective mechanism against pathogen-induced apoptosis. Moreover, in the presence of *V. anguillarum* 2133, GP21 and GP12 induced apoptosis in rectum epithelial cells and suppressed apoptosis in epithelial cells isolated from fore, mid and hind intestine [41]. On the other hand, there are no previous studies on the effect of probiotics on apoptosis in fish immune cells; however, it has been reported a down-regulation of apoptotic caspase-3 and -9 in sea bass HK leucocytes infected with *V. anguillarum* thus evading the host immune response [42]. Understanding of the mechanisms involved in immune cells apoptosis modulation may provide additional insights into the mechanism of immune homeostasis and tolerance to microorganisms [43] and may offer new options for the treatment of different diseases [44]. Previous studies with mammalian cells have reported that probiotics may allow cells to survive from different stresses and stimulate cell survival [20], while the induction of apoptosis may be a beneficial effect in the therapy of inflammatory diseases [43,45,46]. However, further studies are needed to determine how the modulation of apoptosis can affect fish leucocytes *in vivo* and the health status of the animals.

Probiotics can interact with humoral and cellular components of the immune system to enhance the innate and specific immune responses [8]. Probioic LAB, like other Gram-positive bacteria, express conserved microbe associated molecular patterns (MAMPs), which are recognized by receptors expressed by epithelial and immune cells [21], the pattern recognition receptors (PRRs). This interaction drives the activation of the innate immune response, including the respiratory burst activity and phagocytosis [8]. In this sense, different inactivated bacteria have showed immunostimulatory capability under *in vitro* [15,23] and *in vivo* [47,48] conditions. However, viable bacteria have demonstrated a better stimulatory effect on fish immune response using comparative studies [49,50]. This work shows that eight different LAB of aquatic origin are able to activate, *in vitro*, these two innate immune parameters in turbot HK leucocytes. The activation of respiratory burst activity was observed regardless of bacterial viability; however, at higher concentrations (10^5 and 10^6 CFU/ml) the viable LAB showed a more marked effect. In addition, with seven out of the eight studied LAB strains, a significant increase of phagocytic activity and phagocytosis index was only observed for the viable forms. It is worthy to note that viable LAB strains, such as *Vibrio* SMF110, *Lc. remorius* SMM69 and *W. cibaria* P71, which stimulated a high respiratory burst activity and phagocytosis, induced a higher death in phagocytes (Tables 1 and 2) compared to other strains, as *E. faecium* CV1, *P. pentosaceus* SMM73 and *P. pentosaceus* TPP3 with lower respiratory burst activity at 10^5 and/or 10^7 CFU/ml. These results suggest a correlation between the production of reactive oxygen species (ROS) by phagocytic cells and death cell as indicated by other studies in mammals and fish [51–54]. During phagocytosis macrophages and other phagocytic cells produce ROS through activation of a multicomponent NADPH oxidase [52]. These reduced metabolites of oxygen are very useful to protect against pathogens but, when they are present at high levels, multiple intracellular signaling pathways controlling oxidative stress-induced apoptotic/necrotic cell death can be activated [55]. However, we cannot establish in all cases a direct correlation between oxidative burst and phagocyte death, since with heat-inactivated *Lc. remorius* SMM69, we observed a lower oxidative burst activity at 10^5 CFU/ml compared with *P. pentosaceus* SMM73, but there is a significant induction of phagocytes death. These results suggest that other intrinsic factors of LAB strains may be involved in leucocyte death induction or protection, such as the different antioxidant properties of LAB strains. Further studies are needed to confirm these hypotheses.

Based on the obtained results, the immunomodulatory properties of the eight studied LAB strains are altered by heat treatment. Similar observations were also reported for *Lb. rhamnosus* JC1 1136, whose capacity to enhance phagocytic and complement activity in rainbow trout was affected by heat treatment [49]. The viability-dependent induction of immune response may be due to the effects on structural integrity of the bacteria during the exposition to high temperatures and the alteration of molecules that play an important immunomodulatory role, like MAMPs. It is known that the composition and length of MAMPs is a strain-specific trait, which could explain some strain-dependent effects observed, such as the burst respiratory activity and death induction effect exerted by the LAB strains of aquatic origin on turbot HK leucocytes. Nevertheless, further in vivo studies have to be carried out to gather evidences of these effects in the presence of turbot pathogens. In this regard, most of these LAB have shown to display antimicrobial activity against the turbot pathogens *Tenacibaculum maritimum* and *Vibrio splendidus*, good seawater survival, tolerance to low pH and turbot bile, adhesion to turbot skin mucus, and
in vivo respiratory burst and phagocytic activities. Finally, our work sug-
turbot leucocytes. Moreover, our results indicate that the viability
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.02.021.

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