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Evaluation of Immunomodulatory Effects of Lactic Acid Bacteria in Turbot (*Scophthalmus maximus*)

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In the present work, the effects of several lactic acid bacteria on the immune response of turbot (*Scophthalmus maximus*) macrophages have been studied both in vitro and in vivo. Out of six lactic acid bacterial strains tested, only heat-killed *Lactococcus lactis* significantly increased the turbot head kidney macrophage chemiluminescent (CL) response after 24 h of incubation. Nitric oxide (NO) was also significantly enhanced by this bacterium after 72 h of incubation with either viable (10^3 and 10^6 cells/ml) or heat-killed (10^6 cells/ml) bacteria. Viable *Leuconostoc mesenteroides* (10^6 cells/ml) was also capable of significantly increasing NO production. Since *L. lactis* proved to be the strain with more effects on the host immune function, further in vivo and in vitro experiments were conducted with this bacterium. The in vitro capacity of *L. lactis* to adhere to turbot intestinal mucus was positively confirmed. When orally administered, *L. lactis* significantly increased the macrophage CL response and the serum NO concentration after 7 days of daily administration. The antibacterial effect of the extracellular products from the six LAB strains against the fish-pathogenic bacterium *Vibrio anguillarum* was also demonstrated in vitro.

The increased intensification of aquaculture has led to a high number of disease outbreaks with an increasing range of pathogens. Consequently, the extensive use of broad-spectrum antibiotics in aquaculture has led, as in other fields, to drug resistance problems (32). In order to improve health and welfare in the rearing of these animals, several alternatives such as improved husbandry, nutrition, and water quality; lower stocking densities; and use of vaccines, nonspecific immunostimulants (7), and bacterial probiotics such as lactic acid bacteria (LAB) (9, 26) have been proposed.

Probiotics are defined as microbial dietary adjuvants that beneficially affect the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract (23).

The role of LAB within the digestive tract of endothermic animals and humans has been extensively studied (14, 18, 28), while only a few studies have demonstrated that LAB are part of the normal intestinal microflora in larvae and juvenile fish (25). Most studies with probiotics conducted to date with fish have been undertaken with strains isolated and selected from aquatic environments and cultured animals. The main bacteria tested belonged to the genera *Bacillus*, *Vibrio*, and *Pseudomonas* (9, 22). Few authors have tested in vivo the protection conferred by probiotics in fish experimentally infected with pathogens. Gastesoupe (8) found that the *Lactobacillus* or *Carnobacterium* strain isolated from rotifers increased the resistance of turbot larvae against a pathogenic *Vibrio* sp. Gildberg et al. (10) demonstrated that *Carnobacterium divergens* decreased the mortality rate of Atlantic cod fry challenged with *Vibrio anguillarum* but not the mortality of salmon fry challenged with *Aeromonas hydrophila*. Robertson et al. (27) reported that another strain of *Carnobacterium*, administered to fingerlings and fry of Atlantic salmon, reduced the mortality caused by *Aeromonas salmonicida*, *Vibrio ordallii*, and *Yersinia ruckeri* but not *V. anguillarum*.

Although the role of LAB as immunomodulators improving nonspecific defenses is well known for mammals (11, 16, 17), this role, as far as we know, still has to be shown for fish.

In the present work, we have studied for the first time the effects of several LAB on the nonspecific immune response of turbot (*Scophthalmus maximus*). The in vitro effect of viable or heat-killed bacteria on the release of reactive radicals (both nitrogen and oxygen radicals) by turbot head kidney (HK) macrophages was determined. In the case of *Lactococcus lactis*, in vivo experiments to determine its effect on nonspecific immune functions were also performed, as well as experiments to determine its capacity for binding turbot intestinal mucus. Furthermore, in vitro inhibitory activities of filtered LAB culture supernatants were assayed against the pathogenic bacterium *V. anguillarum*.

MATERIALS AND METHODS

Fish. Healthy turbot (*S. maximus* L.) of 40 to 60 g were obtained from a commercial fish farm. The animals were acclimatized in seawater aquarium tanks for 2 weeks, maintained at 18°C, and fed daily with a commercial diet.

Bacterial strains. LAB strains used in the present study were obtained from the Spanish Type Culture Collection (CECT). The strains were *Lactobacillus casei* (CECT 4043), *Lactobacillus brevis* (CECT 815), *Lactobacillus helveticus* (CECT 541), *Lactococcus lactis* subsp. *lactis* (CECT 539), and *Leuconostoc mesenteroides* subsp. *mesenteroides* (CECT 4046). *Pediococcus acidilactici* (NRRL B-5627) was obtained from the Northern Regional Research Laboratory.

All the strains were grown overnight at 30°C in DeMan, Rogosa, and Sharpe (MRS) broth; washed in sterile saline buffer (phosphate-buffered saline [PBS]); and resuspended in Leibovitz medium (L-15; Gibco) supplemented with 5% fetal calf serum (FCS) at concentrations of 10^6 and 10^3 cells/ml. In some experiments LAB were heat killed in boiling water for 2 h.

The pathogenic strain *V. anguillarum* DC11R2 was kindly provided by J. J. Borrego from the University of Málaga, Malaga, Spain (2).

Isolation of HK macrophages. Turbot HK macrophages were isolated according to the method of Chung and Secombes (5). The anterior kidney was removed

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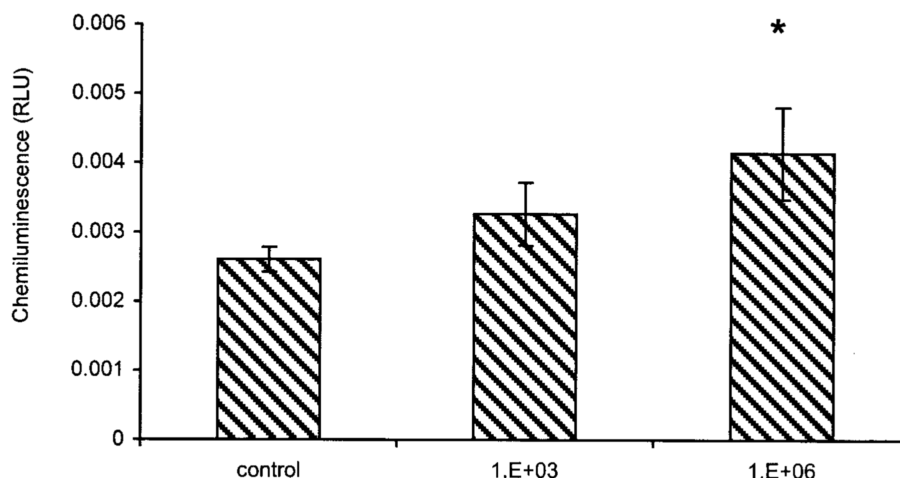

FIG. 1. CL response of HK macrophages with heat-killed *L. lactis* (10^3 and 10^6 cells/ml) after 24 h of incubation at 18°C. Data are expressed as mean RLU ± standard deviations. *, stimulation significantly higher than the mean response observed in nontreated controls ($P < 0.05$). $n = 4$.

aseptically and passed through a 100-μm-pore-size mesh with L-15 supplemented with heparin (10 IU/ml) and 2% FCS. The resulting cell suspension was placed on a 34 to 51% Percoll density gradient. Gradients were centrifuged for 30 min at 500 × *g* at 4°C. The interface cells were then collected and washed at 500 × *g* for 10 min in L-15 containing 0.1% FCS, and the viable cell concentration was determined by trypan blue exclusion. Cells were resuspended in L-15 with 0.1% FCS and dispensed into 96-well plates at a concentration of 10^6 cells/ml. After 3 h, the nonadherent cells were washed off and the monolayers were maintained in L-15 with 5% FCS or the different treatments.

In vitro macrophage stimulation. For the in vitro experiments, HK macrophages dispensed in 96-well plates were treated with 100 μl of the viable or heat-killed LAB strain (10^6 and 10^3 cells/ml). In the case of *L. lactis*, *L. casei*, and *P. acidilactici* the effect of combinations of the bacterial strains was also tested (10^6 cells/ml, final concentration). In all cases, after 24 or 72 h of incubation at 18°C, 50 μl of the macrophage supernatants was removed to determine the nitric oxide (NO) concentration. The remaining cells were used to measure the chemiluminescence (CL) response.

CL assay. The CL response of turbot HK macrophages was measured as the emission of relative luminescence units (RLU) produced by stimulation of cell membranes with phorbol myristate acetate (PMA; Sigma) and amplified by the addition of 5-amino-2,3-dihydro-1,4-phthalazinediedione (Luminol; Sigma) prepared in dimethyl sulfoxide at a final concentration of 10^{-4} M.

Briefly, macrophage monolayers were washed in phenol red-free Hanks' balanced salts solution (HBSS), and 100 μl of 1× HBSS containing Luminol (10^{-4} M) and PMA (2 μg/ml) was added to each well. Controls without PMA were also included. After 30 min of incubation in the dark, the luminescent emission was measured in a luminometer (Fluoroskan Ascent; Labsystems) with 2,000 ms of integration time in each measure. Triplicate wells were used in all experiments.

NO assay. NO production was assayed according to the method described by Tafalla and Novoa (33). This method is based on the Griess reaction, which quantifies the nitrite content of the macrophage supernatant or serum, since NO is an unstable molecule and degrades to nitrite and nitrate (12). After incubation of macrophages at 18°C, 50 μl of macrophage supernatants was removed from individual wells and placed in a separate 96-well plate per triplicate. One hundred microliters of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid was added to each well, followed by 100 μl of 0.1% *N*-naphthylethylenediamine (Sigma) in 2.5% phosphoric acid. Optical density was determined with a multiscan spectrophotometer (Labsystems) at 540 nm. The molar concentration of nitrite in the sample was determined from standard curves previously generated with known concentrations of sodium nitrate.

In vitro adhesion of *L. lactis* to turbot intestine. In order to determine the affinity and capacity of an exogenous *L. lactis* strain to adhere to turbot intestinal mucus, an in vitro adhesion experiment was carried out.

The intestine was excised from the fish, carefully overturned to leave the mucus layer on the surface, and posteriorly tied on both sides with surgical thread to a fixed support. The whole intestine was then introduced for 1 h into a tube containing isotonic solution at 15°C with *L. lactis* (10^8 cells/ml) or without bacteria in the case of control intestines. The intestines were aired every 10 min with an air pump.

After 1 h of treatment, intestines were taken out, placed over sterile petri dishes, and washed with physiological solution. Each intestine was then placed in a clean petri dish to remove the entire mucus surface, and the mucus was weighed, homogenized, and adjusted to a concentration of 100 mg of mucus/ml with physiological solution.

The solution was plated at different dilutions in MRS agar to determine the concentration of LAB. Mucus obtained from control intestines was also plated to determine the amount of LAB present in a normal juvenile turbot. The difference between the numbers of LAB counted in treated intestines and LAB counted in control intestines was attributed to the adherence of *L. lactis*.

In vivo experimental design. To determine the immunomodulatory effects of *L. lactis* when administered in vivo, two groups of five turbot each were force-fed daily. One group was treated with 100 μl of a 10^6-cell/ml suspension, and the other was treated with 100 μl of PBS (control). At day 7, HK macrophages were obtained from each turbot as described above. Blood obtained from the caudal vein was used for serum extraction. The blood was left to clot for 2 h at room temperature and incubated overnight at 4°C. After this time, the plasma obtained after centrifugation was frozen at −80°C until used.

The HK macrophages were used to determine the CL response, NO production, and phagocytosis, while the serum was used to determine the antibacterial index, lysozyme activity, and NO concentration.

The intestines from each fish were also obtained to determine the colonization capacity of *L. lactis* in turbot.

Several assays were performed to determine the differences between *L. lactis*-treated and nontreated turbot.

(i) Phagocytosis assay. A suspension of zymosan A (Sigma) in sterile PBS was heated at 100°C for 30 min, washed twice, and resuspended in L-15 to make a particle/macrophage ratio of approximately 100:1. Four hundred microliters of isolated macrophages (10^6 cells/ml in L-15 with 0.1% FCS) from treated and control fish was placed in each of the wells of an eight-well slide (Nunc). Slides were placed in moist chambers for 30 min at 20°C to allow the macrophages to adhere to the bottom, L-15 was then removed, and zymosan was added. The slides were then incubated under the same conditions for 30 min to allow phagocytosis to take place. Slides were washed twice in 1× phenol red-free HBSS, fixed in ethanol, stained with Haemacolor, and mounted with DePex.

(ii) Serum bactericidal activity. The serum bactericidal activity was measured by comparing the growth of bacteria in serum with that observed in tryptone soy broth (TSB). Briefly, serum samples (33 μl) were placed in triplicate in each well of a 96-well plate. A suspension of *Escherichia coli* (ATCC 13706) (100 μl of 10^8 cells/ml in TSB) was then added to each well, shaken, and incubated for 6 h at 18°C. The plate was then centrifuged for 10 min at 200 × *g*, the supernatant was discharged, and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) was added to each well. After 15 min in the dark, the optical density (600 nm) of the viable bacteria was measured, and the

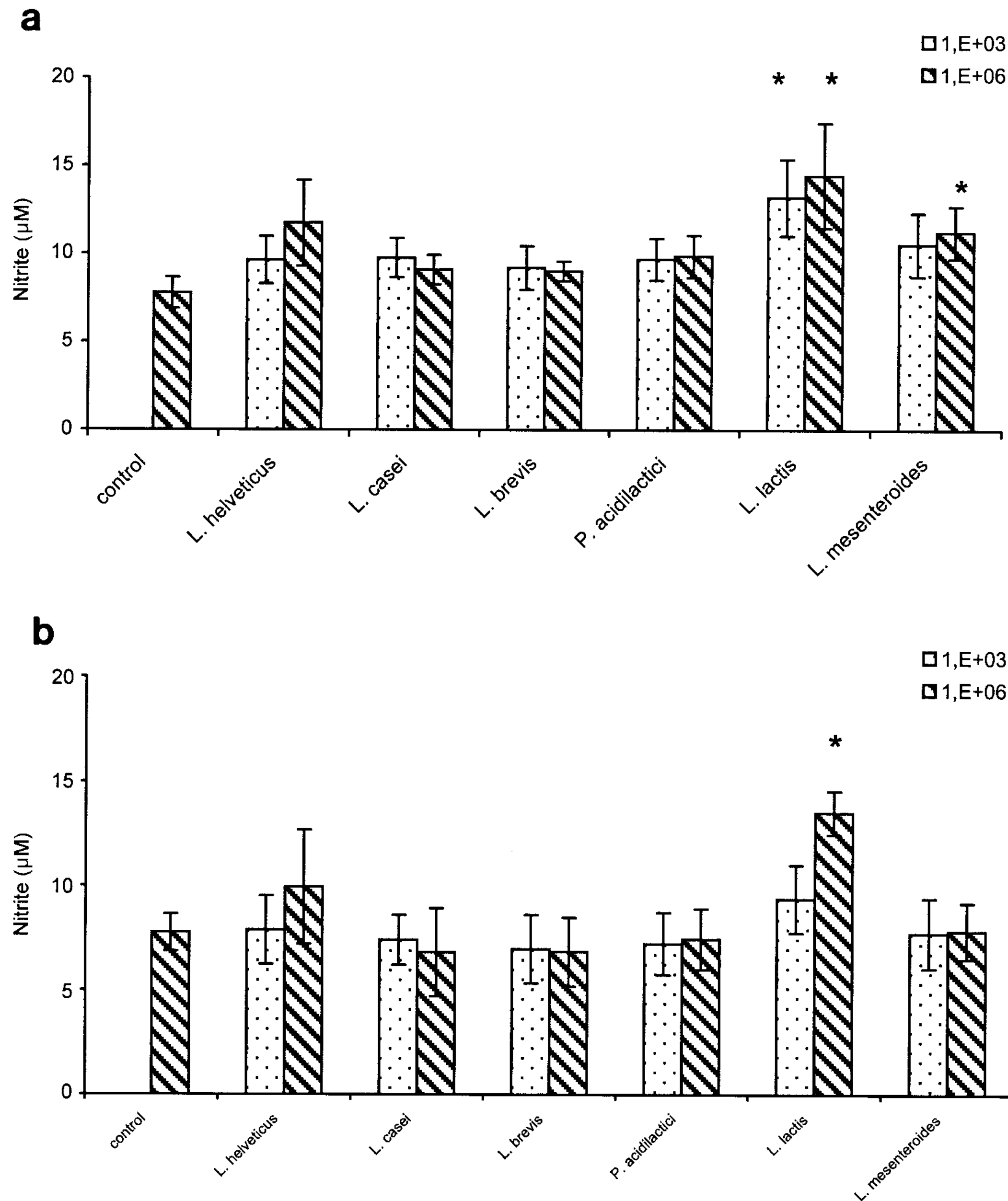

FIG. 2. Effect of incubation with viable (a) and heat-killed (b) LAB for 72 h on NO production of HK macrophages. Data are presented as mean micromolar concentrations of nitrite ± standard deviations. *, stimulation significantly higher than the mean response observed in nontreated controls ($P < 0.05$). $n = 4$.

antibacterial index was calculated as follows: antibacterial index = sample ABS_{600}/control ABS_{600}, where ABS is absorbance.

(iii) Lysozyme activity. Serum (25 µl/well) was placed in triplicate in a 96-well plate, and 175 µl of a suspension of *Micrococcus lysodeikticus* (75 mg/ml in 0.1 M phosphate buffer with 0.09% NaCl, pH 5.8) was added. After the plate was shaken, the decrease in absorbance at 450 nm was recorded for 5 min. Lysozyme activities were converted to lysozyme concentration by using hen egg white lysozyme as a standard.

(iv) *L. lactis* intestinal colonization. In order to determine in vivo the ability of *L. lactis* to colonize turbot after oral exposure, intestines from control and treated fish were aseptically extracted and placed individually in sterile petri dishes. The mucus was extracted and plated as described for the in vitro adhesion experimental assay.

Antibacterial activity of ECPs of LAB. We also determined the in vitro capacity of extracellular products (ECPs) from the six LAB strains mentioned above to inhibit the growth and proliferation of *V. anguillarum* (DC11R2), a fish pathogen.

The ECP extraction was performed according to the method of Cabo et al. (3). Briefly, all the LAB strains were grown overnight at 30°C in MRS broth. After the incubation, the pH was adjusted to 3.5 with 2 N ClH, then heated at 80°C for 3 min, and centrifuged at 500 × *g* for 30 min. The supernatant was filtered through a 45-µm-pore-size filter, buffered at pH 6.0, and stored in aliquots at −80°C.

Bactericidal assay. The tested bacterial strain, a *V. anguillarum* strain, was grown overnight at room temperature in TSB supplemented with 1% NaCl. The assay was performed in triplicate in a 96-well plate by dispensing 50 µl of the

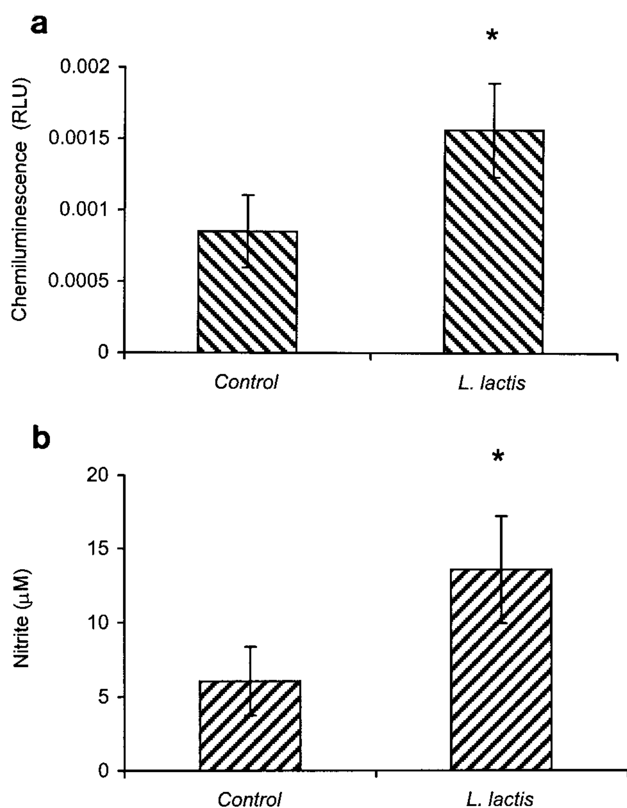

FIG. 3. Effect of in vivo administration (daily force-feeding) of *L. lactis* during 7 days on CL response (a) and serum nitrite content (b). Data are presented as mean RLU ± standard deviations (a) and as mean micromolar concentrations of nitrite ± standard deviations. *, stimulation significantly higher than the mean response observed in nontreated controls ($P < 0.05$). $n = 5$ in each group.

bacterial suspension (10^9 cells/ml) per well and 50 μl of the ECPs (direct and previously diluted 1:2).

Controls were included by incubating the bacteria with PBS and MRS broth instead of ECPs. After 15 h of incubation, changes in optical density (600 nm) were measured.

Statistical analysis. The data were compared by using the Student *t* test and an analysis of variance. Results were expressed as the means ± standard deviations, and the differences were considered significant at $P < 0.05$. All treatments were assayed in triplicate for each fish.

RESULTS

In vitro effects of LAB on the production of NO and oxygen radicals by turbot macrophages. After 24 h of incubation of turbot macrophages with the different viable strains of LAB (10^3 and 10^6 cells/ml), no significant effects were observed on the CL response or NO production (data not shown). When the macrophages were incubated with heat-killed LAB, only *L. lactis* (10^6 cells/ml) was able to significantly stimulate macrophage CL (Fig. 1). In the case of NO production by macrophages, no modifications were observed when they were incubated for 24 h with viable or heat-killed *L. lactis* at any concentration used (data not shown).

Treatment of macrophages for 24 h with combinations of some viable and heat-killed LAB did not significantly stimulate the CL response; only heat-killed *L. lactis* alone elicited the CL response. No significant differences between treatments with combinations of LAB were observed for NO production by macrophages (data not shown).

After 72 h of incubation, the macrophage NO production was significantly increased with the viable *L. lactis* treatment (10^6 and 10^3 cells/ml) and *L. mesenteroides* (10^6 cells/ml) (Fig. 2a). Furthermore, the higher dose of heat-killed *L. lactis* was capable of significantly stimulating NO production (Fig. 2b). With this incubation period the CL response was not significantly modified by treatments with the different LAB (data not shown).

In vitro adhesion of *L. lactis* to turbot intestine. In vitro results showed an important capacity of *L. lactis* to bind to turbot intestinal mucus. An average of 10 CFU/ml was counted in MRS agar in control intestines, whereas for intestines treated with *L. lactis* an average of 2×10^6 CFU/ml was isolated. This difference between treated and control intestines was attributed to *L. lactis* adherence.

In vivo immunomodulatory effects of *L. lactis* in turbot. After 7 days of force-feeding, the CL response (Fig. 3a) as well as NO levels in serum (Fig. 3b) of turbot treated with *L. lactis* was significantly enhanced in comparison to that of the control group.

The phagocytic rate and index, serum lysozyme concentration, and bactericidal activity were not modified by the *L. lactis* treatment (data not shown).

In vivo, the *L. lactis* adhesion to and colonization of turbot intestine was low, since the bacteria were recovered in only one fish (1.5×10^3 cells/ml). For control fish, no LAB were isolated.

Antibacterial activity of ECP from LAB. Incubation of *V. anguillarum* with LAB ECPs resulted in a significant inhibition of growth of the pathogenic bacteria. When the ECPs were not diluted, all bacterial strains significantly inhibited the growth of the pathogenic bacteria. When the ECPs were half diluted, treatments with *L. casei* and *L. mesenteroides* were the only ones not capable of inhibiting the growth of *V. anguillarum* (Fig. 4).

DISCUSSION

The use of LAB in fish culture has been associated in some cases with an improvement in disease resistance and fish survival; however, the immune mechanisms that may be related to this process have never been studied. The results presented in this paper demonstrate that *L. lactis*, an exogenous LAB, is able to modulate the turbot immune system both in vitro and in vivo. Of all the LAB strains tested, heat-killed *L. lactis* significantly increased CL response after 24 h of incubation in vitro. Structural differences in the cell wall compositions of different LAB strains are suggested elsewhere to be responsible for these differences in efficacy (11).

Poor synergism between LAB strains was also observed, since no significant modifications were shown in the CL response or NO production when macrophages were incubated with combinations of viable or heat-killed LAB. It has been shown elsewhere for fish that exogenous molecules can modulate macrophage activity and also can act synergistically or antagonistically to influence the overall outcome for the cell function (24).

The results obtained in vivo are well correlated with in vitro

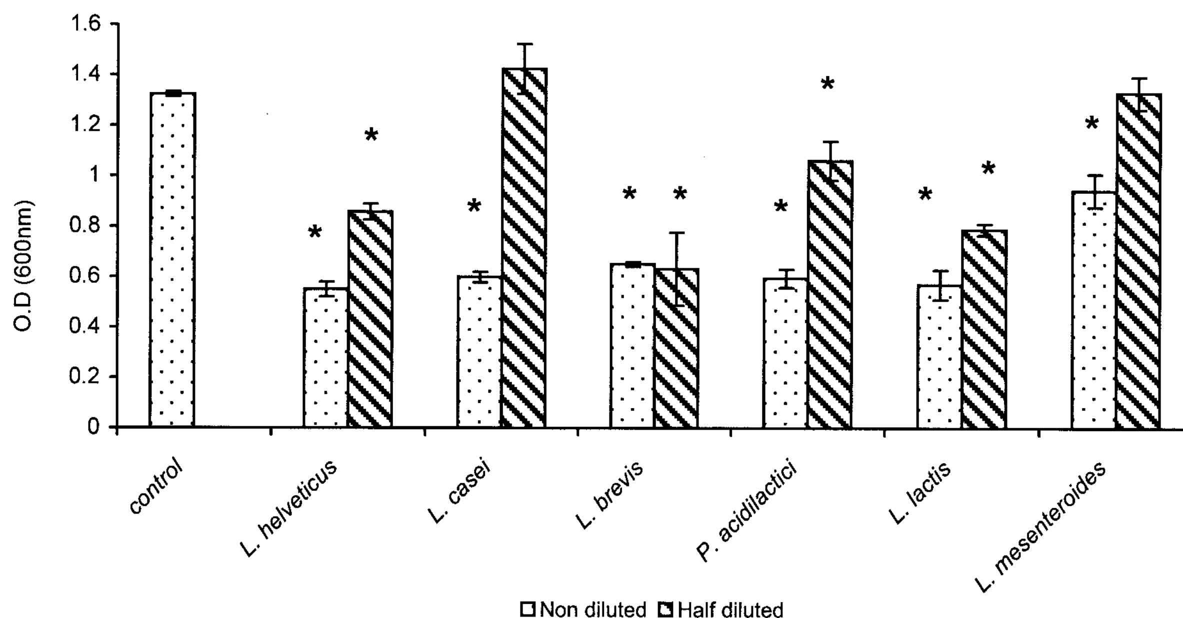

FIG. 4. Growth inhibition of *V. anguillarum* (DC11R2) by incubation with ECPs from LAB for 15 h, nondiluted or half diluted. Data are presented as mean optical densities (600 nm) ± standard deviations. *, significantly lower growth than the mean growth observed in controls incubated with MRS broth ($P < 0.05$).

observations. A significant increase in the CL response of HK macrophages in the group of *L. lactis*-treated fish was noted after 7 days of force-feeding. The mechanism by which *L. lactis* stimulates the CL response is still unclear. It has been reported elsewhere that peptidoglycan present in the gram-positive bacterial cell wall induces inflammation (6). In mammals, administration of LAB resulted in an increased phagocytic activity of peripheral blood leukocytes and macrophages (14, 15, 29).

The enhancement of the CL response in *L. lactis*-treated turbot may possess biological importance since it is related to the phagocytic activity of macrophages, which can improve the host's disease resistance and survival (30). However, this fact still has to be shown in subsequent studies.

The effect of different LAB on NO production was also studied. In vitro experiments showed that, after 72 h of incubation with the lower dose of viable *L. lactis* (10^3 cells/ml) and the higher dose (10^6 cells/ml) of either viable or heat-killed bacteria, the macrophage NO production was significantly enhanced. It was also observed that the higher dose of viable *L. mesenteroides* caused a significant augmentation of NO production. As observed in vitro, daily force-feeding with *L. lactis* also enhanced NO levels in serum.

For mammals, it has been recently shown that incubation of macrophages with LAB also induced NO and cytokine production (34). It may be that in vivo cytokines or other soluble factors may act in synergy with *L. lactis* and therefore induce NO production.

To our knowledge this is the first report of the effect of LAB on NO production in fish. In recent years, NO has been shown to be a very important molecule in regulating immune functions as well as having a direct antimicrobial effect (13, 20). In cell-free assays, it has been shown that the NO donors have inhibitory properties against the fish-pathogenic bacteria *A. salmonicida*, *Renibacterium salmoninarum*, and *Y. ruckeri* (4).

The adherence and survival capacity of LAB are also thought to be important in probiosis, since strains that are able to adhere and survive in the gut mucosa are more efficient at stimulating phagocytic cells (29). Our results suggest that, even though *L. lactis* is able to adhere in vitro to turbot intestinal mucus without specificity problems, in vivo adherence and persistence in the intestinal mucus are more variable. It may be that the adherence capacity of *L. lactis* is affected by environmental factors such as gastrointestinal acids, competition for surface with other bacteria, presence of marine water, and others.

Another desirable property of a probiotic candidate is the capacity to inhibit the growth of pathogens. In our experiments, nondiluted ECPs from *L. lactis* and the other LAB studied were capable of significantly reducing the growth of the pathogenic *V. anguillarum*. ECPs from LAB include several substances that could damage bacterial growth such as lactic acid (1), organic acids (21), hydrogen peroxide, carbon dioxide (23), and bacteriocins (19, 31). More studies are necessary to understand in vivo relationships between probiotic LAB and pathogenic bacteria and to consider other ways to inhibit the growth of undesirable bacteria, such as competition for nutrients and adhesion sites in the intestine (9).

In summary, the in vitro experiments suggest that, out of the six LAB strains studied, *L. lactis* is the one with the strongest stimulation and antibacterial properties for turbot and, therefore, a future candidate for probiotic use in turbot culture.

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