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In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens

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Abstract

The present study describes the screening of five lactic acid bacteria (LAB) for use as probiotics based on their competitive adhesion and production of antagonistic substances against some fish pathogens. A reduction of adhesion of all pathogenic strains tested was obtained with three of the LAB strains (*Lactococcus lactis* subsp. *lactis* CLFP100, *Lactococcus lactis* subsp. *cremoris* CLFP102 and *Lactobacillus curvatus* CLFP150). With the exception of fish pathogens *Flavobacterium psychrophilum* and *Renibacterium salmoninarum* that were not inhibited by LAB strains, production of antagonistic compounds by all tested LAB was observed against at least one of the indicator strains. Based on mucus adhesion, competitive exclusion, and suppression of fish pathogen growth, the selected LAB strains can be considered for future challenge experiments in fish as a very promising alternative to the use of chemotherapeutic agents.

Keywords: adhesion, antimicrobial activity, nonspecific adhesion, probiotics, fish pathogens

1. Introduction

The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular, since the chemotherapeutic agents traditionally used may introduce potential hazards to public health and to the environment because of the evolution of antimicrobial resistance not only in bacteria, but also in fungi, viruses, and parasites (McKeegan et al., 2002).

Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2001). They are usually members of the healthy indigenous microbiota and their addition can assist in returning a disturbed microbiota to its normal beneficial composition. The beneficial effects of probiotics may be mediated by competition for specific pathogen receptor sites on the mucosal surface, production of inhibitory compounds, competition for nutritional substrates, or by an enhancement of the host's innate and adaptive immune responses (Verschuere et al., 2000; Balcázar et al., 2006).

When selecting a potential probiotic strain for beneficial health effects on the host, many criteria must be considered. In order to colonize the gastrointestinal tract, potential probiotics should express high tolerance to acid and bile and have the ability to adhere to host tissue. Colonization of specific microbiota in the gastrointestinal tract may provide protection by competition for host extracellular matrix-binding sites, thereby blocking adhesion and spread of the pathogens; stimulation of host-cell immune defences; or triggering cell-signalling events that deactivate the production of virulence factors and the subsequent onset of sepsis (Reid et al., 2001).

We have earlier shown that fish that were not exhibiting signs of disease during a furunculosis outbreak had a high number of lactic acid bacteria (LAB), especially *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus*

curvatus, *Leuconostoc mesenteroides*, and *Lactobacillus sakei* (Balcázar et al., 2007a). The present study therefore was designed to investigate the ability of selected LAB strains to adhere to two different fish mucus preparations and their activity against several fish pathogens, with the aim of providing a basis for the selection of probiotic strains.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

The fish pathogens *Aeromonas salmonicida* subsp. *salmonicida* CLFP 501, *Carnobacterium piscicola* CLFP 601, *Lactococcus garvieae* CLFP LG1 and *Vagococcus salmoninarum* CLFP 602 were isolated from rainbow trout (*Oncorhynchus mykiss*) by our laboratory, during natural outbreaks. *Yersinia ruckeri* ATCC 29473 and *Flavobacterium psychrophilum* NCIMB 1947 were obtained from Dr. José Guijarro, Department of Functional Biology, University of Oviedo, Spain. *Vibrio anguillarum* La 192 and *Renibacterium salmoninarum* Rs 146 were obtained from Dr. Eva Jansson, National Veterinary Institute, Sweden. With the exception of *F. psychrophilum* and *R. salmoninarum*, all pathogen strains were grown in brain heart infusion broth (BHI, Scharlau Chemie, Barcelona, Spain) overnight at 22 ºC, while *F. psychrophilum* was grown in Anackel-Ordal broth (0.5 % tryptone, 0.05 % yeast extract, 0.02 % sodium acetate, 0.02 % beef extract, adjusted to pH 7.4) for 2 days at 15 ºC with agitation (150 rpm), and *R. salmoninarum* was grown in modified KDM2 broth (1 % bacto peptone, 0.05 % yeast extract, 0.05 % L-cysteine; adjusted to pH 6.5) for 6 days at 15 ºC with agitation (150 rpm).

The following LAB strains were used: *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, *Lb. curvatus* CLFP 150, *Leuc. mesenteroides* CLFP 196, and *Lb. sakei* CLFP 202. The strains were previously isolated from the intestines of

healthy salmonids and genetically identified by 16S rRNA gene sequencing (Balcázar et al., 2007a). LAB strains were grown in BHI broth overnight at 22 ºC. Tritiated thymidine ([*methyl*-1,2-^{3}H]thymidine; 10 µl ml^{-1}, 117 Ci mmol^{-1}) was added to the medium to metabolically label the bacteria. After incubation, bacteria were harvested by centrifugation (2000 *g*), washed twice with phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2), and resuspended in PBS. Bacterial suspensions were adjusted to an absorbance (600 nm) of 0.25 ± 0.05 that corresponded to 10^{7}-10^{8} CFU ml^{-1}. Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU per millilitre.

2.2. Mucus preparation and characterization

Healthy rainbow trout were obtained from a commercial fish farm in the Autonomous Community of Aragon, Spain. Fish with body weights of 250-750 g were sacrificed by immersion in a tank containing tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd., Canada) at a concentration of 150 mg l^{1} of water for 15 min, according to the instructions given by the Zaragoza University Ethics Committee. The skin mucus then was collected from the whole body by scraping the surfaces with a rubber spatula into a small amount of PBS. For intestinal mucus, the intestine was separated and mucus was collected and homogenized in PBS. All mucus preparations were centrifuged twice at 12000 *g* for 5 min at 4 ºC to remove particulate and cellular material. Preparations were then adjusted at a protein concentration of 0.5 mg ml^{-1} in PBS. The protein concentration was determined by a modification of the method of Lowry et al. (1951) as described by Miller and Hoskins (1981), using bovine serum albumin (BSA; Sigma, St. Louis, Mo.) as a standard.

2.3. In vitro adhesion assay

Adhesion of the radioactively labelled bacteria was determined as described by Nikoskelainen et al. (2001a). Briefly, 100 µl (0.5 mg ml^{-1}) of skin or intestinal mucus was immobilized on polystyrene microtiter plate wells by overnight incubation at 4 °C. The wells were washed twice with 250 µl of PBS to remove unbound mucus. A suspension of 100 µl radioactively labelled bacteria (see above) was added to each well. After incubation for 1 h at 22 °C, the wells were washed twice with 250 µl PBS to remove unbound bacteria. Bound bacteria were released and lysed by incubation at 60 °C for 1 h with 1% sodium dodecyl sulfate (SDS) in 0.1 M NaOH. Adhesion was assessed by quantifying the amount of radioactivity by liquid scintillation and was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity in the bacterial suspension added to the immobilized mucus. Adhesion of the bacteria was determined in at least three independent experiments for each mucus type, and each assay was performed in triplicate to correct for intra-assay variation.

2.4. Nonspecific adhesion of LAB and pathogenic strains

To determine if the observed mucus adhesion of the LAB and pathogenic strains was due to nonspecific adhesion, adhesion of the strains to BSA was determined as described above for mucus. Adhesion to polystyrene was determined as an indicator of cell surface hydrophobicity. High hydrophobicity has been suggested to be an indicator for good adhesive abilities (Wadström et al., 1987; Ouwehand et al., 2003).

2.5. Competitive exclusion assay

Labelling and culture conditions were as described above for the *in vitro* adhesion assay. Nonlabelled LAB strains (100 µl; 10^7 to 10^8 CFU ml^{-1}) were allowed to bind to the immobilized mucus, for 1 h at 22 °C. Nonbound LAB strains were washed away with PBS. Subsequently, 100 ml of labelled pathogen strains (*A. salmonicida* subsp. *salmonicida*, *C. piscicola*, *Lc. garvieae*, and *Y. ruckeri*) were added to the wells and

incubated for 1 h at 22 °C. After unbound labelled bacteria were washed away, bound bacteria were released and lysed by incubation at 60 °C for 1 h with 1 % SDS in 0.1 M NaOH. The adhesion of *A. salmonicida* subsp. *salmonicida*, *C. piscicola*, *Lc. garvieae* and *Y. ruckeri* was determined as described above for the *in vitro* adhesion assay. Since mucus adhesion of *F. psychrophilum*, *R. salmoninarum*, *V. anguillarum*, and *V. salmoninarum* was found to be very low (2 % or less), competitive exclusion by the LAB strains was not assessed for these strains.

2.6. Growth inhibition by spent culture liquid

In order to assess the production of possible antimicrobial substances, the five LAB strains listed above were grown in 25 ml of De Man Rogosa and Sharpe broth (MRS, Pronadisa, Madrid, Spain) at 24 °C for two days. After incubation, an inhibition assay was performed as described by Nikoskelainen et al. (2001a). In brief, the bacterial suspensions were removed by centrifugation (2000 *g*), and spent culture supernatants were sterilized by passage through 0.45-µm-pore-size filters. After sterilization, half (5 ml) of each spent culture supernatant was neutralized (pH 6.8) with 5 M NaOH. The type LAB strains *Lc. lactis* subsp. *lactis* DSM 20481^T, *Lc. lactis* subsp. *cremoris* DSM 20069^T, and *Lc. lactis* subsp. *hordniae* DSM 20450^T were used as negative controls against Gram-negative pathogens.

The fish pathogens *A. salmonicida* subsp. *salmonicida*, *C. piscicola*, *Lc. garvieae*, *Y. ruckeri*, *V. salmoninarum*, and *V. anguillarum* were grown in 1 ml of BHI broth overnight at 22 °C, while *F. psychrophilum* was grown in Anackel-Ordal broth for 2 days at 15 ºC, and *R. salmoninarum* was grown in modified KDM2 broth for 6 days at 15 ºC. The cells were harvested by centrifugation (2000 *g*), washed twice with PBS, and resuspended in 1 ml of PBS. The bacterial suspensions were transferred evenly on TSA plates, with the exception of *F. psychrophilum* and *R. salmoninarum* which were

transferred on Anackel-Ordal and KDM2 plates, respectively. Four wells were made in each agar plate with a sterile pasteur pipette; 10 µl of neutralized and 10 µl of untreated spent culture supernatant from the LAB strains were added to the wells. In two wells, neutralized MRS and MRS (pH 5.59) were added to determine possible inhibitory activity of the medium. After aerobic incubation for 2 days at 22 °C, clearing zones were determined. It is important to point out that *F. psychrophilum* and *R. salmoninarum* were incubated for 2 and 6 weeks at 15 ºC, respectively. These assays were carried out in duplicate and independently repeated at least three times.

2.7. Statistical analysis

All results are shown as the average of at least three independent experiments; variation is expressed as standard deviation. Student's *t* test, analysis of variance (ANOVA) and the Duncan's multiple range tests were used to determine if strains and mucus tested differed significantly ($P < 0.05$). The Pearson correlation coefficient was calculated to determine the possible relation between the adhesion to polystyrene and to intestinal mucus. All statistics were performed using SPSS for Windows version 11.5 (SPSS, Chicago, IL).

3. Results and discussion

The ability of bacterial strains to adhere is often considered one of the main selection criteria for potential probiotics (Ouwehand et al., 1999b). Adhesion to the mucus intestinal may prolong persistence (Alander et al., 1999) and contribute to the elimination of pathogenic bacteria (Lee et al., 2003). Moreover, adhesion to the mucus intestinal also has been suggested to enhance the ability to stimulate the immune system (Shi and Walker, 2004). Balcázar et al. (2007b) observed a correlation between colonization with probiotic bacteria and nonspecific humoral response such as

alternative complement pathway activity and lysozyme activity in brown trout (*Salmo trutta*).

3.1. Adhesion of pathogenic strains

All eight fish pathogenic strains tested tended to adhere in relatively low number to the two different fish mucus preparations (ranging from 0.5 to 12.2 % adhesion [Fig. 1]). However, only *A. salmonicida* subsp. *salmonicida*, *C. piscicola*, and *Lc. garvieae* showed preferential binding to mucus preparations since they were found to adhere significantly better to intestinal mucus than to skin mucus. This suggests that they have the ability to bind to intestinal mucus, which may help explain their virulence. In fact, it has been suggested that the intestine is a site of colonization and a possible route of infection for some pathogens such as *A. salmonicida* (Jutfelt et al., 2006) as well as a target for protective treatments such as feed containing probiotic bacteria (Nikoskelainen et al., 2001b; Irianto and Austin 2002).

3.2. Adhesion of LAB strains

The LAB strains *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, and *Lb. curvatus* CLFP 150 tended to adhere in large numbers to mucus from the intestine (16.2 to 19.5% adhesion [Fig 2]), although, these strains tended to adhere significantly less ($P < 0.05$) to skin mucus (11.3 to 5.0 % adhesion). No significant difference in adhesion to the two different mucus preparations was observed for the other tested LAB strains. These results confirm our earlier observations that a high number of LAB were isolated from intestinal mucus of healthy salmonids during furunculosis outbreaks, while the number of these bacterial species in the mucus from skin was very low or, in some cases, they were absent (Balcázar et al., 2007a).

3.3. Nonspecific adhesion

Each tested fish pathogenic strain tended to adhere in similar percentages to BSA and the two different mucus preparations ($P > 0.05$) with the exception of *A. salmonicida* subsp. *salmonicida*, *C. piscicola*, *Lc. garvieae*, and *Y. ruckeri*, which tended to adhere significantly better to intestinal mucus in comparison to BSA (Fig. 1).

All LAB strains adhered significantly less to BSA in comparison to intestinal mucus. In addition, the strains adhered well to polystyrene, although the results were not statistically significant (Fig. 2). No correlation was observed between adhesion to mucus preparations and polystyrene. This suggests that hydrophobic interactions are only partially involved in the binding process. In fact, previous studies have suggested that the microbial adhesion process of LAB include passive forces, electrostatic interactions, steric forces, lipoteichoic acids, and specific structures such as external appendages covered by lectins (Servin and Coconnier, 2003).

3.4. Competitive exclusion

The adhesion capacity of *Y. ruckeri* to intestinal mucus was significantly reduced (from 68.80 to 35.54 %, $P < 0.05$) after exposure of intestinal mucus to LAB strains (Table 1). The adhesion of *C. piscicola* was significantly reduced ($P < 0.05$) between 51.82 and 45.11 % by strains *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, *Lb. curvatus* CLFP 150, and *Lb. sakei* CLFP 202. *Leuc. mesenteroides* CLFP 196 also reduced the adhesion of *C. piscicola* to 27.45 %, although the difference was not statistically significant ($P > 0.05$). Moreover, the adhesion of *A. salmonicida* subsp. *salmonicida* and *Lc. garvieae* was only significantly reduced ($P < 0.05$) by *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, and *Lb. curvatus* CLFP 150. An interesting observation was that *Leuc. mesenteroides* CLFP 196 enhanced the adhesion of *A. salmonicida* subsp. *salmonicida* to intestinal mucus, although the result was not statistically significant ($P > 0.05$). In an earlier study, two strongly adhesive

222 strains of lactobacilli significantly increased *in vitro* adhesion of *Salmonella*
223 *typhimurium* to human intestinal mucus (Tuomola et al., 1999). This clearly
224 demonstrates that each probiotic strain should be judged on its own merits and that
225 extrapolation from related strains is not acceptable, as suggested by previous studies
226 (Ouwehand et al., 1999a).

227 ***3.5. Growth inhibition by spent culture liquid***

228 With the exception of *F. psychrophilum* and *R. salmoninarum* which were not inhibited
229 by LAB strains, measurable clearing zones were detected around the well filled with
230 neutralized or non-neutralized spent culture liquid from LAB strains against at least one
231 of the indicator strains. The fish pathogenic strains *A. salmonicida* subsp. *salmonicida*
232 and *Y. rukeri* were inhibited by all LAB strains, while antimicrobial activity against
233 other pathogenic strains was very variable (Table 2). In addition, neutralized spent
234 culture liquid from the type LAB strains demonstrated that only *Lc. lactis* subsp.
235 *cremoris* DSM 20069^{T} produces an inhibitory effect against *Lc. garvieae*.
236 Although, the inhibitory mechanism of the interaction was not characterized in this
237 study, the source of the antimicrobial activity cannot be attributed to the acidity of the
238 culture, since the supernatants were neutralized (pH 6.8). Previous studies have
239 suggested that the inhibitory effects of LAB may be due to either individual or joint
240 production of organic acids, hydrogen peroxide, or bacteriocins (Klaenhammer, 1993;
241 Vandenbergh, 1993). Bacteriocins of LAB are commonly active against Gram-positive
242 spoilage and pathogenic bacteria, but these bacteriocins exhibit limited or no
243 antimicrobial action against Gram-negatives (Abee et al., 1995). However, a nisin-like
244 bacteriocin produced by *Lc. lactis* subsp. *lactis* A 164, isolated from kimchi, was active
245 against closely related LAB and *Salmonella typhimurium* (Choi et al., 2000). Chung and
246 Yousef (2005) reported that *Lb. curvatus* OSY-HJC6 produces a bacteriocin-like agent

247 active against Gram-negative strains such as *Escherichia coli* p220, *E. coli* O157:H7,
248 *Salmonella enteritidis*, *S. typhimurium*, and *Pseudomonas fluorescens*.
249 In conclusion, the use of probiotics is an important management tool, but its efficiency
250 depends on understanding the nature of competition between species or strains. Based
251 on mucus adhesion, competitive exclusion, and suppression of fish pathogen growth,
252 *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, *Lb. curvatus*
253 CLFP 150, *Leuc. mesenteroides* CLFP 196, and *Lb. sakei* CLFP 202 can be considered
254 as probiotic strains, although more information on the host/microbe interactions in vivo
255 and development of monitoring tools (e.g. molecular biology) are still needed, in order
256 to finalize selection criteria for potential use in aquaculture.

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Fig 1. Adhesion of pathogenic bacteria to mucus preparations (intestine and skin), BSA, and polystyrene. Adhesion is expressed as percentage of radioactivity recovered from wells compared to radioactivity of the added bacteria; error bars indicate SD. *Adhesion to skin mucus is significantly ($P < 0.05$) different from intestinal mucus.

Fig 2. Adhesion of LAB strains to the mucus preparations (intestine and skin), BSA, and polystyrene. Adhesion is expressed as percentage of radioactivity recovered from wells compared to radioactivity of the added bacteria; error bars indicate SD. *Adhesion to skin mucus is significantly ($P < 0.05$) different from intestinal mucus.

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372

373 Table 1. Competitive exclusion of fish pathogens by LAB strains

LAB strains	Adhesion reduction (%)[a], mean ± S.D.			
	A. salmonicida	*C. piscicola*	*Lc. garvieae*	*Y. ruckeri*
Lc. lactis subsp. *lactis* CLFP 100	68.15 ± 16.64*	45.11 ± 13.82*	48.57 ± 20.07*	59.09 ± 19.21*
Lc. lactis subsp. *cremoris* CLFP 102	74.03 ± 16.78*	51.82 ± 19.96*	52.98 ± 26.29*	68.80 ± 9.50*
Lb. curvatus CLFP 150	65.28 ± 10.90*	49.33 ± 15.93*	51.02 ± 17.77*	65.30 ± 8.68*
Leuc. mesenteroides CLFP 196	– 10.47 ± 14.49	27.45 ± 21.88	19.33 ± 20.15	38.64 ± 10.12*
Lb. sakei CLFP 202	30.70 ± 19.23	45.30 ± 11.71*	13.02 ± 27.11	35.54 ± 10.74*

374

375 [a]Data are expressed as the mean percentage reduction of adhesion ± S.D. (standard

376 deviation).

377 *Significant reduction of pathogen adhesion ($P < 0.05$).

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379

380 Table 2. Antimicrobial activities of the LAB strains towards indicator bacteria

LAB strains	Indicator bacteria (*)							
	A. salmonicida	*C. piscicola*	*F. psychrophilum*	*Lc. garvieae*	*R. salmoninarum*	*Y. ruckeri*	*V. anguillarum*	*V. salmoninarum*
Lc. lactis subsp. *lactis* CLFP 100	++	–	–	–	–	+	++	–
Lc. lactis subsp. *cremoris* CLFP 102	++	–	–	–	–	+	++	–
Lb. curvatus CLFP 150	++	+	–	–	–	+	–	+
Leuc. mesenteroides CLFP 196	++	–	–	+	–	+	–	–
Lb. sakei CLFP 202	++	–	–	–	–	+	–	–
Lc. lactis subsp. *lactis* DSM 20481	–	–	–	–	–	–	–	–
Lc. lactis subsp. *cremoris* DSM 20069	–	–	–	+	–	–	–	–
Lc. lactis subsp. *hordniae* DSM 20450	–	–	–	–	–	–	–	–

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382 Source of the bacterial strains: CLFP, Collection of Laboratory of Fish Pathology (Zaragoza, Spain); DSM, German Collection of

383 Microorganisms and Cell Cultures (Braunschweig, Germany).

384 *Clear zones with neutralized spent culture liquid from the tested LAB strains: +, clear zone of 10 mm or more; + +, clear zone of 15 mm or

385 more; –, no inhibition.

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