



HAL
open science

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

José Luis Balcázar, Daniel Vendrell, Ignacio de Blas, Imanol Ruiz-Zarzuela, Olivia Gironés, José Luis Múzquiz

► **To cite this version:**

José Luis Balcázar, Daniel Vendrell, Ignacio de Blas, Imanol Ruiz-Zarzuela, Olivia Gironés, et al.. In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. *Veterinary Microbiology*, 2007, 122 (3-4), pp.373. <10.1016/j.vetmic.2007.01.023>. <hal-00532199>

HAL Id: hal-00532199

<https://hal.science/hal-00532199v1>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

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

Authors: José Luis Balcázar, Daniel Vendrell, Ignacio de Blas, Imanol Ruiz-Zarzuela, Olivia Gironés, José Luis Múzquiz



PII: S0378-1135(07)00061-2
DOI: doi:10.1016/j.vetmic.2007.01.023
Reference: VETMIC 3578

To appear in: *VETMIC*

Received date: 29-12-2006
Revised date: 25-1-2007
Accepted date: 29-1-2007

Please cite this article as: Balcázar, J.L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Gironés, O., Múzquiz, J.L., In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.01.023

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

3 José Luis Balcázar*, Daniel Vendrell, Ignacio de Blas, Imanol Ruiz-Zarzuola, Olivia

4 Gironés, and José Luis Múzquiz

5 *Laboratory of Fish Pathology, Faculty of Veterinary Sciences, University of Zaragoza.*

6 *50013 Zaragoza, Spain*

7 *Corresponding author: telephone: +34 976761569; fax: +34 976761612; e-mail:
8 balcazar@unizar.es

9 **Abstract**

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

22
23 **Keywords:** adhesion, antimicrobial activity, nonspecific adhesion, probiotics, fish
24 pathogens

25 **1. Introduction**

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

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

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

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

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

55 **2. Materials and Methods**

56 **2.1. Bacterial strains and growth conditions**

57 The fish pathogens *Aeromonas salmonicida* subsp. *salmonicida* CLFP 501,
58 *Carnobacterium piscicola* CLFP 601, *Lactococcus garvieae* CLFP LG1 and
59 *Vagococcus salmoninarum* CLFP 602 were isolated from rainbow trout (*Oncorhynchus*
60 *mykiss*) by our laboratory, during natural outbreaks. *Yersinia ruckeri* ATCC 29473 and
61 *Flavobacterium psychrophilum* NCIMB 1947 were obtained from Dr. José Guijarro,
62 Department of Functional Biology, University of Oviedo, Spain. *Vibrio anguillarum* La
63 192 and *Renibacterium salmoninarum* Rs 146 were obtained from Dr. Eva Jansson,
64 National Veterinary Institute, Sweden. With the exception of *F. psychrophilum* and *R.*
65 *salmoninarum*, all pathogen strains were grown in brain heart infusion broth (BHI,
66 Scharlau Chemie, Barcelona, Spain) overnight at 22 °C, while *F. psychrophilum* was
67 grown in Anackel-Ordal broth (0.5 % tryptone, 0.05 % yeast extract, 0.02 % sodium
68 acetate, 0.02 % beef extract, adjusted to pH 7.4) for 2 days at 15 °C with agitation (150
69 rpm), and *R. salmoninarum* was grown in modified KDM2 broth (1 % bacto peptone,
70 0.05 % yeast extract, 0.05 % L-cysteine; adjusted to pH 6.5) for 6 days at 15 °C with
71 agitation (150 rpm).

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

75 healthy salmonids and genetically identified by 16S rRNA gene sequencing (Balcázar et
76 al., 2007a). LAB strains were grown in BHI broth overnight at 22 °C. Tritiated
77 thymidine ([*methyl*-1,2-³H]thymidine; 10 µl ml⁻¹, 117 Ci mmol⁻¹) was added to the
78 medium to metabolically label the bacteria. After incubation, bacteria were harvested by
79 centrifugation (2000 g), washed twice with phosphate-buffered saline (PBS; 10 mM
80 phosphate, pH 7.2), and resuspended in PBS. Bacterial suspensions were adjusted to an
81 absorbance (600 nm) of 0.25 ± 0.05 that corresponded to 10⁷-10⁸ CFU ml⁻¹. Dilution
82 plating was used to verify the relationship between absorbance at 600 nm and CFU per
83 millilitre.

84 ***2.2. Mucus preparation and characterization***

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

98 ***2.3. In vitro adhesion assay***

99 Adhesion of the radioactively labelled bacteria was determined as described by
100 Nikoskelainen et al. (2001a). Briefly, 100 μl (0.5 mg ml^{-1}) of skin or intestinal mucus
101 was immobilized on polystyrene microtiter plate wells by overnight incubation at 4 °C.
102 The wells were washed twice with 250 μl of PBS to remove unbound mucus. A
103 suspension of 100 μl radioactively labelled bacteria (see above) was added to each well.
104 After incubation for 1 h at 22 °C, the wells were washed twice with 250 μl PBS to
105 remove unbound bacteria. Bound bacteria were released and lysed by incubation at 60
106 °C for 1 h with 1% sodium dodecyl sulfate (SDS) in 0.1 M NaOH. Adhesion was
107 assessed by quantifying the amount of radioactivity by liquid scintillation and was
108 expressed as the percentage of radioactivity recovered after adhesion relative to the
109 radioactivity in the bacterial suspension added to the immobilized mucus. Adhesion of
110 the bacteria was determined in at least three independent experiments for each mucus
111 type, and each assay was performed in triplicate to correct for intra-assay variation.

112 **2.4. Nonspecific adhesion of LAB and pathogenic strains**

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

118 **2.5. Competitive exclusion assay**

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

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

131 **2.6. Growth inhibition by spent culture liquid**

132 In order to assess the production of possible antimicrobial substances, the five LAB
133 strains listed above were grown in 25 ml of De Man Rogosa and Sharpe broth (MRS,
134 Pronadisa, Madrid, Spain) at 24 °C for two days. After incubation, an inhibition assay
135 was performed as described by Nikoskelainen et al. (2001a). In brief, the bacterial
136 suspensions were removed by centrifugation (2000 g), and spent culture supernatants
137 were sterilized by passage through 0.45- μ m-pore-size filters. After sterilization, half (5
138 ml) of each spent culture supernatant was neutralized (pH 6.8) with 5 M NaOH. The
139 type LAB strains *Lc. lactis* subsp. *lactis* DSM 20481^T, *Lc. lactis* subsp. *cremoris* DSM
140 20069^T, and *Lc. lactis* subsp. *hordniae* DSM 20450^T were used as negative controls
141 against Gram-negative pathogens.

142 The fish pathogens *A. salmonicida* subsp. *salmonicida*, *C. piscicola*, *Lc. garvieae*, *Y.*
143 *ruckeri*, *V. salmoninarum*, and *V. anguillarum* were grown in 1 ml of BHI broth
144 overnight at 22 °C, while *F. psychrophilum* was grown in Anackel-Ordal broth for 2
145 days at 15 °C, and *R. salmoninarum* was grown in modified KDM2 broth for 6 days at
146 15 °C. The cells were harvested by centrifugation (2000 g), washed twice with PBS, and
147 resuspended in 1 ml of PBS. The bacterial suspensions were transferred evenly on TSA
148 plates, with the exception of *F. psychrophilum* and *R. salmoninarum* which were

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

157 **2.7. Statistical analysis**

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

165 **3. Results and discussion**

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

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

175 **3.1. Adhesion of pathogenic strains**

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

186 **3.2. Adhesion of LAB strains**

187 The LAB strains *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP
188 102, and *Lb. curvatus* CLFP 150 tended to adhere in large numbers to mucus from the
189 intestine (16.2 to 19.5% adhesion [Fig 2]), although, these strains tended to adhere
190 significantly less ($P < 0.05$) to skin mucus (11.3 to 5.0 % adhesion). No significant
191 difference in adhesion to the two different mucus preparations was observed for the
192 other tested LAB strains. These results confirm our earlier observations that a high
193 number of LAB were isolated from intestinal mucus of healthy salmonids during
194 furunculosis outbreaks, while the number of these bacterial species in the mucus from
195 skin was very low or, in some cases, they were absent (Balcázar et al., 2007a).

196 **3.3. Nonspecific adhesion**

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

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

209 **3.4. Competitive exclusion**

210 The adhesion capacity of *Y. ruckeri* to intestinal mucus was significantly reduced (from
211 68.80 to 35.54 %, $P < 0.05$) after exposure of intestinal mucus to LAB strains (Table 1).
212 The adhesion of *C. piscicola* was significantly reduced ($P < 0.05$) between 51.82 and
213 45.11 % by strains *Lc. lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP
214 102, *Lb. curvatus* CLFP 150, and *Lb. sakei* CLFP 202. *Leuc. mesenteroides* CLFP 196
215 also reduced the adhesion of *C. piscicola* to 27.45 %, although the difference was not
216 statistically significant ($P > 0.05$). Moreover, the adhesion of *A. salmonicida* subsp.
217 *salmonicida* and *Lc. garvieae* was only significantly reduced ($P < 0.05$) by *Lc. lactis*
218 subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, and *Lb. curvatus* CLFP
219 150. An interesting observation was that *Leuc. mesenteroides* CLFP 196 enhanced the
220 adhesion of *A. salmonicida* subsp. *salmonicida* to intestinal mucus, although the result
221 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. ruckeri* 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 Vandenberg, 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.

257 **Acknowledgments**

258 This study was supported by a grant from the National Adviser Body of Continental
259 Cultures (JACUCON). J. L. Balcázar was supported by a fellowship from the Spanish
260 International Cooperation Agency (AECI). We thank E. Jansson and J. Guijarro for
261 providing us with bacterial pathogens for this study. We also thank M.C. Uriel and J.
262 Puertas for skillful technical assistance; W. Aguirre and D.B. Wilson for helpful
263 comments and suggestions.

264 **References**

- 265 Abee, T., Krockel, L., Hill, C., 1995. Bacteriocins: Mode of action and potentials in
266 food preservation and control of food poisoning. *Int. J. Food Microbiol.* 28, 169–185.
- 267 Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-
268 Sandholm, T., von Wright, A., 1999. Persistence of colonisation of human colonic
269 mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption.
270 *Appl. Environ. Microbiol.* 65, 351–354.
- 271 Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Girones, O., Múzquiz, J.L.,
272 2007a. Sequencing of variable regions of the 16S rRNA gene for identification of lactic

- 273 acid bacteria isolated from the intestinal microbiota of healthy salmonids. Comp.
274 Immunol. Microbiol. Infect. Dis. in press.
- 275 Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A.C., Márquez, I.,
276 Girones, O., Múzquiz, J.L., 2007b. Changes in intestinal microbiota and humoral
277 immune response following probiotic administration in brown trout (*Salmo trutta*). Br.
278 J. Nutr. in press.
- 279 Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz,
280 J.L., 2006a. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173–186.
- 281 Choi, H.J., Cheigh, C.I., Kim, S.B., Pyun, Y.R., 2000. Production of a nisin-like
282 bacteriocin by *Lactococcus lactis* subsp. *lactis* A 164 isolated from kimchi. J. Appl.
283 Microbiol. 88, 563–571.
- 284 Chung, H.J., Yousef, A.E., 2005. *Lactobacillus curvatus* produces a bacteriocin-like
285 agent active against gram-negative pathogenic bacteria. J. Food Safety 25, 59–79.
- 286 FAO/WHO, 2001. Report of a joint FAO/WHO expert consultation on evaluation of
287 health and nutritional properties of probiotics in food including powder milk with live
288 lactic acid bacteria. Córdoba, Argentina.
- 289 Irianto, A., Austin, B., 2002. Use of probiotics to control furunculosis in rainbow trout,
290 *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 25, 333–342.
- 291 Jutfelt, F., Olsen, R.E., Glette, J., Ringø, E., Sundell, K., 2006. Translocation of viable
292 *Aeromonas salmonicida* across the intestine of rainbow trout, *Oncorhynchus mykiss*
293 (Walbaum). J. Fish Dis. 29, 255–262.
- 294 Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria.
295 FEMS Microbiol. Rev. 12, 39–86.

- 296 Lee, Y.-K., Puong, K.-Y., Ouwehand, A.C., Salminen, S. 2003. Displacement of
297 bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *J. Med.*
298 *Microbiol.* 52, 925–930.
- 299 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement
300 with the Folin phenol reagent. *J. Biol. Chem.* 19, 265–275.
- 301 McKeegan, K.S., Borges-Walmsley, M.I., Walmsley, A.R., 2002. Microbial and viral
302 drug resistance mechanisms. *Trends Microbiol.* 10, S8–S14.
- 303 Miller, R.S., Hoskins, L.C., 1981. Mucus degradation in human colon ecosystems.
304 Faecal population densities of mucus degrading bacteria estimated by a ‘most probable
305 number’ method. *Gastroenterology* 81, 759–765.
- 306 Nikoskelainen, S., Salminen, S., Bylund, G., Ouwehand, A.C., 2001a. Characterization
307 of the properties of human- and dairy-derived probiotics for prevention of infectious
308 diseases in fish. *Appl. Environ. Microbiol.* 67, 2430–2435.
- 309 Nikoskelainen, S., Ouwehand, A., Salminen, S., Bylund, G., 2001b. Protection of
310 rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*.
311 *Aquaculture* 198, 229–236.
- 312 Ouwehand, A.C., Batsman, A., Salminen, S.J., 2003. Probiotics for the skin: a new area
313 of potential application. *Lett. Appl. Microbiol.* 36, 327–331.
- 314 Ouwehand, A.C., Kirjavainen, P.V., Grönlund, M.-M., Isolauri, E., Salminen, S.J.,
315 1999a. Adhesion of probiotic micro-organisms to intestinal mucus. *Int. Dairy J.* 9, 623–
316 630.
- 317 Ouwehand, A.C., Kirjavainen, P.V., Shortt, C., Salminen, S.J., 1999b. Probiotics:
318 mechanisms and established effects. *Int. Dairy J.* 9, 43–52.
- 319 Reid, G., Howard, J., Gan, B.S., 2001. Can bacterial interference prevent infection?
320 *Trends Microbiol.* 9, 424–428.

- 321 Servin, A., Coconnier, M.H., 2003. Adhesion of probiotic strains to the intestinal
322 mucosa and interaction with pathogens. *Best Pract. Res. Clin. Gastroenterol.* 17, 741–
323 754.
- 324 Shi, H.N., Walker, W.A., 2004. Bacterial colonization in the developing gastrointestinal
325 tract: role in the pathogenesis of intestinal diseases. *Biosci. Microflora* 23, 55–66.
- 326 Tuomola, E.M., Ouwehand, A.C., Salminen, S.J., 1999. The effect of probiotic bacteria
327 on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol. Med.*
328 *Microbiol.* 26, 137–142.
- 329 Vandenberg, P., 1993. Lactic acid bacteria, their metabolic products and interference
330 with microbial growth. *FEMS Microbiol. Rev.* 12, 221–238.
- 331 Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as
332 biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671.
- 333 Wadström, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S., Gullmar, B.,
334 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. *J. Appl.*
335 *Bacteriol.* 62, 513–520.
- 336
- 337
- 338
- 339
- 340
- 341
- 342
- 343
- 344

345

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

350

351

352

353

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

358

359

360

361

362

363

364

365

366

367

368

369

370

371

372

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

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

374

375 ^aData are expressed as the mean percentage reduction of adhesion ± S.D. (standard
376 deviation).

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

378

379

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

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

381

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.

386

387



