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Characterization of the beneficial properties of lactobacilli isolated from bullfrog (*Rana catesbeiana*) hatchery

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Abstract The present work addresses the isolation and partial identification of the microbial population of a *R. catesbeiana* hatchery in spring and summer as well as some beneficial properties of *Lactobacillus* strains isolated in different seasons and hatchery areas. The bacterial population was grouped into the following taxa: *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus faecalis* and *Ent. faecium*, and Enterobacteriaceae (*Enterobacter* spp., *Escherichia coli*) while *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were isolated from frogs displaying red-leg syndrome. The *Lactobacillus plantarum* and *L. curvatus* strains isolated showed to inhibit the growth of red-leg syndrome associated pathogens and food-borne bacteria by organic acids. While *L. plantarum* CRL 1606 also inhibited red-leg syndrome related pathogens by hydrogen peroxide, meat spoilage bacteria were only inhibited by acidity. However, by using a MRS medium added with tetramethylbenzidine and peroxidase, a high percentage of H_2O_2-producing lactobacilli were detected. The surface properties of *Lactobacillus* strains showed that a few strains were able to agglutinate ABO human erythrocytes, while the highest number of strains had a low to medium degree of hydrophobicity. This paper constitute the first study related to the beneficial properties of *Lactobacillus* isolated from a bullfrog hatchery, as well as the selection criteria applied to a group of strains, which could help to control or prevent bacterial infectious diseases in raniculture.

Keywords *Rana catesbeiana* · Red-leg syndrome · Prevention · Probiotics · *Lactobacillus*

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Introduction

Rana catesbeiana—commonly known as bullfrog—hatcheries have developed all around the world based on the increase of the international use and consumption of meat market and by-products. The Food and Agriculture Organization (2001) indicates that bullfrog meat has a high proportion of essential amino acids, a large degree of absorbable proteins, low contents of sodium chloride and lipids (mainly cholesterol). Similarly, the by-products, such as skin, liver and gut, are required by different industries (Texeira et al. 2002). These demands cause an

intensive bullfrog production process where the animals are more susceptible to infectious diseases, such as red-leg syndrome (RLS); RLS is one of the most common and economically devastating diseases described in *R. catesbeiana* hatcheries. The syndrome affects animals at different stages of growth with tadpoles being more susceptible (Glorioso et al. 1974; Bühler et al. 2000; Mauel et al. 2002). The etiological agents include *Proteus vulgaris*, *Pr. mirabilis*, *Citrobacter freundii*, *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus iniae*, *Chryseobacterium meningosepticum*, *Chr. indolgenes* (Glorioso et al. 1974; Mauel et al. 2002; Pasteris et al. 2006). Poor husbandry practices, as well as the presence of potentially pathogenic bacteria in the environment, have been recognized as predisposing factors to infectious diseases (Mauel et al. 2002).

When the first signs of RLS appear, a common practice is to sacrifice the infected specimens and to apply antibiotic therapies, which increase the cost of production. The antibiotics can alter the meat quality and induce bacterial resistance (Verschuere et al. 2000). Based on these situations and on the subsequent reluctance to using antibiotics, the application of probiotics in aquaculture is becoming increasingly popular (Ringø and Gatesoupe 1998; Ringø et al. 2005; Nikoskelainen et al. 2001a, b; Vine et al. 2006).

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Reid et al. 2003). However, Gatesoupe (1999) redefined probiotics for aquaculture as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to keep alive, with the aim of improving health". This is an alternative way to control diseases, by supplementing or, even in some cases, replacing the use of antimicrobial compounds.

The selection of probiotics requires different in vitro screening experiments, as assays for the production of antagonist compounds (organic acids, bacteriocin-like metabolites, diacetyl and hydrogen peroxide), and their growth on or attachment to intestinal mucus (Verschuere et al. 2000; Vine et al. 2006) or eukaryotic cell surfaces. Therefore, a wide range of Gram-positive (*Bacillus*, *Carnobaterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Micrococcus* and *Weisella*) and Gram-negative bacteria (*Pseudomonas*, *Aeromonas*, *Alteromonas*, *Photorhodobacterium* and *Vibrio*), yeast, microalgae, and bacteriophages have been evaluated as probiotics for fish aquaculture. Their mode of action includes competitive exclusion, production of antagonistic substances, stimulation of host immunity (Irianto and Austin 2002), biofilm formation, competition for nutrients, and also some nutritional effects, as stimulation of appetite and the improvement of nutrition status in fish and bullfrogs (Lara-Flores et al. 2003; de Carla Dias et al. 2007).

Based on the host specificity exerted by the members of the indigenous microbiota of human and animals (Savage and Kotarski 1979; Vine et al. 2006), the design of a probiotic for aquaculture includes that the microorganism must be isolated from healthy animals or their environment (Vine et al. 2006). In previous work, the microbial population of *R. catesbeiana* hatchery in autumn was described. It includes *Lactobacillus* spp., *Enterococcus faecium*, *Ent. faecalis*, *Pediococcus* spp., *Micrococcus* spp and *Streptococcus* spp. Enterobacteriaceae family was mainly represented by *Enterobacter* spp and *Proteus vulgaris*, a RLS-associated pathogen which was also isolated from tissues of animals displaying the syndrome (Pasteris et al. 2006).

Keeping in mind that the involvement of probiotics in fish aquaculture is now recognized as one of the preventive measures for diseases prevention and control (Nikoskelainen et al. 2001a, b), the purpose of this work was to complete the evaluation of the microbiota isolated from a bullfrog hatchery at different seasons, and to study some beneficial properties of *Lactobacillus* strains. The final objective is to proceed further in the selection of potential probiotic microorganisms that can be used in raniculture.

Materials and methods

Animals and samples collection

Samples of ten specimens of both healthy and non-healthy *R. catesbeiana* (fattening phase of growth), freshwater, and balanced feed were taken in a hatchery located at the Northwest of Argentina during the spring and summer (September and

December), respectively and kept at 8°C until use. Frogs were taken from separate areas of the hatchery. Samples from the skin were obtained under aseptic conditions by scraping 1 cm^2 of ventral surfaces of living animals using sterile cotton swabs. The samples were collected in LAPT medium supplemented with 0.7% agar (w/v) (Raibaud et al. 1963). Freshwater samples were taken from tanks and stored on ice; 200 ml of water were centrifuged at 7,000×*g* and 4°C for 15 min and the pellet was suspended in 10 ml of peptone-water [meat peptone, 0.1% (w/v); Britania, Argentina]. Ten-gram samples of balanced feed were taken from tanks and washed with 10 ml of peptone-water.

Isolation and partial identification of microbial populations from a *R. catesbeiana* hatchery

The samples were plated on MRS agar (de Man et al. 1969) for lactic acid bacteria, LBS agar (Lactobacillus Selection Media) for *Lactobacillus*, MSA (Mannitol Salt Agar) for *Staphylococcus*, MC agar (MacConkey) for *Enterobacteriaceae*, CATCA (Citrate Azide Tween Carbonate Agar) for *Enterococcus faecalis* and *Ent. faecium* (Reuter 1992), PCA (plate count agar) for total aerobic heterotrophic microorganisms, and SAB (Sabouraud agar) for mycelial fungi and yeast. Plates were incubated aerobically for 48–72 h at 30°C, except those from SAB, which were incubated for 15 days. The identification of microorganisms was performed by morphologic and phenotypic characteristics, using the following biochemical assays: Gram staining, catalase reaction, nitrate reduction, indole production, citrate and urea utilization, mobility, coagulase, arginine and hipurate hydrolysis, Voges–Proskauer reaction, haemolysis test, growth in 6.5% NaCl, growth at different pH and temperatures and fermentation patterns of some sugars (Holt et al. 1994). The strains were stored at −20°C in MRS medium supplemented with 20% (v/v) glycerol. All culture media were obtained from Britania (Argentina).

Bacterial strains and culture conditions

Lactobacillus strains were isolated from a *R. catesbeiana* hatchery during the autumn (Pasteris et al. 2006), summer and spring and were identified by phenotypic and genotypic (Pasteris et al. 2008) approach. The strains were grown in MRS broth medium for 12 h at 37°C. RLS-associated pathogens (*Proteus vulgaris*, *Pr. mirabilis*, *Citrobacter freundii*, *Pseudomonas aeruginosa,* and *Staphylococcus epidermidis*) and *S. aureus* were grown in nutritive broth for 8 h at 37°C. Other meat spoilage bacteria (*Listeria monocytogenes* Scott A and *Salmonella enteritidis*) were grown in brain heart infusion (BHI) broth in the same conditions.

Screening of antagonistic activity

Inhibitory properties of Lactobacillus strains

The inhibition of bacterial growth was used to test the production of antimicrobial metabolites by the isolates. The antibacterial activity was evaluated by the agar-well diffusion assay (Strasser de Saad et al. 1995; Juárez Tomás et al. 2003). Different concentrations of indicator strains (1×10^2 and 1×10^5 CFU/ml) were inoculated in nutritive or BHI soft agar (0.7% w/v) at 45°C and poured into Petri dishes. After solidification, wells of 10 mm diameter were made into the agar plates and 100 µl of overnight *Lactobacillus* supernatant were added to each well. The supernatants were obtained by centrifuging (3,000 *g* for 10 min) *Lactobacillus* cultures and filtered through a 0.22 µm pore-size filter (Millipore). About 2 ml fractions were adjusted to pH 7.0 with sterile 1 N NaOH and treated with 0.5 mg/ml catalase (SIGMA) at 25°C for 30 min. Untreated and treated (neutralized and neutralized + catalase) cell-free supernatants placed in the wells were allowed to diffuse into the agar for 1 h at room temperature. The plates were then incubated at 37°C in microaerophilic conditions for 24 h.

The antagonistic metabolites in the supernatants of *Lactobacillus* strains inhibit the growth of the pathogens by producing an inhibitory area around the well. The inhibition was expressed as degree (in millimetres) of inhibition.

Hydrogen peroxide production

The hydrogen peroxide (H_2O_2) production in lactobacilli cultures was qualitatively determined by the plate method, employing horseradish peroxidase incorporated in tetramethyl-benzidine (TMB) agar medium (Juárez Tomás et al. 2004). Peroxidase

catalyses the oxidation of TMB (chromogenic substrate) to a purple-blue pigment evidenced in those colonies that produce H_2O_2.

The *Lactobacillus* strains were grown in MRS broth and inoculated in MRS plates containing 1 mM TMB (3,3′, 5,5′-tetramethyl-benzidine, from Sigma Chemical Co, Saint Louis, MO, USA, dissolved in methanol), and 2 U/ml of peroxidase (Peroxidase EC 1.11.1.7, Type II: From Horseradish, Sigma Chemical Co., Saint Louis, MO, USA). The plates were incubated at 37°C in a 5% CO_2 atmosphere. After incubation for 48 h, the plates were exposed to air. Colonies able to produce H_2O_2 developed a blue or brown colour. According to the colour intensity, the strains were classified as strong (blue), medium (brown), weak (light brown) or negative (white colonies) producers.

Cell surface properties of *Lactobacillus* strains

Haemagglutination ability

Bacterial cells were collected by centrifuging and suspended to 10^9 CFU/ml by using pH 7.1 phosphate buffered saline (PBS) solution. Haemagglutination (HA) was carried out at room temperature in a 96-well round-bottom micro titter plates by using PBS as diluents. About 50 µl of two fold diluted samples were mixed with 50 µl of 2% (v/v) suspension of both human (ABO blood group system) and amphibian red blood cells obtained by venipuncture and cardiac puncture, respectively, previously centrifuged and washed twice with PBS. The plates were shaken and incubated at 37°C for 30 min and then at 4°C for 6 h. The HA titter was visually determined. The results were expressed as the inverse of the highest dilution of bacteria producing red blood cell agglutination (Ocaña et al. 1999).

Degree of hydrophobicity

Bacterial surface hydrophobicity was determined by the MATH (microbial adhesion to hydrocarbon) assay described originally by Rosenberg and Doyle (1990). The *Lactobacillus* cells were grown in MRS broth at 37°C and later collected by centrifugation at early logarithmic growth phase, washed twice and resuspended in physiological solution (PS) to an optical density (OD 600 nm) 0.6–0.7. Hexadecane (0.45 ml) was added to test tubes containing washed cells (2.7 ml). The samples were gently agitated in a vortex for 90 s. The tubes were left to stand for 15 min for separation of the two phases and the OD of the aqueous phase was determined. The degree of hydrophobicity was calculated from three replicates by using the following (Ocaña et al. 1999):

$$\%\text{Hydrophobicity} = [(\text{OD}_{600}\text{ before mixing} - \text{OD}_{600}\text{ after mixing})/\text{OD}_{600}\text{ before mixing}]\text{x}100$$

The score of hydrophobicity applied is the following: high (60–100%), medium (30–60%), low (0–30%).

Statistical analysis

Experiments were carried out in triplicate. Significant differences (Tukey's test) (Rossman and Chance 1998) and the correlations were tested by using the Minitab Student R12 software.

Results

Bacterial population of a *R. catesbeiana* hatchery

The microorganisms present in a *R. catesbeiana* hatchery in spring and summer belong to the following genera: *Lactobacillus*, *Pediococcus*, *Enterococcus*, members of the Enterobacteriaceae family as well as to the genera *Pseudomonas* and *Staphylococcus*.

In spring, a similar number of *Lactobacillus* spp., *Ent. faecalis* and *Enterobacter* spp. were present in the skin of healthy animals with a low degree of isolation of *Pediococcus* spp., *Streptococcus* spp., *Ent. faecium* and *Escherichia coli*. These findings were also observed in summer, but no *Ent. faecium* were isolated.

In non-healthy frogs, only *Lactobacillus* spp. and *Pediococcus* spp. were isolated in spring, while *Lactobacillus*, *Ent. faecium* and *Ent. faecalis* were present in summer, although the *Lactobacillus* population was higher in summer than in spring. This microbiota was also constituted by members of the Enterobacteriaceae family, mainly *Enterobacter* spp. and *E. coli. Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were only isolated in summer from frog's abdominal skin ulceration.

Freshwater samples of healthy animals in spring showed the presence of *Enterobacter* spp. and *Ent. faecalis* with a low number of *Lactobacillus* spp., while in summer the lactobacilli population increased with the isolation of *Pediococcus* spp. and *Ent. faecalis*. The number of *E. coli* isolates was higher than spring. Freshwater from non-healthy frogs in spring was a source of a low number of *E. coli* and *Pediococcus* spp. In summer, a similar proportion of Enterobacteriaceae (*Enterobacter* spp. and *E. coli*) was isolated. A *Lactobacillus* population was present in a low number, but the isolation was higher in samples from healthy frogs.

Balanced feed in summer presented a similar proportion of *Lactobacillus* spp., *Pediococcus* spp., *Ent. faecium* and *Ent. faecalis*. Low number of *E. coli* was detected.

Under our assay conditions, neither mycelial fungi nor yeasts were isolated.

Inhibitory activity of Lactobacillus strains

Seventy-nine *Lactobacillus* strains isolated at different seasons (spring, autumn, and summer) were evaluated for their inhibitory properties against RLS-associated pathogens and meat spoilage bacteria. Sixty-four *Lactobacillus* strains were able to inhibit the growth with at least one of the assayed pathogens. The strains that presented the widest inhibitory spectrum were selected to display in the results. Table 1 shows the final pH and the antagonist activity of culture supernatants of *Lactobacillus* strains isolated from different areas of a *R. catesbeiana* hatchery in autumn and spring (Pasteris et al. 2006). The inhibitory activity of *Lactobacillus plantarum* CRL 1676 and CRL 1678 isolated from healthy frogs (in autumn) against autochthonous *Ps. aeruginosa* GRB, *Pr. vulgaris* MIB10, and *S. epidermidis* strains, RLS-related pathogens isolated from human source (*Ps. aeruginosa* ATCC 27853, *Pr. vulgaris*, *Pr. mirabilis*, and *C. freundii*) and *S. aureus* was similar, while *Salmonella enteritidis* and *Listeria monocytogenes* Scott A growth was less affected. The inhibitory effect was also observed for *L. plantarum* CRL 1677 but without inhibition on *Listeria* and *Salmonella* growth.

Any of the *Lactobacillus* strains isolated from non-healthy animals in autumn were able to inhibit the growth of RLS-related pathogens and meat spoilage bacteria. This behaviour was also observed in the strains of *Lactobacillus* from freshwater samples from both healthy and non-healthy frogs in autumn and spring.

Five *Lactobacillus* strains isolated from healthy animals in spring were not able to inhibit the growth of any of the pathogen strains assayed. However, from four *Lactobacillus* strains isolated from non-healthy frogs, only *L. plantarum* CRL 1679 inhibited the growth of indicator strains, mainly autochthonous *Ps. aeruginosa* GRB and *S. epidermidis*. The strain was also able to inhibit food-borne bacteria, mainly *Listeria*.

A total of 24 *Lactobacillus* strains isolated from balanced feed in autumn exerted an inhibitory activity against both RLS-associated pathogens and food-borne bacteria. *L. plantarum* CRL 1680 and CRL 1606 strains showed the highest antagonistic activity.

The final pH reached was between 3.5 and 4.4. The antimicrobial activity disappeared when the supernatants were neutralized, which indicates that the inhibition was produced by organic acids, except for *L. plantarum* CRL 1606 whose inhibitory activity disappeared when the neutralized supernatants were treated with catalase, indicating that H_2O_2 was also responsible for the antimicrobial activity. This effect was observed against RLS-associated pathogens and *S. aureus*, while *L. monocytogenes* and *S. enteritidis* were only inhibited by organic acids.

The final pH of culture supernatants and the inhibitory spectrum of *Lactobacillus* strains isolated from bullfrog's hatchery in summer are summarized in Table 2. Ten *Lactobacillus* strains inhibited the growth of the indicator strains. Among them, *L. curvatus* CRL 1682, *L. plantarum* CRL 1607, and CRL 1684 isolated from healthy frogs exhibited the highest inhibitory activity against the assayed indicator strains, while *L. curvatus* CRL 1681, isolated from the same source was not able to inhibit *S. enteritidis* and *L. monocytogenes*.

The antimicrobial activity of six *Lactobacillus* strains isolated from the freshwater of healthy frogs was also evaluated (Table 2). *L. plantarum* CRL 1687 and CRL 1608 showed the higher inhibitory effect on both RLS-associated pathogens and food-borne bacteria growth.

L. plantarum CRL 1688 and 1689 isolated from balanced feed in summer (Table 2) evidenced a

Table 1 Inhibitory activity of *Lactobacillus* strains isolated from *R. catesbeiana* hatchery in autumn and spring against RLS-related pathogens and meat spoilage bacteria

Origin	*Lactobacillus* strains	pH[b]	Indicator strains																			
			Inhibition (mm)																			
			Ps. aeruginosa		*Ps. aeruginosa* GRB		*Pr. vulgaris*		*Pr. vulgaris* MIB10		*Pr. mirabilis*		*C. freundii*		*S. enteritidis*		*L. monocytogenes*		*S. aureus*		*S. epidermidis*	
			A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
HA	*L. plantarum* CRL 1676	3.9	11	11	11	11	12	10	8	8	10	10	8	7	5	3	6	4	9	7	10	8
	L. plantarum CRL 1677	4.4	6	6	10	9	8	7	6	6	9	7	5	4	–	–	–	–	7	3	8	7
	L. plantarum CRL 1678	3.8	11	11	10	10	11	10	10	10	12	12	7	6	5	5	6	6	11	7	12	10
NHA[a]	*L. plantarum* CRL 1679	3.8	5	4	10	9	3	3	4	4	5	4	2	1	2	2	5	4	2	2	10	10
BF	*L. plantarum* CRL 1680	3.9	13	12	12	10	11	10	13	9	12	11	9	8	5	3	6	6	11	8	11	9
	L. plantarum CRL 1606	3.7	9/4/0	9/3/0	10/2/0	9/2/0	8/2/0	6/1/0	7/3/0	6/2/0	8/4/0	7/3/0	8/4/0	7/3/0	6/0/0	3/0/0	4/0/0	4/0/0	8/3/0	7/1/0	7/3/0	6/3/0

Lactobacillus strains isolated in autumn from the skin of healthy animals (HA) and balanced feed (BF)

[a] Strain isolated from the skin of non-healthy animal in spring

[b] End-pH of the lactobacilli cultures after 24 h of incubation at 37°C

Initial concentrations of indicator strains: A. 1×10^2; B. 1×10^5. (–), without inhibition. The values indicate the size (in mm) of the inhibitory hales produced by crude supernatants. *L. plantarum* CRL 1606: untreated supernatant/neutralized supernatant/neutralized supernatant+catalase. The results represent the means of three separate experiments

Table 2 Inhibitory activity of *Lactobacillus* strains isolated from *R. catesbeiana* hatchery in summer against RLS-related pathogens and meat spoilage bacteria

Origin	*Lactobacillus* strains	pH[a]	Indicator strains																			
			Inhibition (mm)																			
			Ps. aeruginosa		*Ps. aeruginosa* GRB		*Pr. vulgaris*		*Pr. vulgaris* MIB10		*Pr. mirabilis*		*C. freundii*		*S. enteritidis*		*L. monocytogenes*		*S. aureus*		*S. epidermidis*	
			A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
HA	*L. curvatus* CRL 1681	4.0	10	10	10	10	8	3	8	6	5	4	7	5	–	–	–	–	9	6	7	6
	L. curvatus CRL 1682	4.0	9	9	8	6	10	9	6	4	6	7	6	5	3	1	1	1	6	8	8	8
	L. curvatus CRL 1683	4.1	9	9	9	9	8	4	6	4	8	5	8	8	–	–	5	5	7	4	7	5
	L. plantarum CRL 1607	3.7	13	6	12	12	8	7	9	9	10	10	9	3	6	4	7	6	10	7	9	9
	L. plantarum CRL 1684	3.6	15	9	13	13	12	7	11	10	10	10	8	4	5	2	7	7	10	10	10	8
	L. plantarum CRL 1685	3.6	10	10	10	10	8	6	8	7	9	7	10	6	4	3	2	2	10	8	8	6
FW	*L. plantarum* CRL 1687	4.2	9	9	9	9	6	5	5	4	7	6	7	5	–	–	2	–	10	6	5	5
	L. plantarum CRL 1608	4.2	9	9	10	10	8	5	8	8	10	8	7	5	4	1	5	4	9	8	9	7
BF	*L. plantarum* CRL 1688	4.4	8	4	9	7	–	–	4	3	3	–	3	2	–	–	–	–	2	1	–	–
	L. plantarum CRL 1689	4.4	8	3	9	5	6	2	6	5	2	2	6	1	–	–	–	–	2	2	2	–

HA, healthy animals; FW, freshwater; BF, balanced feed

[a] End-pH of the lactobacilli cultures after 24 h of incubation at 37°C

Initial concentrations of indicator strains: A. 1×10^2; B. 1×10^5. (–), without inhibition. The results represent the means of three separate experiments

similar inhibitory effect against autochthonous *Ps. aeruginosa* and *Pr. vulgaris*, with a lower antimicrobial activity on *Staphylococus* species. However, RLS-related pathogens isolated from human sources were inhibited by the strain CRL 1689 to a higher degree. No *Lactobacillus* strains affected the growth of *Salmonella* and *Listeria*.

The antagonistic effect observed in this group of *Lactobacillus* strains was also abolished when cultures supernatant (final pH between 3.6 and 4.4) were neutralized, indicating that the inhibition is attributed to the organic acids produced.

Hydrogen peroxide production

The ability of seventy-nine *Lactobacillus* strains to produce H_2O_2 in TMB–MRS plates was evaluated (Fig. 1). 63 and 83% of *Lactobacillus* strains isolated from the skin of both healthy and non-healthy animals showed some level of H_2O_2 production, while 21 and 33%, respectively, were strong producers. 75% of *Lactobacillus* strains from freshwater of healthy animals were shown to be H_2O_2 producers, 25% of them as strong producers. With balanced feed, 81% of the *Lactobacillus* strains were shown to produce the oxidative metabolite, 4% of them as strong. None of the *Lactobacillus* strains from non-healthy animals freshwater were shown to produce H_2O_2 under the experimental conditions assayed (data not shown).

Cell surface properties of *Lactobacillus* strains

Haemagglutination

Haemagglutination (HA) test were performed in seventy-nine *Lactobacillus* strains. Only three strains were able to haemagglutinate human red blood cells, being the highest HA titres (64) observed in *L. curvatus* CRL 1683, *L. plantarum* CRL 1607 isolated from healthy frogs, and *L. plantarum* CRL 1606 isolated from balanced feed.

Hydrophobicity

Forty-five strains of *Lactobacillus* isolated from a *R. catesbeiana* hatchery that showed the highest antimicrobial activity against RLS-related pathogens and meat spoilage bacteria were selected to study for their degree of surface hydrophobicity (Fig. 2). Regardless of the area from which the strains were isolated (skin mucus of healthy and non-healthy frogs, freshwater samples and balanced feed) most of the lactobacilli showed a low degree of hydrophobicity; only a few number of strain presented medium or high hydrophobicity. When analyzing the values from *Lactobacillus* strains isolated from different areas, those from freshwater samples presented a wider interquartile range (IQ = 17) of hydrophobicity with a median value of 24%. *Lactobacillus* from balanced feed showed an IQ = 9.5 with a median value of

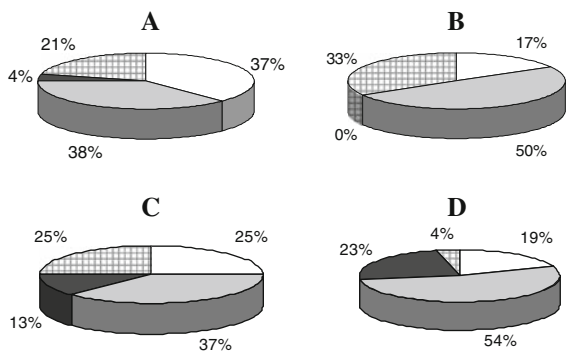

Fig. 1 Hydrogen peroxide production by *Lactobacillus* strains isolated from a *R. catesbeiana* hatchery. **a** Healthy animals; **b** non-healthy animals; **c** freshwater from healthy animals, and **d** balanced feed. Production of hydrogen peroxide: negative (□), weak (▨), medium (■), and strong (▤)

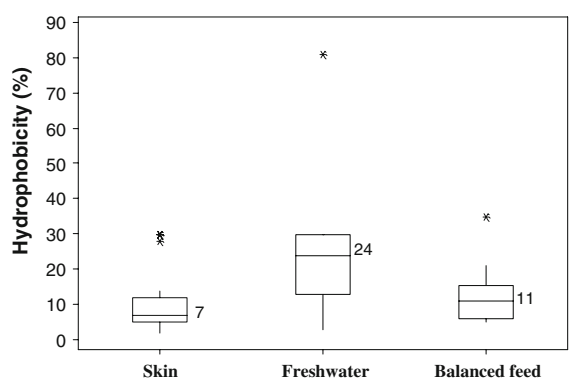

Fig. 2 Hydrophobicity of *Lactobacillus* strains isolated from a *R. catesbeiana* hatchery. Digits represent the median value of hydrophobicity obtained. * Indicates the hydrophobicity values out of the interquartile range

11%, while those from skin mucus presented the lowest values (IQ = 7.0) with a median of 7%.

Correlation between beneficial properties of *Lactobacillus* strains

The selection of potentially probiotic strains requires studying the correlation between their surface and inhibitory properties, looking for strains that share some properties or characteristics. The matrix plot for hydrophobicity and inhibitory activity against RLS-related pathogens shown in Fig. 3, demonstrate that there is no correlation between these properties (Pearson correlation value = 0.0001). The relationship between the inhibitory activities against the different autochthonous RLS-associated pathogens shows a weak correlation between the size of inhibitions hales of *Ps. aeruginosa* and *Pr. vulgaris* (Pearson correlation = 0.592, $P < 0.0001$). A similar behaviour was observed between *Pr. vulgaris* and *S. epidermidis* (Pearson correlation = 0.687, $P < 0.0001$). These studies were also performed for food-borne bacteria, where no correlation was observed (Fig. 4).

Selection of *Lactobacillus* strains

The potentially probiotic *Lactobacillus* strains were selected from those that showed the highest inhibitory activity, production of hydrogen peroxide, titres of haemagglutination, and medium to low values of hydrophobicity (Figs. 3, 4; Table 3).

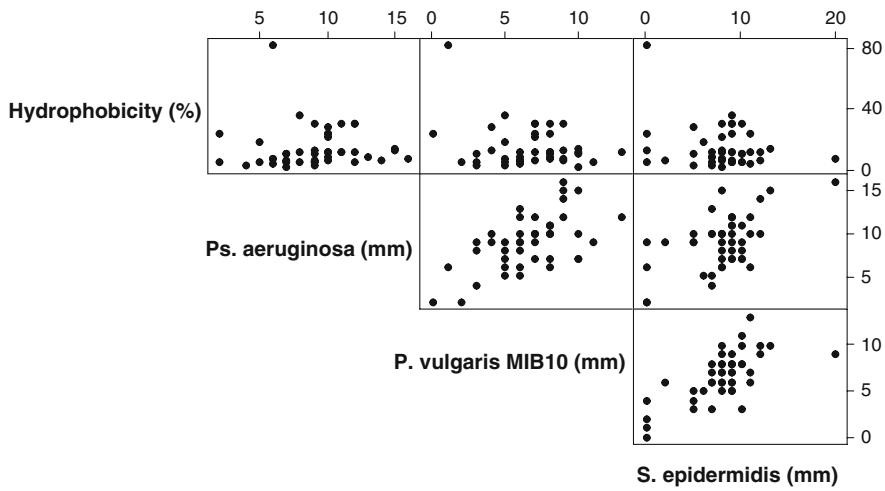

Fig. 3 Relationship between the probiotic properties measured by hydrophobicity degree and the diameters of the inhibition halos on RLS-related pathogens

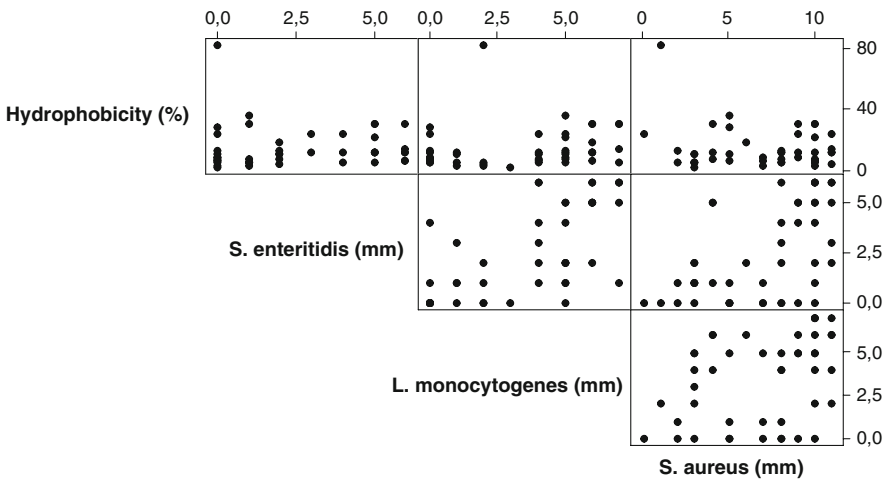

Fig. 4 Relationship between the probiotic properties measured by hydrophobicity degree and the diameters of the inhibition halos on food-borne bacteria

Table 3 Relationship between inhibitory and surface properties of selected *Lactobacillus* strains

Source	Strain	Inhibition by organic acids	H_2O_2 production	HEHA	Hydrophobicity (%)
HA	*L. curvatus* CRL 1683	++	+++	64	5
	L. plantarum CRL 1607	+++	+	64	30
FW	*L. plantarum* CRL 1608	+++	+++	–	23
BF	*L. plantarum* CRL 1606	+++	+++	64	13

HA, healthy animals; FW, freshwater from healthy animals; HEHA, haemagglutination of human erythrocytes

Score of inhibition: ++ (medium), +++ (high)

Ps. aeruginosa GRB was used as indicator strain

Score of H_2O_2 production in TMB–MRS: +++ (strongly positive), ++ (moderately positive), + (weakly positive)

Score of hydrophobicity: high (60–100%), medium (30–60%), low (0–30%)

Discussion

Intensive *R. catesbeiana* production increases the risk of bacterial infectious diseases and mortality, with the aquatic environment being the most significant source of potentially pathogenic microorganisms (Glorioso et al. 1974; Mauel et al. 2002). Thus, antibiotics or vaccines are frequently used to control and prevent infectious diseases in aquaculture (Bühler et al. 2000; Verschuere et al. 2000; Romalde et al. 2005). However, the increasing political and environmental pressure to decrease the use of antibiotics has stimulated the research directed toward controlling the spread of disease (Farzanfar 2006). The application of probiotics is a valuable tool as an alternative therapy to avoid or decrease the use of antimicrobials in aquaculture (Balcázar et al. 2006).

The origin of the bacterial strains for probiotic is sometimes from foods or food-related environment, or either those already included in marketed products that are evaluated or studied for their beneficial properties. However, when looking for probiotic products to increase the colonization, the inclusion of indigenous microorganisms to reconstitute the natural ecosystems of the bullfrog's hatcheries is recommended. Then, the possibilities of recolonization and reestablishment of the autochthonous microbiota in different *R. catesbeiana* mucosal surfaces can be achieved more efficiently. Based on this hypothesis, the selection of strains to be included in a probiotic product for use in a defined ecological niche and host should be done from strains belonging to the indigenous microbiota (Reid et al. 2003).

The present work was not performed as an epidemiological study but rather to complete the description of the microbiota in a *R. catesbeiana* hatchery in summer and spring, and to study the beneficial properties of lactobacilli. All sampled frogs were fattening phase animals in order to avoid variations in the microbial population due to the physiological changes that occur during their biological cycle. In previous research there have been antecedents of the variations of the microbiota produced in some tracts during the different host ages (Otero et al. 2000).

LAB are constituent of *R. catesbeiana* hatchery microbiota and its population was modified according to the season, the area of the hatchery, as well as the health status of the animals. Similar results were reported when the microbiota from a *R. catesbeiana* hatchery was examined in autumn (Pasteris et al. 2006). Consequently, it could be assumed that some environmentally growth conditions (e.g., temperature) are controlled. However, RLS outbreak does occur in summer as was demonstrated by the isolation of *Ps. aeruginosa* and *S. epidermidis*.

Lactobacilli are present in both healthy and non-healthy frogs and are not able to translocate to target organs during an episode of RLS (Pasteris et al. 2006). This research provides one of the arguments to select this LAB group to further study some of the beneficial properties.

Although LAB are generally considered to be non-pathogenic and classified as GRAS (generally regarded as safe; Reid et al. 2003) and Food Grade Microorganisms, an increasing episodes of diseases

that appeared with the worldwide development of fish aquaculture could be attributed to some LAB strains of the genera *Streptococcus*, *Lactococcus*, and *Carnobacterium* (Ringø and Gatesoupe 1998). The use of lactobacilli has been the focus of a renewed interest for the development of probiotic products for aquaculture (Verschuere et al. 2000; Nikoskelainen et al. 2001a, b; Irianto and Austin 2002; Vazquez et al. 2005).

Taking into account the beneficial properties that a probiotic should exhibit (Reid et al. 2006), the inhibitory activity of *Lactobacillus* strains against RLS-associated pathogens and meat spoilage bacteria was tested. *L. plantarum* and *L. curvatus* inhibited the growth of some indicator strains by organic acids. The antimicrobial activity was strain-dependent and pH-independent, but, in some cases, showed modifications according to the initial concentration of the indicator strains used.

Under our experimental conditions, no bacteriocin-producing *Lactobacillus* strain was detected.

L. plantarum CRL 1606 isolated from balanced feed inhibited the growth of RLS-associated pathogens by both acidity and H_2O_2 by using the agar well diffusion method. However, with a more sensitive method (MRS–TMB), it was possible to find a higher percentage of H_2O_2-producing lactobacilli. Many of these strains were also able to inhibit the growth of RLS-related pathogens by organic acids. These results support the possibility of the existence of a synergic effect between inhibitory metabolites produced and excreted into the medium, as it was reported for bovine lactobacilli against *S. aureus* (Otero and Nader-Macías 2006). It is interesting to point out that a few *L. plantarum* strains isolated from the skin ulcerations were also able to inhibit pathogens by both acidity and H_2O_2. These results are suggestive of the hypothesis that lactobacilli could be present in the ulceration trying to control the infection by competitive exclusion.

Although the isolation of H_2O_2-producing lactobacilli in aquaculture is being reported for the first time in this paper, they are involved in the control of infectious diseases in human and endothermic animals (Kullisaar et al. 2002; Naaber et al. 2004; Ljungh and Wadstrom 2006; Otero and Nader-Macías 2006). Other authors (Irianto and Austin 2002; Balcázar et al. 2006) have reported the production of antagonistic molecules by probiotics for aquaculture. Coculture experiments of human probiotic *L. rhamnosus* ATCC 53101 showed significant inhibition of the growth of *Aeromonas salmonicida*, which was mediated by competition for nutrients rather than by production and secretion of inhibitory substances. However, *L. rhamnosus* administered in rainbow trout reduced mortalities after the challenge with *A. salmonicida* (Nikoskelainen et al. 2001a, b). Moreover, the benefit of using *L. plantarum* and *L. helveticus* in turbot, *Scophthalmus maximus* (L.), has been reported leading to their enhanced growth (Gatesoupe 1999; Irianto and Austin 2002), while *Carnobacterium divergens* is able to prevent, to some extent, pathogen-induced damage in the Atlantic salmon foregut (Ringø et al. 2007).

Looking at the cell surface properties of lactobacilli isolated from a *R. catesbeiana* hatchery, most of the *Lactobacillus* strains showed a low degree of hydrophobicity. This fact could be explained by the chemical nature of the skin mucus that has a high content of water and glycoproteins (Ringø et al. 2007). Further studies should be performed to determine the degree of correlation between the low level of hydrophobicity and the ability of *Lactobacillus* strains to adhere to the mucus.

The port of entry for RLS-related pathogens is associated with skin lacerations or the gastrointestinal tract (Glorioso et al. 1974). Consequently, the possibility of using hydrophilic lactobacilli to colonize the skin mucus and probably the intestinal mucus could be a valid alternative to control the adhesion of pathogenic bacteria by competitive exclusion.

Our findings indicate that the hydrophobicity values of *Lactobacillus* strains isolated from skin are included in a lower range than those isolated from freshwater samples. This could indicate the highest specificity of the strains from skin, since lactobacilli present in freshwater could be originated from different frog mucosal surfaces and balanced feed.

Haemagglutination (HA) reaction is a tool of monitoring bacterial adhesion because the erythrocyte surface shares the ontogenetic origin of some of the cells normally colonized by the organisms (Ofek and Doyle 1994). In particular, epithelial cells express outer molecules similar to the red blood cell membrane (Madigan et al. 2006). A low percentage of lactobacilli showed HA by using human erythrocytes, which was suggested to have the same ABO group as some anuran amphibians (Ashhurst 1956;

Balding and Gold 1976). However, no results were found with amphibian erythrocytes, since the rate of sedimentation was higher than that for agglutination. This report constitutes the first study on the surface properties of potentially probiotic lactobacilli for aquaculture, though others have been studied probiotics from endothermic animals (Draksler et al. 2004; Otero and Nader-Macías 2006).

By combining the antagonistic activity and surface properties of *Lactobacillus* (Table 3), we selected four strains: *L. curvatus* CRL 1683 and *L. plantarum* CRL 1607, from healthy animals, *L. plantarum* CRL 1608 from freshwater sample and *L. plantarum* CRL 1606 from balanced feed as potentially candidates to be used as probiotics in *R. catesbeiana* hatchery. Further studies will be performed to evaluate the suitability to include these strains in products to be assayed in in vivo experimental trials.

There are not previous reports about the isolation of *Lactobacillus* strains from others *R. catebesbeina* hatcheries. However, our research group is also evaluating the microbiota in hatcheries from other geographical areas (Montel Mendoza et al. 2008) to compare the biodiversity and, thus to evaluate the possibility to design an universal probiotic to be applied in bullfrog hatcheries. This approach is also supported by the last scientific descriptions on the role of the indigenous microbiota on specific host, areas and segments (Zoetendal et al. 2008).

Since raniculture and the production of frog meat and by-products represents an important and increasing economic activity in various countries (Texeira et al. 2002; Ferreira et al. 2006) and since animals are more susceptible to infectious diseases, this work reports the beneficial properties of lactobacilli isolated from a *R. catesbeiana* hatchery for first time and introduces the basis to design a probiotic with selected *Lactobacillus* strains which could contribute to avoid the use of antibiotics. The use of probiotic LAB represents an effective way to prevent infectious diseases, such as RLS, which affect the production costs in bullfrog hatcheries.

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