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Cultivable microbiota of *Lithobates catesbeianus* and advances in the selection of lactic acid bacteria as biological control agents in raniculture

Gabriela Montel Mendoza [a], Sergio E. Pasteris [a], Cesar E. Ale [a], María C. Otero [a,b], Marta I. Bühler [a], María E. Fátima Nader-Macías [b,*]

[a] *Instituto Superior de Investigaciones Biológicas (INSIBIO-CONICET)-Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia-Universidad Nacional de Tucumán, Chacabuco 461, CP T4000ILI, San Miguel de Tucumán, Argentina*
[b] *Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, CP T4000ILC, San Miguel de Tucumán, Argentina*

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ABSTRACT

The cultivable microbiota of skin and cloaca of captive *Lithobates catesbeianus* includes microorganisms generally accepted as beneficial and potentially pathogenic bacteria. In order to select a group of potentially probiotic bacteria, 136 isolates were evaluated for their surface properties and production of antagonistic metabolites. Then, 11 lactic acid bacteria (LAB) strains were selected and identified as *Lactobacillus plantarum*, *Lb. brevis*, *Pediococcus pentosaceus*, *Lactococcus lactis*, *L. garvieae* and *Enterococcus gallinarum*. Studies of compatibility indicate that all the strains could be included in a multi-strain probiotic, with the exception of *Ent. gallinarum* CRL 1826 which inhibited LAB species through a bacteriocin-like metabolite. These results contribute to the design of a probiotic product to improve the sanitary status of bullfrogs in intensive culture systems, to avoid the use of antibiotics and thus to reduce production costs. It could also be an alternative to prevent infectious diseases during the *ex situ* breeding of amphibian species under threat of extinction.

1. Introduction

During the last decades, the culturing of many amphibian species has grown substantially in an effort to repopulate devastated environments where specific populations have declined to near extinction (Rollins-Smith, 2009). On the international market some species are of great interest to obtain food and by-products (Texeira et al., 2002). In fact, in pharmacological areas, some amphibian derived molecules are being studied as anti-tumor or antimicrobial drugs (Lu et al., 2008; Libério et al., 2011).

The American bullfrog or *Lithobates catesbeianus* is one of the species selected for raniculture because of its desirable biological attributes mainly their muscle mass and by-products such as skin, liver and gut (Texeira et al., 2002). In intensive growth systems, animals are exposed to a wide variety of microorganisms (Lauer et al., 2008; Woodhams et al., 2007). Thus, the indigenous microbiota of skin and gastrointestinal tract could be affected by many factors such as microbial interactions, water flows, husbandry techniques and disinfection, which could alter the equilibrium of the microbial ecosystems. These aspects, together with the stress produced by crowding, may overwhelm immune barriers, and microbial opportunists can cause the outbreak of infectious diseases (Glorioso et al., 1974; Mauel et al., 2002).

In raniculture, research activities are focused on the isolation of pathogens associated with bacterial dermatosepticemia or red-leg syndrome (RLS), an infectious disease that mainly affects bullfrog hatcheries (Glorioso et al., 1974; Mauel et al., 2002; Densmore and Earl Green, 2007) and causes high mortality and significant economic losses. Pathogens include *Enterobacteriaceae*, *Aeromonas hydrophila*, *Elizabethkingia meningoseptica*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Glorioso et al., 1974; Mauel et al., 2002; Schadich et al., 2010). However, RLS has been implicated in a localized die-off of *Bufo americanus*. Pathogenic species belong to the indigenous microbiota and could affect amphibians in the wild under different environmental conditions (Carey et al., 1999).

Treatment of the captive animals with chemotherapeutics contributes both to modifications of the indigenous microbiota and to the spread of antibiotic resistant bacteria (Verschuere et al., 2000; Vine et al., 2004; Ringø et al., 2010). Thus, an alternative therapy is being developed worldwide, associated with the use of probiotics to restore beneficial microbial populations that could help to control potentially pathogenic microorganisms (Reid et al., 2003). Consequently, knowledge of the main components of the indigenous microbiota in the normal physiological state is needed. Previously, the cultivable autochthonous microbiota of an *L. catesbeianus* hatchery located in the northwest of Argentina and composed of

* Corresponding author. Tel.: +54 381 4310465; fax: +54 381 4311720.
E-mail address: fnader@cerela.org.ar (M. E. Fátima Nader-Macías).

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lactic acid bacteria (LAB), *Micrococcus* spp., and *Enterobacteriaceae* was evaluated (Pasteris et al., 2006). Among RLS-associated pathogens, *C. freundii*, *Ps. aeruginosa* and *S. epidermidis* were isolated (Pasteris et al., 2006, 2009a). A study of the beneficial properties of LAB was also carried out and some strains were selected as probiotic candidates for raniculture (Pasteris et al., 2009a,b). However, the microbiota of *L. catesbeianus* hatcheries could be modified according to climatic variations. Keeping in mind the design of a multi-strain probiotic to be applied in different geographical regions of the country, the first aim of this work was to evaluate the cultivable microbiota of another hatchery, with special interest in those genera recognized as beneficial (LAB, *Bifidobacterium* and *Bacillus*) and potentially pathogens associated with RLS. Since the use of probiotics in aquaculture is becoming increasingly popular (Vine et al., 2004; Ringø et al., 2010), the second aim was to study some beneficial properties of the potentially beneficial bacteria and select strains to promote the design of a probiotic for raniculture.

2. Materials and methods

2.1. Animals and samples collection

Samples were taken from twenty *L. catesbeianus* specimens without any apparent signs or symptoms of RLS and therefore considered as healthy frogs. Animals in the fattening phase (12 months of age, weight between 200 and 250 g) were sampled in a hatchery located in the center of Argentina (Río Cuarto, Córdoba) during the autumn (April 2009). Frogs were taken from different areas of the hatchery and skin samples were obtained by scraping 1 cm^2 of the ventral and dorsal skin (VS, DS) and cloaca of live animals by using sterile cotton swabs. The samples were collected in LAPT medium that contains in g/l: yeast extract, 10; peptone, 15; tryptone, 10 and Tween 80, 1 ml/l, pH 6.8 (Raibaud et al., 1963) and kept at 4 °C for 6 h until laboratory processing.

2.2. Isolation of microbial populations from Lithobates catesbeianus

The quantification of the microorganisms was carried out by the serial dilution method using 0.1% (vol./vol.) peptone as a dilution medium. 100 µl samples were plated on selective or differential culture media. Isolation of LAB was performed using Man, Rogosa, Sharpe (MRS) agar (de Man et al., 1969), *Lactobacillus* Selection Media (LBS) agar and M17 agar. Bilis Esculine Agar (BEA) was employed for the isolation of group D *Streptococcus* and *Enterococcus*, while Reinforced *Clostridium* Agar (RCA) supplemented with 1% aniline blue and 0.05% sodium propionate (Bullen et al., 1973) was used to reveal the presence of *Bifidobacterium*; Mannitol Salt Agar (MSA) for *Staphylococcus*, Cetrimide (CET) agar for *Pseudomonas* and MacConkey (MC) agar for coliform bacteria. Plate Count Agar (PCA) was used to evaluate the total number of mesophilic microorganisms and *Bacillus cereus* Agar (BA) for spore-forming bacteria. To remove the vegetative forms of the associated microbiota, samples plated in BA were previously heated at 80 °C for 15 min, and then incubated at 37 °C for 3 h before plating (Leuschner et al., 2003). Sabouraud (SAB) agar was employed for yeast and mycelial fungi isolation. All the plates were incubated in microaerophilic conditions at 37 °C for 48–72 h, except samples plated on SAB that were incubated during 15 days. Only RCA plates were incubated for 1 week, at 37 °C in anaerobic conditions (Anaero-Gen™ – Oxoid – United Kingdom).

Selected colonies of each microbial group were grown in different broth culture media: MRS (for LAB), LAPT + 1% glucose (for group D *Streptococcus*/*Enterococcus*, *Staphylococcus* spp., *Bacillus* spp., *Enterobacteriaceae* and yeast/fungi), LAPT + 1% glucose + 1% lactose (for *Bifidobacterium* spp.) or Brain Heart Infusion (BHI) (for *Pseudomonas* spp.) for 18 h at 37 °C. Cells collected by centrifugation at 3000*g* for 5 min at 4 °C were resuspended in MRS broth + 20% (vol./vol.) glycerol, and stored at −20 °C.

All the culture media were obtained from Britania (Argentina) except for MRS and BA, which were purchased from Merck (Germany) and BHI from Difco (USA).

2.3. Partial phenotypic identification of members of the indigenous microbiota

Bacterial identification was performed by morphological and phenotypic characteristics using standard biochemical assays for the different groups of microorganisms: Gram staining; catalase, urease, phosphatase and coagulase activities; nitrate reduction; indole production; citrate utilization, mobility, arginine, hypurate, starch and casein hydrolysis; bacitracin and novobiocin susceptibility, growth in cetrimide; growth in 6.5% and 7% (w./vol.) NaCl, as well as at different pH and temperatures (4, 42, 50 and 60 °C), and some sugars to determine the fermentation patterns. Gluconate utilization, gas production in Gibson medium and Voges–Proskauer reaction were used to determine the homo- and hetero-fermentative characteristics of the isolates (Holt et al., 1994; Murray et al., 2003).

2.4. Screening of probiotic characteristics

2.4.1. Bacterial strains and culture conditions

The selection of 136 isolates to evaluate the beneficial properties was performed according to the following criteria: (a) colonies that grew in specific media for LAB (MRS, M17, LBS and BEA), and showed rod or cocci morphology, Gram-positive staining and catalase negative activity. (b) isolates from BA medium that showed rod morphology, Gram-positive staining and catalase positive activity and (c) isolates from RCA medium that showed typical cell morphology compatible with *Bifidobacterium*, Gram-positive staining and catalase negative activity. Overall, similar numbers of isolates were taken from each area of isolation (dorsal and ventral skin and cloaca). Thus, 130 LAB, five *Bifidobacterium* spp. and one *Bacillus* spp. were selected to study the surface properties (hydrophobicity and autoaggregation) and the production of antagonistic substances: organic acids, hydrogen peroxide (H_2O_2) and bacteriocin-like metabolites.

The potentially beneficial bacteria were grown in MRS broth for 18 h at 37 °C, while indigenous RLS-associated pathogens (*C. freundii*, *Ps. aeruginosa*, and *S. epidermidis*), *S. aureus* and *Escherichia coli* were grown in nutritive broth for 12 h at 37 °C (Vanderzant and Splittstoesser, 1992). *Listeria monocytogenes* Scott A and *Salmonella enteritidis* were grown in BHI broth in the same conditions. LAB strains were deposited in the Culture Collection of CERELA.

2.4.2. Surface properties

The degree of hydrophobicity of the bacterial surfaces was determined by MATH (microbial adhesion to hydrocarbon) assay described originally by Rosenberg and Doyle (1990). LAB, *Bifidobacterium* spp. and *Bacillus* spp. were grown in MRS broth at 37 °C and later collected by centrifugation at early logarithmic growth phase, washed twice and resuspended in sterile distilled water to an optical density of 0.6 ($OD_{600\ nm}$). 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. In addition, cell suspensions were used to determine the degree of autoaggregation according to Ocaña and Nader-Macías (2002). Briefly, cells obtained as described previously were adjusted to give an $OD_{600\ nm}$ of 0.6. Autoaggregation

was determined spectrophotometrically over 4 h, leaving the suspensions to sediment during the assay. The degree of hydrophobicity and autoaggregation was calculated from three replicates determinations and expressed as follows:

% Hydrophobicity = [(OD before mixing − OD after mixing)/OD before mixing] × 100

% Autoaggregation = $[(1 - D_{final})/OD_{initial}] \times 100$

The degree of bacterial hydrophobicity and autoaggregation was classified as low (0–29%), medium (30–59%) or high (60–100%).

2.4.3. Production of antagonistic metabolites

The inhibitory activity of the potentially beneficial bacteria was assayed by the agar-well diffusion test as described previously (Pasteris et al., 2009a) using RLS-related pathogens isolated from different bullfrog hatcheries and food-borne bacteria as indicator strains. The pathogens were plated at a final concentration of 10^6 CFU/ml. Moreover, H_2O_2 production was also evaluated by the qualitative plate method using peroxidase (Type II: from horseradish, Sigma–Aldrich Chemical) and TMB (3,3′,5,5′-tetramethyl-benzidine from Sigma–Aldrich Chemical) added to MRS agar medium as described previously (Pasteris et al., 2009a). According to the intensity of the color, the strains were classified as strong (blue), medium (brown), weak (light brown) or negative (white colonies) producers.

2.5. Genotypic identification of selected beneficial microorganisms

In order to identify and classify the selected LAB isolates, the variable region VI of the 16S rDNA gene was amplified and sequenced. DNA amplifications were carried out by colony PCR. Strains were cultured in MRS agar (Hébert et al., 2000). The reaction was performed with PCR buffer (1×) (Invitrogen, CA, USA), 2.5 mM $MgCl_2$ (Invitrogen, CA, USA), 0.2 mM dNTPs (Invitrogen, CA, USA), 1 μM MLB16 (5́GGCTGCTGGCACGTAGTTAG) and PLB16 (5́AGAGTTTGATCCTGGCTCAG) primers, *Taq* DNA polymerase (2.5 U) (Invitrogen, CA, USA) and MilliQ water. The final volume was 50 μl. The PCRs were performed in a Bio-Rad MyCycler™ under the following conditions: 4 min at 94 °C of initial denaturation, 30 cycles consisting of 30 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C and a final extension at 72 °C for 7 min. PCR products were electrophoresed in 0.8% agarose gels, purified and sequenced using the DNA sequencing service of CERELA (Tucumán, Argentina). Identification was performed by comparing the 16S rDNA sequences obtained with those deposited in the Genbank database using the BLAST algorithm.

2.6. Compatibility among lactic acid bacteria strains

In order to determine if the selected LAB could be included in a multi-strain probiotic product, compatibility among the strains was evaluated. LAB were grown as indicated above and the fluid supernatants without treatment were used to determine the inhibitory effect by the agar-well diffusion method using 10^6 CFU/ml of indicator LAB strains.

2.7. Statistical analysis

All the experiments were carried out in triplicate and the means of the data were calculated. The statistical analysis of the data was performed using the Minitab 15 and the InfoStat (InfoStat 2008) statistical softwares. ANOVA-general linear model was applied for residues analysis. When they showed normality, significant differences between the mean values were determined (LSD Fisher's test, $P < 0.05$). When they did not, significant differences between the mean values were determined through non-parametrical analysis by the Kruskal Wallis test (95% confidence interval). Significant differences in both surface properties and inhibitory activity were statistically determined by using Student's *t*-test. Correlation between the surface properties was calculated by the Pearson coefficient.

3. Results

3.1. Cultivable bacterial communities of Lithobates catesbeianus

The aerobic mesophilic microorganisms recovered from the ventral skin (VS), dorsal skin (DS) and cloaca of a total of 20 fattening phase bullfrog showed a mean number of around 10^6 CFU/sample, and there were no significant differences (Kruskal Wallis test, $P \leqslant 0.05$) among the areas under study (VS: 6.67, DS: 6.85 and cloaca: 6.66 log CFU/sample).

Each bacterial group or genus was quantified and similar numbers were obtained in the three areas, since no significant differences between the means of each area were detected. The microbiota of both VS and DS and cloaca included microorganisms generally accepted as beneficial (LAB, *Bifidobacterium* spp. and *Bacillus* spp.) and potentially pathogenic bacteria (*Enterobacteriaceae*, *Pseudomonas* spp., *Staphylococcus* spp. and group D *Streptococcus*). The average values of each microbial population are shown in Table 1. LAB and group D *Streptococcus*/*Enterococcus* were the predominant microorganisms in the microbiota of the areas under study and there were no significant differences in the number of viable cells. *Staphylococcus* and *Enterobacteriaceae* also belonged to the major groups, the former being significantly higher. *Bacillus* and *Pseudomonas* were present in smaller numbers and their contribution to the microbiota was similar, while *Bifidobacterium* was found at significantly lower concentrations. However, there were significant differences between viable cell counts in each group or genus of both potentially beneficial and pathogenic microorganisms in the same area (Fisher's test, $P \leqslant 0.05$) (Table 1).

With respect to the *Enterobacteriaceae* family (that includes RLS-associated pathogens), their genera or species were identified and expressed as percentage with respect to the total number of isolates: *Escherichia blattae* (18.6%), *Erwinia* spp. (15.8%), *Klebsiella* spp. (10.5%), *Citrobacter freundii* (8%), *Escherichia coli* (7.9%), *Edwarsiella ictaluri* (7.9%), *Yersinia aldovae* (7.9%) and *Providencia stuartii* (5.2%). A few isolates were identified as *Enterobacter* spp., *Hafnia alvei*, *Leminorella* spp., *Shigella* spp., *Yersinia intermedia*, *Providencia heimbachae* and *P. rettgeri* (2.6% each).

3.1.1. Potentially beneficial bacteria from Lithobates catesbeianus

Among the 650 Gram-positive bacteria isolated from the three areas, there was a weak predominance of cocci versus rods. The isolates were evaluated for their catalase activity, most of them being negative (87%, 85% and 88% from VS, DS, and cloaca, respectively). Thus, the last group was identified as *Leuconostoc* spp., *Pediococcus* spp., *Aerococcus* spp., *Enterococcus* spp., *Lactococcus* spp. and *Lactobacillus* spp. *Bifidobacterum* spp. and *Bacillus* spp. were also identified. The distribution of the different LAB genera within the three areas is not homogeneous, as shown in Fig. 1.

Additional tests were performed to determine the metabolic groups of the LAB genera. Thus, the presence of homo- and hetero-fermentative species was detected in all the areas, the number of obligate heterofermentative species being higher (VS: 75%, DS: 65% and cloaca: 77%). However, facultative heterofermentative species were isolated only in samples from DS (22%).

3.2. Surface properties of potentially beneficial bacteria

Most of the isolates from all the areas showed low percentages of hydrophobicity, with mean values in each area lower than 50% (*t*-test, $P < 0.0001$). However, a wide range of values were ob-

Table 1
Cultivable microbiota in *L. catesbeianus*.

Microbial populations	Microbial counts (log CFU/sample)			
	Total[1]	Skin		Cloaca
		Ventral	Dorsal	
Lactid acid bacteria	6.15 ± 0.66[a]	6.06 ± 0.58[a]	6.01 ± 0.77[a]	6.38 ± 0.59[a]
Bacillus spp.	2.19 ± 1.53[d]	1.23 ± 1.68[cd]	2.65 ± 0.90[d]	2.69 ± 1.67[b]
Bifidobacterium spp.	0.92 ± 0.72[e]	0.76 ± 0.70[d]	1.15 ± 0.68[e]	0.81 ± 0.65[b]
Group D *Streptococcus*/*Enterococcus*	6.10 ± 0.47[a]	6.09 ± 0.62[a]	5.85 ± 0.60[a,b]	6.34 ± 3.32[a]
Staphylococcus spp.	5.02 ± 0.93[b]	5.24 ± 1.41[a,b]	4.40 ± 1.40[c]	5.44 ± 2.08[a]
Enterobacteriaceae	4.02 ± 2.19[c]	4.75 ± 0.51[b]	4.88 ± 1.30[b,c]	2.40 ± 0.52[b]
Pseudomonas spp.	1.90 ± 1.68[d]	2.45 ± 0.32[c]	0.98 ± 0.54[e]	2.28 ± 0.48[b]

[a–e] Means within a column with different superscripts differ (Fisher's test, $P < 0.05$).
[1] Means counts of each microbial group in 20 animals.

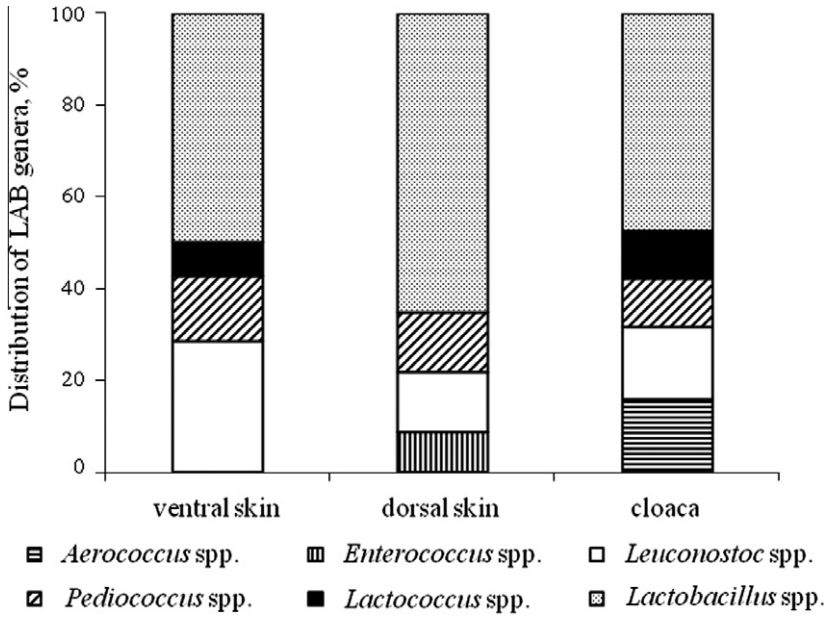

Fig. 1. Distribution of lactic acid bacteria in different areas of *L. catesbeianus*. Different LAB in ventral (100% = 141 isolates) and dorsal (100% = 231 isolates) skin and cloaca (100% = 191 isolates) in 20 frogs.

served, particularly in those isolates from DS and cloaca, which agrees with interquartile (IQ) range (48.24 and 47.17, respectively). The microorganisms isolated from VS showed less variability (IQ: 25.51) (Fig. 2A).

With respect to autoaggregation ability, the behavior was similar to hydrophobicity (mean values lower than 50%, $P < 0.0001$). The autoaggregation index values were included in close ranges according to their low IQ values: 12.72, 12.57 and 10.02 for VS, DS and cloaca, respectively. In all cases, a few isolates from different areas showed autoaggregation values higher than 50% (Fig. 2A).

When comparing the surface properties, there were no significant differences among the areas (*t*-test, $P > 0.05$).

Overall, there was no correlation between the surface properties studied, which agrees with the low Pearson correlation factor (0.215). Nevertheless, there was a group of LAB that exhibited autoaggregation and hydrophobicity values higher than 60% (one strain from VS, four strains from DS and one strain from cloaca). A group of isolates showed a different behavior, with hydrophobicity values higher than 50% and autoaggregation values lower than 25% (two isolates from VS, nine from DS and six from cloaca) (Fig. 2B).

3.3. Antimicrobial activity of potentially beneficial bacteria

One hundred and thirty-six isolates were selected to evaluate the production of inhibitory metabolites against RLS-associated pathogens and food-borne bacteria. Thus, *Ps. aeruginosa* strains were inhibited by most of the potentially beneficial bacteria. The values of the inhibition halos showed a wide range in the microbial groups isolated from the three areas of *L. catesbeianus*. Overall there were no significant differences of the inhibitory activity among the means of the three areas evaluated *t*-test, ($P > 0.05$), although the median values obtained from the bacterial groups isolated from cloaca were slightly higher than those isolated from skin (box plot, Fig. 3A).

The inhibitory values against *C. freundii* were significantly lower than those against *Ps. aeruginosa* (*t*-test, $P < 0.0001$). A small group of potentially beneficial bacteria showed a low inhibitory activity against *S. epidermidis* (median: 0 mm). However, *Enterococcus* spp. 334 was highlighted in this group by the high inhibitory effect (halo size: 13 mm) (Fig. 3A).

Antimicrobial activity against food-borne bacteria was low, and no significant differences among the areas were detected (*t*-test, $P > 0.05$). The LAB isolates that showed inhibitory halos higher than the IQ ranges were later considered for the selection (included in Fig. 3B).

Final pH of the potentially beneficial microorganism cultures was between 3.74 and 5.54 and antimicrobial activity disappeared when the supernatants were neutralized, which indicates that organic acids are responsible of the inhibition. However, *Enterococcus* spp. 334 maintained its inhibitory effect against *S. epidermidis* and

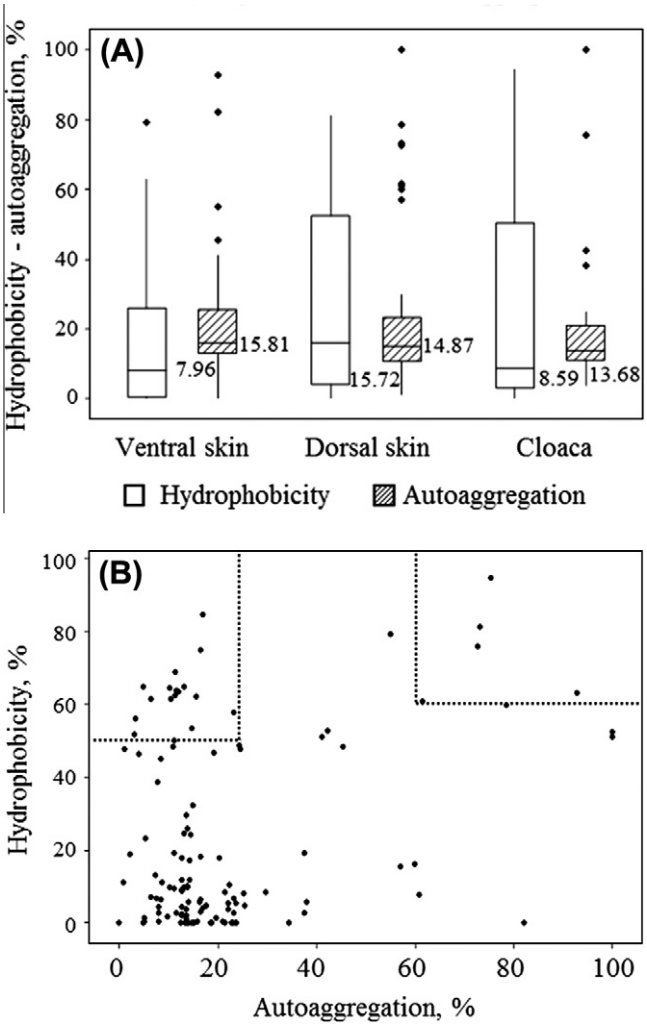

Fig. 2. Surface properties of potentially beneficial bacteria isolated from *L. catesbeianus*. (A) Values represent median results. Symbols indicate the values out of the interquartile range. (B) Correlation between hydrophobicity and autoaggregation. Boxed areas include group of strains used to select for hydrophobicity or autoaggregation.

L. monocytogenes when the neutralized supernatants were treated with catalase, indicating that H_2O_2 and/or a bacteriocin-like metabolite could also participate in the antimicrobial activity.

The ability of 136 isolates to produce H_2O_2 on TMB-MRS plates was studied (Table 2). Most of the isolates from VS, DS and cloaca were negative for H_2O_2 production. However, the percentage of H_2O_2-producer bacteria varied according to the area of isolation. Thus, 17% of the isolates from VS showed a certain level of H_2O_2 production with 3% of strong producers, 20% from DS (11% strong producers) and 11% from cloaca (3% strong producers). The genera *Bifidobacterium* and *Bacillus* were unable to produce H_2O_2 under the experimental conditions.

3.4. Selection criteria of lactic acid bacteria strains

The following criteria were applied to select the potentially probiotic LAB strains: isolates not previously reported as pathogens for raniculture, isolated from healthy frogs, able to inhibit at least seven RLS-related pathogens, and extreme hydrophobicity values. Also, the isolates that showed a high autoaggregation index and inhibited at least two pathogens were included. The eleven LAB strains selected for further studies are summarized in Table 3.

3.5. Identification of lactic acid bacteria strains

The LAB strains selected for their beneficial properties, identified by 16S rDNA sequence analysis, showed a high percentage of identity with *Lb. brevis* (100%), *P. pentosaceus* (98.7–100%), *L. lactis* (100%) and *L. garvieae* (98.5%) and a similar identity to *Lb. plantarum*/*Lb. pentosus* (96.8–100%) and *Ent. gallinarum*/*Ent. casseliflavus* (98.3–100%). Therefore, differential sugar pattern fermentation (Holt et al., 1994) was carried out for the correct identification of these last four species, which were characterized as *Lb. plantarum* and *Ent. gallinarum* strains.

3.6. Compatibility among lactic acid bacteria strains

Evaluation of compatibility among the eleven LAB strains indicated that most of them could be included in a multi-strain probiotic product, with the exception of *Ent. gallinarum* CRL 1826, which inhibited the growth of *Lactobacillus* and *Pediococcus* species through the production of a bacteriocin-like metabolite (data not shown).

4. Discussion

Amphibians may play host for many bacterial species considered as mutualists that are often essential for animal survival (Lauer et al., 2008). The resident microbiota of mucosal surfaces can be affected by different causes, including host environmental factors. Thus, since the final aim of our research group is to design a multistrain probiotic composed of indigenous beneficial bacteria isolated from different bullfrog hatcheries to be applied in diverse geographical regions of Argentina, the autochthonous cultivable microbiota of bullfrogs (skin and cloaca) from the central region of the country in fattening phase, without apparent signs or symptoms of RLS was determined in this work. Previous studies were performed by sampling animals, freshwater and balanced feed from a hatchery located in the northwest of the country, where RLS outbreaks are frequent (Pasteris et al., 2006, 2009a).

Although the present study does not provide a description of the whole microbial diversity in bullfrogs, it was focused to evaluate the cultivable microbiota particularly in those bacterial genera recognized as potentially beneficial and pathogenic, while other authors determined the microbiota associated with infectious diseases in bullfrogs (Glorioso et al., 1974; Mauel et al., 2002).

Even though bullfrogs are ectothermic, the isolation was performed at 37 °C to promote the recovery of all the cultivable bacterial species of main interest. Thus, the skin and cloaca of healthy frogs are mostly composed of LAB (Fig. 1), while *Bifidobacterium* and *Bacillus* were isolated in a smaller proportion. These last two genera are recognized as beneficial in aquaculture (Farzanfar, 2006; Balcázar and Rojas-Luna, 2007) and are reported for the first time in bullfrogs in this work. The microbiota also includes RLS-related pathogens (*Ps. aeruginosa*, *S. epidermidis* and *C. freundii*), other *Enterobacteriaceae*, and yeasts.

There are some studies concerning the evaluation of indigenous microbiota in amphibian species and their participation in the control of emergent infectious diseases (Culp et al., 2007; Harris et al., 2009) but they do not include bullfrogs in captivity. Thus, our group has previously demonstrated that the cultivable autochthonous microbiota of a bullfrog hatchery located in the northwest of Argentina is composed mainly of LAB, *P. pentosaceus* and *Lb. plantarum* being the representative species. *Ent. faecalis*, *Ent. faecium*, *Micrococcus* spp., *E. coli* and *Enterobacter* spp. were also isolated (Pasteris et al., 2006, 2008, 2009a). The variations observed between both hatcheries could be associated with different environmental and hatchery conditions. *C. freundii*, *Ps. aeruginosa* and *S.*

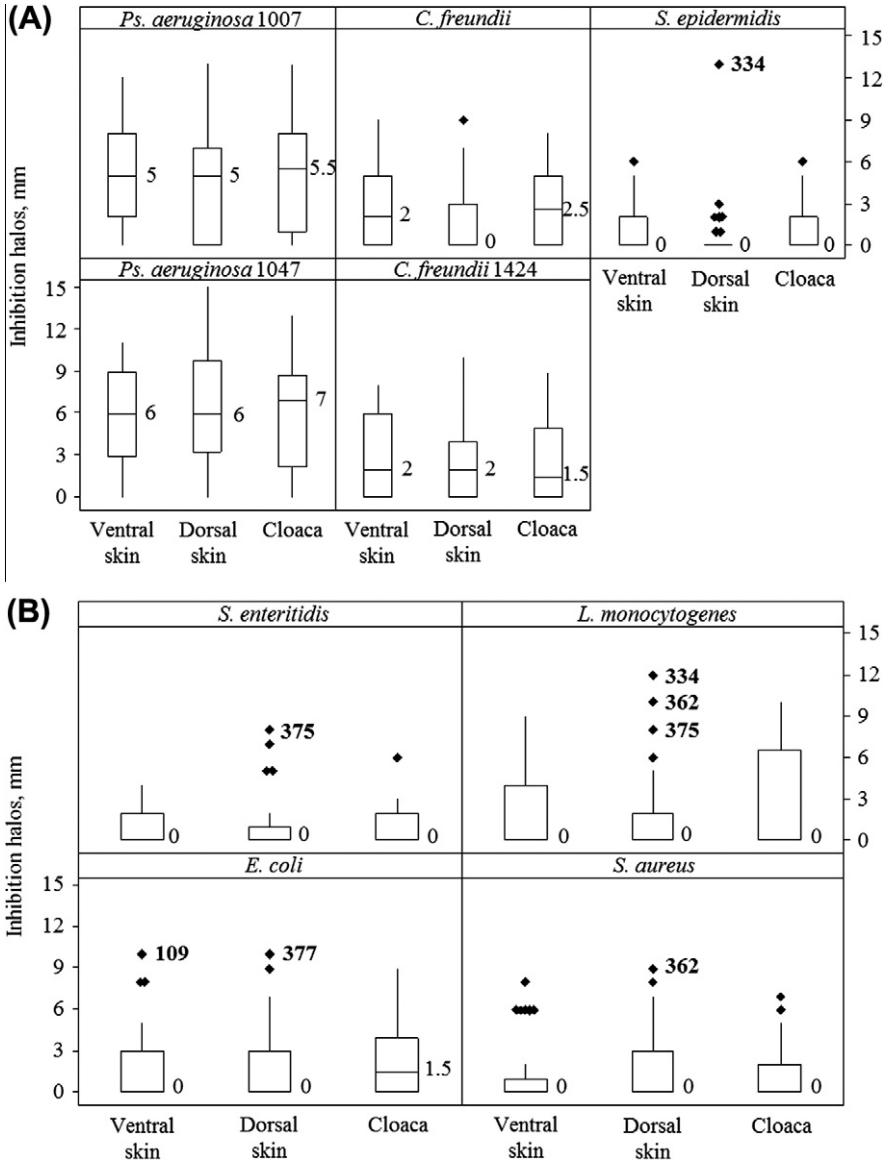

Fig. 3. Inhibitory activity of potentially beneficial bacteria isolated from *L. catesbeianus*. Inhibitory effect against: (A) RLS-associated pathogens, (B) food-borne bacteria. Digits close to the boxes represent the median values. Symbols indicate the values out of the interquartile range. *Enterococcus* spp. 334 and *Lactobacillus* spp. 375, 362, 109 and 377 represent the strains with higher antimicrobial activity.

Table 2
Hydrogen peroxide (H_2O_2) production by potentially beneficial bacteria isolated from *L. catesbeianus*.

Origin	H_2O_2-producing bacteria (%)			
	Strong	Medium	Weak	Negative
Ventral skin[a]	3	12	2	83
Dorsal skin[b]	11	7	2	80
Cloaca[c]	3	8	0	89

[a] 100% = 42 isolates.
[b] 100% = 56 isolates.
[c] 100% = 38 isolates.

epidermidis were isolated from skin ulcerations, but were not detected from the skin of healthy frogs (Pasteris et al., 2006, 2009a).

The use of probiotics in *L. catesbeianus* hatcheries was proposed by de Carla Dias et al. (2007) but they did not specify the origin of the strains evaluated. Since the design of probiotics should include strains with specific characteristics, the first criterion to be applied is the host specificity, taking into account that the autochthonous microorganisms should colonize the specific ecological niche (Zoetendal et al., 2008). Thus, the evaluation of the beneficial properties of LAB, *Bifidobacterium* and *Bacillus* isolates was the next step towards the design of a probiotic. Microbial adhesion constitutes the first and key step in host colonization that can be affected by non-specific interactions (Ofek and Doyle, 1994). Probiotics can prevent pathogen colonization by different mechanisms such as competitive exclusion, steric interactions or blockage of specific cell receptors, and immune system stimulation (McGroarty, 1993; Reid et al., 2003). Therefore, the physicochemical properties of the bacterial cell surface are used to predict adhesion (Rosenberg and Doyle, 1990). Hydrophobicity and autoaggregation are properties related to adhesion, colonization and biofilm formation (Walter et al., 2008). These properties could increase bacterial permanence on the host and therefore their protective effect. In this work, most of the LAB isolates showed low hydrophobicity, which could be related to the aquatic environment and to the chemical nature of the mucus (Fontana et al., 2006). Most of the isolates

Table 3
Selection of potentially probiotic lactic acid bacteria strains.

Strains	Area[a]	Surface properties (%)[b]		H_2O_2[c]	Production of antagonistic substances[d] Indicator strains[e]								
					RLS-related pathogens					Food-borne bacteria			
		HP	AA		Ps 1007	Ps 1047	Cfc	Cf 1424	Sep	Lm	Se	Ec	Sa
Lb. plantarum CRL 1817	VS	2.9	16.6	–	A+++	A+++	A++	A++	A+	A++	A+	A++	A++
Lb. brevis CRL 1820	VS	1.3	5.1	–	A+++	A+++	A++	A++	A+	A++	A+	A+++	A++
Ent. gallinarum CRL 1826	DS	59.8	78.6	+	–	–	–	–	A,P,B+++	A,P,B+++	–	–	–
Lb. plantarum CRL 1819	DS	60.7	61.5	–	A++	A++	A++	A++	A+	A+	–	A++	–
Lb. plantarum CRL 1821	DS	74.6	16.6	+	A+++	A+++	A++	A++	–	A+++	A+	A++	A++
P. pentosaceus CRL 1825	C	5.5	22.1	++	A++	A++	A+	A+	A+	A++	A+	A+	A+
P. pentosaceus CRL 1824	C	5.6	16.3	–	A++	A++	A++	A++	A+	A++	A+	A++	A++
L. lactis CRL 1827	C	61.4	6.6	–	A+++	A++	–	A++	A+	A++	A+	A+	A++
L. garvieae CRL 1828	C	51.0	100	–	–	A+	A+	A+	–	–	–	–	–
Lb. plantarum CRL 1816	C	94.4	75.4	–	A++	A+++	A++	A++	A+	A++	A+	A++	–
Lb. plantarum CRL 1818	C	47.7	24.7	–	A++	A++	A+	A+	A+	A+	A+	A+	–

[a] Source of the isolates: VS = ventral skin; DS = dorsal skin; C = cloaca.
[b] Surface properties: HP = Hydrophobicity; AA = Autoagreggation.
[c] H_2O_2 production in MRS-TMB medium: negative (−), weak (+), medium (++) and strong (+++).
[d] Inhibition of sensitive strains by the production of antagonistic substances: A = organic acids; P = hydrogen peroxide; B = bacteriocin. Score according to the size of the halo (mm) of inhibition of sensitive strains: $\leqslant 4$ (+); $5 \geqslant 9$ (++); $\geqslant 10$ (+++).
[e] Indicator strains: Ps1007 = *Ps. aeruginosa* 1007; Ps1047 = *Ps. aeruginosa* 1047; Cfc = *C. freundii* CFc; Cf1424 = *C. freundii* 1424; Sep = *S. epidermidis*; Lm = *L. monocytogenes* Scott A; Se = *S. enteritidis*; Ec = *E. coli*; Sa = *S. aureus*.

were not autoaggregating, with the exception of some of them with an index higher than 80%. However, autoaggregation was taken into account as a selection criterion because this property is closely related to the probiotic effect (Cesena et al., 2001; Walter et al., 2008).

Evaluation of the surface properties in the same isolates indicated that there was no correlation between hydrophobic and autoaggregating characteristics of individual isolates (Fig. 2B), suggesting that these properties are related to different cell wall components. Most of the isolates showed low hydrophobicity and autoaggregation percentages. These findings could help in the building of new criteria to select probiotics for raniculture, and possibly in general aquaculture. In these environments, probiotics should present hydrophilic rather than hydrophobic features. Similar results were observed in LAB isolated from *L. catesbeianus* (Pasteris et al., 2009a,b).

Microbial interactions play an important role in the maintenance of an equilibrated population between beneficial and potentially pathogenic microorganisms. Many studies have shown the ability of LAB (Verschuere et al., 2000; Vázquez et al., 2005; Balcázar et al., 2007), *Bacillus* (Aly et al., 2008; Qi et al., 2009) and *Bifidobacterium* (Irianto and Austin, 2002; Ringø et al., 2010) to control pathogenic bacteria in aquaculture through the production of antimicrobial metabolites (organic acids, H_2O_2 and bacteriocins). The inhibitory activity of LAB, *Bifidobacterium* and *Bacillus* isolates against RLS-related pathogens from different bullfrog hatcheries and meat spoilage bacteria was evaluated. All LAB isolates inhibited the growth of at least one pathogenic bacterium by organic acids, with the exception of *Enterococcus* spp. 334, which inhibited the growth of *S. epidermidis* and *L. monocytogenes* by organic acids, H_2O_2 and a bacteriocin-like metabolite by the agar-well diffusion method. However, a more sensitive method (TMB + peroxidase) enabled the detection of a higher percentage of H_2O_2-producing LAB isolates, although in smaller numbers than those previously reported (Pasteris et al., 2009a,b).

Bifidobacterium spp. and *Bacillus* spp. were hydrophilic, non-autoaggregating and unable to inhibit any pathogenic bacteria under the experimental conditions.

In this work, different criteria were applied to select indigenous LAB strains to contribute to the design of a probiotic for raniculture. They included the health status of the animals from which the isolates were obtained, the bacterial species regarded as safe, the surface properties and the inhibitory activity. Moreover, the inclusion of microorganisms in a probiotic product requires their correct taxonomic identification, basically because frog meat is used for human consumption (FAO/WHO, 2001). Thus, a group of selected LAB was identified by 16S rDNA sequence analysis and phenotypic approaches (API CHL50) as *Lb. plantarum*, *Lb. brevis*, *P. pentosaceus*, *L. lactis*, *L. garvieae* and *Ent. gallinarum*. Most of these strains are generally recognized as safe (GRAS), although the *Enterococcus* strain requires the evaluation of virulence factors and vancomycin resistance.

The LAB strains selected showed different antimicrobial spectra and can be included in a multi-strain probiotic to increase the possibilities of inhibition of a higher number of RLS-related pathogens and others associated to food quality. Therefore, evaluation of the compatibility of these strains was carried out. All the strains could be combined, with the exception of *Ent. gallinarum* CRL 1826, which inhibited the growth of selected *Lactobacillus* and *Pediococcus* strains.

In this work, the cultivable microbiota of healthy bullfrogs was characterized, and potentially probiotic microorganisms were selected. These results will enable progress in the design of a multi-strain probiotic to improve the sanitary status of animals in intensive growth systems. Nowadays, an experimental animal model to evaluate the probiotics action (adverse effects, protection against RLS-related pathogens) on *L. catesbeianus* embryos development and growing animals is being performed. The application of probiotics could be a valuable tool to avoid the use of antibiotics and antiseptics and thus to reduce production costs in raniculture. Moreover, these investigations could represent an interesting starting point to evaluate the potential application of probiotics to prevent infectious diseases associated with the global decline of amphibian species.

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