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Beneficial properties of lactic acid bacteria isolated from a *Rana catesbeiana* hatchery

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Abstract

This work addresses the selection of potentially probiotic lactic acid bacteria (LAB) to be used in raniculture. Thus, strains belonging to the genera *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Enterococcus faecium* isolated from a *Rana catesbeiana* hatchery were evaluated for their inhibitory properties against RLS-associated pathogens (*Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*) and food-borne bacteria. Cell-free supernatants of LAB strains inhibited the growth of at least one of the pathogens by organic acids, but *L. lactis* CRL 1584 also produced a bacteriocin-like metabolite. The ability of LAB strains to produce H_2O_2 in MRS+TMB medium was also studied. Seventy-eight to ninety six per cent of the strains showed some level of H_2O_2 production. Moreover, different organic solvents were used to determine the hydrophobicity and Lewis acid/base characteristic of LAB strain surfaces. Most of the strains presented hydrophilic properties, but no acidic or basic surface characters. However, some strains isolated from the skin showed a high degree of hydrophobicity and basic components in the cell surface due to their adhesion to chloroform. These properties were not observed in LAB from balanced feed and freshwater. Taking into account general guidelines and the beneficial properties studied, five strains were selected as potential candidates to be included in a probiotic for raniculture.

Keywords: aquaculture, *Rana catesbeiana*, red-leg syndrome, lactic acid bacteria, probiotics

Introduction

The *Rana catesbeiana* market has grown substantially due to the increasing demand for frog products in the food industry and for live laboratory material (Olvera-Novoa, Ontiveros-Escutia & Flores-Nava 2007). Domestic farming of frogs is a growing area because of frogs' biological attributes, especially their muscle mass and the use of their by-products. For instance, the skin of frogs and toads is a rich source of pharmacological and antimicrobial peptides, which play various roles in the regulation of physiological functions of the skin and in the defense against predators or micro-organisms (Goraya, Knoop & Conlon 1998; Urbán, Nagy, Pál, Sonnevend & Conlon 2007). Frog and toad skin is also used for the fabrication of wallets and purses, their fat used in the cosmetic industry and the abdominal organs for pate (liver) and thread for surgery (gut) (Texeira, Pereira Mello & Lima dos Santos 2002). These facts have increased the demand of *R. catesbeiana* products, mainly in Europe, and have lead to its more widespread cultivation in Thailand, Taiwan and Brazil (Texeira *et al.* 2002). Of the total worldwide production, only an estimated 15% comes from bullfrog hatcheries, whereas the remaining proportion comes from the capture of animals in the wild (Olvera-Novoa *et al.* 2007). Farming operations require frogs to be placed in captivity; this confinement increases the risk of epizootics, such as encephalitis, mycobacteriosis and red-leg syndrome (RLS) (Glorioso, Amborsky, Amborsky & Culley 1974; Bühler, Sánchez Toranzo & Zaltz 2000; Ferreira, de Souza Fonseca, Muñiz Afonso, Gomes da Silva, Saad & Lilenbaum 2006). Red-leg syndrome is the main cause of mortality and significant economic losses in raniculture (Mauel, Miller, Frazier & Hines II 2002) with Enterobacteriaceae (*Proteus vulgaris*, *Proteus mirabilis*, *Citrobacter freundii*, *Edwardsiella tarda*), *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus iniae*, *Chryseobacterium meningosepticum*, *Chryseobacterium*

indolgenes being its main pathogens (Glorioso *et al.* 1974; Mauel *et al.* 2002).

Currently, the prevention and control of aquaculture diseases have focused on good husbandry practices and the use of vaccines or antibiotics (Bühler *et al.* 2000; Verschuere, Rombaut, Sorgeloos & Verstraete 2000; Romalde, Ravelo, López-Romalde, Avendaño-Herrera, Magariños & Toranzo 2005). Treating or feeding frogs with antibiotics may cause the development of resistant bacteria (Akinbowale, Peng & Barton 2006). An alternative method of prevention is the use of probiotics (Reid, Sanders, Rex Gaskins, Gibson, Mercenier, Rastall, Roberfroid, Rowland, Cherbut & Klaenhammer 2003), which are able to inhibit colonization of and to exert inhibitory effects against undesired micro-organisms, as well as to support the natural host microbial defense mechanisms (Gatesoupe 1999; Gram, Melchiorsen, Spanggaard, Huber & Nielsen 1999; Ringø, Schillinger & Holzapfel 2005). Thus, a wide range of Gram (+) (*Bacillus, Carnobaterium, Lactobacillus, Enterococcus, Streptococcus, Lactococcus, Pediococcus, Micrococcus* and *Weisella*) and Gram (-) bacteria, yeast, microalgae and bacteriophages have been evaluated as probiotics for fish aquaculture (Irianto & Austin 2002).

Taking into account that homologous and specific species of a probiotic have shown to be more effective in the temporary colonization needed for beneficial effect, such as immunostimulation (Salminen, Deighton, Benno & Gorbach 1998; Vaughan, Heilig, Ben-Amor & De Vos 2005), the autochthonous microbial population of a *R. catesbeiana* hatchery from Argentina was studied at different seasons. The microbiota includes lactic acid bacteria (LAB): *Lactobacillus plantarum, Lactobacillus curvatus, Pediococcus pentosaceus, Leuconostoc mesenteroides* and *Enterococcus faecium Micrococcus* spp., *Enterobacter* spp. and *Escherichia coli* were also found. *P. vulgaris, P. aeruginosa* and *S. epidermidis* were isolated from tissues of animals displaying RLS (Pasteris, Bühler & Nader-Macías 2006; Pasteris, González, Van Schoor, Bühler, Nader-Macías, Vandamme & De Vuyst 2008). In a previous work, the evaluation of inhibitory and surface properties of *Lactobacillus* species was performed and some strains were proposed as potentially probiotic micro-organisms to be used in raniculture (Pasteris, Bühler & Nader-Macías 2004; Pasteris, Vera Pingitore, Roig Babot, Bühler & Nader-Macías 2007).

Given that probiotics in fish aquaculture are now recognized as one of the measures needed for disease prevention and control (Nikoskelainen, Ouwehand, Salminen & Bylund 2001; Nikoskelainen, Salminen, Bylund & Ouwehand 2001; Irianto & Austin 2002; Balcázar, de Blas, Ruiz-Zarzuela, Cunnigham, Vendrell & Múzquiz 2006; Vine, Leukes & Kaiser 2006) and Gram (+) cocci belonging to the LAB species are parts of the autochthonous microbiota of a bullfrog hatchery (Pasteris *et al.* 2006, 2008), the purpose of this work was to evaluate some of the beneficial properties of different LAB genera and to later have the scientific support required to propose their potential application as probiotics in raniculture.

Materials and methods

Bacterial strains and culture conditions

The LAB strains belonging to the genera *P. pentosaceus, L. mesenteroides, Lactococcus lactis* and *E. faecium* were isolated from an *R. catesbeiana* hatchery in autumn, summer and spring and identified by phenotypic and genotypic approaches (Pasteris *et al.* 2006, 2008). The strains were grown in MRS (de Man, Rogosa & Sharpe 1969) and LAPTg (Raibaud, Galpin, Ducluzeau, Mocquot & Oliver 1963) broth media for 12 h at 37 °C. Indigenous RLS-associated pathogens (*P. vulgaris* MIB10, *P. aeruginosa* GRB and *S. epidermidis*) as well as those from other ecological niches (*P. aeruginosa* ATCC 27853, *P. vulgaris, P. mirabilis* and *C. freundii*) and *Staphylococcus aureus* were grown in nutritive broth for 8 h at 37 °C. Other food-borne bacteria (*Listeria monocytogenes* Scott A and *Salmonella enteritidis*) were grown in brain–heart infusion (BHI) broth in the same conditions. The strains were stored at − 20 °C in MRS medium supplemented with 20% (v v^{-1}) glycerol. All culture media were obtained from Merck (Darmstadt, Germany).

Lactic acid bacteria strains belong to the bacterial culture collection of CERELA.

Inhibitory activity of LAB strains

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 (Juárez Tomás, Ocaña, Wiesse & Nader-Macías 2003). Different concentrations of indicator strains (1×10^2 and 1×10^5 CFU mL^{-1}) were inoculated in nutritive or BHI soft agar (0.7% w v^{-1}) at 45 °C and poured into Petri dishes. After solidification, wells of 10 mm diameter were performed into the agar plates and 100 μL of overnight LAB supernatants were added to each well. Cell-free supernatants of LAB cultures

were obtained by centrifugation at 3000 *g*, at 4 °C for 20 min and filtered through a 0.22 μm pore-size filter (Millipore, St. Louis, MO, USA). Two millilitres fractions were adjusted to pH 7.0 with sterile 1 N NaOH and treated with 0.5 mg mL^{-1} catalase (Sigma-Aldrich Chemical, St Louis, MO, USA) at 25 °C for 30 min. Crude 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 for 24 h in microaerophilic conditions.

The antagonistic metabolites present in the LAB strains supernatants inhibited 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 (H_2O_2) production by LAB strains

The H_2O_2 production in LAB cultures was qualitatively determined by the plate method, employing horseradish peroxidase incorporated in tetramethylbenzidine (TMB) agar medium (Juárez Tomás, Otero, Ocaña & Nader-Macías 2004). Peroxidase catalyses the oxidation of TMB (chromogenic substrate) to a purple-blue pigment evidenced in those colonies that produce H_2O_2. The LAB strains were grown in MRS broth and inoculated in MRS plates containing 1 mM TMB (3,3′, 5,5′-TMB, from Sigma-Aldrich Chemical, dissolved in methanol) and 2 U mL^{-1} of peroxidase (Peroxidase EC 1.11.1.7, Type II: From horseradish, Sigma-Aldrich Chemical). 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.

Physicochemical characterization of the bacterial surfaces

The hydrophobicity and Lewis acid/base properties of LAB strains were determined by the microbial adhesion to hydrocarbon assay (Rosenberg & Doyle 1990) by using different solvents: hexadecane (apolar), chloroform (electron acceptor) and ethyl acetate (electron donor). The LAB strains were grown in MRS broth at 37 °C and collected by centrifuging at early logarithmic growth phase, washed twice and resuspended in 0.9% (w v^{-1}) NaCl to an optical density ($OD_{600\,nm}$) between 0.6 and 0.7. 0.45 mL of each organic solvent was added to test tubes containing 2.7 mL of washed cells. 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 bacterial adhesion to organic solvent (Ly, Vo, Le, Belin & Waché 2006) was calculated as follows:

Adhesion (%) = [(OD_{600} before mixing − OD_{600} after mixing)/OD_{600} before mixing] × 100. The score of adhesion applied was: high (60–100%), medium (30–59%), low (0–29%).

Organic solvents were purchased from Cicarelli, Buenos Aires, Argentina.

Statistical analysis

All the experiments were performed by triplicate and the means of the data were determined. To analyse the surface properties results, medians and interquartile ranges (IQ) were calculated using software MINITAB (version 14).

Results

Inhibitory activity of LAB strains

One hundred and four LAB strains belonging to the genera *P. pentosaceus*, *L. mesenteroides*, *L. lactis* and *E. faecium* isolated from a *R. catesbeiana* hatchery at different seasons were evaluated for their inhibitory properties against RLS-associated pathogens and food-borne bacteria. The strains were able to inhibit the growth with at least one of the assayed pathogens. However, those strains that showed the widest inhibitory spectrum were selected to be included in the tables. Consequently, Table 1 shows the final pH and the antimicrobial activity of culture supernatants of *L. mesenteroides* and *P. pentosaceus* strains isolated in autumn and spring when growing in MRS broth. *L. mesenteroides* strains isolated from the skin of healthy frogs were found to inhibit the growth of autochthonous RLS-related pathogens and food-borne bacteria, with the strain 2M presenting the highest inhibitory activity. Among the *P. pentosaceus* strains isolated from this ecological area, 6D, 7q, 7j and MCH 9′V strains showed the maximal antimicrobial activity. However, *P. pentosaceus* 7j showed the highest inhibitory effect against RLS-related pathogens.

Table 1 Inhibitory activity of lactic acid bacteria strains isolated from a *Rana catesbeiana* hatchery in autumn and spring

			Indicator strains											
			Inhibition (mm)											
			Proteus vulgaris MIB10		*Pseudomonas aeruginosa* GRB		*Staphylococcus epidermidis*		*Salmonella enteritidis*		*Listeria monocytogenes*		*Staphylococcus aureus*	
Origin	LAB strains	pH*	A	B	A	B	A	B	A	B	A	B	A	B
HA	*Leuconostoc mesenteroides* 6K	3.8	4	3	9	4	10	8	1	1	4	4	3	2
	L. mesenteroides 2M	3.5	10	9	15	15	13	11	6	6	7	6	11	9
	Pediococcus pentosaceus 6D	3.5	8	8	6	5	9	9	1	1	2	2	5	2
	P. pentosaceus 7q	3.5	6	4	10	10	7	5	3	2	4	4	2	2
	P.pentosaceus 7J	3.5	10	9	12	10	14	10	3	1	5	4	10	7
NHA	*P. pentosaceus* 1L	3.8	11	10	11	10	11	8	4	4	4	4	10	8
	P. pentosaceus 1F	3.4	5	4	7	4	14	7	1	1	1	1	3	2
	P. pentosaceus 1Q	3.4	3	3	5	2	5	3	2	1	2	2	8	8
BF	*P. pentosaceus* 9A	3.5	12	10	12	12	10	8	5	1	5	5	11	8
	P. pentosaceus 9K	3.5	7	7	10	8	14	12	3	1	4	3	8	8
HA†	*P. pentosaceus* MCH9'V	3.5	4	4	10	8	10	10	5	1	5	2	4	4
NHA†	*P. pentosaceus* MIB18	3.8	4	4	10	9	10	10	2	2	5	4	2	2

LAB strains were isolated from the skin of healthy (HA), and non-healthy (NHA) animals, and balanced feed (BF) in autumn and †spring.
*Final pH of the LAB cultures after 24 h of incubation at 37 °C on MRS broth. The initial concentrations of indicator strains were: A, 1×10^2; B, 1×10^5. The values indicate the size (in mm) of the inhibitions produced by crude supernatants.
The results represent the means of three separate experiments.
LAB, lactic acid bacteria.

Pediococcus pentosaceus 1L, 1F, 1Q and MIB18 isolated from non-healthy frogs inhibited the growth of RLS-associated pathogens and food-borne bacteria, mainly 1L strain.

Pediococcus pentosaceus 9A and 9L, isolated from balanced feed, showed the widest inhibitory activity against the assayed pathogens.

Lactic acid bacteria from freshwater samples of both healthy and non-healthy frogs showed the lowest inhibitory effect, with indigenous *P. aeruginosa* being the most sensitive strain (results not shown).

The antagonistic effect observed in this group of LAB strains was abolished when cultures supernatants (final pH between 3.4 and 3.8) were neutralized, indicating that the inhibition could be attributed to the organic acids produced.

The antimicrobial activities of some LAB isolated in summer are summarized in Table 2. From the different LAB species isolated from healthy frogs, *P. pentosaceus* SEP 7B′ and SEP 35A showed the widest antibacterial spectrum.

Enterococcus faecuim GST23, *L. lactis* GST13 and *L. mesenteroides* FZ46A isolated from freshwater samples of healthy frogs mainly inhibited the growth of *P. aeruginosa*. However, *P. pentosaceus* AM19 is the strain that showed the highest inhibitory effect against *P. vulgaris*, *S. epidermidis* and *S. aureus*.

The final pH reached was between 3.5 and 4.3 and the antimicrobial activity disappeared when the supernatants were neutralized, which indicates that the inhibition could be produced by organic acids.

In reference to the LAB strains isolated from balanced feed, *Pediococcus*, *Lactococcus* and *Enterococcus* showed a low antibacterial activity. However, fluid supernatants of *L. lactis* CRL 1584 inhibited a vancomycin-resistant *E. faecium* strain, *L. monocytogenes* Scott A and *P. aeruginosa* ATCC 27853 when growing in LAPTg medium. This antimicrobial activity was due to organic acids, H_2O_2, and a bacteriocin-like metabolite (Table 2). However, when *L. lactis* was grown in MRS medium, a low degree of inhibition by organic acids against *Proteus* and *Listeria* was observed.

H_2O_2 production

The ability of 104 LAB strains to produce H_2O_2 in TMB–MRS plates was evaluated (Fig. 1). Seventy-eight per cent of LAB strains isolated from the skin of healthy animals showed some level of H_2O_2 production. All the LAB strains from the skin of non-healthy frogs were able to produce the oxidative metabolite. However, 8–17% of the strains were strong producers as e.g., *P. pentosaceus* FZ28 isolated from healthy animals, included in Table 2.

Ninety-four per cent of the LAB strains from the freshwater of healthy frogs were shown to be H_2O_2 producers, with 19% of them being strong producers. In the LAB strains from balanced feed, 96% of them were shown to produce the oxidative metabolite, but none of them were strong producer strains. Under our experimental conditions, all of the strains from the freshwater of non-healthy animals were not able to produce H_2O_2.

The majority of H_2O_2-producing LAB strains were also able to synthesize organic acids.

Physicochemical characterization of the bacterial surfaces

The diversity of the bacterial surface characteristics of all the LAB strains isolated from a *R. catesbeiana* hatchery was evaluated (Fig. 2). The adhesion to hexadecane was very low and indicates that the higher hydrophobicity was shown by the strains isolated from the skin (median value = 3.8%, IQ = 9.96). The strains isolated from freshwater and balanced feed exhibited median values of 1.76 (IQ = 6.34) and 2.0% (IQ = 5.45) respectively. Among the strains isolated from the skin, there was a group with a different behaviour that shows values of hydrophobicity index higher than 40% (e.g., *P. pentosaceus* Sep 35A and FZ6, and *L. mesenteroides* 2M). Although the median and IQ range values of adhesion to ethyl acetate (skin: 4.27%, IQ = 12.50; water: 4.04%, IQ = 9.92 and balanced feed: 2.34%, IQ = 7.67) were higher than hexadecane, there were no strains exhibiting different behaviour. The strains also showed low affinity to chloroform (median values between 0.98% and 0.08%) but exhibited the widest IQ ranges (skin: 15.71; water: 5.19 and balanced feed: 25.42). However, a group of strains isolated from the skin presented a degree of adhesion higher than 50%, and only *P. pentosaceus* 9K isolated from balanced feed exhibited more than 75% of adhesion to chloroform.

Selection of LAB

The criteria applied to select a group of LAB strains to be further studied are as follows: (1) strains isolated from the skin or water of healthy animals and/or balanced feed; (2) presence of cell-surface properties: strains with high (> 50%) or low (< 5%) degree of

Table 2 Inhibitory activity of lactic acid bacteria strains isolated from a *Rana catesbeiana* hatchery in summer

			Indicator strains											
			Inhibition (mm)											
			***Proteus vulgaris* MIB10**		***Pseudomonas aeruginosa* GRB**		***Staphylococcus epidermidis***		***Salmonella enteritidis***		***Listeria monocytogenes***		***Staphylococcus aureus***	
Origin	**LAB strains**	**pH***	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
HA	*Leuconostoc mesenteroides* FZ 13	3.7	6	6	6	6	5	1	0	0	0	0	6	5
	Pediococcus pentosaceus SEP2C	3.9	6	5	10	9	7	5	1	1	3	1	8	7
	P. pentosaceus SEP 35A	3.8	9	9	15	13	8	6	2	1	5	5	8	8
	P. pentosaceus SEP7B′	3.5	10	8	13	13	12	11	4	3	7	7	12	10
	P. pentosaceus FZ 6	3.6	7	6	9	6	8	5	5	3	6	6	4	4
	P. pentosaceus FZ28	3.6	1	1	3	1	4	2	1	1	1	1	0	0
FW	*Enterococcus faecuim* GST23	4.2	5	5	10	9	2	2	0	0	0	0	6	5
	Lactococcus lactis GST13	4.3	3	2	10	10	5	3	0	0	0	0	8	8
	L. mesenteroides FZ46A	3.8	5	3	11	7	0	0	0	0	4	2	2	2
	P. pentosaceus AM19	3.9	8	8	12	9	10	6	1	0	3	3	9	7
BF	*L. lactis* CRL 1584†	4.2	0	0	1	0	0	0	0	0	1	1	0	0
	L. lactis CRL 1584‡	4.2	3	0	8/6/0	8/6/0	0	0	0	0	8/7/6	8/7/3	1	0

LAB strains were isolated from the skin of healthy (HA), freshwater samples of healthy animals (FW), and balanced feed (BF) in summer.

*Final pH of the LAB cultures after 24 h of incubation at 37 °C on MRS broth. Initial concentrations of indicator strains: A, 1×10^2; B, 1×10^5. The values indicate the size (in mm) of the inhibitions produced by crude supernatants. For *L. lactis* CRL 1584: cell-free supernatants from

†MRS and

‡LAPTg media respectively.

Numbers indicate: crude supernatant/neutralized supernatant/neutralized supernatant+catalase. The results represent the means of three separate experiments.

LAB, lactic acid bacteria.

hydrophobicity and basic characters (> 50% of adhesion to chloroform); and (3) production of inhibitory substances: H_2O_2 production and ability to inhibit at least one of the assayed RLS-associated pathogens and food-borne bacteria.

According to these criteria, five strains were selected as probiotic candidates that are indicated in Table 3 with their specific properties.

Discussion

Aquaculture has become an important economic activity but specific bacterial pathogens can be a significant cause of mortality in both fish and bullfrog hatcheries because intensive husbandry practices often result in the breakdown of natural host barriers (Mauel *et al.* 2002; Ringø *et al.* 2005). Commercial hatcheries have attempted to overcome this problem by disinfection, stimulation of host resistance and prophylactic or therapeutic treatment with antibiotics (ATB) (Bühler *et al.* 2000; Verschuere *et al.* 2000; Romalde *et al.* 2005), which has led to selective pressure of ATB resistance in bacteria (Akinbowale *et al.* 2006). An alternative approach is manipulating the gut microbiota by adding antagonistic bacteria (Ringø *et al.* 2005). In the last decade, LAB have received increasing attention as probiotic as an effective way to reduce the use of ATB in both endothermic and poikilothermic animals (Draksler, González & Oliver 2004; Balcázar *et al.* 2006).

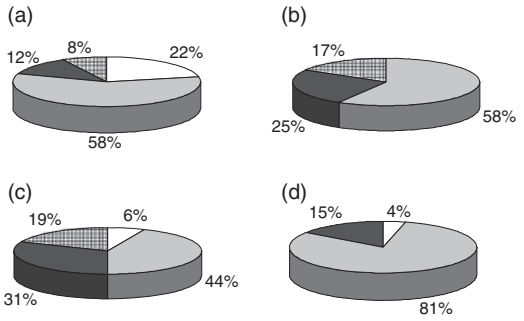

Figure 1 Hydrogen peroxide production by lactic acid bacteria strains isolated from a *Rana catesbeiana* hatchery. (a), healthy animals; (b), non-healthy animals; (c), freshwater from healthy animals and (d), balanced feed. Production of hydrogen peroxide: negative (□), weak (▒), medium (■), and strong (▦).

Different modes of action of probiotics were proposed, such as competitive exclusion by oxygen availability, nutrients and adhesion sites in the host; enhancement of the innate or non-specific immune system (Irianto & Austin 2002) and antiviral effects, and improvement of the water quality (Balcázar *et al.* 2006; Farzanfar 2006). Probiotics may also stimulate appetite and increase the nutritional status in fish and bullfrogs (Lara-Flores, Olvera-Novoa, Guzman-Mendez & Lopez-Madrid 2003; de Carla Dias, de Paiva Badiz Furlaneto, da Silva Ayroza, Menezes França, Ferreira & Verardino de Stéfani 2007).

Since the beneficial strains to be included in a probiotic product for use in a defined ecological niche and host should be isolated from the same area where it will be applied to increase colonization properties

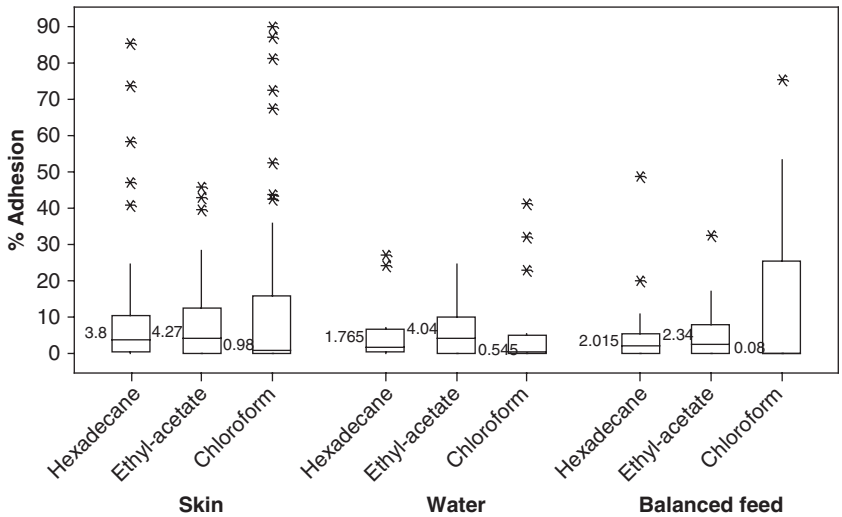

Figure 2 Physicochemical characterization of the lactic acid bacteria strains surfaces isolated from a *Rana catesbeiana* hatchery. ⁎ Indicates hydrophobicity values out of the interquartile range.

Table 3 Relationship between inhibitory activity, hydrogen peroxide production and surface properties of selected lactic acid bacteria

Strain	Source	Inhibition of pathogens				H_2O_2 production	Surface properties		
		Proteus vulgaris	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*	*Listeria monocytogenes*		Hexadecane	Ethyl acetate	Chloroform
Leuconostoc mesenteroides 2M (CRL 1759)	HA	++	++	++	+	w	58.12	39.50	86.97
Pediococcus pentosaceus Sep 35A (CRL 1762)	HA	+	+	++	+	w	85.58	45.98	89.98
P. pentosaceus FZ6 (CRL 1763)	HA	+	+	+	+	+++	73.63	23.70	72.37
P. pentosaceus 7j (CRL 1761)	HA	++	++	++	+	++	0.0	0.0	0.0
P. pentosaceus 9K (CRL1760)	BF	+	++	++	+	+	48.78	17.02	75.53
Lactococcus lactis CRL 1584	BF	+	–	+*	+*,†	+	1.42	0.0	0.0

*Inhibition by hydrogen peroxide,

†Inhibition by bacteriocin-like metabolite.

Score of inhibition: +(halo $<$ 10 mm), ++ (halo $\geq$ 10 mm).

Score of H_2O_2 production in TMB-MRS: +++, strongly positive; ++, moderately positive; +, weakly positive.

Score of adhesion: high (60–100%), medium (30–59%), low (0–29%).

HA, healthy animals; BF, balanced feed; CRL, Centro de Referencia para Lactobacilos Culture Collection; H_2O_2, hydrogen peroxide; TMB, tetramethyl-benzidine.

(Reid *et al.* 2003), the first step of our research was focused on the isolation of autochthonous LAB that resulted in representative members of the *R. catesbeiana* hatchery microbiota (Pasteris *et al.* 2006). In this paper, the evaluation of some beneficial properties of LAB strains isolated from this particular ecological niche was performed, in order to go further in the design of a probiotic product to be used in raniculture.

Although disease outbreak produced by many genera as *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Lactococcus* and *Vagococcus* has been documented in fish aquaculture (Ringø & Gatesoupe 1998), there is no information in raniculture. In previous work, we demonstrated that LAB were not able to translocate to target organs either in *R. catesbeiana* specimens displaying RLS or in healthy animals (Pasteris *et al.* 2006), which is an interesting result to show that LAB strains do not have adverse effects. These findings provided one of the arguments to support the selection of LAB to further study some of their beneficial properties. Therefore, a group of *L. plantarum* and *L. curvatus* strains was selected as potentially probiotic candidates for raniculture (Pasteris *et al.* 2004, 2007). Since other LAB genera are parts of the native microbiota of bullfrog hatchery (Pasteris *et al.* 2006, 2008), the purpose of this work was to perform a screening to evaluate the beneficial properties of Gram (+) and catalase (−) cocci. There were no references in the literature on the evaluation of such properties in these specific LAB groups in bullfrog hatcheries.

To study the beneficial properties of the isolated strains, three *in vitro* assays were used to determine their surface characteristics and their inhibitory effect against RLS-related pathogens and food-borne bacteria.

The antimicrobial effect of bacteria in aquaculture results from the production of bacteriocins, H_2O_2 or organic acids (Verschuere *et al.* 2000). Our results showed that *P. pentosaceus*, *L. mesenteroides* and *E. faecium* strains were able to inhibit the pathogens mainly by acid production. The same effect was observed against RLS-associated pathogens from other ecological niches (e.g., *P. aeruginosa* ATCC 27853, *C. freundii* and *P. vulgaris* – results not shown). This antimicrobial effect was strain-dependent and pH-independent, but, in some cases, showed modifications according to initial concentration of the indicator strains. Alakomi, Skyttä, Saarela, Mattila-Sandholm, Latva-Kala and Helander (2000) reported that the outer membrane of Gram (-) bacteria was disrupted by lactic acid and that this permeabilization of the Gram (-) cell wall increased its susceptibility to other

antimicrobial substances, e.g., bacteriocins such as nisin (Cutter & Siragusa 1998).

None of the evaluated LAB were able to inhibit the growth of RLS-associated pathogens and food-borne bacteria by H_2O_2 production by using the agar well diffusion method, except for *L. lactis* CRL 1584. With a more sensitive method (MRS–TMB), it was possible to find a higher percentage of H_2O_2-producing strains. Many authors have proposed that the release of the oxidative metabolite to the aquatic environment could reduce potentially opportunistic pathogens (Verschuere *et al.* 2000; Farzanfar 2006). This report is the first to evaluate these characteristics in strains isolated from this specific environment.

Lactic acid bacteria strains with a low acidification capability showed a wider spectrum of inhibition, which could be related to some other metabolites released to the media, for example, H_2O_2. This fact could support the possibility of the existence of a synergic effect as it has been reported for bovine lactobacilli against *S. aureus* (Otero & Nader-Macías 2006).

It is interesting to point out that a few LAB strains isolated from the skin ulcerations of bullfrogs displaying RLS were able to inhibit pathogens by acidity and were also H_2O_2-producing strains. This fact could indicate that the environmental conditions in the skin ulceration could exert some type of environmental pressure that modifies the ability of LAB to produce inhibitory metabolites with antimicrobial effect. These results also would support the possibility that a probiotic could be applied as a preventive strategy.

From the 104 LAB strains studied, only *L. lactis* CRL 1584 isolated from balanced feed produced a bacteriocin-like metabolite, which inhibited the growth of a vancomycin-resistant *E. faecium* strain, *P. aeruginosa* ATCC 27853 and *L. monocytogenes* Scott A. *L. lactis* has been suggested as a bacteriocin-producing strain and also as potentially probiotic in turbot (Campos, Rodriguez, Calo-Mata, Prado & Barros Velásquez 2006), but there are no references in raniculture. The bacteriocin-like metabolite was only produced in LAPTg medium, but not in MRS medium, possibly due to a catabolite repression mechanism as was reported by Hernández de Rojas, Suárez and Rodríguez (2004) for a lactococcin.

Bacterial adhesion to tissues is considered the first and key step in host colonization and can be influenced by non-specific interactions based on hydrophobic, Lewis acid/base and ionic interactions (Ofek & Doyle 1994). Probiotic micro-organisms can prevent pathogen access either by steric interactions or by specific blockage of cell receptors (McGroarty 1993). Bacterial adhesion has been associated with the attachment to a variety of substrates and the physicochemical properties of the bacterial cell surface were used to predict adhesion (Rosenberg & Doyle 1990). Therefore, in this paper different organic solvents were used to determine the hydrophobicity and Lewis acid/base characteristic of the bacterial surface of LAB strains. Most of the strains studied presented hydrophilic properties, but not acidic or basic surface characters. However, some LAB strains isolated from the skin showed different behaviour, such as *P. pentosaceus* Sep 35A and FZ6 and *L. mesenteroides* 2M, which exhibited a high degree of hydrophobicity and contained basic components in the cell surface, according to their adhesion to chloroform. These properties were not detected in the strains isolated from balanced feed and water (except *P. pentosaceus* 9K), which could indicate that these characteristics could be associated with the interaction with components of mucosal surfaces, such as epithelial cells and mucus that contain acid glycoconjugates (Els & Henneberg 1990).

From the 104 LAB isolated, five strains were selected as potentially probiotic candidates for raniculture (Table 3) taking into account general guidelines (Reid *et al.* 2003) and the beneficial properties studied, including *L. lactis* CRL 1584, in their inhibitory activity against RLS-related pathogens and their hydrophilic properties. This strain also inhibited *L. monocytogenes*, which is a causative agent of listeriosis (Tompkin 2002). *Lactococcus lactis* strain should be used as a single probiotic culture since this strain is able to inhibit the growth of the other selected micro-organisms.

Even though the inhibitory properties due to organic acid or bacteriocins have been reported from fish and fish farming, for example, *Aerococcus*-like strains, *Pediococcus acidilactici*, *Weisella hellenica*, *E. faecium* and *Enterococcus mundtii* (Ringø 2004; Campos *et al.* 2006; Gatesoupe 2008), our research represents the first statement on the beneficial properties of a specific group of cocci into the LAB species in a bullfrog hatchery.

Although the selection of probiotic micro-organisms must be initially carried out through the application of *in vitro* characteristics (as performed in this work), the definitive application and clinical evidence of their *in vivo* effects should be evaluated through the use of animal models or specific hosts, which is the next step in our work. Despite the fact that these results might be considered preliminary, they represent the bases for the next step and are valuable

because it is the first approach specific to raniculture. Further studies are being performed to determine the antibiotic susceptibility, the presence of extrachromosomal elements and some other functional and technological properties of selected LAB strains.

The final objective of our group is to design a probiotic that could be included in a veterinary product or balanced feed for the prevention of RLS and to avoid carcase contamination by *Listeria* and *Staphylococcus*.

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