Aquaculture Research

Aquaculture Research, 2017, **48**, 2157–2172 doi: 10.1111/are.13053

Preliminary assessment of *in vivo* safety of potentially probiotic lactic acid bacteria for American bullfrog culture

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Abstract

The effect of the administration of beneficial lactic acid bacteria (LAB): *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827, *Enterococcus gallinarum* CRL 1826 and combined CRL 1584+ CRL 1826 on the development of bullfrog embryos from the hatching stage until 31 days (tadpole) was evaluated. *In vitro* assays indicated that 10^3, 10^6 and 10^9 CFU mL^{-1} single LAB strains remained viable until 24 h in 10% Ringer. Around 10^9 CFU mL^{-1} LAB (individually and combined) were used in an experimental design built to evaluate their effect when administrated at different intervals (three 7-day cycles with 5-day rest periods in between) to embryos until day 31. The highest potentially beneficial population (LAB) numbers were detected in the LAB-treated groups. All the LAB-treatments increased it and were significantly higher than the controls. Although the highest, potentially pathogenic, population (Red-Leg Syndrome-RLS-related pathogens) numbers were detected in the control and the lowest in the CRL 1584+ CRL 1826-treated group, they did not differ significantly. Stereoscopic studies showed no malformations in any LAB-treated group and all the specimens reached the same stage of their biological cycle with a survival >94%. The histological structure of target organs for RLS-associated pathogens (intestine and skin) and stomach was not affected and the spleen was developed. Only the LAB-treated groups showed microorganisms associated with the intestinal mucus, without inflammatory reaction in the lamina propria. This is the first report on the evaluation of the absence of adverse effects after LAB administration to bullfrog embryos using indigenous microorganisms.

Keywords: lactic acid bacteria, probiotics, bullfrog culture, morphological studies, raniculture

Introduction

In the international market, some specific amphibian species are of main interest to obtain foodstuff and by-products (Texeira, Pereira Mello & Lima dos Santos 2001). For instance, the skin is used in pharmacology to obtain molecules for their use as antimicrobial (Goraya, Knoop & Conlon 1998; Giacometti, Cirioni, Barchiesi & Scalise 2000; Rollins-Smith, Reinert, O'Leary, Houston & Woodhams 2005), antiviral (VanCompernolle, Taylor, Oswald-Richter, Jiang, Youree, Bowie, Tyler, Conlon, Wade, Aiken, Dermody, KewalRamani, Rollins-Smith & Unutmaz 2005) and anti-tumour (Libério, Joanitti, Azevedo, Cilli, Zanotta, Nascimento, Sousa, Pires Júnior, Fontes & Castro 2011) drugs. During the last few years, the culture in captivity of many amphibian species has grown substantially in an effort to repopulate devastated environments, where the specific population has progressively declined close to their extinction

(Gagliardo, Crump, Griffith, Mendelson, Ross & Zippel 2008; Rollins-Smith, Ramsey, Pask, Reinert & Woodhams 2011).

The American bullfrog (*Lithobates catesbeianus*) meat possesses a high proportion of essential amino acids, a broad degree of absorbable proteins and low contents of sodium chloride and lipids (mainly cholesterol) (FAO/WHO 2001) that make this species one of the most often selected for raniculture. In intensive growth systems, bullfrogs are exposed to a wide variety of microorganisms. Thus, the indigenous microbiota of the skin and gastrointestinal tract could be affected by intrinsic (gastric pH, pancreatic enzymes, bile) and extrinsic (chemotherapeutics) factors 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 may cause the outbreak of infectious diseases (Mauel, Miller, Frazier & Hines 2002). The bacterial dermatosepticaemia or Red-Leg Syndrome (RLS) is one of the main infectious diseases that affect bullfrog hatcheries, causing high mortality and significant economic losses (Densmore & Earl Green 2007). The aetiological agents associated with RLS include Enterobacteriaceae, *Aeromonas hydrophila*, *Elizabethkingia meningoseptica*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Glorioso, Amborski, Larkin, Amborski & Culley 1974; Mauel *et al.* 2002; Schadich, Cole, Squire & Mason 2010).

In the hatchery conditions, the treatment or prevention of infectious diseases using chemotherapeutic substances contributes to disturbances in the indigenous microbiota and to the spread of antibiotic resistance to many bacterial genera (Verschuere, Rombaut, Sorgeloos & Verstraete 2000). Therefore, a novel and alternative therapy is being developed, which is associated with the application of probiotic microorganisms to restore the microbial populations, in order to exert a physiological effect on the host and to protect it from potentially pathogenic microorganisms (Reid, Sanders, Rex Gaskins, Gibson, Mercenier, Rastall, Roberfroid, Rowland, Cherbut & Klaenhammer 2003). The use of lactic acid bacteria (LAB) as probiotics has been reported in ectotherm animals, mainly in fish and shrimp aquaculture (Farzanfar 2006; Vine, Leukes & Kaiser 2006; Balcázar, de Blas, Ruiz Zarzuela, Vendrell & Girones 2007; Gatesoupe 2008; Ringø, Løvmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010). With respect to raniculture, some researchers have used commercial probiotics to increase bullfrog weight, but the prevention of infectious diseases has not been evaluated (de Carla Dias, Meneses França, Ferreira, De Stéfani, Martins, Marcantonio, Teixeira & Hipolito 2005; de Carla Dias, De Stéfani, Ferreira & Meneses França 2008). According to host-microbiota specificity (Chabrillón, Rico, Arijo, Díaz-Rosales, Balebona & Moriñigo 2005; Zoetendal, Rajilic-Stojanovic & de Vos 2008; Lee, Tomita, Kleerebezem & Bron 2013), probiotics are preferably formulated with beneficial microorganisms isolated from the same ecological niches or mucosal tract where they will be applied in order to promote adhesion, permanence and colonization, and thus to increase the possibilities of exerting a beneficial effect on the host. On this basis, our research group previously selected *Lactococcus lactis* CRL 1584 and 1827, and *Enterococcus gallinarum* CRL 1826 isolated from *L. catesbeianus* hatcheries in Argentina (Pasteris, Bühler & Nader-Macías 2006; Pasteris, González, Van Schoor, Bühler, Nader-Macías, Vandamme & De Vuyst 2008; Pasteris, Vera Pingitore, Roig Babot, Otero, Bühler & Nader-Macías 2009; Montel Mendoza, Pasteris, Ale, Otero, Bühler & Nader-Macías 2012) as potentially probiotic candidates. The LAB strains were selected according to their beneficial properties. *L. lactis* CRL 1584 and *E. gallinarum* CRL 1826 produce bacteriocins able to inhibit indigenous *C. freundii* and *P. aeruginosa* (RLS-related pathogens) together with organic acids and hydrogen peroxide while *L. lactis* CRL 1827 exerts its inhibitory effect only by organic acids. The selection was also based on surface properties (*L. lactis* strains were hydrophilic while *E. gallinarum* was hydrophobic), resistance to antiseptics ($CuSO_4$, $KMnO_4$, methylene blue, malachite green) and antibiotics (chloramphenicol, gentamicin, penicillin, ceftazidime, amikacin, ciprofloxacin, oxytetracycline, vancomicin) usually used in raniculture and absence of virulence factors (Pasteris, Roig Babot, Otero, Bühler & Nader-Macías 2009; Pasteris, Guidoli, Otero, Bühler & Nader-Macías 2011; Montel Mendoza 2014; Pasteris, Vera Pingitore, Ale & Nader-Macías 2014; Montel Mendoza, Ale, Nader-Macías & Pasteris 2015). Technological characteristics of some of the beneficial LAB, such as compatibility and resistance to lyophilization were evaluated. The compatibility studies indicated that selected LAB strains could be included in a mixed probiotic product (Montel Mendoza 2014), while LAB viability after freeze-

drying was strain specific and depended on the lyoprotectant used. Highest viability was obtained when stored at 4°C, and the beneficial properties remained stable for 18 months independently of storage temperature (Montel Mendoza, Pasteris, Otero & Nader-Macías 2014).

Even though the above LAB strains have shown *in vitro* beneficial properties that supported their selection, probiotic characteristics must be evidenced through *in vivo* assays involving evaluation of their physiological effect on the host and protection against pathogens colonization in animal models. In the case of new beneficial strains, they should be assayed in the specific host to determine their possible adverse or harmful effect. Therefore, the aim of this work was to determine the effect of the administration of potentially probiotic LAB strains during the development of *L. catesbeianus* embryos from the hatching until the tadpole stages. The studies were focused on the evaluation of cultivable microbial populations during administration and rest periods, on the morphological characteristics of the specimens and on the histological structure of organs from the gastrointestinal tract and skin (main RLS-pathogens port of entry) of both control and LAB-treated specimens. There are no reports on the histology of the cited organs in bullfrog tadpoles that also support our studies on control specimens.

Materials and methods

Lactic acid bacteria strains and culture conditions

Autochthonous *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827 and *Enterococcus gallinarum* CRL 1826 from raniculture were used to perform the *in vivo* studies. The strains were selected according to their beneficial properties (Pasteris, Roig Babot *et al.* 2009; Pasteris, Vera Pingitore *et al.* 2009; Montel Mendoza *et al.* 2012; Montel Mendoza 2014).

Lactococcus lactis CRL 1584 was grown in LAPTg (in g L^{-1}: glucose, 10; yeast extract, 10; peptone, 15; tryptone, 10 and Tween 80, 1 mL L^{-1} (Raibaud, Gapin, Ducluzeau, Mocquot & Oliver 1963) broth, pH 6.8 for 6 h at 37°C in a 5% CO_2-enriched chamber (microaerophilia), while *L. lactis* CRL 1827 and *E. gallinarum* CRL 1826 were cultured in MRS (in g L^{-1}: meat peptone, 10; meat extract, 10; yeast extract, 5; glucose, 20; K2HPO4, 2; MgSO4, 2; MnSO4.4H2O, 0,05; sodium acetate, 5; triammonium citrate , 2; tween 80, 1 mL) (de Man, Rogosa & Sharpe 1969) broth for 10 h in the conditions cited above.

Bacterial strains were stored at −20°C in their specific growth media supplemented with 20% (w/v) glycerol.

Survival of lactic acid bacteria in the experimental growth conditions of bullfrog specimens

To determine if the LAB were able to survive in the laboratory conditions in which bullfrog specimens carry out their biological cycle, cells from LAB strain cultures on LAPTg or MRS broth were harvested by centrifugation (8000 *g* at 4°C for 10 min), washed twice with 10% Ringer solution (R10) and suspended to get 10^3, 10^6 and 10^9 CFU mL^{-1}. These suspensions were incubated at 26°C for 48 h and samples were taken at 12-h intervals to determine the number of viable cells by plating on LAPTg agar (1.5% w/v).

Ringer solution composition (in g L^{-1}): NaCl, 6.6; $CaCl_2$, 0.15; KCl, 0.15; Tris-HCl, 10 mM (pH 7.4).

Lactic acid bacteria administration to *Lithobates catesbeianus* embryos

Bullfrog specimens obtained from a local hatchery (Tucumán, Argentina) were fed *ad libitum* with boiled lettuce from 79 h post fertilization (gill circulation-open mouth stage) up to the end of the assay (31 days).

For the experiments of LAB administration, 10 plastic bowls containing 300 mL R10 and 100 randomly selected embryos at the hatching stage were prepared. The LAB-treated groups included: (1) *L. lactis* CRL 1827 (10^9 CFU mL^{-1}); (2) *L. lactis* CRL 1584 (10^9 CFU mL^{-1}); (3) *E. gallinarum* CRL 1826 (10^9 CFU mL^{-1}); (4) mix [*L. lactis* CRL 1584 (10^9 CFU mL^{-1}) + *E. gallinarum* CRL 1826 (10^9 CFU mL^{-1})]. Control groups included specimens without LAB administration. Duplicate assays were performed each year (2013 and 2014).

All LAB were administered in three 7-day cycles (1, 2 and 3) separated by two rest periods (5 days each) (Fig. 1). During the first cycle, embryos were incubated with the LAB suspensions during 24 h. At this time, complete R10 + LAB renovations were carried out daily. For the second and third cycle of administration, specimens were placed in larger plastic bowls (30 mL water/animal) with aeration systems. In these cycles, specimens were incubated with the LAB suspensions only during

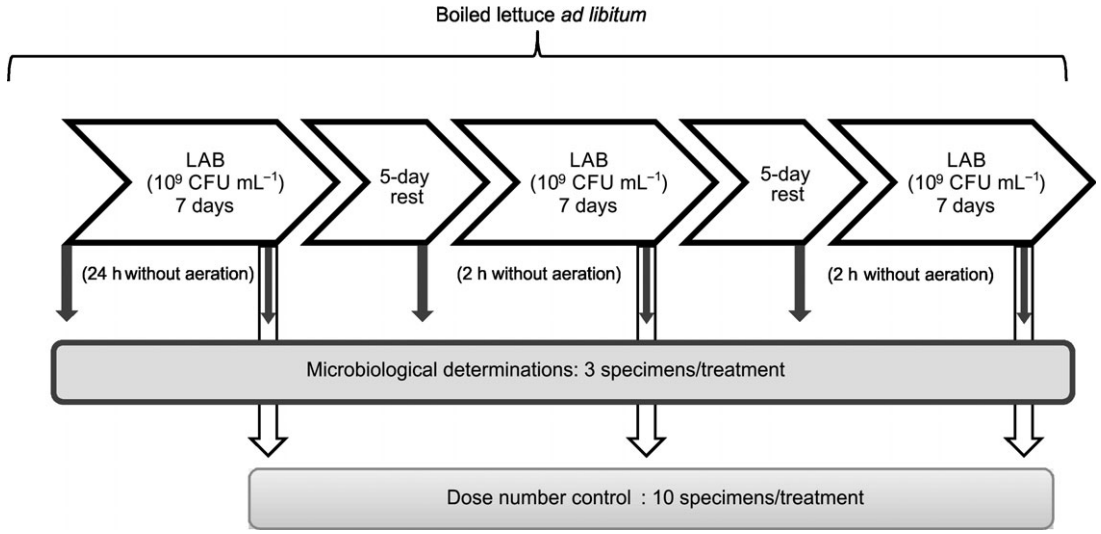

Figure 1 Protocol of the lactic acid bacteria administration to *Lithobates catesbeianus* embryos.

2 h day^{-1} without aeration. Immediately after these 2 h of incubation, a complete water replacement was performed. Twenty-four hours later, the LAB strains were again administered for 2 h without aeration and with previous water replacement.

After the second cycle the water volume was increased to 80 mL/animal to favour tadpole growth. The bowls were maintained at 26°C.

At the end of cycles 1, 2 and 3, 10 animals/bowl were taken out, humanely euthanized by transdermal exposure to buffered benzocaine hydrochloride (Miller, Gray, Rajeev, Chandler Schmutzer, Burton, Merrill & Baldwin 2009) and immersed in 10% buffered formaldehyde and Bouin's fluid for 24 h at room temperature.

The Institutional Laboratory Animal Care and Use Committee of CERELA approved the experimental protocol (CRL-BIOT-LMP-2012/1A) used in this work.

Quantification of cultivable microbial populations during lactic acid bacteria administration to *Lithobates catesbeianus* embryos

At the end of each administration cycle (7, 19 and 31 days) and rest period (12 and 24 days), three *L. catesbeianus* specimens/bowl were randomly selected for microbiological quantifications. Animals were previously euthanized, washed twice and homogenized in 3 mL sterilized distilled water. The microbial quantification was carried out by the serial dilution method using 0.1% (v/v) peptone water as a dilution medium. One hundred microlitre samples were plated on selective or differential culture media. Lactic acid bacteria quantification was performed using M17 agar for *Lactococcus* species; MRS agar pH 5.5 for total LAB counts and Bilis Esculine Agar (BEA) for group D *Streptococcus* and *Enterococcus* counts. MacConkey (MC) and Cetrimide (CET) agar were utilized for coliform bacteria and *Pseudomonas* counts respectively. Plate Count Agar (PCA) was employed to determine the total number of mesophilic microorganisms. All plates were incubated in microaerophilia at 37°C for 48–72 h and the number of viable cells (CFU mL^{-1}) was determined (Montel Mendoza *et al.* 2012). The same protocol was carried out in 2013 and 2014.

The components for culture media preparation were supplied by Britania laboratories (Argentina) while MRS was purchased from Merck (Germany).

Survival and morphological evaluations of *Lithobates catesbeianus* after lactic acid bacteria administration

Bullfrog survival after the LAB administration was determined by quantifying the number of live specimens at the end of each LAB administration cycle and rest period.

For morphological evaluations, six randomly selected specimens were euthanized and structural modifications were evaluated in both controls and LAB-treated tadpoles by using stereoscopic microscopy. Later, specimens were fixed, embedded in Histowax and serial longitudinal sections (7-μm thickness) from the dorsal to the ventral area were

obtained with a Microm HM 325 (Zeizz, Walldorf, Germany) microtome. Sections were then stained with Haematoxylin–Eosin, Mallory trichrome and Brown and Brenn and viewed under a light microscope (Leicca DM1000 with a Leica ICC5HD photograph camera; Leica Microsystems CMS GmbH, Wetzlar, Germany). Haematoxylin–Eosin and Mallory trichrome stains were applied to evaluate the general organization of the specimens. The studies were focused on organs of the gastrointestinal tract (stomach and intestine), spleen and skin. Brown and Brenn stain (Leaver, Evans & Corrin 1977) was used to differentiate Gram-positive and Gram-negative bacteria in the intestinal and stomach lumen, skin and lamina propria (translocation).

Chemicals for staining were purchased from Sigma-Aldrich: Ehrlich's haematoxylin, crystal violet and malachite green; Merck (Germany): Eosin Y, azocarmine G, aniline and pyronin Y; Anedra (Argentina): Aniline blue and Cicarelli (Argentina): Orange G.

Statistical analysis

Microbiological studies were performed in duplicate each year (2013 and 2014). The mean values were calculated considering two replicates/assay (each replicate was obtained from three repeated measures from each bowl). The number of microorganisms of each population under different LAB-treatments was subjected to a nonparametric ANOVA (Kruskal–Wallis test); multiple comparisons were performed to establish significant differences ($P < 0.05$) between the results from control and each LAB-treatment at the same time (day). The INFOSTAT (2015 version, Agricultural College of the National University of Córdoba, Argentina) software was used.

The LSD Fisher test was used to compare the evolution of potentially beneficial and pathogenic populations and their differences between the LAB-treated groups; a probability of $P < 0.05$ was considered statistically significant. A MINITAB (version 15; Minitab Inc., State College, PA, USA) software was used.

Results

Survival of lactic acid bacteria in the experimental growth conditions of bullfrog specimens

Since LAB strains will be administered to the water in the same conditions in which embryos perform their biological cycle and as most reproduction studies are carried out in R10, the viability of the LAB strains was evaluated when 10^3, 10^6 and 10^9 CFU mL^{-1} were maintained in R10 for 48 h (Fig. 2). The results obtained indicated that *L. lactis* CRL 1584 kept its viability independently of the initial cell concentration (Fig. 2A). A similar behaviour was observed with 10^9 CFU mL^{-1} *L. lactis* CRL 1827 (Fig. 2B); however, a decrease in cell viability was detected between 12–36 h, especially with 10^3 and 10^6 CFU mL^{-1}. A complete loss of LAB viability was determined with low cell concentrations (10^3 CFU mL^{-1}) at 48 h.

The viability of *E. gallinarum* CRL 1826 decreased since 24 h by three log units at the end

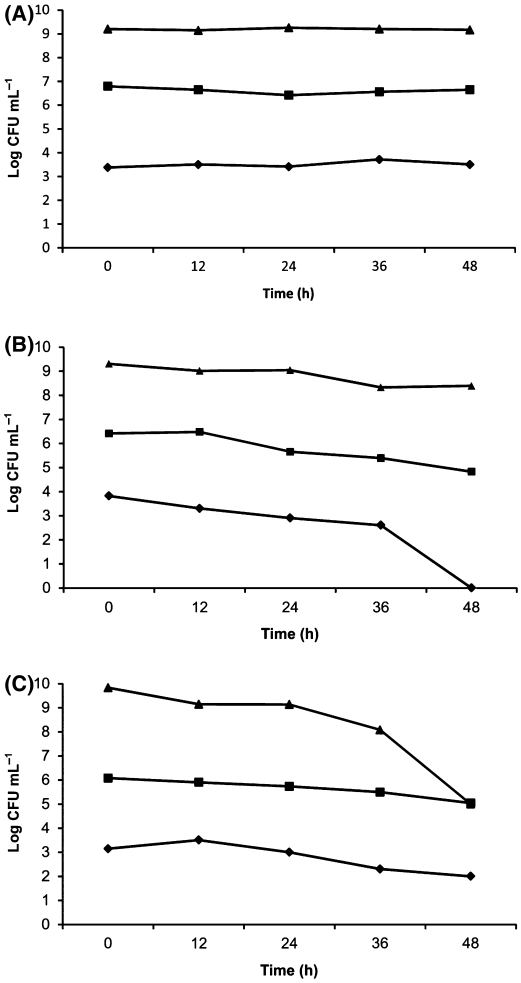

Figure 2 Viability of lactic acid bacteria strains in Ringer 10%. (A) *Lactococcus lactis* CRL 1584, (B) *Lactococcus lactis* CRL 1827, (C) *Enterococcus gallinarum* CRL 1826. Dose 1: 10^3 CFU mL^{-1}; Dose 2: 10^6 CFU mL^{-1}; Dose 3: 10^9 CFU mL^{-1}.

of the assay (48 h) when the initial population was 10^9 CFU mL^{-1} (Fig. 2C).

On the basis of these results, the effect of LAB administration on the development of *L. catesbeianus* embryos was evaluated with 10^9 CFU mL^{-1} added every 24/2 h in three 7-day cycles with 5-day rest periods in between (Fig. 1).

Quantification of cultivable microbial populations during lactic acid bacteria administration to *Lithobates catesbeianus* embryos

A nonparametric ANOVA (Kruskal–Wallis test) was applied to determine the evolution of potentially beneficial and pathogenic microorganisms when bullfrog embryos from the hatching stage (50–60 h) up to 31 days (tadpole stage) were administered with LAB (individually and CRL 1584+ CRL 1826). The results showed significant differences ($P < 0.005$) in all microbial populations evaluated with the exception of Enterobacteriaceae.

Therefore, multiple comparisons were carried out to determine the significant differences ($P < 0.05$) between the LAB-treated groups and its control at each time (Fig. 3).

The viability (log CFU mL^{-1}) of potentially beneficial microorganisms (LAB) was evaluated in M17, MRS and BEA media. Thus, in M17 at day 7 significant differences were determined for CRL 1584+ CRL 1826-treated group and its control (mean ± SD = 6.93 ± 0.57 and 4.60 ± 0.71 respectively). At day 12 significant differences were observed in *L. lactis* CRL 1584-treated group with respect to the control (6.77 ± 1.16 and 5.34 ± 0.35 respectively) (Fig. 3A); however, at days 12 and 24, the significant difference was established by the *L. lactis* CRL 1827-treated group (6.70 ± 0.96 and 5.85 ± 0.10-control; 7.24 ± 0.30 and 4.59 ± 1.23-control respectively) (Fig. 3A). With respect to MRS medium, the significant differences in potentially beneficial populations were detected at day 12 for *E. gallinarum* CRL 1826, *L. lactis* CRL 1584 and CRL 1584 + CRL 1826-treated groups (5.94 ± 1.88, 6.37 ± 1.38 and 6.66 ± 0.59 respectively. Control = 1.65 ± 0.49) (Fig. 3B). However, the significant differences were shown by *L. lactis* CRL 1827-treated group at both days 24 (6.86 ± 1.00. Control = 3.77 ± 0.33) and 31 (6.82 ± 0.66. Control = 3.89 ± 0.13) (Fig. 3B). The microbial populations grown in BEA medium at day 12 were significant different in the CRL 1584 + CRL 1826-treated group than its control (6.19 ± 0.43 and 1.73 ± 0.38) while at day 19, *L. lactis* CRL 1584, *E. gallinarum* CRL 1826 and CRL 1584+ CRL 1826-treated groups showed significant differences with respect to its control (5.89 ± 1.13, 6.46 ± 0.61 and 6.38 ± 0.25 respectively. Control = 2.44 ± 0.40) (Fig. 3C).

In MC medium, the mean values of viable bacteria were similar ($P > 0.05$) in all treatments and times (Fig. 3D) while in CET agar significant differences were only observed day 19 for CRL 1584+ CRL 1826-treated groups (1.34 ± 1.30) with respect to the control (3.56 ± 0.80) (Fig. 3E).

The total mesophilic populations showed significant differences only at day 12 when specimens were treated with *E. gallinarum* CRL 1826 and CRL 1584+ CRL 1826 (7.12 ± 0.45 and 7.11 ± 0.33 respectively, control = 5.63 ± 0.65) (Fig. 3F).

In all cases, in the control groups no significant differences were observed between the evaluated times ($P < 0.05$).

Dynamics of beneficial and potentially pathogenic populations during the LAB-treatments of *Lithobates catesbeianus* specimens

The mean values (log CFU mL^{-1}) of potentially beneficial (obtained from M17, MRS and BEA media) and pathogenic (obtained from MC and Cetrimide media) populations were calculated at each time and treatment and multiple comparisons were performed. The general mean for potentially beneficial population (LAB) was 5.6 log CFU mL^{-1} (Fig. 4A). This population increased up to day 19 and remained between 6.03 and 6.21 ± 0.14 up to 31 days. All the LAB-treatments induced an increase in this population above the general mean and was significantly ($P < 0.05$) higher than the controls (Fig. 4A).

The potentially pathogenic population (RLS-related pathogens) showed a general mean of 1.9 log CFU mL^{-1} (Fig. 4B). This population achieved 2.31 log CFU mL^{-1} at day 19. Although the highest potentially pathogenic population numbers were detected in the control and the lowest one in the CRL 1584 + CRL 1826-treated group, no significant differences were observed (Fig. 4B).

Survival and morphological evaluations of *Lithobates catesbeianus* after lactic acid bacteria administration

The survival of the bullfrog embryos was determined during the assay (31 days). The highest

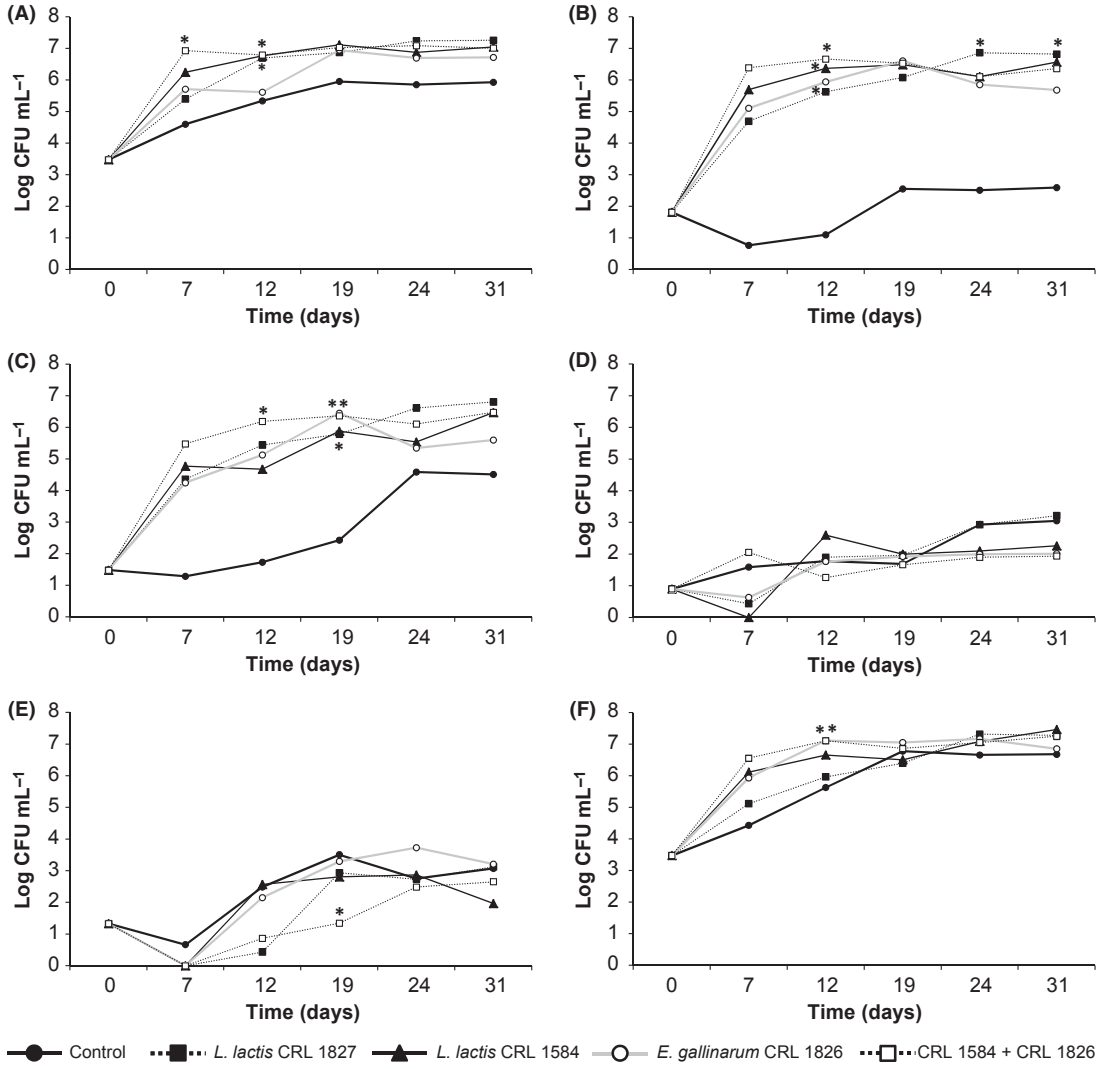

Figure 3 Cultivable bacterial populations during lactic acid bacteria administration to *Lithobates catesbeianus* embryos. (A) M17 agar (*Lactococcus* species), (B) MRS agar (LAB), (C) Bilis Esculine Agar (group D *Streptococcus* and *Enterococcus*), (D) MacConkey agar (coliform bacteria), (E) Cetrimide agar (*Pseudomonas* species), (F) Plate Count Agar (total number of mesophilic microorganisms). *Indicates significant differences between the treatments and its control for each time (Kruskal–Wallis test, $P < 0.05$).

values (98 ± 1%) were observed when specimens were administered with the mixed cultures (*E. gallinarum* CRL 1826+ *L. lactis* CRL 1584) and the lowest when administered with *L. lactis* CRL 1827 (94 ± 3%) (Table 1).

Macroscopic anatomy

Lithobates catesbeianus tadpoles at the end of the treatment (31 days) representing the 20–60 of Gosner's stage (Gosner 1960) were selected to evaluate the effect of LAB administration. No malformations were observed and all the specimens were at the same stage of their biological cycle, even though some variations in body size were observed. On the basis of these results, specimens with mean size (2 cm) were selected for histological evaluations.

Histological studies

Histological evaluations were performed in both control and LAB-treated specimens to evaluate the effect of the administration of different LAB strains on specific organs. As there is no description of bullfrog tadpole histology, all studies were per-

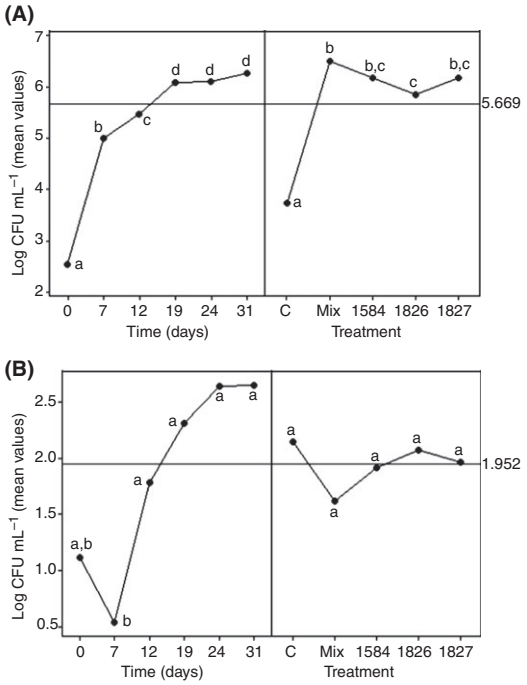

Figure 4 Effect of LAB-treatment on the dynamic of potentially beneficial (A) and pathogenic (B) populations during 31 days. Different letters indicate significant differences (LSD Fisher test, $P < 0.05$).

Table 1 Survival of *Lithobates catesbeianus* tadpoles after lactic acid bacteria administration

Treatment	Survival (%) ± SD
Control	97 ± 3
L. lactis CRL1584	96 ± 2
L. lactis CRL1827	94 ± 3
E. gallinarum CRL1826	98 ± 2
L. lactis CRL1584 + *E. gallinarum* CRL1826	98 ± 1

formed compared with controls at each stage. Figure 5 shows a longitudinal histological slice of a tadpole control. In the cephalic area, ocular cavities, skeletal muscle fibres and cartilaginous tissue represented the main structures. In the mean portion, internal gills could be seen while in the abdominal area the liver, a large and looped intestine and spleen were observed. In the caudal region, the kidney and skeletal muscle fibres inserted in the tadpole tail were observed. The embryo was coated by a cubic nonkeratinized stratified epithelium, with some keratinization areas (mouth region).

Histological sections of the intestine, stomach and skin of untreated (control) tadpoles are shown in Figs 6A, B, 7A1, B1 and 8A1, B1). The intestinal epithelium varied between low and high cubic and was coated with mucus layer. Abundant dietary fibres were observed in the intestinal lumen (Figs 6B and 7A1). The lamina propria was small, with low proportion of cells (fibroblasts) and collagen fibres and no inflammatory reaction was detected (Figs 6A and 7B1). The spleen was well developed, but no red and white pulps were distinguished. The organ showed high amount of haematopoietic cells and a rich vascularity (Figs 6A and 7B1). The Mallory trichrome method highlighted the mucus layer and the basal lamina of the intestinal epithelium (Fig. 7A1). The Brown and Brenn stain showed scarce bacteria associated with both, intestinal mucus and lumen (Fig. 7B1). The stomach presented a single cubic epithelium coated with a mucus layer; no submucosa was evidenced, and a serous layer was observed. The lumen showed abundant dietary fibres (Fig. 8A1). The skin presented a cubic epithelium with a varying degree of stratification coated with a profuse mucus layer (Fig. 8B1). In the lamina propria low numbers of fibroblasts and collagen fibres were observed (Fig. 7B1).

The effect of *L. lactis* CRL 1584 administration on the histology of stomach, intestine, spleen and skin of tadpoles was evaluated to determine if the LAB strain caused structural modifications or had adverse effects on the organs (Figs 6–8). Overall, the organs maintained their histological characteristics. High microorganism amounts were associated with the intestinal mucus layer; the subjacent connective tissue being similar to the control (Figs 6C, D; 7A2 and 8A2). Brown and Brenn stain (Fig. 7B2) evidenced a high proportion of Gram-positive cocci. The stomach mucosa (Fig. 8A2) and skin (Fig. 8B2) did not show modifications compared to the control. Also, the stomach lumen showed a high proportion of dietary fibres and a low amount of microorganisms (Fig. 8A2).

The histological sections of tadpoles treated with *L. lactis* CRL 1827 and with *E. gallinarum* CRL 1826 are shown in Figs 6–8. In both LAB-treatments, the intestine showed similar characteristics to the control (Fig. 6E, F, G, H; 7A3, B3, A4 and B4). The mucus layer evidenced associated bacteria and dietary fibres (Fig. 6E, F, G, H; 7A3, B3, A4 and B4). The connective tissue showed fibrob-

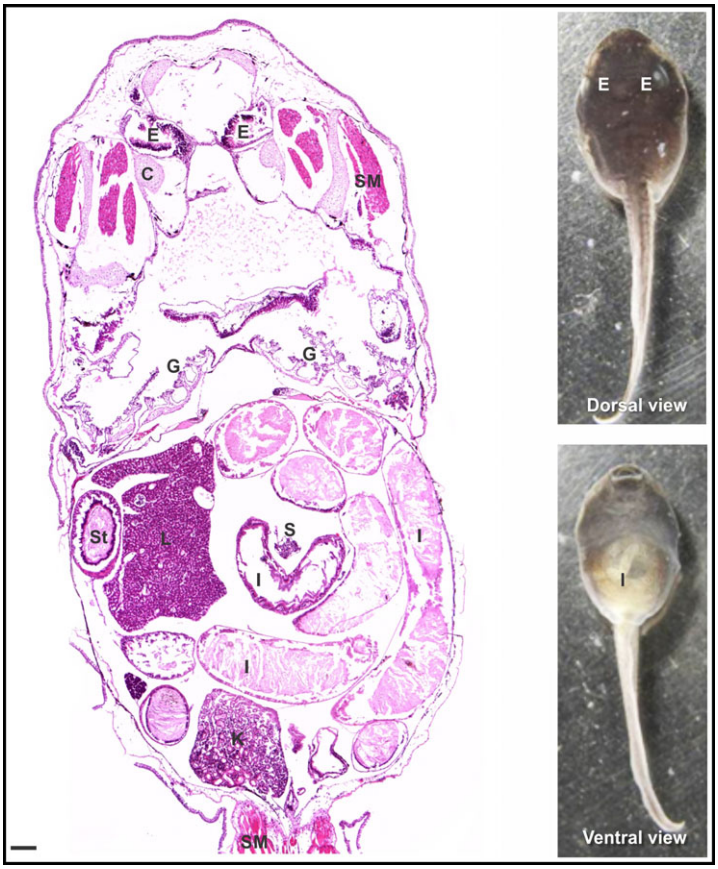

Figure 5 Histological section of *Lithobates catesbeianus* tadpole stained with Haematoxylin–Eosin (E), eyes; (C), cartilages; (SM), skeletal muscle fibres; (G), gills; (St), stomach; (L), liver; (S), spleen; (I), intestine; (K), kidney. Bar = 199 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

lasts, lymphocytes and low amount of collagen fibres (Fig. 6E, H). The Mallory tricrome stain indicated no modifications in the mucus layer, basal lamina or spleen (Fig. 7A3, A4). High amounts of microorganisms were observed in the intestinal mucus layer (Fig. 6F, H, and 7B3, B4). No changes in the histological characteristics of the stomach or skin were detected (Fig. 8A3, A4, B3, B4).

When tadpoles were treated with *L. lactis* CRL 1827 and *L. lactis* CRL 1584 plus *E. gallinarum* CRL 1827 (Figs 6–8), no histological differences were observed compared to the control.

No inflammatory reaction was observed in the underlying connective tissue of stomach, intestine or skin (Figs 6–8) in any of the LAB-treated specimens.

Discussion

The cultivable microbiota of two *L. catesbeianus* hatcheries of Argentina comprises LAB, and RLS-associated pathogens, *C. freundii* being isolated from nonhealthy bullfrog while *P. aeruginosa* and *S. epidermidis* were identified from healthy specimens (Pasteris *et al.* 2006; Pasteris, Roig Babot *et al.* 2009; Montel Mendoza *et al.* 2012). *In vitro* studies such as the evaluation of antagonistic activity of LAB metabolites on indigenous RLS-related pathogens and surface properties previously performed allowed us to select LAB strains with beneficial characteristics for raniculture (Pasteris, Roig Babot *et al.* 2009; Pasteris, Vera Pingitore *et al.* 2009; Montel Mendoza *et al.* 2012).

However, some international organizations and government rules have recently incorporated guidelines to support the probiotic denomination of different formulas or products. In addition, there are some recent publications indicating that *in vitro* results are not always similar to those obtained through *in vivo* studies (Muench, Kuch, Wu, Begum, Veit, Pelletier, Soler-García & Jerse 2009; De Gregorio, Juárez Tomás, Leccese Terraf & Nader-Macías 2014; Arora & Baldi 2015; Bratz, Gölz, Janczyk, Nöckler & Alter 2015). Therefore, the probiotic effect must be demonstrated on a

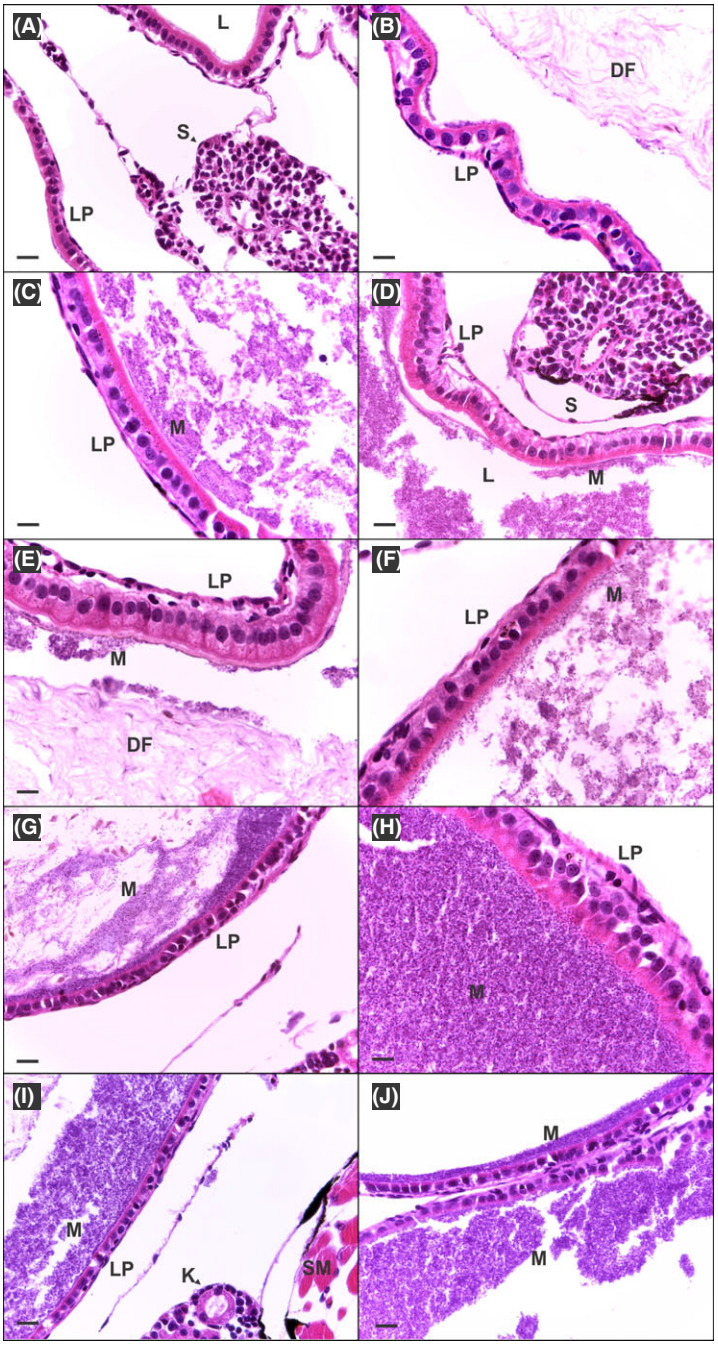

Figure 6 Light microscopy photograph of histological section of *Lithobates catesbeianus* intestine stained with Haematoxylin–Eosin. A and B: control; C and D: specimens treated with *L. lactis* CRL 1584; E and F: specimens treated with *L. lactis* CRL 1827; G and H: specimens treated with *E. gallinarum* CRL 1826; I and J: specimens treated with *L. lactis* CRL 1584 + *E. gallinarum* CRL 1826. (L), lumen, (LP), lamina propria; (S), spleen; (K), kidney; (SM) skeletal muscle; (DF), dietary fibres and (M), microorganisms. Bars = 20 and 12 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

specific host to determine if there are no adverse or harmful effects, and to study the physiological effect of the strains on the host. Other aspects to take into consideration are host development and environmental factors. In this work, the effect of potentially probiotic LAB administration (single

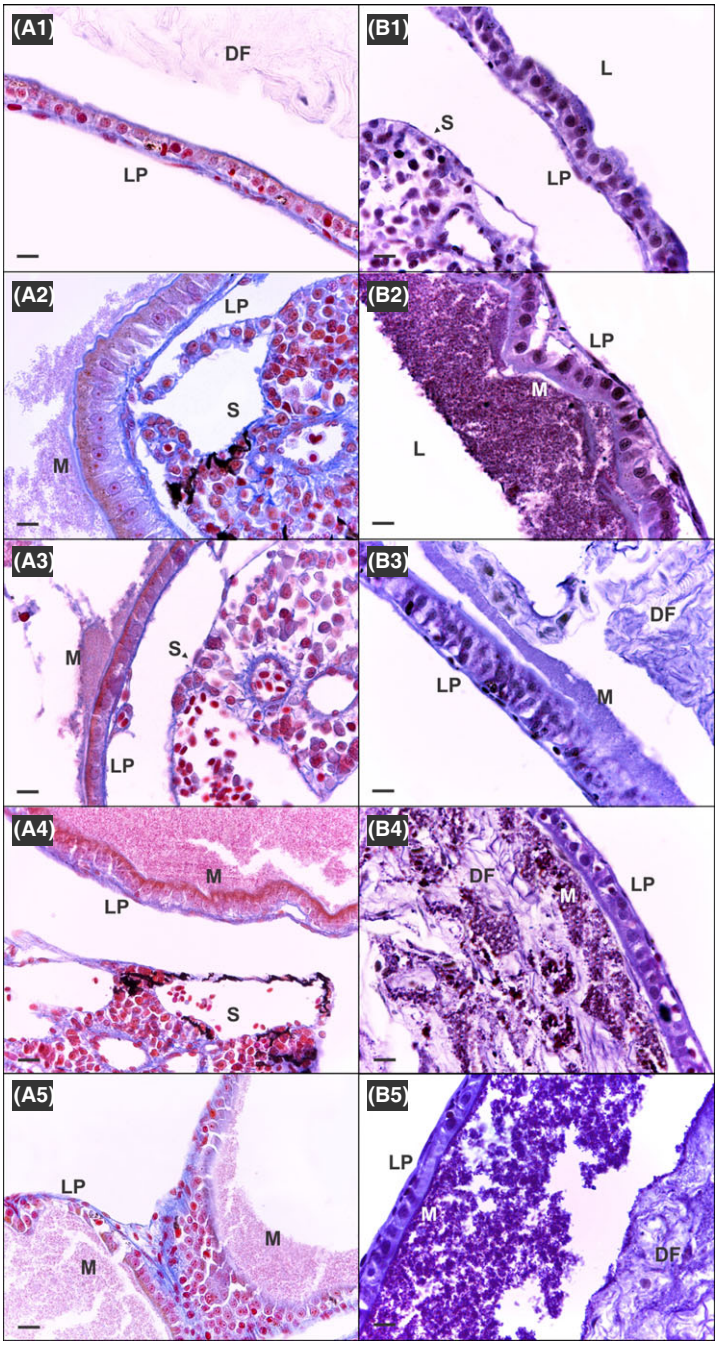

Figure 7 Light microscopy photograph of histological section of organs from *Lithobates catesbeianus* intestine stained with Mallory trichrome (A) and Brown and Brenn (B). A1 and B1: control; A2 and B2: specimens treated with *L. lactis* CRL 1584; A3 and B3: specimens treated with *L. lactis* CRL 1827; A4 and B4: specimens treated with *E. gallinarum* CRL 1826; A5 and B5: specimens treated with *L. lactis* CRL 1584 + *E. gallinarum* CRL 1826. (L), lumen, (LP), lamina propria; (S), spleen; (DF), dietary fibres and (M), microorganisms. Bars = 20 and 12 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

and in one combination-CRL 1584 + CRL 1826) during the development of *L. catesbeianus* embryos from the hatching stage (50–60 h) was evaluated. These studies were performed in 2013 and 2014 since the reproduction period of *L. catesbeianus* in Tucumán (Argentina) comprises the rainy season (December-January). The *in vivo* assays were carried out by using embryos obtained from a local bullfrog hatchery.

Many LAB species are recognized as safe and therefore they are considered for the design or formulation of probiotic products (Reid *et al.* 2003). Although probiotics containing LAB are being applied in aquaculture activities (Farzanfar 2006; Vine *et al.* 2006; Balcázar *et al.* 2007; Gatesoupe 2008; Ringø *et al.* 2010), our research group advanced in the formulation of probiotics for raniculture by using indigenous

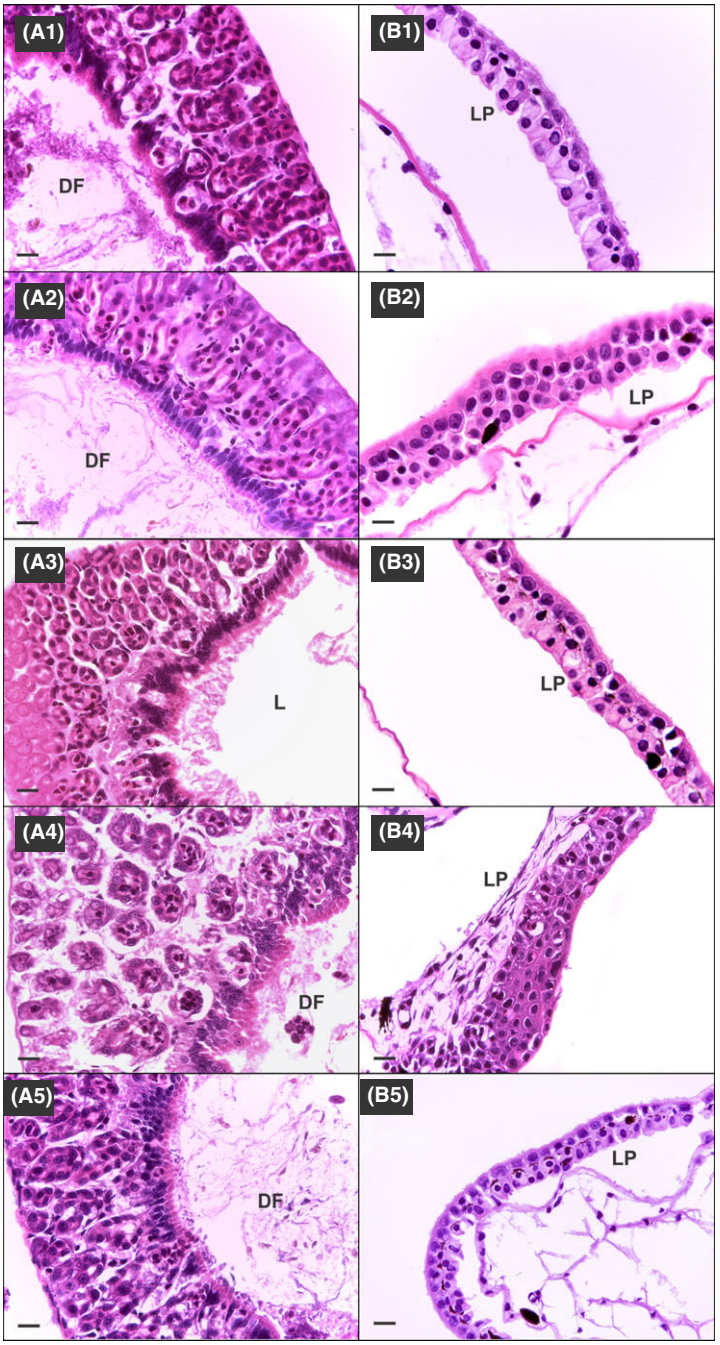

Figure 8 Light microscopy photograph of histological section of organs from *Lithobates catesbeianus* stomach (A) and skin (B) stained with Haematoxylin–Eosin. A1 and B1: control; A2 and B2: specimens treated with *L. lactis* CRL 1584; A3 and B3: specimens treated with *L. lactis* CRL 1827; A4 and B4: specimens treated with *E. gallinarum* CRL 1826; A5 and B5: specimens treated with *L. lactis* CRL 1584 + *E. gallinarum* CRL 1826. (L), lumen, (LP), lamina propria and (DF), dietary fibres. Bars = 20 and 12 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

LAB according to host-species specificity (Zoetendal *et al.* 2008). In raniculture, probiotics could be added to water ponds for tadpoles or to balanced feed for juvenile and adults specimens. Although RLS affects all *L. catesbeianus* stages, this species is particularly sensitive during metamorphosis stage (Mauel *et al.* 2002). In this work, the effect of indigenous *L. lactis* CRL 1827, *L. lactis* CRL 1584, *E. gallinarum* CRL 1826 and *L. lactis* CRL 1584 plus *E. gallinarum* CRL 1826 administration was evaluated only up to 31 days (tadpole stage). Considering that most reproduction and embryo development studies are performed in R10, the maintenance of LAB viability in this solution was first determined. Single LAB strain suspensions maintained their viability until

24 h incubation in R10. Therefore, during the first cycle of administration (7 days), 10^9 CFU mL^{-1} of each of the LAB strains and one combination (CRL 1584 + CRL 1826) were administered for 24 h. This LAB dose was selected to evaluate its effect on the host, considering that some probiotics are formulated with this bacterial concentration since it will help to reach the target organs in an appropriate viable cell amount. To simulate the bullfrog hatchery conditions, tadpoles were then transferred to larger bowls with chlorine-free water and aeration; 10^9 CFU mL^{-1} LAB strains were administered for 2 h day^{-1} without aeration. Lactic acid bacteria strains maintained their viability during these time periods (data not shown).

The results indicate that the administration of indigenous LAB to bullfrog embryos did not affect the survival, although no other growth parameters were included in this study. The effect of the commercial probiotics, *Lactobacillus acidophillus*, *E. faecium* and *Bifidobacterim bifidum*-Probiotic 1 and *Bacillus subtilis*-Probiotic 2, on bullfrog survival and growth (weight gain and feed conversion) was evaluated earlier (França 2007; França, Dias, Teixeira, Marcantônio, De Stéfani, Antonucci, Rocha, Ranzani-Paiva & Ferreira 2008). The authors indicated that the administration of these probiotics did not affect survival either the growth parameters.

In this work, we determined the effect of *L. lactis* CRL 1827, *L. lactis* CRL 1584, *E. gallinarum* CRL 1826 and *L. lactis* CRL 1584 plus *E. gallinarum* CRL 1826 administration on the establishment of the autochthonous microbiota that included potentially beneficial and pathogenic microbial cultivable populations. The highest number of potentially beneficial populations was detected in the LAB-treated groups. All the LAB treatments increased the populations and were significantly higher than the control. The potentially pathogenic populations were low and did not vary between the treated groups, the highest values being detected in the control. Only a diminution in the number of viable bacteria in cetrimide medium was observed for the CRL 1584 + CRL 1826-treated group. In this last case, further studies are needed to demonstrate the presence of RLS-related pathogens such as *C. freundii* and *P. aeruginosa* respectively.

With respect to the effect produced by the LAB administration on bullfrog specimens, no structural changes were observed by stereoscopic microscopy.

Histological studies were carried out to evaluate the structural morphology of different organs. The administration of single and combined (CRL 1584 + CRL 1826) LAB strains did not modify the histological structures of stomach, intestine or skin. It is interesting to point out that the LAB-treated groups showed high levels of microorganisms associated with the intestinal mucus layer, which could be related to the surface properties of the strains administered (Pasteris, Vera Pingitore *et al.* 2009; Montel Mendoza *et al.* 2012). However, no microorganisms associated with the mucus skin were observed, possibly because this mucus layer have a different composition to the intestinal mucus, or because of the fast or easy wash-out of microorganisms during the R10 or water changes.

Since there are no available publications on the ontogeny of *L. catesbeianus* immune system, *Xenopus leavis* was used as a model. In this species, immune system formation starts at the end of the neurula stage. The spleen is formed between days 12 and 14 post fertilization and in adults specimens is constituted by a red pulp (haemopoietic) and a white pulp (lymphopoietic) (Costa, Soto, Chen, Zorn & Amaya 2008; Chen, Costa, Love, Soto, Roth, Paredes & Amaya 2009; Robert & Cohen 2011). These last characteristics were previously described in *L. catesbeianus*, as well as the histological changes in adult specimens displaying RLS (Pasteris *et al.* 2006). In this work, the spleen was well developed in tadpoles (31 days) but without red and white pulp; however, cells from erythroid, lymphoid and myeloid lineages were observed.

On the other hand, it has been demonstrated that peripheral blood of bullfrog tadpoles in hatchery conditions have high levels of lymphocytes (91–93%), followed by neutrophils and/or basophils, eosinophils and monocytes (Ferreira, Bueno-Gumarães, Soares, Ranzani-Paiva, Rivero & Saldiva 2003; França 2007; Rocha, Ferreira, Teixeira, Dias, França, Antonucci, Marcantonio & Lauretto 2010). In our study, mature leucocytes were observed at spleen level in both control and LAB-treated tadpoles; thus specimens would be immunologically competent. This aspect and the absence of inflammatory reaction in the connective tissue of skin and intestine, the main port of entry of RLS-related pathogens (Mauel *et al.* 2002), indicate that the administration of LAB (individually and in one combination) did not exert adverse effects on the development of *L. catesbeianus* embryos until 31 days (tadpole stage).

Further studies are required to determine if LAB administration protects bullfrogs against challenge with RLS-related pathogens which will allow us to advance in the design of probiotic formulations for raniculture.

Conclusion

This work represents the first report on the determination of the absence of adverse effect when potentially probiotic indigenous LAB strains from *L. catesbeianus* were administered from the hatching up to the tadpole stages of bullfrog embryos.

Acknowledgments

This research was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 632; 744), Agencia Nacional de Promoción Científica y Tecnológica (PICT 543; 1187) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (26/D 414 and 528).

The authors thank the Arroyo Mixta hatchery that supplied the bullfrog embryos.

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