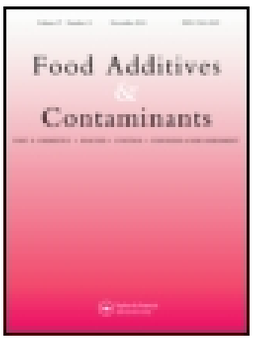

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ARTICLE

Pediococcus acidolactici and *Pediococcus pentosaceus* isolated from a rainbow trout ecosystem have probiotic and ABF1 adsorbing/degrading abilities *in vitro*

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ABSTRACT

Probiotics are being used in biological control of bacterial pathogens, as an alternative to antibiotics, to improve health and production parameters in fish farming. Fish farming production is severely affected by aflatoxins (AFs), which are a significant problem in aquaculture systems. Aflatoxins exert substantial impact on production, causing disease with high mortality and a gradual decline of reared fish stock quality. Some aspects of aflatoxicosis in fish, particularly its effects on the gastrointestinal tract, have not been well documented. The aim of the present study was to evaluate probiotic properties of lactic acid bacterial (LAB) strains isolated from rainbow trout intestine and feed. Moreover, AFB_1-binding and/or degrading abilities were also evaluated to assess their use in the formulation of feed additives. Growth at pH 2, the ability to co-aggregate with bacterial pathogens, inhibition of bacterial pathogens, and determination of the inhibitory mechanism were tested. Aflatoxin B_1 (AFB_1) adsorption and degradation ability were also tested. All strains were able to maintain viable (10^7 cells ml^{-1}) at pH 2. *Pediococcus acidilactici* RC001 and RC008 showed the strongest antimicrobial activity, inhibiting all the pathogens tested. The strains produced antimicrobial compounds of different nature, being affected by different treatments (catalase, NaOH and heating), which indicated that they could be H_2O_2, organic acids or proteins. All LAB strains tested showed the ability to coaggregate pathogenic bacteria, showing inhibition percentages above 40%. *Pediococcus acidilactici* RC003 was the one with the highest adsorption capacity and all LAB strains were able to degrade AFB_1 with percentages higher than 15%, showing significant differences with respect to the control. The ability of some of the LAB strains isolated in the present work to compete with pathogens, together with stability against bile and gastric pH, reduction of bioavailability and degradation of AFB_1, may indicate the potential of LAB for use in rainbow trout culture.

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KEYWORDS
Rainbow trout; acid lactic bacteria; probiotic; aflatoxin B1; adsorption; degradation

Introduction

Aflatoxins are secondary toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Fish farming production is severely affected by aflatoxins (AFs), representing a significant problem in aquaculture systems. Fish are highly sensitive to the effects of AFs, especially aflatoxin B_1 (AFB_1), which may affect health, making animals more susceptible to infectious diseases and immune system depression (Almeida et al. 2011). These aflatoxins exert a substantial impact on production, causing disease and gradual decline of reared fish stock quality (Santacroce et al. 2008). Aflatoxin B_1 is the most commonly found toxin in food and feed (Rustom 1997; CAST, 2003). It is also considered to be the most toxic, having demonstrated carcinogenic, teratogenic and mutagenic effects (IARC, 2002).

Aflatoxicosis occurs as a result of the ingestion of food or feed contaminated with AFs. Aflatoxins are considered unavoidable contaminants in feed, but exposure of animals to a certain level of AFs can be tolerated. The maximum permitted levels in feed are regulated in most countries, and action levels depend on the type of feed and on the animals (CAST Report 2003). Once AFs are ingested, they are absorbed through the gastrointestinal tract (GIT) and are metabolically activated or detoxified in the cells of the intestinal mucosa and in the liver, where they undergo biotransformation by epoxidation, hydroxylation,

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demethylation, conjugation or other spontaneous processes (Urrego Novoa and Díaz, 2006).

Cold freshwater fish such as rainbow trout (*Oncorhynchus mykiss*) are more susceptible to low concentrations of AFB_1 than tropical fish (Deng et al. 2010). The toxic effects of AFs in rainbow trout have been investigated since the discovery of these toxins (Halver 1969; Gallagher and Eaton 1995). The carcinogenic effect of AFB_1 has been studied in fish such as salmon, rainbow trout, channel catfish, tilapia, guppy and Indian major carps (Murjani 2003). However, some aspects of aflatoxicosis in fish, particularly its effects on the GIT, have not been well documented.

During the last decades, antibiotics have been used as a common strategy to manage fish disease and also to improve growth and feed conversion efficiency. However, the development and spread of antimicrobial resistant pathogens associated with this practice is well documented (Kim et al. 2004; Sørum 2006). Therefore, research orientated to the development of new dietary supplementation strategies involving the use of probiotics to improve fish health and growth is necessary (Denev 2008). Live microorganisms can decontaminate feeds by attaching the mycotoxin to their cell wall components or by active internalisation and accumulation (Halász et al. 2009).

Adsorbent agents (substances of high molecular weight) bind with the mycotoxins found in food, and are not dissociated in the digestive tract of the animal. In this way the toxin–adsorbent complex passes through the animal and is eliminated in faeces (Gimeno and Martins 2007). Mycotoxins can adhere to these compounds by physical adsorption (weak van der Waals interactions and hydrogen bonds; this process is easily reversible) and chemical adsorption or chemisorption (strong interactions by ionic or covalent bonds) (Tapia Salazar et al. 2010).

According to the FAO/WHO definition, probiotics are 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001). At present, probiotics are an alternative to antibiotics for improving health and production parameters in fish farming because they are being used as biological controllers in the prevention of bacterial pathogens (Irianto and Austin 2002). Lactic acid bacteria, such as *Lactobacillus* spp., *Leuconostoc* spp., *Streptocccus* spp., *Pediococcus* spp., and *Enterococcus* spp., among others, produce antimicrobial compounds that are important in the bio-preservation of feeds and are of particular interest as probiotics, being of importance in the GIT of fish (Messens and De Vuyst 2002; Tovar Ramirez et al. 2008; Wang et al. 2008; GerLABdo et al. 2012). Lactic acid bacteria have been largely found in the GIT of various animals such as mice, rats, pigs, poultry and humans (Tannock et al. 1982). Moreover, several years ago studies have demonstrated that LAB are part of the normal intestinal microbiota in fish (Ringø et al. 1995).

There has been a significant amount of research done on the ability of LAB to mitigate the effects of aflatoxin-producing strains and the AFB_1 binding *in vivo* and *in vitro* (Dalié et al. 2010, Hernandez-Mendoza et al. 2010; Bovo et al. 2012; Corassin et al. 2013). The aim of the present study was to evaluate probiotic properties of LAB strains isolated from rainbow trout intestine and feed. AFB_1-binding and/or degrading abilities were also evaluated to assess their use in the formulation of feed additives intended to improve productive parameters and prevent mycotoxicosis in aquaculture systems.

Materials and methods

Isolation and identification of lactic acid bacteria

Lactic acid bacteria were isolated from fish feed and from samples of the gastrointestinal content of 10 juvenile rainbow trout from a fish farm located in Las Tapias, province of Córdoba . A sagittal cut was made to all the trout with a scalpel and 7 cm of the small intestine were removed. Five intestines were mixed with Man, Rogosa and Sharp (MRS) broth, and intestinal contents were collected from the remaining five intestinal were deposited in the same media.

For the isolation of LAB from feed, 10 g of sample were inoculated in 90 ml of MRS broth and incubated for 48 h at 25°C under microaerophilic conditions. Strains were streaked on MRS agar for colony isolation and incubated for 48 h at 25°C under microaerophilic conditions.

Morphophysiological identification

Strains of LAB isolated from rainbow trout intestine and feed were characterised on the basis of

morphological, physiological and biochemical tests by *Bergey's Manual of Determinative Bacteriology* (Bergey and Holt 1994). All bacteria were grown aseptically in 10 ml MRS broth for 24 h at 25°C. Cell morphology, Gram staining, catalase and oxidase tests were performed as a preliminary screening for LAB. The isolated LAB strains were identified by catalase, oxidase, 6.5% NaCl tolerance, growth at 10° C and 45°C, pH 9.5 and 40% bile tolerance. The selected LAB strains were maintained as stock cultures at −80°C in 10% skim milk and 20% glycerol.

DNA extraction

For molecular identification of LAB strains, a pure colony of each isolate grown on MRS solid medium was transferred to 3 ml of MRS broth and incubated at 25°C for 24 h under microaerophilic conditions. After incubation, one ml of culture was centrifuged (12,000 g, 15 min). DNA was extracted using 700 µl of extraction buffer (200 mmol l^{-1} Tris-HCl pH 8, 25 mmol l^{-1} EDTA pH 8, 25 mmol l^{-1} NaCl, 1% SDS) and incubated for 30 min at 65°C. Deproteinisation was performed twice using equal volume of chloroform: isoamyl alcohol (24: 1), following the procedure proposed by Leslie and Summerell (2006).

Polymerase chain reaction and 16S rDNA sequencing

PCR assay was performed using the method proposed by Pryde et al. (1999) as a reference protocol. A fragment of approximately 450 bp of 16S rDNA was amplified. Primers used were: F-lac 5′-GCAGCAGTAGGGAATCTTCCA-3′ and R-lac 5′-GCATTYCACCGCTACACATG-3′.

The reaction mixtures contained 20–25 ng of total DNA of the analysed strain, in a total volume of 50 µl of 1 × reaction buffer containing 2 mM $MgCl_2$, 1.25 U Taq DNA polymerase (5 U μl^{-1}, Invitrogen by Life Technologies, Buenos Aires, Argentina), 0.2 mM of each dNTP and 0.3 µM of each primer. A negative control, containing all reagents without DNA, was included in every set of reactions. PCR was conducted according to the following cyclic conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of 94°C for 45 s, 53°C for 45 s and 72°C for 3 min, and a final extension step of 72°C for 10 min, and then held at 4°C indefinitely.

DNA fragments were visualised after electrophoretic run on 1.5% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide and gels were photographed using a MiniBIS Pro analyser (DNR Bio Imaging Systems, Jerusalem, Israel). The fragment sizes were measured by comparison with DNA 100-bp ladder (Invitrogen by Life Technologies, Buenos Aires, Argentina).

For DNA sequencing of both strands, template DNA was send to Macrogen Inc. (Seoul, Korea). Sequences were compared using the local alignment search tool (BLAST) program with the NCBI database (GenBank) (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were submitted to GenBank (ID #1,980,444).

Probiotic properties

Tolerance to gastric pH and bile salts

To test the tolerance of LAB strains to gastric pH, cells were cultured in MRS broth for 48 h at 25°C. Then, the cultures were diluted in sterile phosphate buffered saline (PBS) and cell concentration was adjusted to 10^7 CFU ml^{-1}. The PBS pH was previously adjusted to 6.5 (control) and 2.0 by addition of HCl 1N. One ml aliquots of each pH suspension were sampled immediately (0 h) and after 1.5 h incubation at 25°C. These samples were serially diluted in sterile PBS and viable counts were determined by plate count using MRS agar. The plates were incubated for 48 h at 25°C under microaerophilic conditions and CFU ml^{-1} were evaluated (Balcazar et al. 2008).

The tolerance of LAB strains to bile salts was tested. Lactic acid bacteria strains (100 µl culture aliquots) were inoculated in MRS broth + 40% bile salts (Britania, Buenos Aires, Argentina) and incubated for 24 h at 25°C (Cueto-Vigil et al. 2010).

Antagonistic activity among lactic acid bacteria and against bacterial pathogens

Indicator strains

For this study, the following pathogenic bacterial strains were used: *Escherichia coli, Pseudomonas aeruginosa, S. aureus* and *Salmonella typhimurium*. All of them were isolated from animal clinical cases in a local veterinary diagnostic laboratory and deposited in the culture collection of the National University of Rio Cuarto, Cordoba, Argentina.

Antagonistic activity assays

The antagonistic activity assay was done according to the method described by Campos et al. (2008) with slight modifications. Lactic acid bacteria were grown in MRS broth and incubated for 24 h at 25°C. Similarly, pathogenic strains *S. typhimurium, E. coli, P. aeruginosa* and *S. aureus* (10^5–10^6 CFU ml^{-1}) were grown in brain heart infusion (BHI) agar and incubated for 24 h at 25°C. Then, cultures were adjusted to 0.5 McFarland scale in PBS. One ml of each strain was taken and pour-plated in 1.2% MRS agar. Once the agar solidified, two procedures were followed: (1) the plate was divided into four sections and each fourth was surface-spread with a pathogenic strain using a sterile cotton swab; (2) an overlayer of 10 ml 1.2% brain heart infusion (BHI) agar was placed on the MRS, and once solidified, the plate was divided into four sections and each fourth was surface-spread with a pathogenic strain using sterile cotton swabs. Plates were incubated for 24 h at 25°C. The results are considered positive when there is no growth of the pathogenic strain in interaction with LAB.

Inhibition of bacterial pathogens growth by lactic acid bacteria cell-free supernatants

The inhibition of pathogenic bacteria growth by LAB extracellular products was studied using the well-diffusion method with modifications (Teo and Tan 2005; Marguet et al. 2011). Cell-free culture supernatants (CFCS) were obtained from strains that showed the best antimicrobial activity. Briefly, LAB strains were grown in MRS broth for 18 h at 25°C under microaerophilic conditions and centrifuged at 8500 g for 20 min at 4°C. The supernatants were exposed to chloroform vapours for 30 min and then treated under different conditions. Different aliquots of each supernatant were (i) heated for 30 min, (ii) neutralised with NaOH 10N (Sigma Aldrich, St Louis, MO, USA) or (iii) treated with 0.1 mg ml^{-1} catalase (Sigma Aldrich). The pathogenic strains were cultured in BHI broth for 24 h at 37°C and suspensions of each (adjusted to 0.5 McFarland scale equivalent to <300 UFC ml^{-1}) were inoculated in BHI agar using sterile swabs. Then, 9 mm wells were punched with a sterile cork borer and 100 µl of the colony-forming strains were placed in each well. The plates were incubated for 24 h at 25°C under microaerophilic conditions. Each assay was done in triplicate and all the experiments were repeated three times. The result was evaluated according to the presence of a halo of inhibition around the well.

Coaggregation assay

Lactic acid bacteria strains were tested for their capacity to coaggregate with pathogenic strains (*E. coli, P. aeruginosa, S. typhimurium* and *S. aureus*). The LAB strains were cultured on MRS broth for 24 h at 25°C under microaerophilic conditions and afterwards cultures were centrifuged for 15 min at 5000 g. The pellets were washed three times with sterile PBS and resuspended in sterile PBS to adjust to 0.05 OD_{600} (Collado et al. 2007). Then, equal volumes (2 ml) of the bacterial suspensions and pathogen suspensions were mixed together for 2 h at 25°C. The absorbance (A) of the suspension at OD_{600} was measured after 2 h of incubation at room temperature (Kos et al. 2003). The coaggregation percentage was expressed as:

$$(1)\ DO_M /[(DO_P + (DO_B /2)] \times 100$$

where:

DO_M: LAB + bacterial pathogen mixture absorbance after incubation

DO_P: bacterial pathogen culture absorbance after incubation

DO_B: LAB culture absorbance after incubation

Adsorption of aflatoxin B_1 in vitro

The study of the ability to bind AFB_1 in the animal GIT was performed according to Bueno et al. (2007) with some modifications. LABs were exposed to the gastric pH, then cells were washed twice with PBS and incubated with 1 ml of a 20 $ngml^{-1}$ AFB_1 solution (in PBS) for 1 h at 25°C in a shaking bath. Afterwards, cells were centrifuged and the supernatant containing unbound mycotoxin was collected and stored at −20°C until HPLC analysis. LAB cells not exposed to GIT conditions were included as controls. Aflatoxin B_1 quantification was performed by HPLC Waters Alliance 2695 system coupled to a fluorescence detector (Waters 2487) (Milford, Massachusetts, USA), according to the methodology described by Trucksess and Wood (1994) with some modifications (Cole and Dorner 1994), and the AFB_1

standards solutions were prepared according to AOAC (1995). An aliquot (200 µl) of the samples was derivatised with 700 µl trifluoroacetic acid: acetic acid: water (20:10: 70, v/v) solution. Chromatographic separations were performed on stainless steel, C18 reversed phase column (Luna Phenomenex (Torrance, California, USA), 150 × 4.6 mm id, 5 µm particle size). Water (4 v/v): methanol (1 v/v): acetonitrile (1 v/v) was used as mobile phase at a flow rate of 1.5 ml min^{-1} and the limit of detection (LOD) was 0.5 ng ml^{-1}. The fluorescence of AFB_1 derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. A calibration curve was constructed by injecting AFB_1 standards of 5; 30 and 50 ng ml^{-1} and the toxin levels in samples were quantified by comparison of peak areas. The percentage of mycotoxin bound to the bacteria was calculated using the equation: % Reduction = 100 × (1 – mycotoxin peak area of sample/mycotoxin peak area of control).

Aflatoxin B_1 degradation in vitro

To test the ability of LAB strains to degrade AFB_1 *in vitro* by extracellular enzymes, cell-free culture supernatants (CFCS) were obtained. This assay was done as described by Zhao et al. (2010). A 24 h culture was obtained inoculating 30 ml of MRS broth and incubating under microaerophilic conditions for 24 h at 37°C. The culture was centrifuged at 3000 rpm for 10 min and then filtered through a 0.22 µm Millipore cellulose acetate membrane. Aliquots of 800 µl were transferred to microtubes in triplicates and 200 µl of a 500 ng ml-1 AFB_1 solution added (100 ng AFB_1 per tube). Tubes were incubated for 48 h at 37°C in the darkness and then remaining AFs were extracted three times with 1 ml chloroform. The three chloroform fractions were collected and combined and AFB_1 concentration was quantified by HPLC according to Trucksess and Wood (1994). Control tubes containing AFB_1 and MRS broth without LAB were included. Each treatment was done in triplicate. Degradation percentages were calculated by comparison with the control tubes (100%).

Statistical analyses

Data were analysed with an analysis of variance. The results were compared using a linear mixed model and Fisher's protected least significant difference (LSD) test; $p < 0.05$ was considered statistically significant.

Results

Tolerance to gastric pH and bile salts

Of the total strains isolated, only 40% were able to resist gastric pH and bile salts: those strains of LAB showing high resistance to low pH (2.0) and bile salts. Viable cell counts did not change after 1.5 h exposure to pH 2.0 when compared with the control. The same results were observed when LABs were exposed to 40% of bile salts.

The strains of LAB able to survive in these conditions are those that were used for later tests.

Lactic acid bacteria morphophysiological and molecular identification

Eight isolates were obtained from trout feed and intestine. These formed smooth round colonies in MRS solid medium. Cells were round Gram-positive and catalase-negative, tetrad cocci (1–2.5 µm) with no-spore formation. They produced acid from glucose but not gas. All strains were able to tolerate 6.5% NaCl, 40% bile and grow at 10°C, 45°C and pH 9.5.

Molecular characterisation identified six strains as *Pediococcus acidilactici* and two as *Pediococcus pentosaceus* (Table 1). Sequences were submitted and annotated in GenBank (accession numbers: KY464092, KY464091, KY464093, KY464094, KY464095, KY464096, KY464097, KY464098).

Antagonistic activity among lactic acid bacteria and against bacterial pathogens

The antagonistic activity of LAB strains was tested against pathogenic bacteria. The tested LAB strains showed inhibitory activity mainly against Gram negative bacteria. Seven out of eight LAB strains (87.5%) were able to inhibit *P. aeruginosa*, five

Table 1. Nomenclature and identification of lactic acid bacteria strains isolated from rainbow trout intestine and fish feed.

Strain	Identification	Origin
RC001	*Pediococcus acidilactici*	Fish feed
RC002	*Pediococcus acidilactici*	
RC003	*Pediococcus acidilactici*	
RC004	*Pediococcus acidilactici.*	
RC005	*Pediococcus acidilactici*	
RC006	*Pediococcus pentosaceus*	
RC007	*Pediococcus pentosaceus*	Intestine
RC008	*Pediococcus acidilactici*	

(67%) inhibited *S. typhimurium* growth, two (25%) inhibited *E. coli* growth and three (37.5%) inhibited *S. aureus* growth. *Pediococcus acidilactici* RC001 (isolated from fish feed) and *Pediococcus acidilactici* RC008 (isolated from trout intestine) showed the strongest antimicrobial activity, since they were able to inhibit all the tested pathogens (Figure 1).

Inhibition of bacterial pathogens growth by lactic acid bacteria cell-free supernatants

Inhibition of bacterial pathogen growth by LAB extracellular products present in the cell-free culture supernatant (CFCS) of the different strains is described in Table 2. Four of the tested strains showed extracellular antimicrobial activity. The strains produced antimicrobial compounds of different nature being affected differently by the treatments (catalase, NaOH and heating), indicating that they could be H_2O_2, acids or proteins. Cell-free culture supernatants of RC001 and RC008 were able to inhibit *P. aeruginosa* in all cases, being unaffected by any of the treatments. Strains *Pediococcus acidolactici* RC002, RC003,RC004 and *Pediococcus pentosaceus* RC006 CFCSs did not inhibit any of the pathogens tested. None of the CFCSs tested were able to inhibit the growth of *S. typhimurium*.

Table 2. Inhibition of bacterial pathogens growth by LAB extracellular products present in the cell-free culture supernatant (CFCS).

		CFCS treatment			
CFCS	Pathogen	Control	Catalase	NaOH	Heating
320–15	*P. aeruginosa*	+	+	+	+
	S. typhimurium	-	-	-	-
	E. coli	+	-	+	-
	S. aureus	-	-	-	-
225–7	*P. aeruginosa*	-	-	-	-
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	-	-	-	-
225–8	*P. aeruginosa*	-	-	-	-
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	-	-	-	-
702–4	*P. aeruginosa*	+	+	-	-
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	-	-	-	-
702–6	*P. aeruginosa*	-	-	-	-
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	-	-	-	-
703–2	*P. aeruginosa*	-	-	-	-
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	-	-	-	-
M2	*P. aeruginosa*	+	+	-	+
	S. typhimurium	-	-	-	-
	E. coli	-	-	-	-
	S. aureus	+	+	+	-
M8	*P. aeruginosa*	+	+	+	+
	S. typhimurium	-	-	-	-
	E. coli	+	+	-	+
	S. aureus	+	-	+	-

Co-aggreggation assay

All LAB strains tested showed ability to coaggregate pathogenic bacteria, showing inhibition percentages above 40% (Figure 2). The strain RC001 presented higher ability to coaggregate pathogenic microorganisms, with percentages >60%. On the other hand the highest co-aggregation percentages were obtained between LAB and *S. aureus* (>50%) while the lowest were observed between LAB and *P. aeruginosa* (37 to 66%).

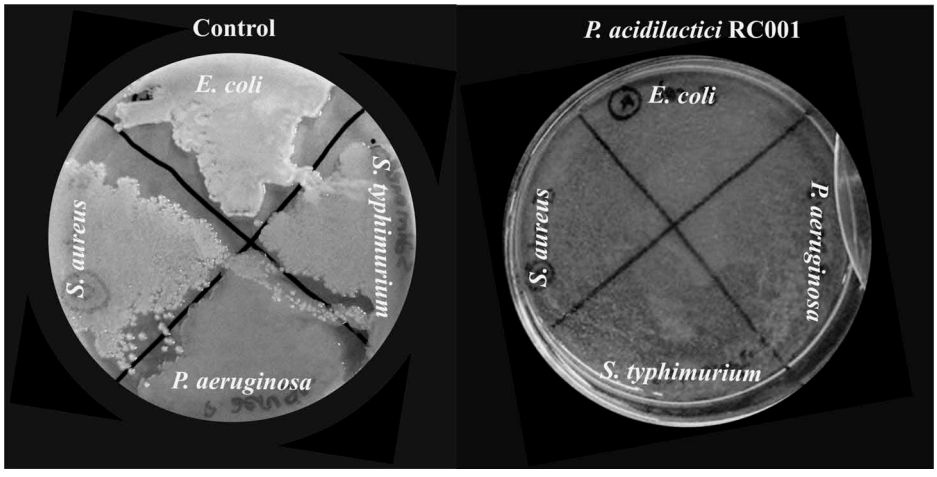

Figure 1. Antagonistic activity of LAB strains against bacterial pathogens: inhibition of *E. coli, P. aeruginosa, S. typhimurium* and *S. aureus* growth by LAB strain RC001 isolated from fish feed.

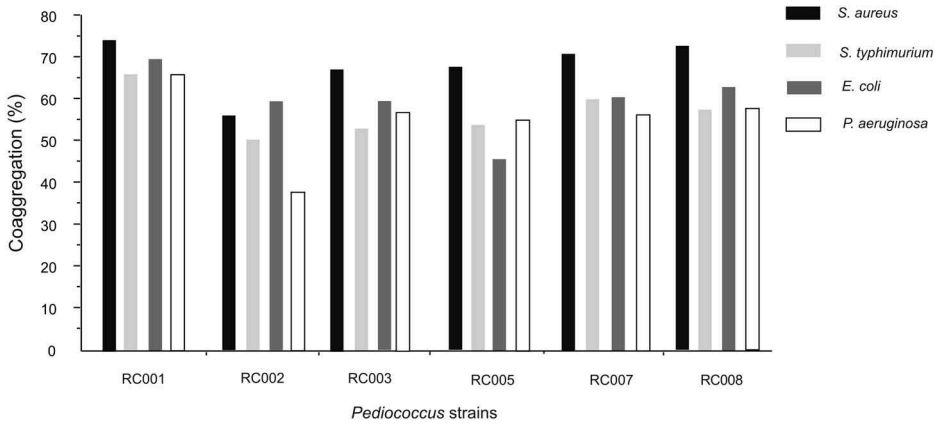

Figure 2. Coaggregation percentage of LAB strains isolated from rainbow trout intestine and fish feed with bacterial pathogens.

Aflatoxin B_1 binding

All LAB strains tested showed *in vitro* AFB_1 binding ability and the adsorption percentages varied among strains. Adsorption percentages did not exceed 23% when cells were exposed to 20 ng ml^{-1} AFB_1. *Pediococcus acidilactici* strain RC003 (22.49%) was the one with the highest adsorption capacity, followed by RC005 (19.53%) and RC002 (19.36%). Other strains showed adsorption percentages between 10 and 16%. The LAB strains that showed higher adsorption capacity were isolated from feed (Figure 3).

Aflatoxin B_1 degradation

All LAB strains were able to degrade AFB_1 with percentages higher than 15%, showing significant differences with respect to the control. *Pediococcus pentosaceus* strain RC006 and *Pediococcus acidilactici* strain RC005 were the ones that had the highest degrading capacity, with degrading percentages of 36 and 34%, respectively. Other strains showed degradation percentages between 19 and 29% (Figure 4).

Discussion

Beneficial bacteria from rainbow trout intestine and feed were screened for their potential as probiotics and AFB_1 binders in the present work. Lactic acid bacteria are frequently used as probiotics in the animal food industry since they represent an important part of the normal GIT animal microbiota (Drisko et al. 2003).

Fish are exposed to a wide range of microorganisms present in the environment. Gatesoupe (1999) stated that most bacteria in the fish intestine are

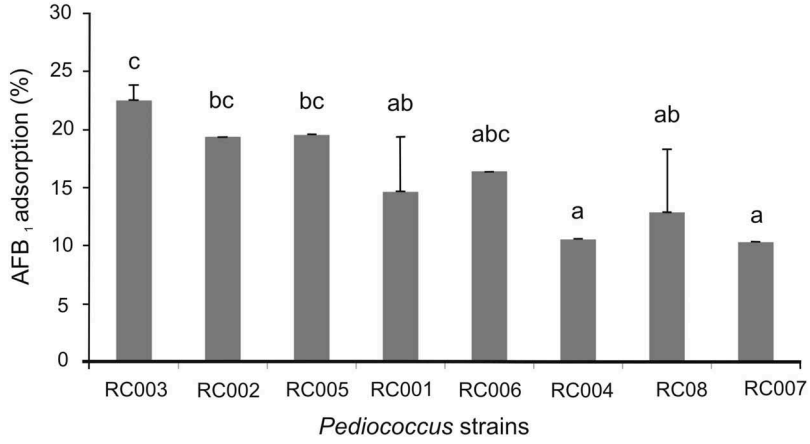

Figure 3. Aflatoxin B_1 adsorption percentage of LAB strains isolated from rainbow trout intestine and fish feed. Different letters indicate statistically significant differences between adsorption percentages achieved by different strains according to ANOVA ($p < 0.05$).

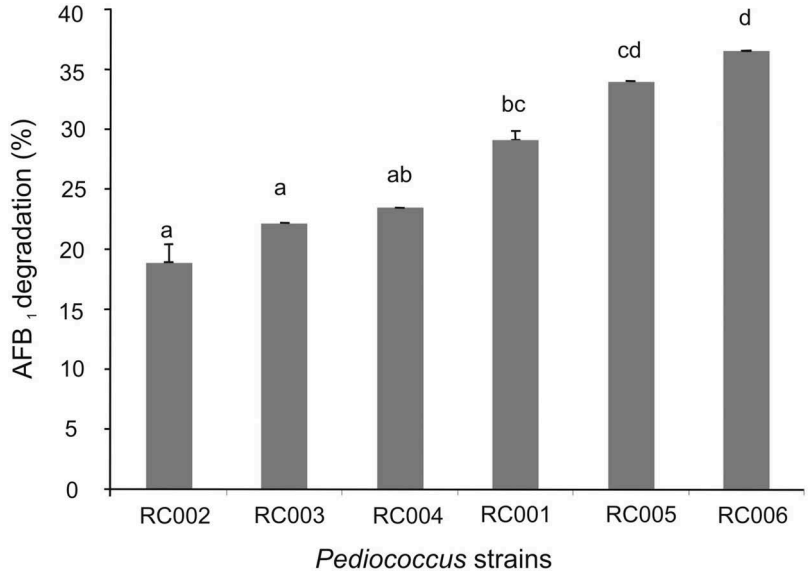

Figure 4. Aflatoxin B_1 degradation percentage of LAB strains isolated from rainbow trout intestine and fish feed. Different letters indicate statistically significant differences between adsorption percentages achieved by different strains according to ANOVA ($p < 0.05$).

transitory, since exchange of microorganisms with water and feed is continuous. Several investigations have demonstrated that LAB microorganisms have received considerable attention due to their probiotic properties, which are important for the cultivation of aquatic organisms (fish, molluscs and crustaceans) (Balcázar et al. 2008; Merrifield et al. 2010). In the present study, we observed that rainbow trout intestinal microbiota was composed mainly of *P. pentasoceus* and *P. acidilactici*. Other authors reported that *Pediococcus* spp. as well as *Lactobacillus* spp., *Carnobacterium* spp., *E. faecium* and *Leuconostoc* sp. belonged to the normal microbiota of the GIT in healthy fish (Gatesoupe 2010; Merrifield et al. 2014). *Pedioccocus* species have been isolated from different kinds of samples, from fermented foods, but isolation from fish feed has not previously been reported. In addition, few studies have isolated *Pediococcus* spp. as a part of the normal fish gut microbiota. Also, in the present study, *Pediococcus* species were identified as a part of the normal microbiota. In other studies, *P. acidilactici* was used as alternative treatment to limit the prevalence of the vertebral column compression syndrome (VCCS) in rainbow trout (Aubin et al. 2005). Giannenas et al. (2015) observed that dietary supplementation with a multi-strain probiotic containing *P. acidilactici, Bacillus subtilis, Enterococcus faecium* and *Lactobacillus reuteri* significantly increased growth performance and health status of trout. The probiotic also modulated intestinal microbial communities favouring LAB.

The isolated strains studied here have demonstrated probiotic plus AFB_1 binding and degrading properties, which could have beneficial effects on fish health by reducing impairment caused by this mycotoxin. Different bacteria have been used by the European Union in animal nutrition, such as *Pediococcus* (especially *P. acidilactici*) together with LABs such as *Enterococcus* (*E. faecium*), *Lactobacillus* (*L. acidophilus, L. casei, L. farciminis, L. plantarum* and *L. rhamnosus*) and other Gram-positive bacteria such as *Bacillus* (*B. cereus, B. licheniformis* and *B. subtilis), Streptococcus* species (*S. infantarius*), and yeasts (*Saccharomyces cerevisiae* and *S. boulardii*) (Anadón et al. 2006).

The selection criteria for probiotic microorganisms were established by the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) (FAO/WHO, 2002). The essential characteristics for LAB to be used as probiotics include the following: recognition as safe (GRAS: generally recognised as safe); tolerance to bile and acid, tolerance during processing and storage; antagonistic effect against pathogens (Begley et al. 2005; Vesterlund et al. 2005; Lin et al. 2006). One of the *in vitro* selection criteria tested is

resistance to acid in simulated GIT conditions and bile salts (Park et al. 2006). Tolerance to bile is important for the probiotic strains to grow and survive in the fish intestine. However, there is still no consensus about the precise concentration to which the selected strain should be tolerant (Balcázar et al. 2008). In this work, it was observed that all strains tested showed resistance to acid pH and high concentrations of bile salts, which concurs with Fernández-Juri et al. (2011), where all evaluated strains resisted acid exposure.

In the present work, the ability of LAB to coaggregate with pathogenic bacteria and to inhibit their growth was tested. Coaggregation capacity of probiotics is essential in the protection against gastrointestinal pathogens. According to different investigations, the coaggregation capacity of the probiotic strains allows a barrier to be formed that prevents colonisation by pathogenic microorganisms and can inhibit their growth (Collado et al. 2007; Taheri et al. 2009). All LAB strains tested showed the ability to co-aggregate with pathogenic bacteria at percentages between 40 and 70%. Similarly, Osmanagaoglu et al. (2010) reported *P. pentosaceus* isolated from human breast milk demonstrated 6.26 and 12.99% coaggregation with *Salmonella enterica* serotype *Typhimurium* SL 1344 and *E. coli* LMG 3083. Collado et al. (2007) suggested that coaggregation ability is strain-specific, a result that agrees with ours. Lactic acid bacteria are capable of colonising the intestine and act antagonistically against Gram negative pathogens and may, therefore, act as probiotic microorganisms. In the present study, *Pediococcus* strains were able to inhibit pathogenic Gram negative bacilli (*E. coli, P. aeruginosa* and *S. typhimurium*) and as well as Gram positive cocci (*S. aureus*). Similarly, Jang et al. (2015) showed that *P. pentosaceus* isolated from kimchi was able to inhibit *Listeria monocytogenes* in a salmon based medium and in salmon fillets.

The production of extracellular antimicrobial substances by LAB was also evaluated. Many bacterial strains have the capacity to inhibit pathogenic microorganisms by the production of inhibitory compounds like organic acids, fatty acids, hydrogen peroxide or bacteriocins (Gatesoupe 2010). In the present study, *Pediococcus acidilactici* RC001 and RC008 showed the strongest antimicrobial activity, since their CFCSs were able to inhibit all the tested pathogens. The antimicrobial activity of *Pediococcus* strains tested was associated with extracellular compounds of different nature such as proteins (bacteriocins), organic acids (most probably lactic acid) and H_2O_2, as well as others that were not affected by any of the neutralising treatments. Sica et al. 2012 reported that LAB isolated from wild rainbow trout from Bahía Blanca estuary in Argentina also produced antimicrobial compounds of the same nature that inhibited *L. monocytogenes*. Lactic acid bacteria are known to produce a wide variety of antimicrobial substances, of which bacteriocins have been widely studied (Schnurer and Magnusson, 2005). Bacteriocins are of great interest in the food sector as they can be used as possible natural food preservatives. A large number of pediocines have been isolated and characterised to date. The bacteriocins are produced by various pediococcal species and have gained considerable attention due to their remarkable thermal stability, activity in a broad pH range, broader antimicrobial spectrum, greater specificity and effectiveness at very low concentrations (Kumar et al. 2011). Although the production of pediocines has not yet been tested in this study, future studies in our laboratory will consider this topic.

Aflatoxin B_1 is the main contaminant of feedstuffs (Fernández-Juri et al. 2011); thus, we determined the ability of LAB strains to adsorb this toxin. Our results showed that all strains were able to adsorb some amount of AFB_1 in the tested concentration (20 ng ml^{-1}). However, binding percentages did not exceed 23%. Similar results were obtained by Fernández Juri et al. (2014) who noted AFB_1 adsorption percentages below 30% for enterococci strains isolated from dog faeces. Nevertheless, Campos et al. (2009) and Fernández Juri et al. (2009) stated that strains with these percentages of AFB_1 adsorption could be used as additives in dry pet foods naturally contaminated with low levels of this toxin and still make a difference. On the other hand, other LAB, such as *L. casei* strains isolated from cheese, corn silage and human faeces, have been reported to adsorb up to 50% AFB_1 (Hernández-Mendoza et al. 2010). Other authors have also show that LAB are able to bind AFs (Zinedine et al. 2005; Pizzolitto et al. 2011). In addition, *Pediococcus* strains isolated in the present study were also able to degrade AFB_1. There are no other studies to date reporting such a

characteristic for probiotic *Pediococcus* strains. This could be an advantage since adsorption is a reversible process, while degradation reduces AFB_1 concentration more effectively in contaminated substrates. Other authors suggested that the best approach for mycotoxin decontamination should be degradation by selected microorganisms (Vanhoutte et al. 2016)

The studies performed in the present work allowed us to determine the probiotic characteristics and AFB_1 binding and degrading ability of microbial strains isolated from different animal species and a different environment, since most of our previous works were on strains isolated from pig and poultry (Armando et al. 2011). These results encourage the search of potential probiotics in the aquatic environment to select the best strains for further experiments, such as *in vivo* studies. As stated by Watson et al. (2008), the future application for probiotics in aquaculture is very promising. There is an increasing demand for aquaculture products and alternatives to antibiotics. Probiotics intended for aquacultured animals are now receiving particular attention and many commercial products are now available, particularly directed at shrimp larval culture.

The present study is one of the few reports on the probiotic potential of LAB isolated from a fish farm in Argentina, adding regional relevance to the results, since aquaculture has gained national attention in recent years. As a developing country, the formulation of additives of national industry to improve animal health and performance is crucial to reduce costs, since most products available in the market are imported and expensive.

The ability of some of the LAB strains isolated in the present work to compete with pathogens, together with stability against fish bile and gastric pH, may indicate their potential for use in rainbow trout culture. Animal species specificity is an important factor affecting *in vivo* adhesion and colonisation of the intestine by probiotics. The isolation of probiotic strains from the same species to be used increases the probability of a better performance. However, since variation between individuals can exist, the combination of different strains in the same probiotic additive is convenient to assure better results.

In conclusion, LAB with probiotic characteristics and AFB_1 binding ability and degradation were isolated from fish feed and rainbow trout intestine. Future *in vitro* and *in vivo* studies will enable selection of the most adequate strains to be used in the formulation of feed additives intended to improve fish health and reduce the use of antibiotics in aquaculture systems.

Highlights

- The predominant species isolated from rainbow trout intestine and fish feed were *Pediococcus acidolactici* and *Pediococcus pentosaceus*.
- LAB strains isolated from trout intestine and feed showed probiotic characteristics.
- All strains showed resistance to pH 2.0 and bile salts.
- Strains were able to bind 10–22% AFB_1 *in vitro*.
- All strains were able to degrade 15–36% AFB_1.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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