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Probiotic characteristics and aflatoxin B_1 binding ability of *Debaryomyces hansenii* and *Kazaschtania exigua* from rainbow trout environment

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Abstract

The aim of this study was to evaluate probiotic properties and the aflatoxin B_1 adsorption ability of yeasts isolated from rainbow trout intestine and fish feed to assess their use in the formulation of feed additives. Growth at pH 2, bacterial pathogens inhibition, bacterial pathogens co-aggregation, autoaggregation, homologous and heterologous inhibition against lactic acid bacteria were evaluated. Moreover, aflatoxin B_1 adsorption was tested. All strains were able to maintain viable (10^7 cells/ml) at pH 2. All strains isolated from intestine were identified as *Kazaschtania exigua*, while strains isolated from feed were all identified as *Debaryomyces hansenii*. *Kazaschtania exigua* RC035 and RC037 showed the strongest antimicrobial activity while *K. exigua* RC037 and RC038 were the most efficient co-aggregating bacterial pathogens. All strains exhibited strong autoaggregation. None of the tested yeast strains showed homologous inhibition towards other yeasts and heterologous inhibition towards lactic acid bacteria strains. *Debaryomyces hansenii* RC031 demonstrated aflatoxin B_1 adsorption capacity (21%). The results of the present study indicate that select strains of *Kazaschtania exigua* and *D. hansenii* showed potential to improve the health of rainbow trout by inhibiting pathogens and binding AFB_1 and their use as probiotics may improve the production of rainbow trout in aquaculture systems.

KEYWORDS
aflatoxin B_1 adsorption, *Debaryomyces hansenii*, *Kazaschtania exigua*, probiotic capacities, rainbow trout

1 | INTRODUCTION

Aflatoxins (AFs) cause diseases with high mortality and a gradual decrease in the quality of the stock of bred fish, affecting fish farming and representing a significant problem in aquaculture systems (Santacroce et al., 2008). This is a group of mycotoxins produced by *Aspergillus flavus* and *A. parasiticus* that represent an important source of contamination in foods and feeds worldwide (Murjani, 2003). Aflatoxins have also potent mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties and are of particular importance because adverse effects on animal and human health, generalized as "aflatoxicosis" (CAST, 2003). In terms of toxic potential and occurrence, aflatoxin B_1 (AFB_1) is the most important of AFs, it is a potent carcinogen (IARC, 2002). The carcinogenic effect of AFB_1 has been studied in fish such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), tilapia (*Tilapia sparrmanii*) and guppy (*Poecilia reticulate*) (Murjani, 2003).

Strategies for preventing, eliminating, inactivating or reducing the bioavailability of AFB_1 in food or in contaminated products include physical, chemical and biological methods (Kabak, Dobson, & Var, 2006). Microorganisms have been studied for their potential to decontaminate mycotoxins either by degradation or by reduction of their bioavailability in animal production systems. Live microorganisms can decontaminate mycotoxins by attaching them to their cell wall components or by degradation (Karaman, Basmacioglu, Ortatatli, & Oguz, 2005; Raju & Devegowda, 2000; Shetty, Hald, & Jespersen, 2007).

Probiotics are live microorganism which, when administered in adequate number, confer a health benefit in the host (FAO and WHO, 2001). The inclusion of probiotics in various types of food and feed products has increased significantly during the past three decades. Probiotics are used as biological controllers in the prevention of bacterial pathogens as an alternative to antibiotics to improve health and production parameters in fish farming (Irianto & Austin, 2003). Yeasts are of particular interest as probiotics as they provide β-glucans and nucleotides that stimulate fish's immune system (Sahoo & Mukherjee, 2001). A variety of yeast species have been isolated from rainbow trout intestine including *Debaryomyces hansenii*, *Candida* sp., *Saccharomyces cerevisiae*, *Leucosporidium* sp., and *Rhodotorula* sp. as the dominant yeast mycobiota (Gatesoupe, 2007). However, only two species (*D. hansenii*, *S. cerevisiae* and *S. cerevisiae* var. *boulardii*) have been used as probiotics in aquaculture (Tovar et al., 2002; Waché et al., 2006).

The inclusion of mycotoxin-binding microorganisms in contaminated diets could prevent the absorption of mycotoxins during their passage through the GIT and enhance their excretion in faeces (Bueno, Casale, Pizzolitto, Salvano, & Oliver, 2007).

The aim of the present study was to evaluate the probiotic potential of yeast strains isolated from rainbow trout intestine and fish feed in order to formulate feed additives to improve productive parameters in aquaculture systems. Moreover, AFB_1-binding ability was evaluated.

2 | MATERIALS AND METHODS

2.1 | Isolation and identification of feed-borne and intestine-borne yeasts

Juvenile rainbow trouts (n = 10, average weight 50 g) and high-quality trout feed (composed by 40% protein, fishmeal, fish oil) were collected at a fish farm located in Las Tapias, Córdoba province, Argentina.

Yeasts were isolated from intestine content as follows: a sagittal cut was made to all trouts with a scalpel and the intestines (from the end of the pyloric stomach (ceaca) to the anus) were removed. Intestinal content was squeezed out and transferred to Erlenmeyer flasks containing 150 ml of sterile Yeast-Peptone-Dextrose (YPD) broth (10 g yeast extract (Laboratorios Britania S.A, Buenos Aires, Argentina), 20 g peptone (Laboratorios Britania S.A, Buenos Aires, Argentina), 20 g dextrose (Laboratorios Cicarelli®, Santa Fe, Argentina) and 1 L distilled water.

Yeasts were isolated from feed as follows: 10 g of sample were inoculated in 90 ml of YPD broth and incubated for 48 hr at 25°C. Strains were streaked on YPD agar for colony isolation and incubated for 48 hr at 25°C. Yeast strains were characterized on the basis of morphological, physiological and biochemical tests described by Pitt and Hocking (1999). From each yeast strain, the crossed strike method was used to identify them according to their behaviour against different media. A molecular identification was conducted as follows:

For yeasts DNA extraction, a pure colony of each isolate grown on a solid medium was transferred to 3 ml of YPD agar and incubated for 24 hr at 25°C. One millilitre (1 ml) was centrifuged (12,000 g 15 min) and the obtained pellet was frozen in liquid nitrogen for 5 min. Fungal DNA was extracted using a hexadecyl-trimethyl-ammonium bromide (CTAB) procedure following the methodology proposed by Leslie and Summerell (2006).

The one-step PCR-fingerprinting method was performed using the microsatellite primer $(GTG)_5$ (5′-GTG GTG GTG GTG GTG-3′). PCR reactions were made with 20–30 ng of fungal DNA in a total volume of 25 μl of 1× reaction buffer containing 2 mM $MgCl_2$, 1.25 U Taq DNA polymerase (5 U/μl, Invitrogen by Life Technologies, Buenos Aires, Argentina), 0.2 mM of each dNTP, and 0.6 μM of GTG_5 primer. A negative control, containing all reagents without fungal 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, 54°C for 45 s and 72°C for 1 min, and a final extension step of 72°C for 10 min, and then held at 4°C indefinitely. DNA band patterns were visualized after electrophoretic run on 1.5% agarose gel stained with 0.5 μg/ml ethidium bromide and gels were photographed using a MiniBIS Pro, DNR Bioimaging systems analyser.

The microsatellite-primed PCR results were confirmed by chosen strains for its sequencing at ITS region with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR was set up in a 50-μl reaction mixture containing 5 μl of genomic DNA (10 ng/μl), 1× reaction buffer containing 2 mM $MgCl_2$, 1.25 U Taq DNA polymerase (5 U/μl, Invitrogen by Life Technologies, Buenos Aires, Argentina), 0.2 mM of each dNTP and 0.6 μM of each primer. A negative control, containing all reagents without fungal DNA, was included in every set of reactions. Amplification was performed in a MJ Research PTC-200 thermocycler (GMI Inc. Minnesota, MN, USA) programmed for 5 min at 94°C followed by 35 cycles of 1-min denaturation at 94°C followed by primer annealing 1 min at 55°C and primer extension 1 min at 72°C and a final 5-min elongation step at 72°C. PCR products were visualized after electrophoretic run on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml) (Burgaud, Arzur, Durand, Cambon-Bonavita, & Barbier, 2010).

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

2.2 | Pathogenic bacterial strains

Pathogenic bacteria were used: *Escherichia coli* RC001, *E. coli* RC002, *E. coli* RC003, *Pseudomonas aeruginosa* RC001, *Salmonella typhimurium* RC001, *Streptococcus agalactiae* RC001, *Staphylococcus aureus* RC001 and *Enterococcus faecalis* RC001. All pathogenic bacterial strains were obtained at a local veterinary diagnostic laboratory from animal clinical cases and deposited in the culture collection of the National University of Rio Cuarto, Cordoba, Argentina.

2.3 | Tolerance to gastric pH

Tolerance to gastric pH was determined according to the methodology proposed by Armando et al. (2011). A cell suspension of each yeast strain under study was cultured in YPD broth for 48 hr at 25°C. The cultures were diluted in sterile phosphate buffered saline (PBS) (8.5 g ClNa, 0.295 g KH_2PO_4, 1.37 g $NaHPO_42H_2O$, 1,000 ml distilled water) to 10^7 CFU/ml. The PBS (originally pH 7.2 $\pm$ 2) was adjusted to pH 6.5 (as control) and pH 2.0 by addition of 1N HCl. Aliquots (1 ml) from each pH suspension were taken immediately (0 hr) and after 1.5-hr incubation at 25°C. These samples were serially diluted in sterile PBS and plate counts were determined using YPD agar (100 μl per plate). The plates were incubated for 48 hr at 25°C and CFU/ml were determined. The assay was performed in triplicate.

2.4 | Heterologous inhibition of yeast strains on lactic acid bacteria strains

The antimicrobial activity of yeast cultures over LAB was performed by the well diffusion assay (Lawalata, Sembiring, & Rahayu, 2010). Yeast cultures were grown in YPD broth for 24 hr at 25°C. Lactic acid bacteria were grown in Man Rogosa Sharp (MRS)(Laboratorios Britania, Buenos Aires, Argentina) broth for 24 hr at 25°C. MRS soft agar (10 ml) was inoculated with 50 μl broth culture of LAB. YPD was poured on Petri dish and allowed to solidify, then overlaid with the LAB inoculated MRS soft agar prepared previously and incubated for 1 hr at 4°C. Wells were punched with a sterile cork-borer and filled with 50 μl of each yeast culture (10^7–10^8 cells/ml). Plates were incubated for 24 hr at 25°C and performed in triplicate. The antimicrobial activity was determined by the presence or absence of a clear zone around the wells.

2.5 | Homologous inhibition

Inhibition among yeast strains was tested following the method described by Teo and Tan (2005) with slight modifications. Yeast strains were grown in YPD broth and incubated for 24 hr at 25°C. Central streak of each yeast strain (10^7–10^8 cells/ml) was performed on separate Petri dishes (one central streak each) containing YPD agar, and incubated for 48 hr at 25°C. The interacting yeast strains were streaked perpendicularly across to the previous strain on the same agar plate. After 24-hr incubation, inhibitory effect was determined by the appearance of clear zones surrounding the crossing point of the streak lines, indicating an inhibitory effect of one strain on the other.

2.6 | Inhibition of pathogenic bacterial strains

The ability of the yeast strains to inhibit pathogenic bacterial strains growth was tested by the crossed-streak method according to Teo and Tan (2005) with slight modifications. Yeast strains were grown in YPD broth and incubated for 24 hr at 25°C. Similarly, pathogenic strains, *S. typhimurium* RC001, *E. coli* RC001, *E. coli RC002*, *E. coli RC003*, *P. aeruginosa* RC001, *E. faecal* RC001, *S. agalactiae* RC001 and *S. aureus* RC001 (10^5–10^6 CFU/ml), were grown in brain heart infusion (BHI) agar and incubated for 24 hr at 25°C. Central streaks of each yeast strain were performed on separate Petri dishes (one central streak each) containing YPD agar and incubated for 48 hr at 25°C. Each pathogenic strain was then streaked (perpendicularly) across the central streak. After 24-hr incubation, antagonistic effect was determined by the appearance of clear zones surrounding the junctions of the streak lines, which indicated the inhibitory effect of the yeast against the pathogens.

2.7 | Autoaggregation assay

Aggregation assay was performed according to Del Re, Sgobarti, Miglioli, and Palenzona (2000). Yeast strains were grown for 24 hr at 25°C in YPD broth. Cells were harvested by centrifugation and suspended in buffer phosphate saline (PBS; 8.5 g ClNa, 0.295 g KH_2PO_4, 1.37 g $NaHPO_42H_2O$, 1,000 ml distilled water) to optical density 1 (O.D.) units at 600 nm (T_0). Two ml (2 ml) of yeast suspensions were placed in different tubes and centrifuged. Cells were then resuspended in PBS. After incubation for 2 hr at 25°C (T_1), 1 ml of the upper suspension was transferred to another tube and the O.D. was measured. Aggregation was expressed as 1—(O.D. upper suspension/O.D. total yeast suspension) $\times$ 100.

2.8 | Co-aggregation assay

Yeast strains were tested for their capacity to co-aggregate pathogenic bacterial pathogens (*E. coli* RC001, *P. aeruginosa* RC001, *S. typhimurium* RC001 and *S. aureus* RC001). The inoculum of each pathogenic strain was prepared from a 37°C overnight culture in BHI broth and harvested by centrifugation. The cells were resuspended in PBS (pH 7). The assay was performed as previously reported by Mastromarino et al. (2002), with some modifications, 1 ml of each yeast strain suspension (1×10^7 CFU/ml in PBS) was mixed with a suspension of a pathogenic strain (1 ml) and incubated for 2 hr at 25°C. Suspensions were then observed by optical microscopy ($\times$1,000 magnification) after Gram stain to evaluate the presence or absence of aggregation.

2.9 | Adsorption of aflatoxin B_1

In order to study AFB_1 binding ability, physicochemical conditions of the pH gastric were simulated and yeasts were exposed to them. The study of the ability to bind AFB_1 was performed according to Bueno et al. (2007) with some modifications. Yeast strains were cultured in

YPD broth for 48 hr at 25°C. The cultures were diluted in sterile PBS adjusted to pH 2.0 by addition of HCl 1N and cell concentration was adjusted to 10^7 CFU/ml. Yeast cells were washed twice with PBS and incubated with 1 ml of AFB_1 solution (20 ng/ml in PBS) for 1 hr at 25°C in a shaking bath. The AFB_1 solution was prepared using an AFB_1 analytical standard (Sigma, St. Louis, Missouri, USA). The cells were centrifuged and the supernatant containing unbound mycotoxin was collected and stored at −20°C for HPLC analysis. Yeast cells not exposed to pH conditions were included as controls. The quantification was performed by HPLC Waters Alliance 2695 system coupled to a fluorescence detector (Waters 2487), according to the methodology proposed by Trucksess, Stack, Nesheim, Albert, and Romer (1994) with some modifications (Cole & Dorner, 1994), and the AFB_1 standards solutions were prepared according to AOAC (1995). Aliquots (200 μl) of the samples and the AFB_1 standards (Sigma-Aldrich, St. Louis, MO, USA) used to construct the calibration curve were derivatized with 700 μl trifluoroacetic acid:acetic acid:water (20:10: 70, v/v). Chromatographic separations were performed on stainless steel, C18 reversed phase column (Luna Phenomenex, 150 × 4.6 mm id., 5 μm particle size). A solution of water, methanol and acetonitrile (4:1:1, v/v) was pumped as mobile phase at a flow rate of 1.5 ml/min. The fluorescence of AFB_1 derivatives was recorded at and of 360 nm excitation and 460 nm emission wavelengths respectively. The concentration of this toxin was quantified with the construction of a standard calibration curve. A calibration curve was constructed by injecting AFB_1 standards of 5; 30 and 50 ng/ml and quantification of the toxin levels in samples were calculated by comparison of peak areas. The detection limit of the analytical method was 0.1 ng/g. 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).

2.10 | Statistical analyses

Data were analysed with the general linear and mixed model (GLMM) using InfoStat (version 2012; University of Cordoba, Argentina) software. Data were analysed by two- way ANOVA ($p < .0001$). Means were given with standard deviation (*SD*) and standard error (*SE*) and were compared using Fisher's protected least significant difference (LSD) test ($p < .0001$).

3 | RESULTS

3.1 | Isolation and identification of yeast strains

Yeast strains isolated from feed were identified by morphological, physiological and biochemical tests as *D. hansenii*, *Candida parapsilosis* and *C. tropicalis*, while strains isolated from trout intestine could not be identified in genus and species by this methodology and were identified by a molecular method as described in 2.1. Sequence analysis by the BLAST tool against other sequences from reference strains revealed that all yeast strains isolated from trout intestine had high match with published *Kazaschtania exigua* sequences (99%–100% identity) in GenBank. The obtained sequences were deposited in GenBank under accession numbers MG680914, MG680915, MG680916, MG680917, MG680918, MG680919 and MG680920. Table 1 shows the yeast strains that were selected to conduct the subsequent assays.

3.2 | Tolerance to gastric pH

The isolated yeast strains showed relatively high resistance to low pH. They all survived after 90-min exposure to pH 2. Strains isolated from trout intestine showed higher viability than those from fish feed during simulated gastric pH, maintaining the initial viable counts (10^7 cells/ml); however, strains isolated from feed reduced one log (10^6 cells/ml) after GIT passage.

3.3 | Homologous and heterologous inhibition among yeast and lactic acid bacteria strains

None of the isolated yeast strains showed inhibition towards any other of the studied yeast strains. Moreover, none of the yeast strains showed inhibitory activity against LAB strains.

3.4 | Inhibition of pathogenic bacterial strains

All yeast strains isolated from intestine showed inhibition against at least one pathogenic strain. In contrast, none of the yeast strains isolated from feed showed inhibition of the tested pathogenic strains. *Kazaschtania exigua* RC035 and *Kazaschtania exigua* RC037 showed the strongest antimicrobial activity, as they were able to inhibit all the pathogens tested. Moreover, these strains were not significantly different between them. Comparing among the intestine strains, *Kazaschtania exigua* RC036 had the lowest inhibitory activity (Figure 1; Table 2).

TABLE 1 Nomenclature and identification of yeast strains ($n = 10$) with probiotic characteristics isolated from rainbow trout intestine ($n = 10$ young trouts) and fish feed ($n = 10$, 500 g samples). Other species such as *Candida parapsilopsis* and *C. tropicalis* were also isolated from fish feed but were not included in the studies as they cannot be used as probiotics for being opportunistic pathogens

Strains	Origin	Identification
RC030	Fish feed	*Debaryomyces hansenii*
RC031		
RC032		
RC033		
RC034	Intestine	*Kazaschtania exigua*
RC035		
RC036		
RC037		
RC038		
RC039		

3.5 | Autoaggregation assay

All yeast strains exhibited strong autoaggregation ability after 2 hr of incubation. Eight out of 10 strains showed autoaggregation percentages above 50% (Figure 2; Table 3).

3.6 | Co-aggregation assay

All yeast strains isolated from intestine were able to co-aggregate with at least one of the pathogens tested. *Kazaschtania exigua* RC037 and *K. exigua* RC038 were the most efficient co-aggregating *P. aeruginosa* RC001. *Kazaschtania exigua* RC039 was able to co-aggregate *E. coli* RC001. In contrast, none of the yeast strains isolated from feed (*D. hansenni*) were able to co-aggregate bacterial pathogens (Figure 3).

3.7 | Aflatoxin B_1 binding ability

The ability of yeast strains to bind AFB_1 in vitro varied among yeast strains. Adsorption percentages varied from 4% to 21%. *D. hansenii* strain RC031 demonstrated the highest absorptive capacity (21%; Figure 4).

4 | DISCUSSION

In the present study, selected probiotic properties and AFB_1-binding ability of yeast strains isolated from rainbow trout intestine and fish feed were evaluated in order to investigate their probiotic potential and the possibility to use them in the formulation of feed additives

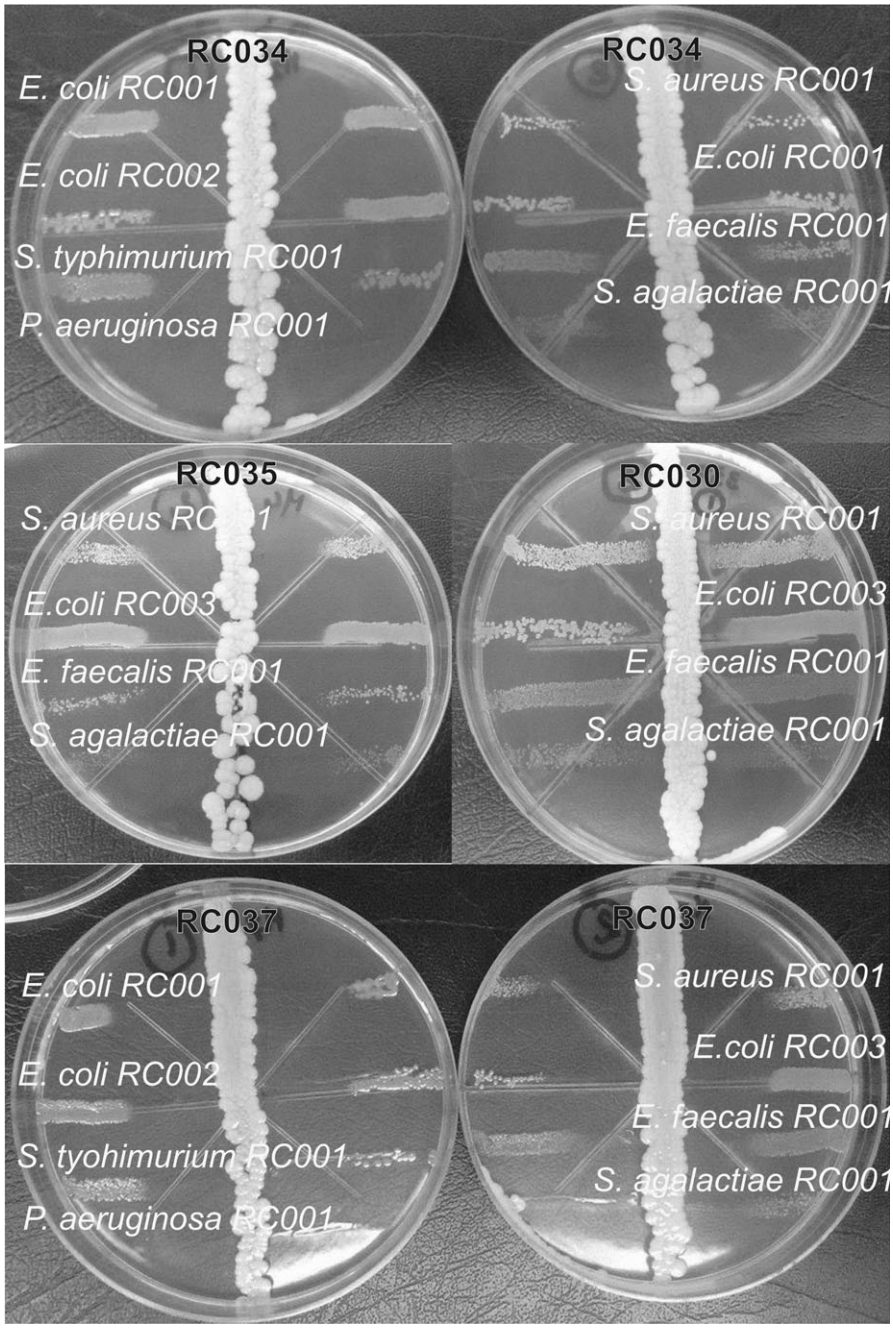

FIGURE 1 Heterologous inhibition of yeast strains against bacterial pathogens. A clear zone around the central streak of the yeast strain tested indicates the inhibition of the bacterial pathogens

TABLE 2 Heterologous inhibition of *Kazachstania exigua* strains ($n = 6$) against bacterial pathogens ($n = 7$). Diameter (cm) of clear zones (halos) in streak intersections was compared using ANOVA ($p < .0001$) and LSD Fisher's tests ($p < .0001$)

Yeast strains	Pathogen strain	Halo diameter (mean − cm)[a,b]	
Kazaschtania exigua RC034	*Escherichia coli* RC001	1.30	efgh
	E. coli RC002	1.30	efgh
	E. coli RC003	1.50	cdefg
	Salmonella typhimurium RC001	1.40	cdefgh
	Streptococcus agalactiae RC001	1.00	hijk
	Staphylococcus aureus RC001	1.00	hijk
	Enterococcus faecalis RC001	0.95	hijk
Kazaschtania exigua RC035	*E. coli* RC001	2.30	a
	E. coli RC002	2.00	ab
	E. coli RC003	1.40	defgh
	S. typhimurium RC001	2.00	ab
	S. agalactiae RC001	1.30	efgh
	S. aureus RC001	1.50	cdefg
	E. faecalis RC001	1.20	fghi
Kazaschtania exigua RC036	*E. coli* RC001	0.80	ijk
	E. coli RC002	0.60	k
	E. coli RC003	0.80	ijk
	S. typhimurium RC001	0.80	ijk
	S. agalactiae RC001	0.70	jk
	S. aureus RC001	0.55	k
	E. faecalis RC001	0.70	jk
Kazaschtania exigua RC037	*E. coli* RC001	1.90	abc
	E. coli RC002	1.80	bcd
	E. coli RC003	1.90	abc
	S. typhimurium RC001	1.60	bcdef
	S. agalactiae RC001	1.20	fghi
	S. aureus RC001	1.60	bcdef
	E. faecalis RC001	1.30	efgh
Kazaschtania exigua RC038	*E. coli* RC001	1.50	cdefg
	E. coli RC002	1.10	ghij
	E. coli RC003	1.80	bcd
	S. typhimurium RC001	1.25	efghi
	S. agalactiae RC001	1.20	fghi
	S. aureus RC001	1.85	abcd
	E. faecalis RC001	1.25	efghi
Kazaschtania exigua RC039	*E. coli* RC001	1.60	bcdef
	E. coli RC002	1.70	bcde
	E. coli RC003	1.60	bcdef
	S. typhimurium RC001	1.90	abc
	S. agalactiae RC001	1.10	ghij
	S. aureus RC001	1.50	cdefg
	E. faecalis RC001	1.20	fghi

None of the strains isolated from feed showed inhibition of the tested pathogenic strains.

[a]*SE* = 0.08.

[b]Different letters indicate statistically significant differences according to Fisher´s protected Least Significant Difference Test ($p < .0001$).

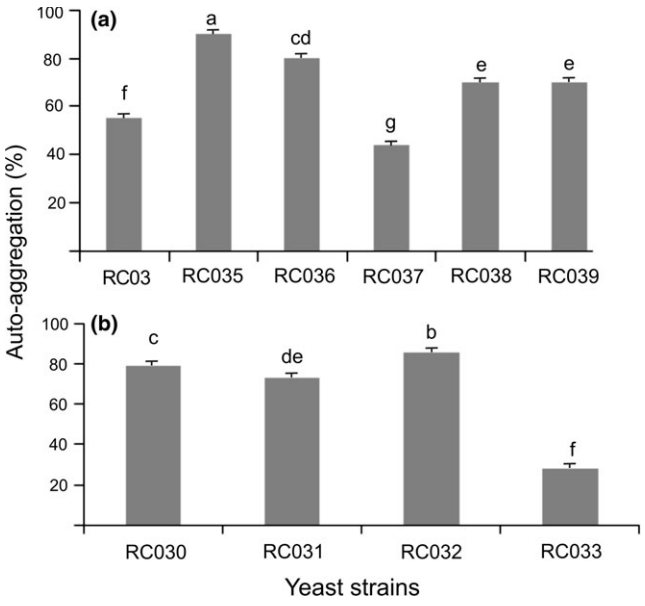

FIGURE 2 Autoaggregation percentages of yeast strains isolated from (a) healthy rainbow trout intestine and (b) fish feed. Different letters indicate statistically significant differences between autoaggregation percentages achieved by different strains according to ANOVA ($p < .0001$)

to improve productive parameters in aquaculture systems. *Kazachtania exigua* were isolated from rainbow trout intestine, whereas *D. hansenii*, *C. parapsilosis* and *C. tropicalis* were isolated from fish feed. Several studies (Aubin, Gatesoupe, Labbé, & Lebrun, 2005; Huyben et al., 2017) have indicated *Rhodotorula* spp. as the dominant intestine yeast in fish—especially rainbow trout, followed by *D. hansenii* and, in a smaller proportion, *S. cerevisiae* and *Leucosporidium* spp. (Eriksson, 2006; Wheeler et al., 2000). *S. cerevisiae* was isolated from neither intestine nor feed fish in the present study. Particularly, *S. cerevisiae* and *D. hansenii* have demonstrated to be able to colonize fish intestine after being experimentally supplied (Gatesoupe, 2007). *Kazachstania exigua* (formerly *Saccharomyces exiguus*) is generally regarded as a safe (GRAS) yeast isolated from different fermented food, feed and beverage sources such as sourdough (Pulvirenti, Solieri, Gullo, & Giudici, 2004; Sugihara, Kline, & Miller, 1970), kefir grains (Vardjan, Mohar Lorbeg, Rogelj, & Čanžek Majhenič, 2013), mezcal (Verdugo Valdez et al., 2011) and liquid piglet feeds (Gori, Bjørklund, Canibe, Pedersen, & Jespersen, 2011). It has also been associated with probiotic potential and inhibition of fungi and mycotoxin production as well as other metabolic properties and functionalities (De Vuyst, Harth, Van Kerrebroeck, & Leroy, 2016). A recent study by Bageri, Manaffar, and Rahimi (2016) is the first report of *K. exigua* as part of the intestinal flora of cultured rainbow trout.

All isolated yeast strains showed high resistance to low pH reducing only 1 log in CFU/ml counts from the initial inoculum. Parada (2013) demonstrated that probiotic *Debaryomyces* spp. could survive GIT passage and be isolated alive from salmon faeces 3 days

TABLE 3 Autoaggregation percentage (%) of yeast strains (n = 10) isolated from rainbow trout intestine (n = 10 young trouts) and fish feed (n = 10, 500 g samples). ANOVA ($p < .0001$) and LSD Fisher's tests were performed to compare medians ($p < .0001$)

Yeast species	Strain	Average autoaggregation %[a]	LSD Fisher test[b]
Kazachstania exigua	RC035	90	a
Debaryomyces hansenii	RC032	85	b
Debaryomyces hansenii	RC030	79	c
Kazachstania exigua	RC036	76	cd
Debaryomyces hansenii	RC031	73	de
Kazachstania exigua	RC038	70	e
Kazachstania exigua	RC039	70	e
Kazachstania exigua	RC034	55	f
Kazachstania exigua	RC037	44	g
Debaryomyces hansenii	RC033	28	f

[a]SE = 0.58.
[b]Different letters indicate statistically significant differences ($p < .0001$).

after administration. Firstly, in order to exert their beneficial effect after consumption, probiotic organisms need to survive the conditions of the manufacturing process of the carrier food and then, GIT conditions and ecosystem. The ability of probiotic strains to survive GIT passage can be mainly attributed to their acid and bile tolerance. This characteristic is intrinsic of strains and can be improved by the protective action of carrier foods and/or by the presence of certain nutrients such as metabolizable sugars (Corcoran, Stanton, Fitzgerald, & Ross, 2005; Valerio et al., 2006).

Composition of commercial probiotic products of veterinary and human use is variable, some comprise various different bacterial or yeast strains and others are made of a single probiotic strain (Ehrmann, Kurzak, Bauer, & Vogel, 2002). The different probiotic strains present in a single probiotic additive or functional food can colonize different sites along the intestine exerting an effective competitive exclusion of bacterial pathogens (Beasley, Manninen, & Saris, 2006). In addition, biological additives with more than one function are most likely to be composed by several strains as, frequently, not all the desirable characteristics can be achieved by a single strain.

Yeast species can synthesize substances that can result antagonistic to accompanying microbiota such as short-chain fatty acids that provide competitive advantages over bacterial pathogens (Golubev, 2006). The inhibition of bacterial pathogens, especially Gram negative bacilli, is another desirable characteristic of probiotic strains. In this work, all yeast strains isolated from trout intestine inhibited the growth of pathogenic tested strains. *Kazaschtania exigua* RC035 and *K. exigua* RC037 showed the strongest inhibitory effect against all pathogenic bacteria.

Inhibition of bacterial growth by the production of extracellular metabolites is another way of probiotic microorganisms to compete against pathogens. Rubio, Hernández, Aguirre, and Poutou (2008) observed no production of extracellular antimicrobial substances by

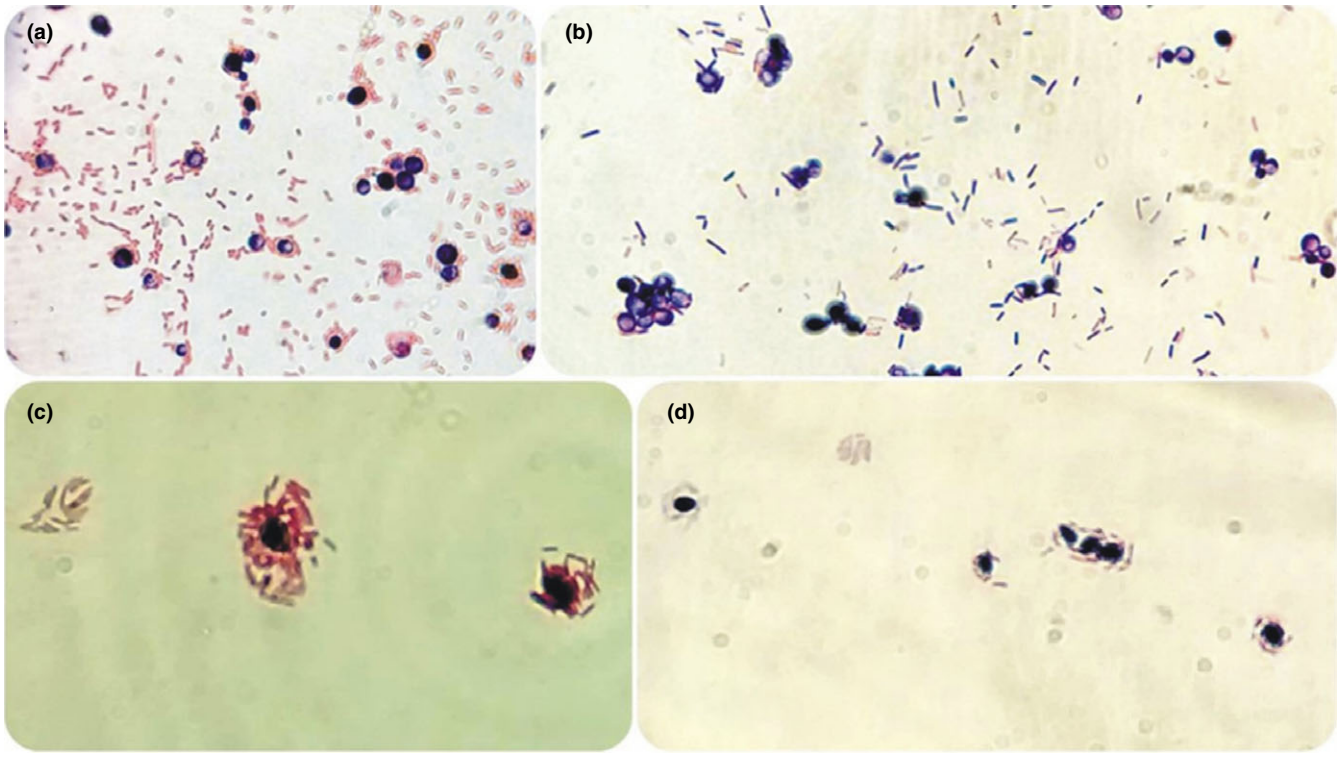

FIGURE 3 Co-aggregation of yeast strains with bacterial pathogens: (a and b) *Debaryomyces hansenii* isolated from fish feed; (c and d) *Kazaschtania* exigua isolated from healthy rainbow trout intestine. The co-aggregation was evaluated according to the presence or absence of clusters

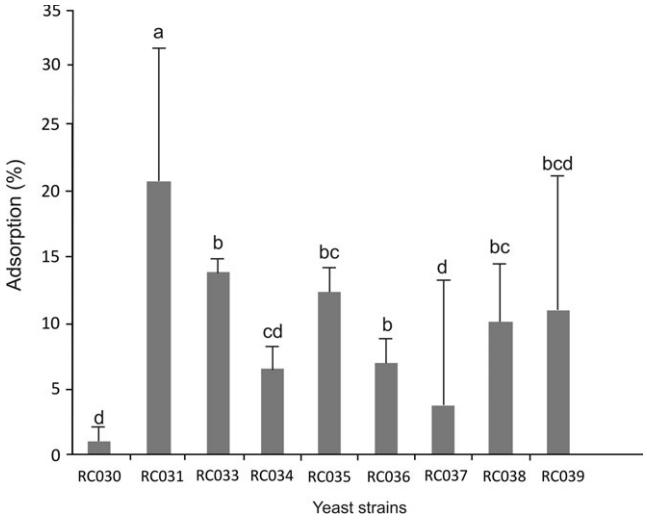

FIGURE 4 Aflatoxin B1 adsorption percentage of yeast strains isolated from rainbow trout intestine and fish feed. Different letters indicate statistically significant differences between adsorption percentages achieved by different strains according to ANOVA ($p < .0001$)

yeasts isolated from different substrates (sugar cane and grapevine) able to inhibit *Salmonella* spp., *E. coli* and *Shigella* spp. growth in vitro. On the contrary, Mantilla and Burgos Portacio (2012), who evaluated the probiotic potential of *S. cerevisiae* strains isolated from chicken faeces, observed they did not produce any antimicrobial substances that could be diffused in the culture media and inhibit bacterial pathogens' growth. Different microorganisms in the same culture may compete for growth nutrients or they may produce metabolic products that inhibit each other's growth. Yeasts may produce vitamins that enhance the growth of LAB (Narvhus & Gadaga, 2003). Therefore, it is necessary to test if these interactions occur in order to prevent inhibition in compound additives, as the composition of the currently used probiotic products usually contain more than one strain (Ehrmann et al., 2002). In the present study, the antimicrobial activity results indicated none of the yeast strains showed inhibitory activity against LAB, indicating that—if the strains show beneficial probiotic properties and they demonstrate no toxicity in further studies—they could be included together in the formulation of mixed feed additives. Similarly, Gadaga, Mutukumira, and Narvhus (2001) studied the interaction of paired co-cultures of nine yeast strains and four LAB strains in milk. The growth of two *Lactococcus* strains was not affected by the co-culture with yeasts, while a third strain grew significantly better ($p < .01$) along with five of the yeast strains tested.

The ability to aggregate and adhere to epithelial cells is a major criterion in the selection of probiotic candidates because they are prerequisite for colonization and has been correlated with adhesion to intestinal epithelial cells (Jankovic, Frece, Abram, & Gobin, 2012). Aggregation is the process of reversible accumulation of cells that causes them to precipitate spontaneously in the medium in which they are suspended (Fletcher, 1987; Gobin, 2011). Autoaggregation is the clumping of yeasts of the same strain, while co-aggregation is the result of cell-to-cell recognition between two different strains. In the present work nearly half of the strains isolated from trout intestine were able to co-aggregate at least with one bacterial pathogen. Pizzolitto et al. (2011) showed that the capacity of yeasts to bind to a microorganism varied according to the yeast strain and the microorganism involved. The co-aggregation ability of a probiotic strain can enhance the formation of a barrier that may prevent the colonization of the intestine by pathogenic bacteria (Collado, Meriluuoto, & Salminen, 2007; Jankovic et al., 2012; Kos et al., 2003). Rubio et al. (2008) qualitatively evaluated the development of *S. cerevisiae* autoaggregates and the pathogenic bacteria around them after 24-hr incubation at 37°C and supposed that this fact would avoid the pathogens adherence to the intestine epithelium.

Trout intestine strains autoaggregate more efficiently. There are no previous studies that inform autoaggregation among yeast strains isolated from fish intestine to date. However, results obtained in the present study can be compared with those reported by Sourabh, Kanwar, and Sharma (2011) who showed autoaggregation lower than 70% of yeast strains isolated from fermented foods. In other animal species such as pigs, Armando et al. (2011) reported autoaggregation percentages higher than 90%.

The ability of yeast strains to bind AFB_1 was also evaluated. AFB_1 adsorption percentages varied from 4% to 21% shown and were comparable to those described by Shetty et al. (2007) who found 10%–40% adsorption in *S. cerevisiae* strains isolated from indigenous fermented foods. Other authors have found high binding percentages using yeast strains (70%–90%) isolated from pig and pet intestines (Fernandez Juri, 2011; González Pereyra et al., 2008).

The present study demonstrated the ability of yeast strains that are member of the native microbiota of healthy animals, to exert beneficial probiotics effects. Besides, some of them such as *D. hansenii RC031* and *K. exigua* RC036 possessed AFB_1 adsorptive ability, another property that could alleviate the toxic effects of chronic mycotoxicosis in fish by reducing the bioavailability of the mycotoxin in the GIT.

The presence of mycotoxins in feed for aquatic animals can significantly reduce growth, feed consumption, survival and increase the feed conversion rate as well as cause alterations in the immune system, which may be responsible for considerable economic losses (Tapia-Salazar et al., 2010). This fact also indicates that could be included together in the formulation of mixed feed additives excretion in faeces (Bueno et al., 2007).

The results obtained with these strains propitiate the development of future studies in order to formulate biological feed additives aimed to improve productive parameters in aquaculture systems to reduce the use of antibiotics as growth promoters in fish production.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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