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Evaluation of *Lactobacillus paracasei* subsp. *tolerans* isolated from Jenyn's sprat (*Ramnogaster arcuata*) as probiotic for juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)

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Summary

A lactic acid bacterial strain, *Lactobacillus paracasei* subsp. *tolerans* F2, isolated from the intestine of *Ramnogaster arcuata*, was evaluated as a growth promoter in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum, 1972) farming. In addition, the safety of the strain was assessed according to the FAO recommendations. Strain F2 was susceptible to most antibiotics tested and no evidence of hemolytic activity was found. When the strain of *Lactobacillus paracasei* subsp. *tolerans* F2 was administered with food, no adverse effects on health were observed and fish biomass increased 12% more in the treatment group than in the control group. Significant differences were detected in the specific growth rate and feed conversion ratio. In the group receiving *Lactobacillus paracasei* subsp. *tolerans* F2-supplemented feed, quantitative differences in the microbial composition of fish feces with respect to the control group were observed. An important decrease in fungi and enterobacteria was observed in feces from the treatment group, coincident with an increase in lactic acid bacteria. This result would indicate a change in the composition of the intestinal microbiota of fish treated with the putative probiotic. These results suggest that the strain of *Lactobacillus paracasei* subsp. *tolerans* F2 has the application potential to improve the performance in rainbow trout farming.

Introduction

With the increasing intensification and commercialization of aquaculture production, infectious disease problems have inevitably emerged (FAO, 2006; Lara-Flores, 2011). A number of approaches have been applied in an attempt to address the disease problem, including vaccines, disinfection and chemotherapy, with particular emphasis on the use of antibiotics (Ringø et al., 2010a). Although vaccines are being developed and marketed, they cannot be used as a universal disease control measure in aquaculture. The use of antibiotics to treat bacterial infection and prevent fish mortality in aquaculture is becoming limited as the pathogens develop resistance to the drugs (Gómez-Gil et al., 2000; González et al., 2000). The massive use of antimicrobials for disease control and growth promotion is on the wane due to the appearance and spread of resistance among harmful microorganisms.

Given the importance of nutrition in maintaining the health of fish, with respect to nutritional involvement on immuno-competence and disease resistance, as well as its role in stress mediation, there is a growing trend towards exploring new strategies in feeding and health management in fish aquaculture (Balcázar et al., 2006a; Lara-Flores, 2011). In addition, the global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies to promote health and growth by using probiotics and other functional dietary supplements (Denev, 2008; Merrifield et al., 2010; Ringø et al., 2010b).

Probiotics, according to the currently adopted definition by the Food and Agricultural Organization and World Health Organization (FAO/WHO, FAO, 2002) are 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'. In aquaculture systems the immediate environment has a great influence on the health status of the host, since those microorganisms present, for example in the intestine of the fish, are the same as those found in their integument, gills, in the environment, and even in the food (Hansen and Olafsen, 1999; Lara Flores et al., 2003). Perhaps the most encompassing definition was proposed by Verschuere et al. (2000) as: a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment.

Of note is that probiotics increase fish growth through an efficient use of the food. These microorganisms synthesize extracellular enzymes such as proteases, amylases and lipases as well as provide the host with vitamins, aminoacids and fatty acids (Martínez Cruz et al., 2012).

Interest in probiotics in aquaculture, particularly in rainbow trout farming, has focused on lactic acid bacteria (LAB), most notably *Lactobacillus* spp., *Carnobacterium* spp., *Enterococcus faecium* and *Pediococcus* spp. (Robertson et al., 2000; Nikoskelainen et al., 2001a,b; Spanggaard et al., 2001; Panigrahi et al., 2005; Kim and Austin, 2006; Balcázar et al., 2007a,b; Brunt et al., 2007; Gatesoupe, 2008; Merrifield et al., 2009). Regarding aquaculture product safety, there is a great concern that commensal bacterial populations from food and the gastrointestinal tract (such as LAB) that are regarded as probiotics could act as a reservoir for antibiotic-resistant genes. Resistance could ultimately be transferred to fish and human pathogenic and opportunistic bacteria, hampering the treatment of infections (Ammor et al., 2007; Egervärn, 2009; Mayrhofer et al., 2010).

The study of probiotics has had a significant development; however, there are few studies on the probiotic potential of LAB isolated from fish of the South Atlantic Ocean and its coastal environments. In a previous study we isolated and identified 22 LAB strains from the sediments and fish from Bahía Blanca Estuary (Buenos Aires Province, Argentina); this research provided the first report on LAB from a coastal environment of South Atlantic Ocean (Sica et al., 2010; Lauzon and Ringo, 2011). Sica et al. (2012) also reported the presence of the probiotic properties of these isolates. Based on the results of these previous studies, one strain isolated from the gut of Jenyn's sprat (*Ramnogaster arcuata*), given the name F2 and identified as *Lactobacillus paracasei* subsp. *tolerans*, was selected for the present study.

The goals of this study were to evaluate the safety of the F2 strain for application in rainbow trout culture and its effects on fish growth parameters when the strain is provided in the diet.

Materials and methods

Bacterial strain

The strain F2, *Lactobacillus paracasei* subsp. *tolerans,* was isolated from the digestive tract of *Ramnogaster arcuata* (*Osteichthyes*, *Clupeidae*) captured in Bahía Blanca Estuary (38°45′–39°25′S; 61°15′–62°30′W) (Argentina) (Sica et al., 2010). The 16S rRNA gene sequences (~1400 bp) were analyzed and showed a 99.9% sequence homology to *Lactobacillus paracase* subsp. *tolerans* JCM 1171T (D16550). The sequence was deposited under the accession number FJ892732 (http://www.ncbi.nlm.nih.gov). The isolation methodology and molecular identification are described in Sica et al. (2010). The strain was selected from 22 candidates for its probiotic properties (Sica et al., 2012).

The strain was stored in Man, Rogosa and Sharpe broth (MRS, BK 070HA; Biokar Diagnostics, Beauvais, France) (De Man et al., 1960) at −70°C supplemented with 20% v v^{-1} glycerol.

Evaluation of safety: antibiotic susceptibility and hemolytic activity

Antibiotic susceptibility profile of strain F2 was studied by employing the method described by Bauer et al. (1966) for clinical isolates, modified by using MRS agar MRS broth amended with 1.2% of purified agar-agar (Merck, Darmstadt, Germany). Clinical and Laboratory Standards Institute (CLSI) type strains, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 from the American Type Culture Collection, were employed as reference strains. All microorganisms were stored in milk-yeast extract at −70°C and cultured in MRS broth prior to the assays.

The suspensions were prepared in Phosphate Buffer Saline (PBS: 150 mM NaCl, 5 mM KH_2PO_4, 5 mM K_2HPO_4, pH 7.2), adjusted to an optical density equivalent to 0.5 turbidity on the McFarland scale and the microorganisms disseminated on the surface of MRS agar plates with embedded swabs.

The antibiotics discs (Britania Laboratories S.A., Argentina) used were: cefotaxime (30 μg), clindamycin (2 μg), erythromycin (15 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg), trimethoprim- sulfamethoxazole (25 μg), penicillin G (10 IU ml^{-1}), cephalothin (30 μg), tetracycline (30 μg), cefuroxime (30 μg), vancomycin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg) and ampicillin (10 μg). Antibiotic discs were placed on the surface of the agar (six discs per plate) and the plates incubated for 24–48 h at 37°C at reduced oxygen tension (Ocaña et al., 2006). The inhibition zone diameters were measured using a sliding caliper and interpreted as resistant (R), intermediate (I), or sensitive (S), according to the CLSI recommendations for Gram-positive bacteria (CLSI, 2005).

For hemolysin production tests, the F2 strain was cultured in MRS broth then streaked on Columbia agar plates (Britania Laboratories, S.A., Argentina) containing 5% v v^{-1} sheep blood. The plates were incubated for 24 h at 37°C (Maurad and Meriem, 2008; Mokhbi et al., 2009). Strains that did not show clear zones around the colonies were classified as non-hemolytic.

The antibiotic susceptibility and hemolytic activity tests were performed in triplicate.

Experimental diet preparation

To provide a viable and stable stock of F2 strain to prepare the experimental diet, putative probiotic cultures were freeze-dried. For this purpose the F2 strain was cultured in MRS broth at 25°C for 48 h. The cells were then harvested by centrifugation at 1200 ***g*** for 10 min and the cell pellet subsequently washed twice with sterile PBS. The washed cells were suspended in 1 ml sterile rehydrated skim milk powder (100 g L^{-1}) as cryoprotectant. Cell density in the suspension was of 10^{12} Colony Forming Units per ml (CFU ml^{-1}). The suspension was frozen at −70°C and lyophilized (Rificor Mod. L-A-B4, Argentina) (Berner and Viernstein, 2006; Kõs et al., 2008; Ming et al., 2009). The viable cell number was monitored periodically by plate counting on MRS agar under reduced oxygen tension.

Commercial trout feed (3 mm pellets, Pilar Company, Argentina, 2700 Kcal kg^{-1} dry matter) was taken as the basal diet to carry the F2 strain; a freeze-dried F2 culture was rehydrated daily with 1 ml PBS. To remove the milk, the cell suspension was centrifuged at 1200 ***g*** for 10 min and the supernatant discarded. Subsequently, the cell pellet was

washed twice by centrifugation with PBS and suspended in 10 ml PBS. The bacterial suspension was then slowly added to the daily ration of commercial feed by gently spraying, mixing it manually little by little and dried at 25°C for 1 h (Balcázar et al., 2007b; Ghosh et al., 2007; Panigrahi et al., 2010). Feed inoculated with F2 was prepared daily. The final F2 viable count in the food was 10^6 CFU g^{-1} of dry food, monitored daily by plate counting on MRS agar. The plates were incubated at 25°C and reduced oxygen tension. Commercial feed with no inoculating was used as control. Due to the very low contribution of nutrients to the bacterial biomass, control and supplemented feed were considered isocaloric.

Feeding trial design

Rainbow trout *Oncorhynchus mykiss* (Walbaum) juveniles (31.25 ± 3.43 g) were reared at an experimental fish farm (Municipality of Necochea, Argentina) and acclimated to our experimental system for 2 weeks before beginning the trial.

Two intensive recirculating systems were employed. Each system included three self-cleaning circular tanks (water volume 0.35 m^3 each), a settling tank with conical bottom (0.03 m^3), one tank provided with a biofilter (1.5 m^3), a water pump, a UV-light unit and a chiller. Each biofilter tank contained submerged net bags; each one contained corrugated plastic pipes cut into pieces 2 cm long and 0.5 cm diameter, to increase the contact surface for the formation of biofilms (total surface of biofilter, 62 m^2). Water was pumped to the top of the biofilter tanks, where it was dispersed via a spray-bar and allowed to trickle by gravity throughout the biofilters. Total water volume of each system was 2580 L. The water flow rate in each fish tank was set at 0.1 L s^{-1}. Water temperature was maintained at 14 ± 1°C and oxygen levels above 80%. Fish were held under a natural photoperiod of latitude 39°S. Water exchanges, approximately 15% of the system volume, were conducted every 72 h.

Each of six tanks was randomly stocked with 28 fish. One system (three tanks) was employed for the probiotic treatment (commercial feed inoculated with the F2 strain) and the other system was used as control (commercial feed).

Fish were fed 2% (dry weight) biomass per day provided in equal rations at 09:00 and 14:00 h for 66 days. The ration was estimated following Leitritz (1959) for dry food, taking into account the body wet weight of the fish. The ration was daily increased assuming conversion 1 : 1. The feed pellets were administered manually so that they were completely consumed and to avoid drift losses.

Fish survival and growth parameters

Trout survival was calculated using the formula% $S = (FN/IN) \times 100$ where FN: final number of individuals and IN: initial number of individuals. Growth performance was assessed in terms of mean weight gain, specific growth rate (SGR) and feed conversion ratio (FCR). The calculation was performed using the formula: $SGR = 100 \times [(\ln FW - \ln IW)/T]$; FCR = FI/WG, where FW is the final weight; IW is the initial weight; T is the duration of the experiment (in days); WG is the wet weight gain; and FI is the feed intake.

Microbiological analysis of solid wastes

Solid wastes, mainly composed of feces, were analyzed to indirectly assess changes in the intestinal content of reared trout due to the treatment. Once a week before feeding the fish and cleaning the settling tanks, solid wastes samples were taken from the bottom of the settling tanks from both systems. Consisting of the accumulated feces over 24 h, the samples were kept in sterile glass bottles, transported to the laboratory at 4°C, and processed immediately. Ten ml of the solid wastes were centrifuged at 1200 ***g*** for 1 min. One gram of the resulting pellet was suspended in 9 ml of sterile distilled water and successive decimal dilutions were prepared. Viable enterobacteriaceae, fungi and LAB were quantified by the plate-counting method. Enterobacteriaceae counts were carried out on Violet Red Bile Dextrose Agar (VRB dextrose-agar, Biokar, France), and incubated aerobically at 37°C for 24 h. Enumeration of molds and yeast was carried out on Potato-Dextrose-Agar (PDA, Merck, Germany) with the addition of 10% citric acid until a final pH of 3.5 then incubated at 25°C over 5 days. The LAB count was carried out on MRS Agar and incubated at 25°C for 72 h under reduced oxygen tension.

A portion of centrifuged solid wastes was dried at 105°C in aluminum boxes, until a constant weight. The boxes were weighed on a precision balance (Ohaus, Pioneer 214, China) and water content of the waste calculated. The counts were transformed to $\log_{10}$ and expressed as Log_{10} CFU g^{-1} of dry waste.

Statistical analysis

The growth performance results are reported as the mean ± standard deviation (SD). Mean values were compared using one-way ANOVA (n = 3). Differences at $P < 0.05$ were considered significant. When significant differences were detected on growth parameters, the effect of size (ES) was calculated, where a value between 0.2 and 0.5 corresponded to a small ES, between 0.5 and 0.8 to a medium ES, and >0.8 to a large ES (Cohen, 1992). To determine the presence of differences in the microbial groups quantified from detritus, a two-way ANOVA without replication was carried out.

Results

Evaluation of safety: antibiotic susceptibility and hemolytic activity

No evidence of hemolytic activity was found in strain F2.

Considering that the zones of inhibition obtained with reference strains tested were comparable to the reference values to determine if the strain F2 was susceptible or resistant, the halos were compared with inhibition halos suggested for the genus *Enterococcus* sp. except for cephalosporins, clindamycin and gentamicin, which were compared with those of the genus *Staphylococcus* sp. (CLSI, 2005). The F2 strain was

Table 1
Growth parameters of rainbow trout after 66 days feeding on *Lactobacillus paracasei* subsp *tolerans* F2-supplemented diet (mean ± SD)

	Control	Experimental diet	P*	ES
Initial mean weight (g)	31.25 (±3.43)	31.20 (±3.15)	0.90	–
Final mean weight (g)	97.80 (±14.86)	101.41 (±14.67)	0.014	1.3
Mean weight gain (%)	213 (±0.67)	225 (±5.5)	0.007	5.0
Specific growth rate (% day^{-1})	1.71 (±0.03)	1.81 (±0.03)	0.008	3.3
Feed conversion ratio (g food g^{-1} fish)	1.25 (±0.10)	1.18 (±0.13)	0.03	1.2
Survival (%)	100	98.8 (±2.06)	0.80	–

*Significance accepted at $P < 0.05$ level. ES: effect of size; values of 0.2–0.5, small ES; 0.5–0.8, medium ES; >0.8 = large ES.

susceptible to all antibiotics tested except vancomycin, trimethoprim-sulfamethoxazole and gentamicin.

Feeding trial

Fish fed with *Lactobacillus paracasei* subsp. *tolerans* F2-supplemented feed showed normal behavior during the experiment and no cannibalism was observed. No adverse effect was attributed to the addition of the putative probiotic. Growth performance of rainbow trout after 66 days feeding on the experimental diet is shown in Table 1.

Microbiological analysis of solid wastes

Enterobacteriaceae, fungi and LAB viable counts from the solid wastes collected from the settling tanks of each system are shown in Figs 1, 2 and 3. In the solid wastes of the treatment system the counts of enterobacteria and fungi were significantly lower than in control as of the third and fourth week of treatment ($P = 0.003$ and $P = 0.0005$, respectively) (Figs 1 and 2). The decline of these microbial groups coincided with significantly higher counts of LAB in solid waste from the treated fish ($P = 0.01$) in the fourth week (Fig. 3).

Discussion

The interest in probiotics as an environmentally friendly alternative is increasing, with an application that is both empirical and scientific. The main strategy in use today is the supplementation of the probiotic bacteria in the feed of fish, in order to improve the growth performance. The positive effect of probiotics depends on both the action mechanisms and the capacity of colonization, that is to say its ability to reach, remain or reproduce in the place where the effect is required. The appropriate use of probiotics in the aquaculture industry has been shown to enhance intestinal microbial balance, and also improve nutrient availability and absorption, thus leading to an increased growth rate and reduced feed conversion ratio (Adineh et al., 2013).

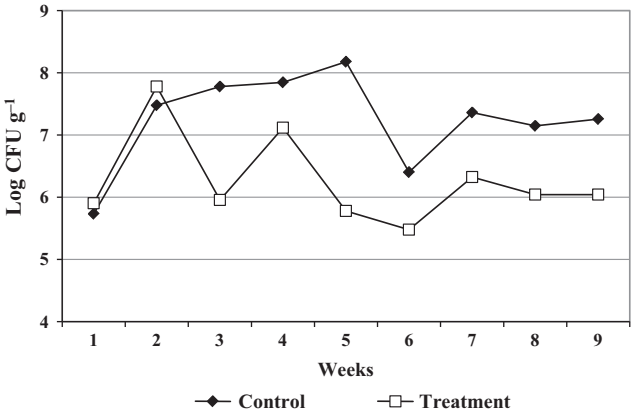

Fig. 1. *Enterobacteriaceae* count in solid wastes from settling tanks. Results expressed as decimal logarithms of colony-forming units per gram of dry waste (Log CFU g^{-1})

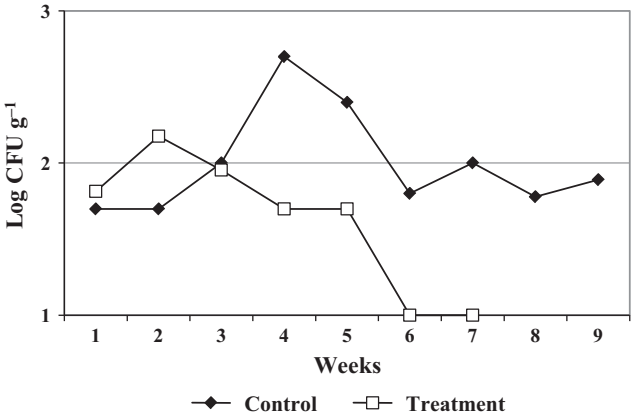

Fig. 2. Fungi count in solid wastes from settling tanks. Results expressed as decimal logarithms of colony-forming units per gram of dry waste (Log CFU g^{-1})

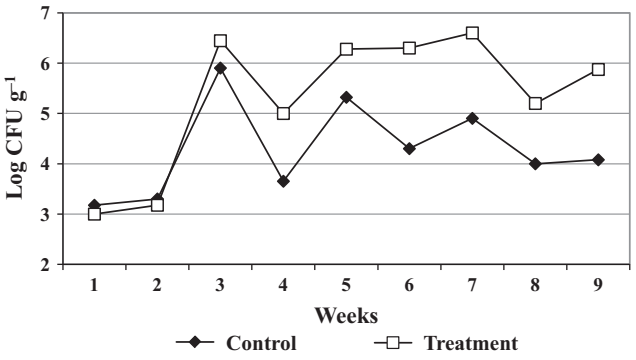

Fig. 3. LAB count in solid wastes from settling tanks. Results expressed as decimal logarithms of colony-forming units per gram of dry waste (Log CFU g^{-1})

Lactobacillus paracasei subsp. *tolerans* F2 is a wild strain isolated from *R. arcuata*, a fish species that spends its entire life cycle in the Bahía Blanca Estuary, which receives the impact of the municipal waste from Bahía Blanca city.

Although *L. paracasei* has a long record of safe usage (Bernardeau et al., 2006; Tsai et al., 2008), F2 is not a commercial strain and does not come from a culture collection. Hence, before the F2 strain could be proposed as a probiotic for rainbow trout culture, it was necessary to test some safety aspects such as hemolytic activity and antibiotic susceptibility (Verschuere et al., 2000; FAO, 2002; Balcázar et al., 2006a; Merrifield et al., 2009).

Lactic acid bacteria (LAB) are commonly used as food processing aids and probiotics, and have a long history of safe use. However, due to their genetic flexibility and widespread occurrence in the food chain and in the intestinal tract, LAB can act as potential reservoirs of antibiotic resistance genes that may be transferred to other bacteria, including human pathogens. Thus, a key requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes (Salminen et al., 1998; Zhou et al., 2005). Several reports are available on the susceptibility of LAB to antibiotics of diverse origins; however, only a few reports can be found on isolates from fish and aquatic environments (Charteris et al., 1998; Salminen et al., 1998; Zhou et al., 2005). Our results indicated that the F2 strain was susceptible to most of the antibiotics tested, including some of the most common antimicrobials used in fish therapy. The F2 strain was resistant to trimethoprim-sulfamethoxazole; however, this resistance may be due to the presence of reactive levels of thymidine or thymine in MRS medium that will antagonize the action of trimethoprim (Danielsen et al., 2004). The strain was also resistant to vancomycin and gentamycin, supporting the usually intrinsic resistance of some *Lactobacillus* species to these antibiotics, which are chromosomally encoded and non-transmissible (Zhou et al., 2005; Otero et al., 2007; Córdoba et al., 2009). Therefore, *L. paracasei* subsp. *tolerans* F2, if administered to fish, will not contribute to a spread of resistance against these antibiotics to the host and to aquatic microbiota. No evidence of hemolytic activity was found in strain F2, an observation that was in agreement with other studies showing that hemolysis is rarely present in the genus *Lactobacillus* (Dávila et al., 2006; Maragkoudakis et al., 2006).

Despite an increasing body of data regarding the rainbow trout health-promoting properties of probiotic species, information is lacking concerning the effects on growth performance and feed utilization of juvenile trout (Merrifield et al., 2009); no information is available on the probiotic potential of LAB strains from the coastal environment of the South Atlantic Ocean.

We supplied feed inoculated with a strain of *L. paracasei* subsp. *tolerans* F2 isolated from *R. arcuata,* to juvenile rainbow trout. The inoculant density of 10^6 CFU g^{-1} of food was considered adequate because it was between the doses recommended by other authors (Balcázar, 2006; Balcázar et al., 2006b; Pérez-Sánchez et al., 2011).

The fish survival results demonstrated that after 66 days consumption of the strain F2 there were no adverse effects regarding their health. The significant improvement in growth performance and in feeding conversion when the fish were fed with a F2-suplemented diet indicated that the strain promotes a better utilization of the feed (Table 1). Slight improvements in feed efficiency can result in an important reduction in the production costs.

During most of the experiment a clear quantitative difference regarding the studied microorganisms in feces was observed between the treated and the control group. Significantly higher counts of LAB observed in the detritus from the treated fish as of the fourth week of the experiment could only be explained by the survival and colonization of F2 in the trout gastrointestinal tract (Fig. 3). In the same period of the trial, the counts of enterobacteria and fungi in the detritus from the treatment system were significantly lower than in control (Figs 1 and 2, respectively). These differences could be caused by an antagonistic effect of strain F2 against these groups of microorganisms. Several authors have demonstrated LAB competitive exclusion of many related and unrelated microorganisms, including pathogenic agents associated with aquaculture (Gatesoupe, 1999; Magnusson et al., 2003; Balcázar et al., 2006a, 2008; Sica et al., 2010, 2012).

On the basis of the basic safety aspects determined, such as absence hemolytic activity and high antibiotic susceptibility, and the observed improvement in growth performance, encourage us to conclude that the use of the *L. paracasei* subsp. *tolerans* F2 has a good prospective for application in the juvenile rainbow trout culture.

Future studies are necessary to optimize dosage rates, supplementation form and/or timing of feeding. Special attention should be focused on expanding the study on immunological effects.

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