The effects of diet supplemented with *Lactobacillus rhamnosus* on tissue parameters of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Natalija Topic Popovic[1], Ivancica Strunjak-Perovic[1], Roberta Sauerborn-Klobucar[1], Josip Barisic[1], Margita Jadan[1], Snjezana Kazazic[2], Inga Kesner-Koren[4], Andreja Prevendar Crnic[3], Jelena Suran[3], Blanka Beer Ljubic[3], Vesna Matijatko[3] & Rozelindra Coz-Rakovac[1]

[1]Laboratory for Ichthyopathology-Biological Materials, Rudjer Boskovic Institute, Zagreb, Croatia

[2]Laboratory for Chemical Kinetics and Atmospheric Chemistry, Rudjer Boskovic Institute, Zagreb, Croatia

[3]Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[4] Dukat Dairy Industry Inc./Lactalis SE Europe, Product Development Department, Zagreb, Croatia

Correspondence: I. Strunjak-Perovic, Rudjer Boskovic Institute, Bijenicka 54, Zagreb, Croatia. E-mail: strunjak@irb.hr

Running title: Effects of *L. rhamnosus* on rainbow trout

Abstract

This study was carried out in order to establish the effects of a 6-week treatment with the diet supplemented with *L. rhamnosus* in concentrations of 10^7 CFU g^{-1} (G1 group) and 10^8 CFU g^{-1} (G2 group) on the condition expressed by condition factors (Fulton's, Clark's and *B*), intestinal microbiology, haematological, histological, and selected antioxidative parameters of rainbow trout. A significantly higher condition factors was found in G1 group indicating that higher concentration of probiotic (10^8 CFU g^{-1}) did not result in the better condition. Cholesterol and urea levels were significantly higher in both G1 and G2 groups, albumin in G1 and creatinine in G2 group with respect to control. A significantly higher liver TBARS level was observed in G2 group. The feeding with supplemented probiont apparently changed the resident microbiota. Three weeks after withdrawal of the supplemented feed, the microflora mostly reverted to the control composition, although *L. rhamnosus* in fecal matter of fish remained inherent. The epithelial structure of the proximal and distal intestine revealed the increased absorptive area in both treated groups, as well as the increase of the mucin-secreting goblet cells. The *L. rhamnosus* treated groups demonstrated the capacity for the augmentation of the innate host defense.

Keywords: *L. rhamnosus*, trout, blood, tissue responses

Introduction

The need for sustainable aquaculture has prompted research regarding the use of probiotics on aquatic organisms for various reasons: promoting growth, improving animal health, inhibiting pathogens, improving reproduction, enhancing stress tolerance, to name the most important traits. Various works evaluated the feasibility of supplementing fish diets with potentially probiotic bacterial strains (Aguirre-Guzman *et al.* 2012; Martinez Cruz *et al.* 2012; Al-Dohail *et al.* 2011; Goncalves *et al.* 2011; Merrifield *et al*. 2010a; Nayak 2010; Frouel *et al.* 2008; Pirarat *et al.* 2006; Nall *et al.* 2004; Panigrahi *et al.* 2005, 2004; Nikoskelainen *et al.* 2003). Along with several favorable criteria for potential probionts, such as ability to adhere and grow within intestinal mucus, ability to colonize the intestinal epithelium, antagonistic properties towards pathogens etc., there are three traits that are essential: 1. they must not be pathogenic, not only with regards to the host species, but also with regards to aquatic animals in general and human consumers; 2. they must be free of plasmid-encoded antibiotic resistance genes; and 3. they must be resistant to bile salts and low pH (Merrifield *et al.* 2010a). Interaction with mucus is the first step in adhesion of bacteria to the intestinal mucosa and other mucosal surfaces. *L. rhamnosus* ATCC 53103 bounds at similar levels to fish mucus as to human intestinal mucus (Ouwehand *et al.* 1999) and penetration through the mucus layer is an important property since the intestinal mucus layer is constantly being synthesized and sloughed off. *L. rhamnosus* was found to easily penetrate and colonize deep within the mucus layer, quicker than the other probiotic bacteria (Nikoskelainen *et al.* 2001a).

Several members of genus *Lactobacillus* have been used as probionts for fish, and previous works (Table 1) provided an array of data on *L. rhamnosus* immune

stimulation and improved survival after challenge with pathogens (Nikoskelainen *et al.* 2003, 2001b), immuno-regulatory stimulation, modulation, and expression of cytokine genes (Panigrahi *et al*. 2007, 2005, 2004), protective effects against induced infections (Pirarat *et al.* 2006), stress coping capacity enhancement (Goncalves *et al*. 2011), fecundity increase (Gioacchini *et al.* 2010). Few studies have, however, been directed at the *L. rhamnosus* influence on the blood biochemistry parameters (Panigrahi *et al.* 2010) its oxidative stress attenuation capacity, and effects on intestinal epithelial structure.

Blood biochemistry reflects physical and chemical changes in organisms, indicating a general metabolic and physiological status. Although various factors (including differences in species, age, sex, water quality, water temperature and handling methods) may contribute to variability in biochemical data that is difficult to interpret, its measurement is a commonly used diagnostic tool in biomonitoring and may be useful for research on nutrient requirements, new diet ingredients and additives, artificial feed effects, respectively (Maita 2007; Coz-Rakovac *et al.* 2008). Also, activities of the antioxidant defense enzymes are often used as sensitive biochemical indicators of fish health status. In this study fish blood biochemistry parameters were assessed, including metabolites and proteins/enzymes, as well as fish oxidative stress parameters (glucose (GLU), urea (URE), creatinine (CRE), cholesterol (CHOL), triglyceride (TRIG), total proteins (TP), albumin (ALB) and activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and paraoxonase activity (PON 1)). Measured blood parameters were correlated to different

concentrations of *L. rhamnosus* in diets of rainbow trout *Oncorhynchus mykiss* (Walbaum).

Lipid peroxidation (LPO) in fish, measured as thiobarbituric acid reactive substances (TBARS), has also been frequently used as a marker of oxidative stress in response to different environmental pollutants (Stepic *et al.* 2012). In addition, TBARS can be used as a marker of increased metabolic and immunological activity (Lushchak 2011). We measured TBARS levels in trout livers to evaluate the adaptive response of liver activity at the end of the feeding period with *L. rhamnosus*.

The purpose of this study was to evaluate the effects of a six-week treatment with the diet supplemented with *L. rhamnosus* ATCC 53103 on condition, intestinal microbiology, haematological, histological, and selected antioxidative parameters of rainbow trout. This paper presents new insights on probiont effect assessment, given that only limited amount of information exists with respect to its potential effects on a number of biological responses.

Material and methods

Bacterial strain, culture and harvest

The bacterium *L. rhamnosus* (ATCC 53103) was used as a freeze-dried powder. The bacterium was cultured in MRS (Oxoid, UK) broth (De Man *et al.* 1960) by cultivating it for 48 h at 37°C. The viability of bacteria was determined by plate counting on MRS agar (Oxoid, UK). The confirmation of the bacterial strain was based on colony and cell morphology, Gram staining, biochemical testing by API 50CH system (BioMerieux, France) and the matrix assisted laser induced desorption ionization (MALDI) connected to the time of flight (TOF) mass spectrometry (MS) channel (Bruker Daltonics, MA,

USA). Subsequently, cultured bacteria were preserved by freezing in glycerol at -85°C. A solution of 30% glycerol (v/v) was prepared by mixing 30 mL glycerol (Gram-Mol, Croatia) with 70 mL deionized water, sterilized by autoclaving at 121°C for 15 min. Equal amounts of 30% glycerol and culture broth were mixed, dispensed into tubes and then frozen.

Diet formulation

The commercial rainbow trout feed (Optiline 2P, Skretting, The Netherlands) was taken as the basal diet for the supplementation of the probiont *L. rhamnosus*. The diet was supplemented with the bacterium to attain two diet groups with concentrations of 10^7 colony-forming units per gram of feed (CFU g^{-1}) (Group 1, G1) and 10^8 CFU g^{-1} (Group 2, G2), respectively. The probiotic-supplemented diet was prepared according to Panigrahi *et al.* (2005). Bacteria were grown in MRS broth over 48 hours in a shaking incubator at 37°C. After incubation, the cells were harvested by centrifugation at 5 000 *g* for 5 min, washed three times with buffer (sterile peptone water: NaCl 0.85% and polypeptone 0.1%) (Kemika, Croatia), and resuspended in the same buffer. Bacterial pellets were measured in the buffer and their densities were determined (Densimat, BioMerieux, France) by converting the measured McFarland scale into bacterial concentration.

The pre-trial feed batch was sprayed with bacterial suspension, mixed manually, air dried on a clean bench for 24 hours and then frozen at -20°C. After several days in the freezer, the feed was defrosted and stored at 4°C. The viability of incorporated *L. rhamnosus* was tested by vortexing 10 g of diet in 90 mL of sterile peptone water, preparing serial dilutions from 10^{-1} to 10^{-12}, and spreading 0.1 mL of each dilution onto

MRS agar. The colony count was determined after 48 hours at 37°C. According to the colony count, the viability and survival rate of *L. rhamnosus* was established and required density to be sprayed on the trial feed adjusted to 10^9 and 10^{10} CFU g^{-1} to finally accomplish desired concentrations in thawed feed of 10^7 and 10^8 CFU g^{-1}, respectfully. Final diet concentrations were thus formulated, G1 and G2 feed groups were stored at -20°C and one day before use, daily rations were placed at 4°C. The G3 feed group consisted of feed sprayed only with sterile peptone water, which was bench dried and stored equally as G1 and G2. The basal diet without any supplementation was regarded as a control feed.

Fish and experimental design

The feeding trial was conducted on a fish farm where 400 rainbow trout (*Oncorhynchus mykiss*) of 100 g initially were sorted and placed in four subsequent runways, 100 trout in each. First runway in line was stocked with the control group (group C) fed only the basal diet, second runway was stocked with G1 (10^7 CFU g^{-1}), following with G2 (10^8 CFU g^{-1}), and G3 (peptone water). The fish were offered the basal control diet for a 4-week adaptation period. At the beginning of probiotic application fish weight was 127.25 ± 16.16 g. During the trial, they were fed the control and three supplemented diets for a period of six weeks. Fish were fed 2% of body weight once a day. After the completion of the experimental feeding, the basal diet was fed to the remaining fish in all groups at the same rate. Water quality parameters were monitored daily.

Fish were sampled at the beginning of the trial, at the end of the six-week trial period (n = 20 from each group), and three weeks after the completion of the feeding trial (n=20 from groups G1 and G2). They were starved 24 h before sampling. Fish were

caught in batches of five, as quickly as possible with a smooth net to avoid injuries, and rapidly transferred to a tank containing 120 mg L^{-1} Ethyl 3-aminobenzoate methanesulfonate (MS-222) anaesthetic (Sigma-Aldrich Inc., MO, USA) to reduce acute stress caused by sampling procedures and easier handling during blood withdrawal and taking biometric data (Topic Popovic *et al.* 2012a).

Total body weight and the weight of the fish without viscera, body length (from the nose to the fork of the tail), body height (measured just before the dorsal fin at the tallest part of the fish), and absolute liver weight were determined to calculate condition factors: Fultons's (K), Clark's (K_C) and B (Rehulka 2000; Jones *et al*. 1999), and hepatosomatic index (Sharifuzzaman *et al.* 2014) using the following equations:

$K = W/L^3 \times 100$

K_C = body weight without viscera/body length^3 × 100

$B = W/(L^2 \times H) \times 1000$

HSI = weight of liver/total body weight × 100

where: W=total fish weight (g); L=fish length (cm); H=body height (cm); HSI=hepatosomatic index.

Liver tissue (0.3 g) was homogenized in 0.9% NaCl at 1 300 rpm and frozen at -85ºC until TBARS analysis. Gill tissue was streaked onto TSA and MRS agars for bacterial identification. The intestines of fish were aseptically dissected and intestinal scrapings were streaked onto TSA and MRS agars for further bacterial identification. Also, 1 g of intestinal content was homogenized with 99 mL of sterile peptone water and serially diluted to plate on MRS agar. Plates were incubated for 48 hours at 37°C. The bacterial count of the rearing water was taken before the trial and immediately after

feeding with feed supplemented with *L. rhamnosus* in order to determine the total plate count and presence of *L. rhamnosus* in the water. Tissues of liver, spleen, and intestine were placed in 4% neutral buffered formalin for histopathology assessment.

Blood withdrawal and sample preparation

Blood was withdrawn from caudal vein and collected in tubes coated with anticoagulant lithium heparin, centrifuged at 12 000 *g* for 90 seconds and resultant plasma was frozen at -85ºC until analysis. Haematocrit (Hct) was determined as packed cells volume by the microhaematocrit method (Wedemeyer & Yasutake, 1977). Heparinized microhaematocrit capillaries were filled with blood, sealed with clay and centrifuged for 120 seconds at 12 000 *g* within 20 minutes from collection. The percentage of packed cells to total volume was determined by direct measurement on StatSpin microhaematocrit capillary tube reader.

Bacterial identification

Bacterial strains were identified based on colony and cell morphology, Gram staining, and phenotypic testing, with standard biochemical tests (API 20E and API 50CH system (BioMerieux, France). Both tests were performed according to the manufacturer's instructions with a few alterations for API 20E in order to adapt the system to the bacteria of freshwater fish: the incubation time was increased to 48-72 hours; the incubation temperature was lowered to 22°C; only the fermentation of sugars was allowed by sealing the cups with sterile mineral oil in the carbohydrate tests (Topic Popovic *et al*. 2007). Bruker Biotyper MALDI-TOF (Bruker Daltonics, Billerica, MA) was used for final bacterial identification. Bacterial isolates (one loopful of each

bacterial culture) were applied as a thin film to a 24-spot steel plate (Bruker Daltonics) in two replicates and allowed to visibly dry at room temperature (referred to as the direct colony technique). Subsequently, 2 μL of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied to the colony and dried in a fume hood. The analysis was performed in a manner that ions generated with a 337-nm nitrogen laser were captured in the positive linear mode in a mass range of 2 to 20 kDa. Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. Each spectrum was the sum of the ions obtained from 200 laser shots performed in five different regions of the same well. Captured spectra were analyzed using MALDI Biotyper automation control and Bruker Biotyper 2.0 software (Bruker Daltonics).

Blood biochemistry

In blood plasma, concentrations of GLU, URE, CRE, CHOL, TRIG, TP, ALB, and activity of ALT, ALP, and GGT were determined by Beckman Coulter commercial kits (Olympus Life and Material Science Europe, Ireland) on the Olympus AU 640 biochemistry analyzer (Olympus, Japan). The activity of SOD and GSH-Px were determined by Randox commercial kits on the Olympus AU 640 biochemistry analyzer. The activity of PON 1 was assayed by modified method of hydrolysis of paraoxon described by Charlton-Menys *et al.* (2006) on the Olympus AU 640. Enzyme activity was presented in U (1 μmol p-nitrophenol min^{-1}) L^{-1}.

Histopathology

Tissues of proximal and distal intestine of fish from all groups at all sampling periods were fixed in 4% neutral buffered formalin, dehydrated through a graded ethanol-xylene series and embedded in paraplast. Sagital and transverse sections (2 μm) were stained with hematoxylin/eosin (H&E) and periodic acid-Schiff (PAS) following the methods described in Pearse (1968). Microphotographs were taken with a digital camera DP70 Olympus connected to an Olympus BX51 binocular microscope, and transferred to Microsoft AnalySIS Soft Imaging System for interpretation.

Lipid peroxidation

Total liver malondialdehyde (MDA) concentration, as thiobarbituric reactive substances, was measured using HPLC with UV detection (Grotto *et al.* 2007) on TSP-130 system (Thermo Separation Products, Inc, Thermo Fisher Scientific, Inc., Waltham, MA, SAD) with reversed–phase analytical column protected with guard column (Waters symmetry® C18 column, 5μm, 150 x 4.6 mm i.d.) maintained at 40°C. 1,1,3,3-tetraethoxypropane was used for calibration (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The mobile phase was a mix of 50 mM phosphate buffer and methanol (50:50, v/v) and the flow rate was maintained isocratically at 1 mL min^{-1}. The UV was programmed at 532 nm. The retention time was 2.9 min.

Statistical analysis

Analyses and correlations between tissue parameters were assessed using the SigmaStat and SigmaPlot Statistical Software ver. 11.0 (Jandel Corp., San Rafael, California). Data are reported as means ± standard deviations. Differences between groups were

evaluated by the *t*-test and Mann–Whitney rank sum test. A level of significance was set at $P = 0.05$.

Results

General observations

During the trial, there was no mortality in any of the four groups. Water temperature ranged from 9.4 to 9.9°C, pH 7.3 to 7.7 and dissolved oxygen 6.5 to 10.5 mg L^{-1}. Fish ingested feed thoroughly and evenly, responding animatedly towards feeding activity. At the end of the experimental feeding with the probiotic, average fish weight and length recorded for C group were 242.25 ± 31.38 g, 27.70 ± 1.07 cm; G1 group 247.45 ± 32.50 g, 26.98 ± 1.39 cm; G2 243.95 + 32.93 g, 27.53 ± 0.86 cm; and G3 245.60 ± 30.95 g, 27.40 ± 1.61 cm. All calculated condition factors K, K_C, B were significantly higher ($P < 0.05$) in the fish treated with 10^7 CFU g^{-1} *L. rhamnosus* supplemented diet (G1 group). The highest HSI was in fish from the control group, while the lowest was in fish fed with diet supplemented with peptone water (G3). However, no significant differences ($P > 0.05$) among the treatments were observed (Table 2).

Microbiological analyses

Before the trial, fish had no detectable lactic acid bacteria (LAB) in the intestines nor in the fecal contents. Also, no LAB was recovered from the rearing water nor control fish at any point. Intestinal scrapings and gill tissues streaked onto TSA revealed mostly *Vibrio fluvials, Aeromonas hydrophila* and *Serratia fonticola* (API 20E). The numbers of viable lactobacilli recovered from fecal contents increased in the *L. rhamnosus* fed G1 and G2 groups from below detection limits (<10 CFU mL^{-1}) at the start of the trial to

levels between 2 x 10^2 and 4 x 10^5 CFU mL^{-1} (G1), between 4.2 x 10^2 and 8 x 10^2 CFU mL^{-1} (G2) by the end of the trial. Interestingly, even G3 group, with no probiotic supplementation in feed, but located downstream from the G1 and G2 groups, demonstrated viable lactobacilli between neglectable limits to 0.6 x 10^4 CFU mL^{-1} in one specimen (Fig. 1). By the end of the 6-week trial period, both from feces and intestinal scrapings, but also from gills, lactobacilli were recovered, mainly *L. rhamnosus* and *L. paracasei* subsp. *paracasei* (MALDI-TOF MS and API 50CH). Also, from gills and intestines, *V. fluvialis, Burkholderia cepacia,* non-fermenter species, and *Pasteurellaceae* were recovered (API 20E), which corresponded with *A. sobria*, *Arthrobacter sp., Pseudomonas corrugata*, and *A. sobria*, respectfully, when identified by MALDI-TOF MS.

Following the replacement of the *L. rhamnosus* feed with unsupplemented feed, three weeks after the end of the trial period, the number of viable lactobacilli recovered from fecal contents decreased slightly in the *L. rhamnosus* fed G1 group from below the detection limits (<10 CFU mL^{-1}) up to levels of 2 x 10^3 CFU mL^{-1}, while in G2 group they ranged between undetectable and levels of 2 x 10^4 CFU mL^{-1}. From intestines of G1 and G2 fish, *V. fluvialis, Ps. aeruginosa, A. hydrophila* were recovered (API 20E). In G3 group, fish had no detectable LAB in the fecal contents, and solely one isolate was recovered from the intestines (*Leuconostoc mesenteroides*) identified by MALDI-TOF MS.

Blood biochemistry

The mean haematocrit values ranged from 35.25 ± 4.51 in G1 to 35.70 ± 3.96 in G2 supplemented feed group and there were no significant differences between the groups

before and after the trial. The plasma metabolites and antioxidative and liver panel enzyme are presented in Table 3. There was no difference in GLU and TRIG concentrations in fish treated with probiont compared to control and group treated with peptone water (G3). Levels of URE and CHOL were significantly higher in fish exposed to both probiont concentrations (G1 and G2) while CRE value was significantly higher only in G2 group exposed to higher concentration of *L. rhamnosus*. The highest TP and ALB levels were recorded in G1 group, but only albumin concentrations revealed significant difference. The activities of antioxidative enzymes did not differ significantly. SOD activity greatly varied between individuals and extensive ranges were noted in all examined groups. Higher but not significant GSHPx level was recorded in G1 group. The PON1 activity was not measurable in all specimens. Obtained data indicated unsignificantly lower values in treated groups in relation to the control. No significant differences were observed in ALT and ALP levels while GGT activity was not measurable and its interpretation was disregarded.

Lipid peroxidation

The levels of LPO (measured by the tissue TBARS level) in liver homogenates are shown in Fig 2. A significantly higher ($P < 0.01$) LPO level was observed in G2 group six weeks after the start of the feeding trial with supplemented diet (10^8 CFU g^{-1} *L. rhamnosus*), compared with the control. There was no significant induction ($P > 0.05$) in LPO formation in any group three weeks after the completion of the supplemented feeding.

Histopathology

The intestinal structure improvement was detected in G1 and G2, such as microvilli length in trout proximal intestine when compared to the control group. Normal microvilli architecture and undamaged enterocytes were observed in the proximal intestine of fish exposed to probiotic bacteria in the feed. In contrast, examinations of intestinal segments of control diet fish documented disintegrated microvilli and tight junctions accompanied by widening of intercellular spaces between enterocytes. The PAS stain highlighted alterations in size and enhanced number of goblet (mucus-secreting) cells in both proximal and distal parts of intestine compared to control diets. The probiotic-supplemented diet increased the level of leukocyte infiltration in the lamina propria of the intestinal mucosa as well of intraepithelial space of enerocytes. The lamina propria and submucosa contained large numbers of wandering eosinophilic granular cells. However, the presence of large absorptive vacuoles in supranuclear space of enterocytes in distal part of intestine was observed mainly in the fish fed control diet, while the fine granular absorptive vacuoles were rarely seen in the probiotic fed fish. Histopathology findings are presented in Fig. 3. No significant histological differences were observed between the G1 and G2 groups.

Discussion

In the present study we observed correlations between colonization of rainbow trout with the probiotic *L. rhamnosus* and its condition, haematological, blood biochemical, antioxidative and histopathological parameters.

Condition is a reliable indicator of energy reserves in fish and general well-being of the fish population. In scientific literature, different factors have been proposed to assess condition of fish including Fulton's condition factor (K), Clark's condition and B

357 factor. While the calculation of Fulton's condition factor is based on the length and total
358 body weight, Clark's condition includes body weight without viscera. However, Jones
359 *et al*. (1999) considered adding height to the traditional calculating models (B factor) as
360 more appropriate for making more concise comparisons of the state between different
361 subgroups and more accurate results over much wider ranges of size and shape than
362 either of the other models. In our study, after six weeks of feeding regime all
363 calculations showed a significantly higher ($P < 0.05$) condition factor in fish from the
364 group treated with *L. rhamnosus* with a concentration of 10^7 CFU g^{-1} than with 10^8 CFU
365 g^{-1} and other groups, suggesting that more probiotic (10^8 CFU g^{-1}) in diets does not
366 necessarily imply a better condition.
367 Former studies of the longevity of *L. rhamnosus* probiont in the digestive tract of
368 fish revealed that it was found to last for a few days after the withdrawal of the
369 supplemented feed, and in the span of a week very few members of this strain were
370 traced in the intestine, to completely disappear by the end of the second week (Panigrahi
371 *et al.* 2005, 2004; Nikoskelainen *et al.* 2003). In this trial, however, the presence of *L.*
372 *rhamnosus* in fecal matter of fish by the end of the third week after withdrawal of the
373 supplemented feed was still significant when correlated to the trial period (G1), and
374 even growing (G2), possibly being related to a longer feeding span during which fish
375 were exposed to the probiont (6 weeks). No count of *L. rhamnosus* or any viable
376 lactobacilli was detected in the rearing water at any sampling point, due to a relatively
377 rapid flow of water in the raceways (10 L s^{-1}; or approx. 40 water exchanges in 24
378 hours). Although the loss of viability of the probiont in the drying, freezing, and
379 defrosting process of supplemented feed was detected and calculated before the onset of
380 the trial, it was compensated for in the diet preparation for both concentrations, as

described in the Material and methods section. All the more, the relatively low counts in the intestines of both groups after six weeks of probiont feeding are somewhat confusing, and may be attributed to the low water temperature throughout the trial (9.4-9.9°C). Interestingly, *in vitro* studies (Ibrahim *et al.* 2004) revealed that *L. rhamnosus* 53103 and LCR 1/83 strains demonstrated high adhesion to both intestinal and skin rainbow trout mucus at temperatures ranging from 4°C to 25°C, and growing significantly in relation to the temperature increase. By contrast, *in vivo* testing by Panigrahi *et al.* (2005) demonstrated that *L. rhamnosus* did not show significant growth in the intestines of fish reared at 16°C, and suggested that using psychrophilic microbes as probionts might have a better colonization potential than the mesophilic *L. rhamnosus*, although the 53103 strain, used in this study, is proved to be acid and bile tolerant and surviving the passage through the gastrointestinal tract, as well as remaining in the intestinal and other mucosal surfaces of the fish (Panigrahi *et al.* 2004).

Although the trial feed was supplemented solely with *L. rhamnosus* and no other LAB were detected in control fish, *L. paracasei* was a frequent finding in the intestines of treated fish, with both standard biochemical methods and MALDI-TOF MS. Although *L. rhamnosus* is apparently included in the MALDI-TOF MS database, the reason for such identification might be that *L. casei*, *L. paracasei* and *L. rhamnosus* form a closely related taxonomic group within the heterofermentative lactobacilli. These three species are difficult to differentiate, especially using traditional fermentation profiles. Ward & Timmins (1999) developed polymerase chain reaction primers specific for each of these species based on differences in the V1 region of the 16S rRNA gene for easier identification. *L. rhamnosus* and *L. paracasei* identified in this way were also

differentiated using a randomly amplified polymorphic DNA (RAPD) primer (Ward & Timmins 1999).

The feeding with supplemented probiont apparently changed the resident microbiota in fish under the study since at the beginning of the trial fish were colonized mostly with motile Gram-negative rods, *V. fluvials, A. hydrophila* and *S. fonticola*, while after the six-week supplemented feeding, the microflora changed into *B. cepacia*, non-fermenter species, and *Pasteurellaceae*, along with *V. fluvialis*. Three weeks after the trial and return to the unsupplemented feed, the flora mostly reverted to the control status, also with the finding of *Ps. aeruginosa.* Balcazar *et al.* (2007) also demonstrated that the LAB added in feed showed an ability to antagonize the resident microbiota, possibly because of a fall in intestinal pH induced by lactic acid or other fermented products produced by LAB strains, slowly decreasing with the withdrawal of the supplemented feed. The probiotic culture must therefore be administered continuously to obtain a balance between the probiotic microorganisms and resident microflora in the intestines of trout. Histopathology findings support this conclusion inasmuch as they comprise positive effects on microstructure of the trout gut, increasing the proximal and distal intestinal absorptive area. A significant increase in microvilli height was also recorded due to administration of diets containing *Bacillus cereus* var. *toyoi* in rainbow trout fingerlings (Gisbert *et al.* 2013). The number of goblet cells, responsible for production and preservation of a protective mucus layer by secreting mucin, also increased in the *L. rhamnosus* fed fish in this work, thus augmenting the capacity of the innate host defense. Such defense was complemented by the increase of the intestinal leukocyte phagocytic and eosinophilic granular cells migration in the G1 and G2 groups. The undisturbed enterocyte architecture in the proximal intestine of the G1 and

G2 groups, tightly bound together, is of great importance, since expanded intercellular spaces between enterocytes facilitate the entrance of the potential pathogens in the bloodstream (Ringø *et al.* 2007).

Not many studies correlated rainbow trout plasma profile and other tissue parameters with probiotic feeding, although measurements of plasma constituent levels can spare the animals from sacrificing and are simpler to perform than conducting complex pathogen-challenge trials (Panigrahi *et al.* 2010). Reference rainbow trout plasma chemistry values are established for several parameters (Bowser 1993): GLU 3.88-14.89 mmol L^{-1}, CRE 17.68-44.2 μmol L^{-1}, TP 28-60 g L^{-1}, ALB 17-19 g L^{-1}, CHOL 3.88-14.89 mmol L^{-1}, ALT 7-12 U L^{-1}, ALP 50-200 U L^{-1}. In our study GLU, CRE, TP, CHOL, and ALB concentrations varied within the referent ranges while the detected ALP were above and ALT levels below the references. The changes in plasma enzymes ALP and ALT can give information about liver and kidney functions (Sandnes *et al.* 1988). Unsignificant differences between treated and control groups indicate normal organ functions irrespective to reference values. The excess energy reserves (such as GLU, CHOL, TRIG) are required by an organism to mediate the effects of potential stress and serve as energy buffers (Topic Popovic *et al*. 2012b), which could partly explain the significant increase of CHOL in this study regarding the control fish, but not regarding the reference values. Lipid components have also previously been found to be influenced by probiotic supplementation (Panigrahi *et al.* 2010). Increases in the plasma URE concentrations may be indicative of branchial epithelial and renal disease in teleost fish (Campbell 2012). In the present study significant elevations of URE and CRE levels were detected in probiotic groups, but these values were within the physiological ranges for freshwater species (Campbell 2012). Plasma TP and ALB

452 levels have frequently been measured as indicators of physiological condition. Their
453 increase in fish treated with lower probiont concentration (10^7 CFU g^{-1}) indicates to a
454 better nutritional status.

455 Cultured fish are exposed to different adverse conditions that can result with
456 oxidative stress. It is a consequence of increased reactive oxygen species (ROS)
457 production while it decreases in the antioxidant defense. The SOD is a group of
458 metalloenzymes playing a crucial antioxidant role and constituting the primary defense
459 against the toxic effects of superoxide radicals (Ural 2013). The GSH-Px catalyses the
460 reduction of hydrogen peroxide and lipid peroxides and is considered an efficient
461 protective enzyme against lipid peroxidation (Ural 2013). Probiotic supplementation can
462 increase plasma antioxidant levels, thus neutralizing ROS (Martinez Cruz *et al.* 2012).
463 However, in present study the activities of antioxidative enzymes did not differ
464 significantly. Although some parameters increased and some decreased regarding to the
465 supplemented feeding (Table 3), for the majority of metabolites and enzymes no
466 significant differences were noted between lower and higher *L. rhamnosus*
467 concentrations at the end of the supplemented feeding. This implies that a concentration
468 difference of LGG between 10^7 and 10^8 CFU g^{-1} had no relevance on plasma
469 biochemistry.

470 In order to evaluate the adaptive response of liver activity after the cessation of
471 the supplemented feed period, the total TBARS levels in livers were measured. As
472 expected, TBARS levels increased after six weeks of feeding, especially in trout fed
473 with 10^8 CFU g^{-1} in comparison with control (Fig. 2). At the end of the feeding trial,
474 three weeks after cessation of the supplemented feeding, liver TBARS from both treated
475 groups decreased to control levels. These results imply that LGG supplemented feed

induced the regulation of cellular processes, enhanced immunity, and consequently adaptive responses to stress and protection from possible infection (Lushchak 2011), during the probiont feeding with a higher *L. rhamnosus* concentration, and the decrease in the TBARS level may be due to the free radical scavenging properties of *L. rhamnosus*. For full confirmation of this conclusion, it is advisable to include a variety of indices of fatty peroxide formation in future studies.

In conclusion, our results indicate that rainbow trout fed with probiotic supplementation of 10^7 CFU g^{-1} *L. rhamnosus* had better condition and nutritional status, revealing that more probiotic (10^8 CFU g^{-1}) in diet does not necessarily enhance biological effects. Plasma metabolite and enzyme levels did not exceed physiological reference ranges although CHOL, CRE, URE and ALB concentrations significantly increased in fish treated with the probiont. The feeding with supplemented probiont apparently changed the resident gut microflora, while three weeks after the trial and return to the unsupplemented feed, the microflora mostly reverted to the control composition. Presence of *L. rhamnosus* in fecal matter of fish by the end of the third week after withdrawal of the supplemented feed was still significant when correlated to the trial period. This study was the first to investigate the effects of *L. rhamnosus* on epithelial histology in the proximal and distal intestine of rainbow trout, providing essential information on the changes influenced by feed supplements.

Acknowledgements

This study was supported by a grant from the Croatian Ministry of Agriculture (VIP Project ur.br. 03-8473/1-2012; 2012-2014). The authors wish to thank the staff of the

Croatian Center for Indigenous Species of Fish and Crayfish in Karstic Waters for taking excellent care of the fish in trial.

References

Aguirre-Guzman G., Lara-Flores M., Sanchez-Martinez J.G., Campa-Cordova A.I., & Luna Gonzalez A. (2012) The use of probiotics in aquatic organisms: A review. *African Journal of Microbiology Research* **6** (23), 4845-4857.

Al-Dohail M.A., Hashim R. & Aliyu-Paiko M. (2011) Evaluating the use of *Lactobacillus acidophilus* as a biocontrol agent against common pathogenic bacteria and the effects of the haematology parameters and histopathology in African catfish *Clarias gariepinus* juveniles. *Aquaculture Research* **42**, 196-209.

Avella M.A., Olivotto I., Silvi S., Place A.R. & Carnevalli O. (2010) Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish. *The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **298**, 359-371.

Balcazar J.L., de Blas I., Ruiz-Zarzuela I., Vendrell D., Calvo A.C., Marquez I., Girones O. & Muzquiz, J.L. (2007) Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *British Journal of Nutrition* **97**, 522-527.

Bowser P.R. (1993) Clinical Pathology in Salmonid Fishes. In: *Fish Medicine.* (ed. By M.K. Stoskopf), pp. 327- 332. WB Saunders Company, Philadelphia, USA.

Campbell T.W. (2012) Clinical Chemistry of Fish and Amphibians. In: *Veterinary Hematology and Clinical Chemistry* (eds. By M.A. Thrall, G. Weiser, R.W. Allison, T.W. Campbell), pp. 607-614. Wiley-Blackwell, Ames, Iowa.

524 Charlton-Menys V., Liu Y. & Durrington P.N. (2006) Semiautomated method for
525 determination of serum paraoxonase activity using paraoxon as substrat. *Clinical*
526 *Chemistry* **52**, 453-457.
527 Coz-Rakovac R., Strunjak-Perovic I., Topic Popovic N., Hacmanjek M., Smuc, T.,
528 Jadan M., Lipej Z. & Homen Z. (2008) Cage culture effects on mullets
529 (Mugilidae) liver histology and blood biochemistry profile. *Journal of Fish*
530 *Biology* **72**, 2557-2569.
531 De Man J.C., Rogosa M. & Sharpe M.E. (1960) A medium for the cultivation of
532 lactobacilli. *Journal of Applied Bacteriology* **23**, 130-135.
533 Frouel S., Le Bihan E., Serpentini A., Lebel J.M., Koueta N. & Nicolas J.L. (2008)
534 Preliminary study of the effects of commercial lactobacilli preparations on
535 digestive metabolism of juvenile sea bass (*Dicentrarchus labrax*). *Journal of*
536 *Molecular Microbiology and Biotechnology* **14** (1-3), 100-106.
537 Gioacchini G., Valle L.D., Benato F., Fimia G.M., Nardacci R., Ciccosanti F.,
538 Piacentini M., Borini A. & Carnevali O. (2013) Interplay between autophagy and
539 apoptosis in the development of *Danio rerio* follicles and the effects of a
540 probiotic. *Reproduction, Fertility and Development* **25** (8), 1115-1125.
541 Gioacchini G., Giorgini E., Merrifield D.L., Hardiman G., Borini A., Vaccari L. &
542 Carnevali O. (2012) Probiotics can induce follicle maturational competence: The
543 *Danio rerio* case. *Biology of Reproduction* **86** (3): 65, 1-11.
544 Gioacchini G., Maradonna F., Lombardo F., Bizzaro D., Olivotto I. & Carnevali O.
545 (2010) Increase of fecundity by probiotic administration in zebrafish (*Danio*
546 *rerio*). *Reproduction* **140** (6), 953-959.

547 Gisbert E., Castillo M., Skalli A., Andree K.B. & Badiola I. (2013) *Bacillus cereus* var.
548 *toyoi* promotes growth, affects the histological organization and microbiota of the
549 intestinal mucosa in rainbow trout fingerlings. *Journal of Animal Science* **91** (6),
550 2766-2774.
551 Gomez G.D. & Balcazar J.L. (2008) A review on the interactions between gut
552 microbiota and innate immunity of fish. *FEMS Immunology and Medical*
553 *Microbiology* **52**, 145-154.
554 Goncalves A.T., Maita M., Futami K., Endo M. & Katagiri T. (2011) Effects of a
555 probiotic bacterial *Lactobacillus rhamnosus* dietary supplement on the crowding
556 stress response of juvenile Nile tilapia *Oreochromis niloticus*. *Fisheries Science*
557 77, 633-642.
558 Grotto D., Santa Maria L.D., Boeira S., Valentini J., Char M.F., Moro A.M.,
559 Nascimento P.C., Pomblum V.J. & Garcia S.C. (2007) Rapid quantification of
560 malondialdehyde in plasma by high performance liquid chromatography – visible
561 detection. *Journal of Pharmaceutical and Biomedical Analysis* **43**, 619-624.
562 Ibrahim F., Ouwehand A.C. & Salminen S.J. (2004) Effects of temperature on *in vitro*
563 adhesion of potential fish probiotics. *Microbial Ecology in Health and Disease*
564 **16**, 222-227.
565 Jones R.E., Petrell R.J. & Pauly D. (1999) Using modified length-weight relationships
566 to assess the condition of fish. *Aquacultural Engineering* **20**, 261-276.
567 Lushchak V.I. (2011) Environmentally induced oxidative stress in aquatic animals.
568 *Aquatic Toxicology* **101**, 13-30.

569 Maita M. (2007) Fish health assessment. In: *Dietary supplements for the health and*
570 *quality of cultured fish* (eds by H. Nakagawa, M. Sato, D.M. Gatlin III), pp 10 –
571 34. CAB International. Oxon, UK.
572 Martinez Cruz P., Ibanez A.L., Monroy Hermosillo O.A. & Ramirez Saad H.C. (2012)
573 Use of probiotics in aquaculture. *ISRN Microbiology*, doi:10.5402/2012/916845.
574 Merrifield D.L., Dimitroglou A., Foey A., Davies S.J., Baker R.T.M., Bogwald J.,
575 Castex M. & Ringo E. (2010a) The current status and future focus of probiotic
576 and prebiotic applications for salmonids. *Aquaculture* **302**, 1-18.
577 Merrifield D.L., Dimitroglou A., Bradley G., Baker R.T.M. & Davies S.J. (2010b)
578 Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) I.
579 Effects on growth performance, feed utilization, intestinal microbiota and related
580 health criteria. *Aquaculture Nutrition* **16** (5), 504-510.
581 Nall N.G., Leukes W.D. & Kaiser H. (2004) *In vitro* growth characteristics of five
582 candidate aquaculture probiotics and two fish pathogens grown in fish intestinal
583 mucus. *FEMS Microbiology Letters* **231**, 145-152.
584 Nayak S.K. (2010) Probiotics and immunity: A fish perspective. *Fish and Shellfish*
585 *Immunology* **29**, 2-14.
586 Ngamkala S., Futami K., Endo M., Maita M. & Katagiri T. (2010) Immunological
587 effects of glucan and *Lactobacillus rhamnosus* GG, a probiotic bacterium, on Nile
588 tilapia *Oreochromis niloticus* intestine with oral *Aeromonas* challenges. *Fisheries*
589 *Science* **76** (5), 833-840.
590 Nikoskelainen S., Salminen S., Bylund G. & Ouwehand A.C. (2001a) Human and
591 dairy-derived probiotics for prevention of infectious diseases in fish. *Applied and*
592 *Environmental Microbiology* **67** (6), 2430-2435.

593 Nikoskelainen S., Ouwehand A.C., Salminen S. & Bylund, G. (2001b). Protection of
594 rainbow trout *Oncorhynchus mykiss* from furunculosis by *Lactobacillus*
595 *rhamnosus*. *Aquaculture* **198** (3-4), 229-236.
596 Nikoskelainen S., Ouwehand A.C., Bylund G., Salminen S. & Lilius E.-M. (2003)
597 Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential
598 probiotic bacteria (*Lactobacillus rhamnosus*). *Fish and Shellfish Immunology* **15**,
599 443-452.
600 Ouwehand A.C., Kirjavainen P.V., Gronlund M.M., Isolauri E. & Salminen, S. (1999)
601 Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy*
602 *Journal* **9**, 623-630.
603 Panigrahi A., Kiron V., Kobayashi T., Puangkaew J., Satoh S. & Sugita H. (2004)
604 Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential
605 probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Veterinary Immunology*
606 *and Immunopathology* **102** (4), 379-388.
607 Panigrahi A., Kiron V., Puangkaew J., Kobayashi T., Satoh S. & Sugita, H. (2005) The
608 viability of probiotic bacteria as a factor influencing the immune response in
609 rainbow trout *Oncorhynchus mykiss*. *Aquaculture* **243**, 241-254.
610 Panigrahi A., Kiron V., Kobayashi T., Puangkaew J., Satoh S. & Sugita H. (2007)
611 Immune modulation and expression of cytokine genes in rainbow trout
612 *Oncorhynchus mykiss* upon probiotic feeding. *Developmental and Comparative*
613 *Immunology* **31**, 372-382.
614 Panigrahi A., Kiron V., Satoh S. & Watanabe T. (2010) Probiotic bacteria *Lactobacillus*
615 *rhamnosus* influences the blood profile in rainbow trout *Oncorhynchus mykiss*
616 (Walbaum). *Fish Physiology and Biochemistry* **36** (4), 969-977.

617 Panigrahi A., Viswanath K. & Satoh S. (2011) Real-time quantification of the immune
618 gene expression in rainbow trout fed different forms of probiotic bacteria
619 *Lactobacillus rhamnosus*. *Aquaculture Research* **42** (7), 906-917.
620 Pearse A.G.E. (1968) Histochemistry, Theoretical and Applied, 3rd ed. J and A
621 Churchill Ltd., London.
622 Pirarat N., Kobayashi T., Katagiri T., Maita M. & Endo M. (2006) Protective effects
623 and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against
624 experimental *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*).
625 *Veterinary Immunology and Immunopathology* **113**, 339-347.
626 Pirarat N., Pinpimai K., Endo M., Katagiri T., Ponpornpisit A., Chansue N. & Maita M.
627 (2011) Modulation of intestinal morphology and immunity in nile tilapia
628 (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. *Research in Veterinary*
629 *Science* **91** (3), 92-97.
630 Rehulka J. (2000) Influence of astaxanthin on growth rate, condition, and some blood
631 indices of rainbow trout *Onchorchynchus mykiss*. *Aquaculture* **190**, 27-47.
632 Ringø E., Salinas I., Olsen R.E., Nyhaug A., Myklebust R. & Mayhew T.M. (2007)
633 Histological changes in intestine of Atlantic salmon (*Salmo salar* L.) following in
634 vitro exposure to pathogenic and probiotic bacterial strains. *Cell Tissue Research*
635 **328** (1), 109-116.
636 Qin C., Xu L., Yang Y., He S., Dai Y., Zhao H. & Zhou Z. (2014) Comparison of
637 fecundity and offspring immunity in zebrafish fed *Lactobacillus rhamnosus* CICC
638 6141 and *Lactobacillus casei* BL23. *Reproduction* **147** (1), 53-64.

639 Sandnes K., Lie Ø. & Waagbø R. (1988) Normal ranges of some blood chemistry
640 parameters in adult farmed Atlantic salmon, *Salmo salar*. *Journal of Fish Biology*
641 **32**, 129-136.
642 Sharifuzzaman S.M., Al-Harbi A.H. & Austin, B. (2014) Characteristics of growth,
643 digestive system functionality, and stress factors of rainbow trout fed probiotics
644 *Kocuria* SM1 and *Rhodococcus* SM2. *Aquaculture* **418-419**, 55-61.
645 Stepic S., Hackenberger B.K., Hackenberger D.K., Velki M. & Loncaric Z. (2012)
646 Impact of Oxidative stress indicated by thiobarbituric acid reactive substances
647 (TBARS) and protein carbonyl levels (PC) on Ethoxyresorufin-O-deethylase
648 (EROD) induction in common carp (*Cyprinus carpio*). *Water Air and Soil*
649 *Pollution* **223** (8), 4785-4793.
650 Topic Popovic N., Strunjak-Perovic I., Coz-Rakovac R., Barisic J., Jadan M., Persin
651 Berakovic A. &, Sauerborn Klobucar R. (2012a) Tricaine methanesulfonate (MS-
652 222) application in fish anaesthesia. *Journal of Applied Ichthyology* **28**, 553-564.
653 Topic Popovic N., Kozacinski L., Strunjak-Perovic I., Coz-Rakovac R., Jadan M.,
654 Cvrtila-Fleck Z. & Barisic J. (2012b) Fatty acid and proximate composition of
655 bluefin tuna (*Thunnus thynnus*) muscle with regard to plasma lipids. *Aquaculture*
656 *Research* **43**, 722-729.
657 Topic Popovic N., Coz-Rakovac R. & Strunjak-Perovic, I. (2007) Commercial
658 phenotypic tests (API 20E) in diagnosis of fish bacteria. *Veterinarni Medicina* **52**
659 (2), 49-53.
660 Ural M.S. (2013) Chlorpyrifos-induced changes in oxidant/antioxidant status and
661 haematological parameters of *Cyprinus carpio carpio*: Ameliorative effect of
662 lycopene. *Chemosphere* **90**, 2059-2064.

663 Ward L.J.H. & Timmins M.J. (1999). Differentiation of *Lactobacillus casei*,
664 *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain
665 reaction. *Letters in Applied Microbiology* **29** (2), 90-92.
666 Wedemeyer G.A. & Yasutake W.T. (1977) Clinical methods for the assessment of the
667 effects of environmental stress on fish health. Technical Papers of the U.S. Fish
668 and Wildlife Service, Washington, DC, pp 1-18.

Table 1 Effects of *Lactobacillus rhamnosus* in aquatic species

Source	Aquatic species	Mode of application	Dose (mL^{-1} water, or g^{-1} feed)	Effect	Reference
CICC 6141	*Danio rerio*	Feed	10^6 CFU mL^{-1}	Fecundity increase (higher rates of egg ovulation, fertilization, hatching); Affecting the innate immunity of offspring	Qin *et al*. (2014)
IMC 501	*Danio rerio*	Water	10^6 CFU mL^{-1}	Reproduction improvement; Inducing oocite maturation	Martinez Cruz *et al*. (2012); Gioacchini *et al.* (2010)
IMC 501	*Danio rerio*	Water	10^6 CFU mL^{-1}	Modulation of microbial communities in the GI tract; Enhancing the presence of *Streptococcus thermophilus*; Affecting the follicle maturation	Gioacchini et al. (2012)

IMC 501	*Danio rerio*	Feed	10^6 CFU g^{-1}	Inhibition of follicular apoptosis; Increase of autophagy in preovulatory follicles	Gioacchini *et al.* (2013)
IMC 501	*Amphiprion ocellaris*	Water and by live prey	10^6 CFU g^{-1}	Faster growth, increased gene expression of growth and development factors; Lessened severity of the general stress response; Improvement of biomineralization	Avella *et al.* (2010)
ATCC 53103	*Oreochromis niloticus*	Feed	10^8 and 10^{10} CFU g^{-1}	Immunostimulation: complement activity, enhanced phagocytosis; Higher serum complement activity; induction of IL-1 and TNF α cytokines; Improved survival after challenge with *Edwardsiella tarda*	Pirarat *et al.* (2011); Nayak (2010); Pirarat *et al.* (2006)
ATCC	*Oreochromis*	Feed	10^{10} CFU g^{-1}	Growth performance improvement;	Goncalves *et al.* (2011)

53103	*niloticus*			Increasing of metabolic support for the crowding stress response	
LGG N/S	*Oreochromis niloticus*	Feed	10^{10} CFU g^{-1}	Reduced mortality, and intestinal damage following *Aeromonas* challenges	Ngamkala *et al.* (2010)
JCM 1136	*Oncorhynchus mykiss*	Feed	10^{11} CFU g^{-1}	Immunostimulation; Immunoglobulin increase, respiratory burst activity, lysozyme, complement activity, plasma immunoglobulin increase	Aguirre-Guzman *et al.* (2012); Nayak (2010); Panigrahi *et al.* (2005)
JCM 1136	*Oncorhynchus mykiss*	Feed	10^{9} CFU g^{-1}	Immunostimulation; Expression of cytokine genes, upregulation of IL-1β1, TNF 1 and 2, TGF-β, complement activity, respiratory burst activity	Aguirre-Guzman *et al.* (2012); Nayak (2010); Panigrahi *et al.* (2007 and 2011)
ATCC	*Oncorhynchus*	Feed	10^{9} and 10^{12}	Disease resistance (*Aeromonas*	Martinez Cruz *et al.* (2012);

53103	*mykiss*		CFU g^{-1}	*salmonicida*); Growth performance	Nikoskelainen *et al.* (2001a)
JCM 1136	*Oncorhynchus*	Feed	10^9 and 10^{11}	Immunostimulation; serum	Nayak (2010); Panigrahi *et*
	mykiss		CFU g^{-1}	lysozyme, phagocytic activity,	*al.* (2004)
				respiratory burst activity,	
				complement activity increase	
ATCC	*Oncorhynchus*	Feed	10^4 up to 10^{10}	Gut microbiota;	Merrifield *et al.* (2010b);
53103	*mykiss*		CFU g^{-1}	Immunological/haematological	Gomez & Balcazar (2008);
				response; Improved survival after	Nikoskelainen *et al.* (2003)
				challenge with *Aeromonas*	
				salmonicida	
JCM 1136	*Oncorhynchus*	Feed	10^9 and 10^{11}	Alterations in blood profile	Panigrahi *et al.* (2010)
	mykiss		CFU g^{-1}	(cholesterol, trygliceride contents,	
				plasma alkaline phosphatase activity,	
				plasma protein, haematocrit values)	

N/S (not stated)

Table 2 Condition factors and hepatosomatic index of rainbow trout fed with probiotic-supplemented or control diets after 6 weeks of continuous feeding (values represent mean ± s.d.)

Parameter	Control group	Feeding regime with feed treated with *L. rhamnosus* in concentrations:		Feed treated with peptone water
	Untreated feed (C)	10^7 CFU g^{-1} (G1)	10^8 CFU g^{-1} (G2)	(G3)
K	1.13 ± 0.07^b	1.26 ± 0.11^a	1.17 ± 0.16^b	1.19 ± 0.086^c
K_C	0.92 ± 0.05^b	1.03 ± 0.09^a	0.88 ± 0.20^b	0.97 ± 0.08^c
B	50.55 ± 2.13^b	54.51 ± 3.30^a	51.49 ± 5.56^b	51.75 ± 3.33^b
HSI	1.51 ± 0.17	1.48 ± 0.17	1.48 ± 0.19	1.41 ± 0.19

Abbreviations: K = Fulton's condition coefficient; K_C = Clark's condition coefficient (based on body weight without viscera); B = condition index based on body height; HSI = hepatosomatic index; differences between the means of the groups in the same row marked with different lowercase lettering are statistically significant ($P < 0.05$)

Table 3 Activities of plasma metabolites and enzymes from rainbow trout (n = 20 fish per group). Groups comprise: C (control with basal diet), 6 weeks of the supplemented feeding with *L. rhamnosus*, G1 (supplemented diets 10^7 CFU g^{-1}), G2 (supplemented diets 10^8 CFU g^{-1}), G3 (diet sprayed with peptone water). Values represent mean ± s.d.

Biochemical parameters	Fish groups			
	C	G1	G2	G3
GLU (mmol L^{-1})	6.63 ± 1.66	6.25 ±1.35	5.71 ± 1.24	5.85 ± 1.56
URE (mmol L^{-1})	0.45 ± 0.09^{b}	0.71 ± 0.15^{a}	0.77 ± 0.16^{a}	0.51 ± 0.15^{b}
CRE (µmol L^{-1})	22.90 ± 2.77^{b}	23.90 ± 4.53^{b}	26.95 ± 5.44^{a}	23.70 ± 5.22^{b}
TP (g L^{-1})	36.65 ± 3.99	39.80 ± 4.89	37.90 ± 3.09	37.20 ± 3.58
ALB (g L^{-1})	14.40 ± 1.93^{b}	16.00 ± 2.05^{a}	15.25 ± 1.74^{ab}	15.00 ± 1.81^{ab}
TRIG (mmol L^{-1})	7.50 ± 2.40	7.41 ± 1.62	8.55 ± 2.38	9.10 ± 3.50
CHOL (mmol L^{-1})	6.40 ± 1.28^{b}	7.50 ± 1.51^{a}	7.43 ± 1.05^{a}	7.08 ± 1.44^{ab}
ALT (U L^{-1})	3.81 ± 3.71	2.21 ± 1.44	2.71 ± 1.38	3.79 ± 5.03
ALP (U L^{-1})	237.00 ± 57.82	235.60 ± 94.26	208.40 ± 65.91	211.20 ± 65.37
SOD (U L^{-1})	288.09 ± 367.30	299.50 ± 426.50	326.13 ± 303.24	350.40 ± 393.62
GSH-Px (U L^{-1})	502.37 ± 51.41	519.42 ± 27.42	480.50 ± 83.35	492.30 ± 64.77
PON 1 (U L^{-1})	3.09 ± 2.09	2.55 ± N/V	2.19 ± N/V	2.18 ± 1.37

N/V (no value); differences between the means of the groups in the same row marked with different lowercase lettering are statistically significant ($P < 0.05$)

Figure 1 The average numbers of viable lactobacilli (log10 based scale) recovered from fecal contents of three experimental rainbow trout groups (G1: 10^7 CFU g^{-1}, G2: 10^8 CFU g^{-1}, G3: peptone water), before the trial (Initial), at the end of the supplemented feeding with *L. rhamnosus* (6 weeks), and 3 weeks after the cessation of the supplemented feeding (Withdrawal). The lactobacilli counts were performed by spreading dilutions on selective medium for lactic acid bacteria (MRS agar). In numerical values, the average numbers of viable lactobacilli (6 weeks and Withdrawal) respectively, were: G1: 160 040 ± 219 052; 444 ± 875.03; G2: 568 ± 380.95; 4 164 ± 8 859.26; G3: 1 200 ± 2 683.28; 0 ± 0 (mean ± standard deviation).

Figure 2 Thiobarbituric acid reactive substances (TBARS) in liver homogenates after 6 weeks of the supplemented feeding with *L. rhamnosus* (6 weeks C, G1 and G2), and 3 weeks after the completion of the supplemented feeding (Withdrawal C, G1 and G2). C: control with basal diet, G1: supplemented diets 10^7 CFU g^{-1}, G2: supplemented diets 10^8 CFU g^{-1}. Data are means ± s.d., n =20. Significant differences compared with control value $^{**}P < 0.01$.

Figure 3 The villi in the proximal and distal parts of intestines from trouts fed diet enriched with *L. rhamnosus*, showing mucosa and a part of submucosa. a) The surface epithelium is continuous with the opening of the goblet cells at the proximal intestinal surface. The goblet cells are secreting and beginning to fill the lumen of the gland with their secretions (arrowhead). The intraepithelial lymphocytes are scattered among and between the epithelial cells (arrow). b) The highly cellular lamina propria in the proximal intestine contains numerous migrating eosinophilic

granular cells (arrowhead). c) Granular absorptive vacuoles in the distal part of the intestine (double arrow).