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

Natalija Topic Popovic¹, Ivancica Strunjak-Perovic¹, Roberta Sauerborn-Klobucar¹,
Josip Barisic¹, Margita Jadan¹, Snjezana Kazazic², Inga Kesner-Koren¹, Andreja
Prevendar Crnic³, Jelena Suran³, Blanka Beer Ljubic³, Vesna Matijatko³ & Rozelinda
Coz-Rakovac¹

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

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

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

⁴Dukat 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 probions, 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 probions 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 $127.25 \pm 16.16$ 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:

\[
K = \frac{W}{L^3} \times 100
\]
\[
K_C = \frac{\text{body weight without viscera}}{\text{body length}^3} \times 100
\]
\[
B = \frac{W}{(L^2 \times H)} \times 1000
\]
\[
\text{HSI} = \frac{\text{weight of liver}}{\text{total body weight}} \times 100
\]

where: \(W=\) total fish weight (g); \(L=\) fish length (cm); \(H=\) body height (cm);

Liver tissue (0.3 g) was homogenized in 0.9% NaCl at 1 300 rpm and frozen at -85°C 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 \textit{L. rhamnosus} in order to determine the total plate count and presence of \textit{L. rhamnosus} in the water. Tissues of liver, spleen, and intestine were placed in 4\% neutral buffered formalin for histopathology assessment.

\textit{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 -85\(^\circ\)C 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.

\textit{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\(^\circ\)C; only the fermentation of sugars was allowed by sealing the cups with sterile mineral oil in the carbohydrate tests (Topic Popovic \textit{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 fluvialis*, *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 $2 \times 10^2$ and $4 \times 10^5$ CFU mL$^{-1}$ (G1), between $4.2 \times 10^2$ and $8 \times 10^2$ 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 negligible limits to $0.6 \times 10^4$ 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 $2 \times 10^3$ CFU mL$^{-1}$, while in G2 group they ranged between undetectable and levels of $2 \times 10^4$ 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 $35.25 \pm 4.51$ in G1 to $35.70 \pm 3.96$ 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 enterocytes. 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
factor. While the calculation of Fulton’s condition factor is based on the length and total
body weight, Clark’s condition includes body weight without viscera. However, Jones
et al. (1999) considered adding height to the traditional calculating models (B factor) as
more appropriate for making more concise comparisons of the state between different
subgroups and more accurate results over much wider ranges of size and shape than
either of the other models. In our study, after six weeks of feeding regime all
calculations showed a significantly higher ($P < 0.05$) condition factor in fish from the
group treated with $L. \text{rhamnosus}$ with a concentration of $10^7$ CFU g$^{-1}$ than with $10^8$ CFU
g$^{-1}$ and other groups, suggesting that more probiotic ($10^8$ CFU g$^{-1}$) in diets does not
necessarily imply a better condition.

Former studies of the longevity of $L. \text{rhamnosus}$ probiont in the digestive tract of
fish revealed that it was found to last for a few days after the withdrawal of the
supplemented feed, and in the span of a week very few members of this strain were
traced in the intestine, to completely disappear by the end of the second week (Panigrahi
et al. 2005, 2004; Nikoskelainen et al. 2003). In this trial, however, the presence of $L. \text{rhamnosus}$ 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 (G1), and
even growing (G2), possibly being related to a longer feeding span during which fish
were exposed to the probiont (6 weeks). No count of $L. \text{rhamnosus}$ or any viable
lactobacilli was detected in the rearing water at any sampling point, due to a relatively
rapid flow of water in the raceways (10 L s$^{-1}$; or approx. 40 water exchanges in 24
hours). Although the loss of viability of the probiont in the drying, freezing, and
defrosting process of supplemented feed was detected and calculated before the onset of
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-
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. fluvialis*, *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
levels have frequently been measured as indicators of physiological condition. Their increase in fish treated with lower probiont concentration (10^7 CFU g⁻¹) indicates to a better nutritional status.

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

In order to evaluate the adaptive response of liver activity after the cessation of the supplemented feed period, the total TBARS levels in livers were measured. As expected, TBARS levels increased after six weeks of feeding, especially in trout fed with 10^8 CFU g⁻¹ in comparison with control (Fig. 2). At the end of the feeding trial, three weeks after cessation of the supplemented feeding, liver TBARS from both treated 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


Table 1 Effects of *Lactobacillus rhamnosus* in aquatic species

<table>
<thead>
<tr>
<th>Source</th>
<th>Aquatic species</th>
<th>Mode of application</th>
<th>Dose (mL⁻¹ water, or g⁻¹ feed)</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CICC 6141</td>
<td><em>Danio rerio</em></td>
<td>Feed</td>
<td>$10^6$ CFU mL⁻¹</td>
<td>Fecundity increase (higher rates of egg ovulation, fertilization, hatching); Affecting the innate immunity of offspring</td>
<td>Qin <em>et al.</em> (2014)</td>
</tr>
<tr>
<td>IMC 501</td>
<td><em>Danio rerio</em></td>
<td>Water</td>
<td>$10^6$ CFU mL⁻¹</td>
<td>Reproduction improvement; Inducing oocite maturation</td>
<td>Martinez Cruz <em>et al.</em> (2012); Gioacchini <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>IMC 501</td>
<td><em>Danio rerio</em></td>
<td>Water</td>
<td>$10^6$ CFU mL⁻¹</td>
<td>Modulation of microbial communities in the GI tract; Enhancing the presence of <em>Streptococcus thermophilus</em>; Affecting the follicle maturation</td>
<td>Gioacchini <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>IMC 501</td>
<td><em>Danio rerio</em></td>
<td>Feed</td>
<td>$10^6$ CFU g$^{-1}$</td>
<td>Inhibition of follicular apoptosis; Increase of autophagy in preovulatory follicles</td>
<td>Gioacchini <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>IMC 501</td>
<td><em>Amphiprion ocellaris</em></td>
<td>Water and by live prey</td>
<td>$10^6$ CFU g$^{-1}$</td>
<td>Faster growth, increased gene expression of growth and development factors; Lessened severity of the general stress response; Improvement of biomineralization</td>
<td>Avella <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>ATCC 53103</td>
<td><em>Oreochromis niloticus</em></td>
<td>Feed</td>
<td>$10^8$ and $10^{10}$ CFU g$^{-1}$</td>
<td>Immunostimulation: complement activity, enhanced phagocytosis; Higher serum complement activity; induction of IL-1 and TNF α cytokines; Improved survival after challenge with <em>Edwardsiella tarda</em></td>
<td>Pirarat <em>et al.</em> (2011); Nayak (2010); Pirarat <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>ATCC</td>
<td><em>Oreochromis</em></td>
<td>Feed</td>
<td>$10^{10}$ CFU g$^{-1}$</td>
<td>Growth performance improvement;</td>
<td>Goncalves <em>et al.</em> (2011)</td>
</tr>
<tr>
<td>Strain</td>
<td>Species</td>
<td>Feed</td>
<td>CFU g⁻¹</td>
<td>Effect</td>
<td>References</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>------</td>
<td>---------</td>
<td>---------------------------------------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>LGG N/S</td>
<td><em>Oreochromis niloticus</em></td>
<td>Feed</td>
<td>10¹⁰</td>
<td>Reduced mortality, and intestinal damage following <em>Aeromonas</em> challenges</td>
<td>Ngamkala <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>JCM 1136</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>Feed</td>
<td>10¹¹</td>
<td>Immunostimulation; Immunoglobulin increase, respiratory burst activity, lysozyme, complement activity, plasma immunoglobulin increase</td>
<td>Aguirre-Guzman <em>et al.</em> (2012); Nayak (2010); Panigrahi <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>JCM 1136</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>Feed</td>
<td>10⁹</td>
<td>Immunostimulation; Expression of cytokine genes, upregulation of IL-1β1, TNF 1 and 2, TGF-β, complement activity, respiratory burst activity</td>
<td>Aguirre-Guzman <em>et al.</em> (2012); Nayak (2010); Panigrahi <em>et al.</em> (2007 and 2011)</td>
</tr>
<tr>
<td>ATCC</td>
<td><em>Oncorhynchus</em></td>
<td>Feed</td>
<td>10⁹ and 10¹²</td>
<td>Disease resistance (<em>Aeromonas</em>)</td>
<td>Martinez Cruz <em>et al.</em> (2012);</td>
</tr>
<tr>
<td>Bact. No.</td>
<td>Host</td>
<td>Feed</td>
<td>Level</td>
<td>Response</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>53103</td>
<td><em>mykiss</em></td>
<td>CFU g⁻¹</td>
<td><em>salmonicida</em></td>
<td>Growth performance</td>
<td>Nikoskelainen et al. (2001a)</td>
</tr>
<tr>
<td>JCM 1136</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>10⁹ and 10¹¹</td>
<td>Immunostimulation; serum</td>
<td>Nayak (2010); Panigrahi et al. (2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>mykiss</em></td>
<td>CFU g⁻¹</td>
<td>lysozyme, phagocytic activity, respiratory burst activity, complement activity increase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>10⁴ up to 10¹⁰</td>
<td>Gut microbiota; Immunological/haematological response; Improved survival after challenge with <em>Aeromonas salmonicida</em></td>
<td>Merrifield et al. (2010b); Gomez &amp; Balcazar (2008); Nikoskelainen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>53103</td>
<td><em>mykiss</em></td>
<td>CFU g⁻¹</td>
<td>Alterations in blood profile</td>
<td>Panigrahi et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>JCM 1136</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>10⁹ and 10¹¹</td>
<td>(cholesterol, trygliceride contents, plasma alkaline phosphatase activity, plasma protein, haematocrit values)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Feeding regime with feed treated with L. rhamnosus in concentrations:</th>
<th>Feed treated with peptone water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated feed</td>
<td>10^7 CFU g^-1</td>
<td>10^8 CFU g^-1</td>
</tr>
<tr>
<td>K</td>
<td>(C) 1.13 ± 0.07^b</td>
<td>1.26 ± 0.11^a</td>
<td>1.17 ± 0.16^b</td>
</tr>
<tr>
<td>KC</td>
<td>0.92 ± 0.05^b</td>
<td>1.03 ± 0.09^a</td>
<td>0.88 ± 0.20^b</td>
</tr>
<tr>
<td>B</td>
<td>50.55 ± 2.13^b</td>
<td>54.51 ± 3.30^a</td>
<td>51.49 ± 5.56^b</td>
</tr>
<tr>
<td>HSI</td>
<td>1.51 ± 0.17</td>
<td>1.48 ± 0.17</td>
<td>1.48 ± 0.19</td>
</tr>
</tbody>
</table>

Abbreviations: K = Fulton’s condition coefficient; KC = 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.

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

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: $160\,040 \pm 219\,052; 444 \pm 875.03$; G2: $568 \pm 380.95; 4\,164 \pm 8\,859.26$; G3: $1\,200 \pm 2\,683.28; 0 \pm 0$ (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).