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The LPS derived from the cell walls of the Gram-negative bacteria *Pantoea agglomerans* stimulates growth and immune status of rainbow trout (*Oncorhynchus mykiss*) juveniles

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ABSTRACT

The effects of oral administration of LPS derived from the cell walls of *Pantoea agglomerans* (LPSp) were tested in rainbow trout fry (10 and 20 μg LPSp kg BW^{-1} day^{-1}) considering their general condition, growth performance, body proximate composition, digestive enzyme activities, histological organization of the intestinal mucosa (number of goblet cells and villi height) and analysis of immunological blood parameters related to the non-specific immune response (hemolytic complement, lysozyme, bacteriolytic activity and respiratory burst). Trout were stocked in a recirculation system and fed three diets: a commercial trout feed (Aller Futura, ALLER AQUA) without the immunostimulant; LPSp, and the same diet in which the top coating the pellets of the control diet with the lyophilized powder of IP-PA1 dissolved in 2% fish oil (final concentrations were 0.06 and 0.12 g LPSp kg^{-1} in order to achieve a feed dosage of 10 and 20 μg LPSp kg BW^{-1} day^{-1}, respectively). Each dietary treatment was tested in triplicate and the trial lasted for 93 days. Diets containing LPSp promoted growth; the final mean body weight and standard length in animals fed the LPSp at 20 μg kg BW^{-1} day^{-1} were 17.4 and 6.4% higher than in the control group ($P < 0.05$). In addition, the oral administration of LPSp enhanced the density of intestinal goblet cells ($P < 0.05$) suggesting an enhancement of the intestinal innate immune function. These results were supported by the enhancement of the blood non-specific immune parameters (lysozyme, bactericidal activity and the levels of complement in serum, and respiratory burst from hemocytes) in fish fed 20 μg LPSp kg BW^{-1} day^{-1}. These results may be of practical significance for fish farmers, since the administration of LPSp at 20 μg kg BW^{-1} day^{-1} might not only prevent disease in aquaculture systems, but also improve at the same time their growth performance and efficiency of the rearing process.

1. Introduction

Immunostimulants are chemical substances which activate transiently elements of the immune response such as white blood cells. Such substances may also, but not necessarily, render animals more resistant to infectious diseases and reduce the risk of disease outbreaks if administrated prior to situations known to result in stress and impaired general performance (*e.g.* handling, change of temperature and environment, weaning to inert diets) or prior to expected increase in exposure to pathogenic microorganisms and parasites (Sahoo, 2007; Magnadóttir, 2010). In addition, aquaculture may benefit from the use of such immunostimulants when they are used prior to and during, developmental phases when the organisms are particularly susceptible to infectious agents (*e.g.* shrimp and fish larval stages, salmon smoltification or sexual maturation). The application of immunestimulants in fish and shrimp aquaculture is increasingly gaining interest as an environmentally safe alternative to antibiotics and chemotherapeutics (LaFrentz et al., in press; Raa, 2000; Song et al., 1997; Magnadóttir, 2010, among others).

Bacterial LPS are the major outer surface membrane components present in almost all Gram-negative bacteria and act as extremely strong stimulators of innate or natural immunity in diverse eukaryotic species (Alexander and Rietschel, 2001; Kohchi et al., 2006). In fact, LPS has been used as an experimental antigen to study the immune reaction in animals (Kadowaki et al., 2013; Kohchi et al., 2006; Ribas et al., 2008, among others). In addition, some studies have reported that the use of different types of immunostimulants both in terrestrial (pigs, poultry and cattle) and aquatic organisms (fishes and shrimps) has shown to exert beneficial effects on growth performance. Immunostimulants, such as LPS, are usually identified by their ability to activate white blood cells in *in vitro* experiments. However, it is important to be

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aware that such experiments provide little information regarding the effect of a given immunostimulant on the whole organism since there is an overall modulation of the immune response involving other regulatory systems such as the endocrine (Mackenzie et al., 2006). Experiments with live animals are needed to reveal the overall effect of an immunostimulant, although its definitive efficiency evaluation would require a challenge with an active pathogen (Djordjevic et al., 2009). In this sense, we studied the long term effects (93 days) of two dietary inclusion levels of the LPS derived from *Pantoea agglomerans* (LPSp) in rainbow trout (*Oncorhynchus mykiss*) fingerlings considering the general condition, growth and immune status of the animals.

2. Material and methods

2.1. Experimental diets

The standard commercial diet Aller Futura (ALLER AQUA; Christiansfeld, Denmark) was used as the control diet (C diet, pellet size: 2 mm). Lipopolysaccharide (IP-PA1) from *P. agglomerans* was used as immunostimulant. Diets containing the immunostimulant (IS) were prepared by ALLER AQUA by top coating the pellets of the C diet with the lyophilized powder of IP-PA1 dissolved in 2% fish oil (999 Fish Oil, TripleNine Fish Protein, Esbjerg, Denmark). Top-coating was done in a 7 kg Forberg mixer (Forberg International, Norway) with a speed of 20 rpm and fish oil was applied for 30 s (retention time: 60 s). The final concentrations of LPSp in IS diets were 0.06 and 0.12 g LPSp kg^{-1} using the commercial diluted product containing 1% LPSp (SOMACY SL100, MACROPHI Inc., Kagawa, Japan) in order to achieve a feed dosage of 10 and 20 μg LPSp kg BW^{-1} day^{-1}, respectively. Experimental diets were named according to the level of LPSp dosage administered to fish: 10 and 20 IS diets, respectively. The proximate biochemical composition of diets is shown in Table 1.

2.2. Animals, experimental conditions and general procedures

Rainbow trout (*O. mykiss*) fingerlings were purchased from a commercial hatchery (Alevines del Moncayo SA, Vozmediano, Spain), transported by road to the IRTA-SCR facilities (Sant Carles de la Rapita, Tarragona, Spain) and acclimated for three weeks in a 3 m^3 rectangular fiberglass tank. During this period, fish were fed twice a day with Microbaq 8 (Dibaq SA, Fuentepelayo, Spain) at 2% of the stocked biomass. Before the onset of the trial, all fish were individually weighted (BW_i) and measured for standard length to the nearest 0.1 g and 1 mm, respectively; and then distributed into 9 fiberglass cylindrical tanks of 400-L (125 fish per tank, initial density = 1.3 kg m^{-3}). During the acclimation and experimental periods, water temperature, conductivity and pH (pH meter 507, Crison Instruments) and dissolved oxygen (OXI330, Crison Instruments) were 13.2 ± 0.2 °C, 1800 ± 200 μS cm^{-1}, 7.5 ± 0.01 and 8.0 ± 0.3 mg L^{-1} (mean ± SD), respectively. Water flow rate in experimental tanks was maintained at approximately 9.0 L min^{-1} by means of a recirculation system (IRTAmar®), which maintained adequate water quality (total ammonia and nitrite ≤0.15 and 0.5 mg L^{-1}, respectively) through UV, biological and mechanical filtration. Photoperiod followed natural changes according to the season of the year (December–March; latitude 40°37′41″ N). Each diet was tested in triplicate (tanks) and was offered for juvenile rainbow trout (BW_i = 4.2 ± 0.1 g) for a period of 93 days. Feeds were distributed four times per day by automatic feeders (ARVO-TEC T Drum 2000TM, Arvotec, Huutokosk, Finland), at the rate of 3.3% of the stocked biomass, which approached apparent satiation.

Sampling to monitor fish growth took place monthly from the onset of the feeding period. For that purpose, 50 fish were randomly sampled from each tank, anesthetized with 150 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma-Aldrich, Madrid, Spain), and their wet body weight (BW, g) and standard length (SL, cm) were determined. At the end of the trial (93 days), all fish from each tank were measured for their final body weight (BW_f, g) and SL_f. Forty five specimens per experimental condition (15 per replicate) were sacrificed with an overdose of anesthetic, blood was withdrawn from the caudal vein of five fish from each tank with 1 mL syringes in less than 2 min, and tissues were excised for histological purposes ($n = 5$), assessing the functionality of the digestive system ($n = 5$) and proximate biochemical composition ($n = 5$). Blood (500 μL) was taken for the NBT assays, whereas the rest of blood was left to clot at 4 °C and immediately centrifuged to obtain serum.

Fish growth and feed utilization from different experimental groups was evaluated by means of the following indices:

$$\text{Feed conversion ratio}\left(\text{FCR, g g}^{-1}\right) = F/(B_f - B_i);$$

where F was the total feed intake during the experimental period (g) and B_f and B_i were the initial and final biomass in grams.

$$\text{Specific growth rate (SGR, \%)} = [(\ln BW_f - \ln BW_i) * 100]/\text{time (days)};$$

where BW_f and BW_i were final and initial body weights in grams.

$$\text{Fulton's condition factor (K)} = \left(BW_f/SL_f^{\,3}\right) * 100;$$

where BW_f and SL_f were the final body weight (8 g) and standard length (cm), respectively. K factor is a morphometric index that estimates fish's body condition, which is determined by measuring the weight and length of individual fish. This approach assumes that heavier fish of a given length are in better condition (Sutton et al., 2000).

Five fish were used for proximate biochemical analysis (whole fish). Fish were homogenized, and small aliquots were dried (120 °C, 24 h) to estimate water content. The total fat content from feed and fish was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of nitrogen followed by vacuum desiccation overnight (Folch et al., 1957). Protein and carbohydrate contents were determined according to Lowry et al. (1951) and Dubois et al. (1956), respectively. Ash contents were determined by keeping the sample at 500–600 °C for 24 h in a muffle furnace (AOAC, 1990). All chemical analyses were performed in triplicate per fish and feed samples.

All animal experimental procedures were conducted in compliance with the experimental research protocol (reference number 4978-T9900002) approved by the Committee of Ethics and Animal Experimentation of the IRTA and in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

2.3. Organization and functionality of the digestive system

For assessing the impact of the LPSp on the digestive system organization and functionality, sacrificed fish were dissected on a glass plate

Table 1
Proximate biochemical composition and energy content of diets used in this study. Data is expressed as mean ± SD.

	C diet	10 IS diet	20 IS diet
Protein (%)	64.2 ± 0.4	64.2 ± 0.2	64.3 ± 0.3
Fat (%)	11.8 ± 0.3	11.9 ± 0.3	11.9 ± 0.2
Ash (%)	11.2 ± 0.2	11.1 ± 0.2	11.1 ± 0.2
Fiber (%)	0.5 ± 0.01	0.5 ± 0.02	0.5 ± 0.01
Gross energy (kcal kg^{-1})[a]	2166	2168	2170

Abbreviations: C diet: commercial feed (Aller Futura, ALLER AQUA; Christiansfeld, Denmark) used as a control; 10 IS diet: control diet with 0.06 g LPSp kg^{-1}; 20 IS diet: control diet with 0.12 g LPSp kg^{-1}.

[a] Gross energy content was estimated as: total carbohydrate × 17.2 J kg^{-1}; fat × 39.5 J kg^{-1}; and protein × 23.5 J kg^{-1}.