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SHORT COMMUNICATION

Effect of a postbiotic on the histopathological features and expression levels of immune-related genes in farmed rainbow trout (*Oncorhynchus mykiss*)

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Aquaculture activity has experienced great diversification and development during the last decades. However, the occurrence of infectious diseases still remains one of the major limiting factors for further intensification, which may be exacerbated by the absence of effective strategies for disease control and prevention. Despite the fact that antibiotic therapy and vaccination have greatly reduced the incidence of diseases, the emergence of antibiotic-resistant bacteria coupled with limited immune protection has emphasized a need for novel treatments (Cabello et al., 2016; Embregts & Forlenza, 2016). The use of postbiotics has recently emerged as a safe alternative to prevent diseases and promote growth in commercial fish and shellfish species (Ang et al., 2020; Pérez-Sánchez et al., 2018). Postbiotics are soluble factors secreted by live bacteria or released after bacterial lysis, which may provide physiological benefit to the host (Aguilar-Toalá et al., 2018). Although the mechanisms implicated have not fully been elucidated, postbiotics possess different functional properties including, but not limited to, antimicrobial, antioxidant and immunomodulatory (Aguilar-Toalá et al., 2018; Puccetti et al., 2020). Moreover, we have recently demonstrated that dietary postbiotic supplementation modifies bacterial communities within the intestinal ecosystem of rainbow trout, thereby providing protection against pathogenic bacteria (Mora-Sánchez et al., 2020; Pérez-Sánchez et al., 2020). The aim of this study was, therefore, to assess the effect of a lactic acid bacteria-based postbiotic on the growth rate, histopathological features, and expression levels of immune-related genes in farmed rainbow trout (*Oncorhynchus mykiss*).

The experiment was conducted in six concrete ponds (30 m × 4 m × 0.8 m) at a commercial fish farm in Spain. Two groups, in triplicate, were randomly assigned to the different ponds, containing 9000 fish each. The first group was fed a commercial feed (Grupo Dibaq; Fuentepelayo, Spain) without any supplement, whereas the second group received the same feed to which the postbiotic was incorporated at a concentration of 3.0 mg/g. The postbiotic was obtained as a fermented product with two lactic acid bacteria belonging to the genus *Lactobacillus* and *Leuconostoc*, which were originally isolated from rainbow trout. The fermented product was then micronized using a cutting mill equipped with exchangeable sieves followed by three and two successive steps with 3.0- and 1.0-mm sieves, respectively. At the start of the experiment, the mean fish weight was 430 ± 16 g and they were fed ad libitum using demand-feeders at a daily maintenance ratio of 1.1% of their estimated body weight. Water quality parameters such as dissolved oxygen (>6.0 mg/L) and temperature (18.5 ± 4.5°C) remained within the expected range for the species.

After twelve weeks of feeding, fish were weighed to evaluate the effect of the postbiotic on the growth rate. Moreover, six fish from each group were sacrificed with an overdose of tricaine methanesulfonate in order to collect head-kidney samples for expression of immune-related genes, as well as organs and tissues for histopathology. All procedures for fish handling and euthanasia were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. Briefly, the total RNA of head-kidney samples was extracted using the RNeasy Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was then synthesized, and expression level of interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α) was determined using a 7500 Real-Time PCR system (Applied Biosystems; Foster City, CA, USA), as previously described (Pérez-Sánchez et al., 2011). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in each reaction as an internal standard, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Moreover, organs and tissue samples were collected for histopathology, which were immediately formalin-fixed and paraffin-embedded, and then stained with haematoxylin and eosin by standard procedures. Lesions were scored semi-quantitatively based on a scale of 0–3 where lesions were 0 = normal, 1 = mild, 2 = moderate, and 3 = severe, as previously described (Chang et al., 2016). PCR-based analyses were also performed on organs and tissue samples to confirm the presence of bacterial fish pathogens due to their prevalence in fish farms, such as *Aeromonas salmonicida*, *Lactococcus garvieae* and *Yersinia ruckeri*, as previously described (Del Cerro et al., 2002; Zlotkin et al., 1998), as well as to evaluate the efficacy of the postbiotic as a preventive tool because the six fish groups have not been vaccinated against these fish pathogens. All statistical analyses were performed using SPSS Statistics version 17.0 (SPSS Inc; Chicago, IL, USA). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test), which were further analysed using an unpaired two-tailed Student's *t*-test. The level of significance was set at $p < 0.05$.

After twelve weeks of feeding, no significant difference ($p = 0.61$) was observed in the final weight between fish fed the postbiotic-enriched diet and control groups, whose mean values were 1177.3 ± 142.5 g and 1237.2 ± 124.9 g, respectively. No significant difference ($p = 0.94$) was also found in the final total biomass between both groups, whose mean values were 6060.0 ± 728.2 kg/farm and 6022.3 ± 322.0 kg/farm in treated and control groups, respectively. Although the expression levels of two pro-inflammatory (IL-1β and TNF-α) cytokines and one anti-inflammatory (IL-10) cytokine were analysed, only IL-1β was significantly upregulated ($p < 0.05$) in the head kidney of fish fed the postbiotic-enriched diet compared to the control groups (Figure 1). These results suggest that postbiotics differentially regulates the expression of pro-inflammatory cytokines. IL-1β has diverse physiological functions and its roles in regulating the inflammatory process are conserved in fish (Secombes et al., 2011). Although previous studies have suggested that TNF-α has overlapping functions with IL-1β, TNF-α plays an important role in diverse host responses, including apoptosis, cell proliferation, differentiation, necrosis and the induction of other cytokines (Reyes-Cerpa et al., 2012; Zou & Secombes, 2016). However, no or mild lesions were observed histologically in fish fed the postbiotic-enriched diet. As IL-1β can respond to a variety of stimuli, it is reasonable to expect that the upregulation of IL-1β is mediated by postbiotics, which are soluble factors (products or metabolic by-products) secreted by probiotic strains or released after bacterial lysis. It should also be noted that fish IL-1β modulates the expression of IL-17 family members, which are important for antibacterial defence (Kono et al., 2011; Secombes et al., 2011). These findings may explain, at least in part, the mechanisms of how postbiotics exert their beneficial effects. In fact, we have recently demonstrated that this postbiotic reduces the susceptibility of rainbow trout to *Lactococcus garvieae*, and its dietary supplementation also modifies the intestinal microbiota composition (Pérez-Sánchez et al., 2020).

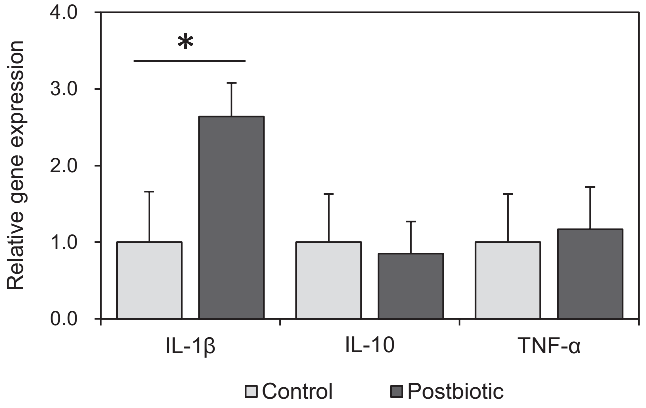

FIGURE 1 Expression levels of IL-1β, IL-10 and TNF-α in head-kidney samples from fish fed the postbiotic-enriched diet (black bars) and control groups (grey bars). The error bars denote standard deviation, and the asterisk indicates the significant difference ($p < 0.05$) between treated and control groups

Interestingly, histopathological examination demonstrated that treated fish had no or mild lesions, whereas moderate lesions were observed in the intestine, gastric mucosa and liver of untreated fish (Figure 2). Lesions such as detachment of intestinal villi (see Figure 3) and inflammatory cell infiltration in the liver were particularly observed in the control group. There was, however, no evidence for the presence of the most common bacterial fish pathogens (*A. salmonicida*, *L. garvieae* and *Y. ruckeri*), either in the control group or in the treated group. Similar results have been reported in fish treated with probiotics (Bunnoy et al., 2019; Pérez-Sánchez et al., 2011; Pirarat et al., 2011); however, unlike previous studies, we have demonstrated the beneficial effects of postbiotics, which should mimic the properties of probiotics, while avoiding the potential risk of administering live microorganisms.

In summary, we observed that dietary postbiotic supplementation modulates the host immune response, as well as reduces tissue damage in fish. To our knowledge, this study reports for the first time the upregulation of fish IL-1β in response to postbiotics, which is involved in the regulation of immune relevant genes. Although further studies are needed to understand the underlying mechanisms by which postbiotics confer a health benefit, these promising results support the use of postbiotics as a sustainable alternative to antibiotics in aquaculture.

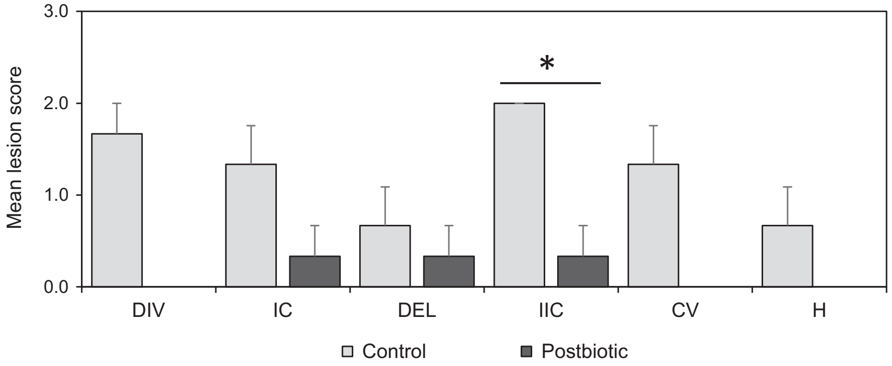

FIGURE 2 Lesion scores in the intestine, stomach, and liver of fish fed the postbiotic-enriched diet (black bars) and control groups (grey bars). Intestinal tissue (DIV, detachment of intestinal villi); gastric mucosa (IC, inflammatory cells; DEL, detachment of the epithelial layer); and liver tissue (IIC, infiltration of inflammatory cells; CV, cytoplasmic vacuolation; H, haemorrhage). The error bars denote standard deviation, and the asterisk indicates the significant difference ($p < 0.05$) between treated and control groups

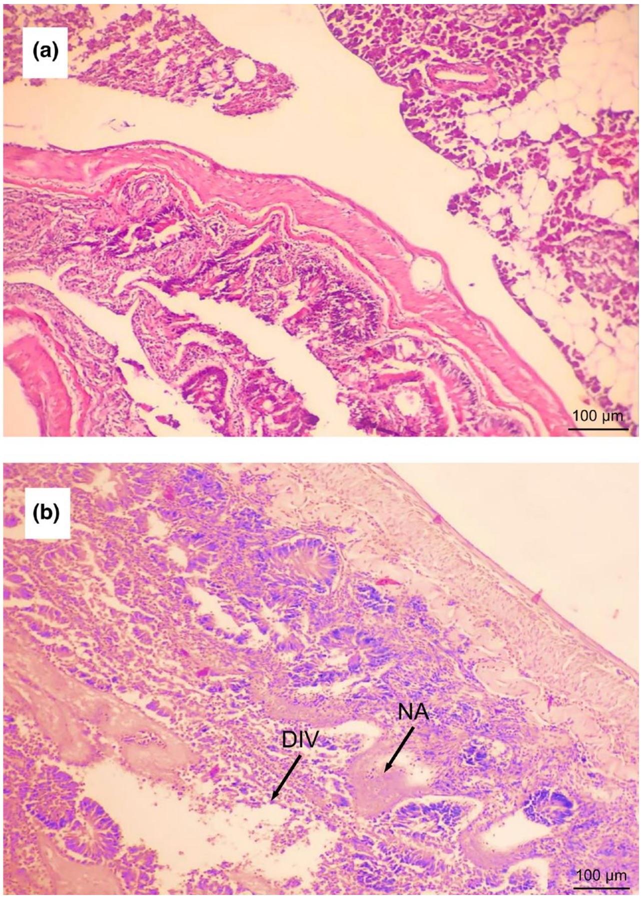

FIGURE 3 (a) Representative intestinal histopathology of a fish fed the postbiotic-enriched diet, which shows normal morphology. (b) Representative intestinal histopathology of an untreated fish, which shows moderate necrotic areas (NA) and detachment of intestinal villi (DIV)

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors made contributions to conception and design, acquisition, and extraction of data and analysis and interpretation of the results. TPS, BMS sampled the animals. TPS, BMS and WJ were involved in the laboratory analysis. BF and JLB did the data analysis. All authors were involved in drafting and revising the manuscript and approved the final version. Each author agrees to be accountable for all aspects of the accuracy or integrity of the work.

DATA AVAILABILITY STATEMENT

Data are available on reasonable request.

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