Microbial Pathogenesis 142 (2020) 104060

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Changes in intestinal microbiota and disease resistance following dietary postbiotic supplementation in rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

Keywords:
Rainbow trout
Lactococcosis
Dysbiosis
Lactic acid bacteria
Postbiotic

ABSTRACT

This experimental study was aimed to investigate whether the dietary supplementation of a postbiotic obtained as a food product fermented with two lactic acid bacteria could induce changes in the intestinal microbiota and prevent the development of *Lactococcus garvieae* infection in rainbow trout (*Oncorhynchus mykiss*). After 30 days of dietary postbiotic supplementation, bacterial community composition and structure was significantly different between the treated and control groups. A higher bacterial diversity and richness in the intestinal samples was found in treated fish, as compared to those samples from untreated fish. Dietary postbiotic supplementation also conferred increased protection against *L. garvieae* infection. These findings suggest that the establishment of a beneficial microbiota is essential to prevent diseases or protect the host from foreign agents.

1. Introduction

Antimicrobial resistance has become a global health threat that requires a coordinated action at the human-animal-environment interface [1]. Aquaculture production is not exempt from these threats because antibiotics are still used for treating fish and shellfish diseases [2–4]. Alternative strategies are therefore urgently needed to tackle this serious and growing threat [5,6]. The use of postbiotics may be a viable alternative approach for preventing and controlling infections. Broadly speaking, the term “postbiotics” refers to soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis, which may provide physiological benefit to the host [7]. Although previous studies have provided plausible evidence of several mechanisms underlying the health-promoting effects of desirable intestinal bacteria or probiotics; recent evidence suggests that bacterial viability is not necessary to develop health-promoting effects [7,8]. As a consequence, the use of postbiotics has emerged as a novel and safe strategy to confer health benefits to the host, while avoiding the potential risk of administering live bacteria. Two lactic acid bacteria belonging to the genus *Lactobacillus* and *Leuconostoc* were selected for this study, which were originally isolated from rainbow trout (*Oncorhynchus mykiss*). These strains exhibited antagonistic activity against *Lactococcus garvieae* under *in vitro* conditions, whose antimicrobial substances were sensitive to proteolytic enzymes. This study was therefore aimed to investigate the effect of a lactic acid bacteria-based postbiotic on intestinal bacterial communities of rainbow trout, as well as to determine its capacity to protect against *L. garvieae* infection.

2. Materials and methods

2.1. Fish and experimental conditions

A total of 160 healthy rainbow trout were obtained from a commercial fish farm. After the acclimatization period for two weeks, the mean fish mass was 24.1 ± 7.4 g. The fish were then randomly assigned to three groups. The first two groups were fed a commercial feed (AQUASOJA, Portugal) without any supplement. The first group (n = 70) served as the control, whereas the second group (n = 20) served to keep fish that were used for experimental infection. The third group (n = 70) received the same feed to which the postbiotic, at a

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https://doi.org/10.1016/j.micpath.2020.104060
Received 12 December 2019; Received in revised form 8 February 2020; Accepted 10 February 2020
Available online 11 February 2020

concentration of 3.0 mg/g, was added by the manufacturer (AQUAS-OJA). The postbiotic was obtained as a fermented food product composed by soy and alfalfa flour, in which two lactic acid bacteria were added in similar concentrations. The fermentation process was then performed, as previously described [9]. The fermented food product was micronized before sending to the manufacturer. All fish were fed daily an amount equal to 1.5% of their biomass.

2.2. Sample collection and sequence analysis

After four weeks of treatment, fish were individually weighed to evaluate the effect of dietary postbiotic supplementation on growth. Moreover, four fish per treatment were sacrificed to collect the intestinal samples, as previously described [10]. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (QIAGEN; Valencia, CA, USA) and submitted to Macrogen Inc. (Seoul, Korea) for high-throughput 16S rRNA gene sequencing on the Illumina MiSeq platform. Analysis of 16S rRNA gene sequences was performed using the MOTHUR software package [11]. Briefly, sequences were aligned and clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff. Selected OTUs were classified using the EzBioCloud database [12]. Alpha diversity was calculated using the Shannon diversity index (H') and the Chao1 richness estimator. Beta diversity was calculated using the Bray-Curtis dissimilarity metric and visualized with principal coordinate analysis (PCoA) plots. Analysis of molecular variance (AMOVA) was used to test for differences in community structure.

2.3. Experimental infection

Immediately after weighing and collecting intestinal samples, fish were challenged with *L. garvieae* by the cohabitation method. A volume of 0.1 ml of a bacterial suspension (*L. garvieae* at 10^4 CFU/ml) was injected intraperitoneally into all fish used for cohabitation (the second group), which were anaesthetized with tricaine methanesulfonate (Tricaine Pharmaq 1000 mg/g) and marked by clipping the adipose fin after injection. Ten infected fish were transferred into each group (the first and third group). All fish were monitored at least three times daily, and dead fish were immediately removed and examined for external disease signs.

2.4. Statistical analysis

All statistical analyses were performed using SPSS Statistics v17.0 (SPSS Inc.; Chicago, IL, USA). Differences in the final weight were analyzed using Mann-Whitney *U* test because data sets were not homogeneous for variance. Alpha diversity (Shannon diversity and Chao1 richness indices) was analyzed using an unpaired two-tailed Student's *t*-test. Survival curves were calculated using the Kaplan-Meier method, and significance was determined using the log-rank test. In all cases, the level of significance was set at $p < 0.05$.

3. Results and discussion

In this study, we initially observed that postbiotic (a food product fermented with two lactic acid bacteria) did not exert any side effects on fish growth. In fact, there was slight increase in the weight of fish treated with postbiotic after four weeks; however, the differences were not significant ($p = 0.07$) between the treated (postbiotic) and control groups, whose mean values were 47.5 ± 12.8 g and 44.2 ± 10.1 g, respectively.

After four weeks of dietary postbiotic supplementation, we also observed that bacterial communities in the intestinal samples from treated fish had significantly ($p < 0.01$) higher diversity (3.7 ± 0.7) and richness (10,544.7 ± 1711.6) than those samples from untreated fish (1.9 ± 0.3 and 3436.8 ± 1114.4, respectively). Moreover, we observed that bacterial community structure was significantly different

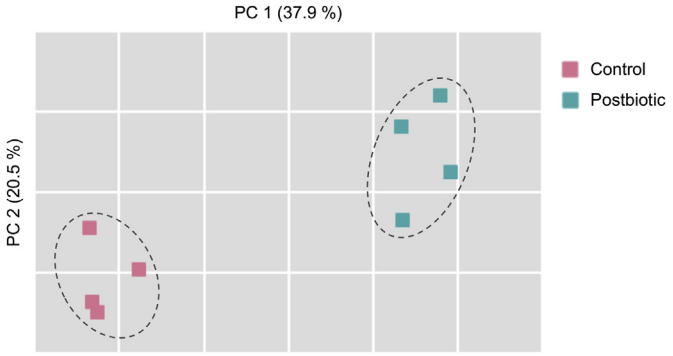

Fig. 1. Bacterial community structure in the intestinal samples from rainbow trout fed a diet with or without postbiotic for 30 days. PCoA plots are based on the Bray-Curtis dissimilarity metric. The first and second axes represent 37.9% and 20.5% of the variation, respectively.

($p = 0.02$) between the treated and control groups, which was determined using a distance matrix based on the Bray-Curtis dissimilarity metric and visualized using PCoA plots (Fig. 1). There were large differences in bacterial community composition between treatment and control groups (Fig. 2). A higher proportion of sequences affiliated to the phylum Proteobacteria (48.9 ± 5.8%), and to a lesser extent, Fusobacteria (6.1 ± 0.5%) was found in the intestinal samples from untreated fish, as compared to those treated with the postbiotic (36.1 ± 14.7% and 2.4 ± 0.9%, respectively). Moreover, sequences affiliated to the phylum Planctomycetes (9.0 ± 5.6%) were only detected in untreated fish. Although bacterial communities in treated fish were also dominated by the phylum Proteobacteria, differences were observed at the OTU level (defined at 97% similarity) between treatment and control groups. For instance, a high proportion of sequences assigned to the genus *Desulfovibrio* was found in the intestinal samples from untreated fish. This reinforces the use of dietary postbiotic because some *Desulfovibrio* species can have important health implications, such as bacteremia caused by *Desulfovibrio desulfuricans* [13]. Likewise, OTUs affiliated to the genera *Enhydrobacter*, *Paracoccus* and *Pseudomonas* were only detected in the intestinal samples from treated fish. Some members belonging to these genera are ubiquitous in aquatic environments and have been found to produce antibacterial compounds and polyunsaturated fatty acids [14,15]. A higher proportion of sequences affiliated to the phyla Firmicutes (19.6 ± 5.3%) and Actinobacteria (9.5 ± 2.7%) was also found in treated fish, as compared to those of the untreated group (5.1 ± 3.7% and 0.6 ± 0.3%, respectively). Interestingly, a relatively high proportion of sequences affiliated to the phylum Firmicutes was found in treated fish, which were classified as belonging to the genus *Lactobacillus* (notably the species *Lb. amylovorus*). Previous studies have demonstrated that *Lactobacillus* species may exert several beneficial effects on the host, including immunomodulation, interference with potential pathogens, and maintenance of a healthy intestinal microbiota [16–18]. Although the antimicrobial substances of the two lactic acid bacteria used for the fermentation process were not identified in this study, these substances are sensitive to proteolytic enzymes, suggesting that they might be bacteriocins. Bacteriocins are ribosomally synthesized peptides or proteins that are generally effective against closely related species [19]. It is therefore expected that dietary postbiotic supplementation can induce or restore a disturbed microbiota to its normal beneficial composition, thereby providing protection through the creation of a hostile environment for potential pathogens.

In order to reinforce the idea that dietary postbiotic supplementation may confer protection against infections, fish were challenged with *L. garvieae* which is a serious fish pathogen responsible for lactococcosis. This disease produces hyperacute and hemorrhagic septicemia in several farmed fish species worldwide [20,21]. Statistical analysis

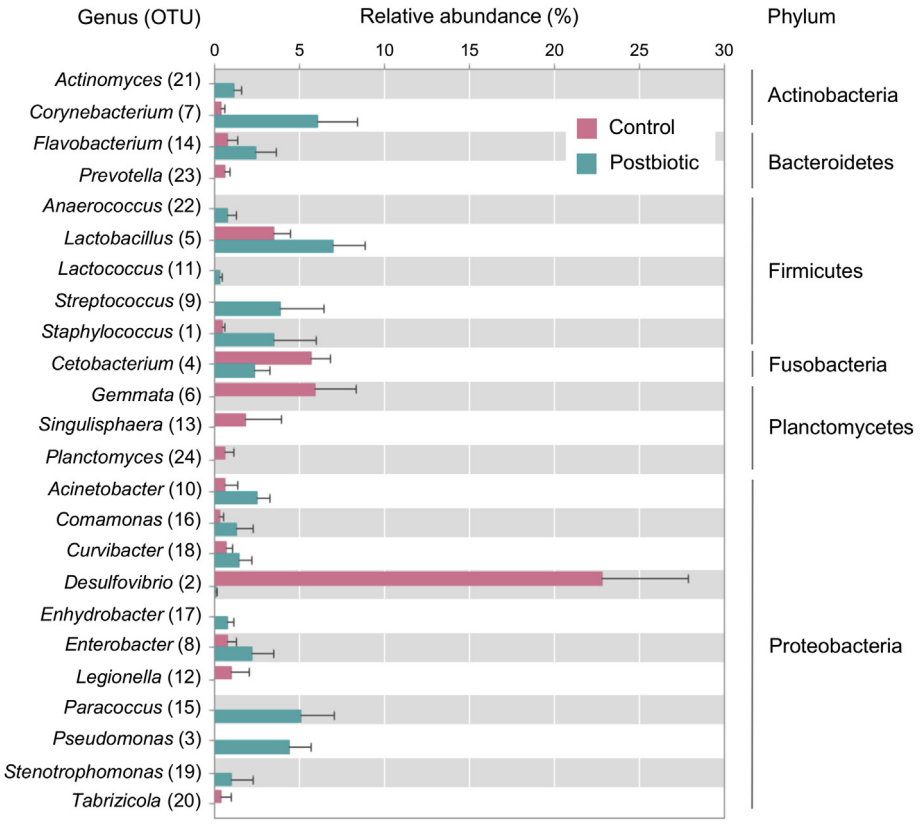

Fig. 2. Relative abundance of dominant genera found in the intestinal samples from rainbow trout fed a diet with or without postbiotic for 30 days.

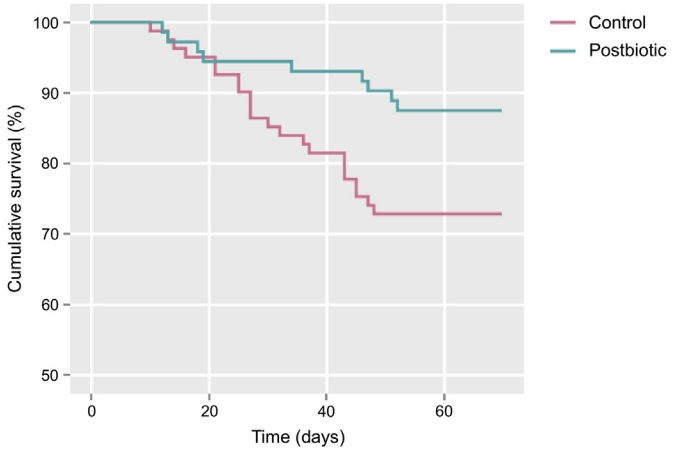

Fig. 3. Cumulative survival of rainbow trout challenged with *L. garvieae* by cohabitation and treated with postbiotic. Survival in the postbiotic group was significantly higher ($p = 0.02$) than the control group according to the Kaplan–Meier method.

revealed significant differences (log-rank test, $p = 0.02$) in fish survival between the treatment and control groups (Fig. 3). Cumulative survivals of the treatment and control groups were 87.5 and 72.8%, respectively. These findings, together with evidence from previous studies, suggest that manipulation of the intestinal microbiota may represent a valuable strategy to increase fish survival rates. To date, manipulation of the intestinal microbiota through probiotics has demonstrated encouraging results to control *L. garvieae* infection [16,17,22]. To our knowledge, however, this is the first study demonstrating beneficial effects of dietary postbiotic administration against *L. garvieae* infection in fish.

4. Conclusions

In summary, we observed that dietary postbiotic supplementation may provide a viable alternative to antibiotics, avoiding the risk of administering live bacterial cells that could transfer antibiotic resistance genes. Postbiotics can both increase bacterial diversity and richness within the intestinal ecosystem, and create a hostile environment for the pathogen by interfering with pathogen colonization, which was demonstrated by high-throughput 16S rRNA gene sequence analysis of fish intestinal samples. This novel dietary strategy has demonstrated promising results to prevent an important disease without the need for handling fish, and confer a beneficial microbiota with the ability to protect the host from pathogens and other foreign agents.

Data availability

All data are available on request from the authors.

Ethics approval

This study was approved by the Ethics Committee on Animal Experimentation (Project License PI57/17) of the Universidad de Zaragoza, Spain.

CRediT authorship contribution statement

Tania Pérez-Sánchez: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **Brenda Mora-Sánchez:** Formal analysis, Investigation, Methodology, Writing - original draft. **Augusto Vargas:** Formal analysis, Methodology. **José Luis Balcázar:** Supervision, Writing - review & editing.

Declaration of competing interest

None.

Acknowledgments

B. Mora-Sánchez was supported by a fellowship from “Banco Santander – Universidad de Zaragoza”. J.L. Balcázar acknowledges the support from the Economy and Knowledge Department of the Catalan Government through Consolidated Research Group (ICRA-ENV 2017 SGR 1124).

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