Effect of a novel postbiotic containing lactic acid bacteria on the intestinal microbiota and disease resistance of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Objective: This study was aimed to assess the effect of a novel postbiotic on bacterial community composition and structure within the intestinal ecosystem of rainbow trout (*Oncorhynchus mykiss*), as well as evaluate its capacity to protect rainbow trout from *Lactococcus garvieae* infection.

Results: After 30 days of dietary postbiotic supplementation, high-throughput 16S rRNA gene sequencing revealed that bacterial community composition, diversity and richness were significantly higher in treated fish than in control fish. The proportion of sequences affiliated to the phylum Tenericutes, and to a lesser extent, the phyla Spirochaetes and Bacteroidetes was increased in fish fed a postbiotic-enriched diet compared to control fish, whereas the abundance of Fusobacteria was higher in control fish. Moreover, the treated fish showed significantly (p<0.05) improved protection against *L. garvieae* compared to control fish.

Conclusions: These findings suggest that dietary postbiotic supplementation may represent an environmentally friendly strategy for preventing and controlling diseases in aquaculture.

Keywords: Rainbow trout; lactococcosis; treatment; postbiotic

1. Introduction

Lactococcosis is a disease caused by *Lactococcus garvieae*, which is responsible for severe economic losses in farmed marine and freshwater fish species (Vendrell et al. 2006; Gibello et al. 2016; Meyburgh et al. 2017). Disease outbreaks are usually treated with vaccines or antibiotics. Although vaccination has proven effective in protecting fish from lactococcosis, this immunity lasts a short period of time or the process is often ineffective when applied to immunologically immature fish (Ravelo et al. 2006; Embregts and Forlenza 2016). Moreover, the use of antibiotics should be limited due to the increasing prevalence of antibiotic-resistant bacteria (Cabello et al. 2013; Santos and Ramos 2018). Consequently, new environmentally-friendly strategies for disease prevention and control are urgently needed. Among, the use of postbiotics is becoming increasingly popular for treatment and/or prevention of diseases (Pérez-Sánchez et al. 2018). Postbiotics are soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis, which may provide physiological benefit to the host (Aguilar-Toalá et al. 2018). Previous studies, including our own, have demonstrated the probiotic effect of some lactic acid bacteria strains under *in vitro* and *in vivo* conditions (Vendrell et al. 2008; Pérez-Sánchez et al. 2011; Zheng et al. 2017). However, the efficiency of a postbiotic obtained as a fermented food product on the lactococcosis prevention has not been previously evaluated. This postbiotic derives from a fermented food, which has been previously evaluated in animal husbandry (HEALTSTOCK project, ID 733627; https://cordis.europa.eu/projects/en).

Given that postbiotics may be potential alternatives to the use of live probiotic microorganisms, it becomes interesting to explore their effects in species of aquaculture interest. The aim of this study was therefore to investigate the effect of a novel postbiotic obtained as a fermented food product containing lactic acid bacteria on bacterial community structure and composition within the intestinal ecosystem of rainbow trout

(*Oncorhynchus mykiss*), as well as evaluate its capacity to protect rainbow trout from *L. garvieae* infection.

2. Materials and Methods

2.1. Postbiotic and experimental conditions

The postbiotic was obtained as a fermented food product composed of soy and alfalfa flour. This fermented food was obtained in two stages. Briefly, a lactic acid bacterium belonging to the genus *Lactobacillus*, previously isolated from rainbow trout and deposited at the Spanish Type Culture Collection (CECT 9882), was grown in de Man, Rogosa and Sharpe broth (MRS; Oxoid, Basingstoke, UK) overnight at 22 °C. The first stage covered the fermentation of the bacterial pre-culture with other minor components. After the incubation period, bacterial cells were collected and non-bitter beer yeast was added to the raw material and the second fermentation process was then performed, as previously described (Cabello-Olmo et al. 2019). The fermented food product was micronized to favor the mixture with commercial feed (Inicio Plus 887; BioMar Iberia, S.A., Dueñas, Spain).

A total of 100 pathogen-free rainbow trout weighting 24.6 ± 5.1 g were obtained from a commercial fish farm, which were acclimatized in our experimental fish facility for two weeks. The fish were then randomly assigned to three experimental groups and maintained in three tanks. Two groups were fed a commercial feed without any supplement: one group (n=40) was used as untreated control, whereas the other group (n=20) was used for the experimental infection as donors. The third group (n=40) received a diet obtained by adding the postbiotic to the commercial diet at 3.0 mg/g. All fish were fed daily at 1.5% of their biomass.

2.2. DNA extraction and sequence analysis

After 30 days of feeding, fish were individually weighed and four fish per treatment were sacrificed to collect the intestinal samples, as previously described (Etyemez and Balcázar 2015). Genomic DNA was extracted using the DNeasy Blood & Tissue kit (QIAGEN; Valencia, CA, USA), and the final concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Genomic DNA samples were then 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 (Schloss et al. 2009). Briefly, paired-end reads were merged into contigs, screened for quality, aligned to the SILVA reference database, and screened for chimeras. Sequences were then randomly subsampled to contain the same number of sequences (99,729) for further comparisons. Curated sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff with the average neighbor clustering algorithm. Alpha diversity was calculated using the Shannon diversity index (H') and the Chao1 richness estimator. Beta diversity was calculated using the Yue & Clayton estimator, which measures the number of shared genera and their relative abundances. The relationship among samples was visualized in a principal coordinate analysis (PCoA) plot based on the Yue & Clayton measure of dissimilarity. Analysis of molecular variance (AMOVA) was used to determine whether the clustering within the ordinations is statistically significant at $p<0.05$.

2.3. Experimental infection

After 30 days of feeding, fish were challenged with *L. garvieae* by the cohabitation method. Briefly, *L. garvieae* strain FLP 33, previously isolated during a natural lactococcosis outbreak in rainbow trout, was grown on tryptic soy agar overnight, resuspended in phosphate-buffered saline (PBS), and adjusted to a concentration of 10^4

CFU/ml. A volume of 0.1 ml of this suspension 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 then transferred into the tanks containing the other two experimental groups. All fish were monitored at least three times daily, and dead fish were immediately removed and examined for external signs of lactococcosis.

2.4. Statistical analysis

All statistical analyses were performed using SPSS Statistics v17.0 (SPSS Inc.; Chicago, IL, USA). Differences in the final weight and alpha diversity (number of OTUs, Shannon diversity index and Chao richness estimators) were 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. The level of significance was set at $p<0.05$.

3. Results

After 30 days of feeding, no significant difference ($p=0.08$) was observed in the final weight between treated (postbiotic) and control groups. Mean final weight of fish was 36.4 ± 7.2 g for treated group and 39.2 ± 6.9 g for control group. Moreover, the number of OTUs observed at a 97% taxonomic cutoff was significantly higher ($p<0.05$) in the intestinal samples from fish treated with the postbiotic (414±59), as compared to those samples from untreated fish (334±16). Shannon diversity index and Chao richness estimators were also determined, demonstrating that the intestinal samples from treated fish (1.2±0.2 and 4,437.9±241.2, respectively) had significantly higher ($p<0.01$) bacterial diversity and richness than those samples from untreated fish (0.9±0.1 and 3,788.4±262.5, respectively).

Overall taxonomic characterization of the bacterial community was conducted at the phylum level (Fig. 1). Although Tenericutes and Fusobacteria were found to be the most abundant phyla, there were differences in their abundance between treated and control groups. A higher proportion of sequences affiliated to the phylum Fusobacteria (notably the genus *Cetobacterium*) was found in the intestinal samples from untreated fish (control), as compared to those treated with the postbiotic. In contrast, a higher proportion of sequences affiliated to the phylum Tenericutes (mainly members belonging to the genus *Mycoplasma*) was found in fish treated with the postbiotic, as compared to those of the control group. A slight increase in the abundance of Spirochaetes (notably the genus *Brevinema*) and Bacteroidetes (particularly members belonging to the genera *Bacteroides*, *Dysgonomonas* and *Flavobacterium*) was also observed in fish treated with the postbiotic. At the phylum level, the abundance of sequences affiliated to Proteobacteria, and to a lesser extent, Actinobacteria and Firmicutes was similar between treated and control groups; however, differences were observed at the OTU level (defined at 97% similarity) between the two groups.

The effect of dietary postbiotic supplementation on bacterial community structure within the intestinal ecosystem was determined using a distance matrix based on the Yue & Clayton measure and visualized using PCoA plots (Fig. 2). The results revealed clear separation between treated and control groups. These observations were further validated using AMOVA tests, as implemented by MOTHUR, which showed that bacterial community structure was significantly different ($p<0.01$) between treated and control groups.

After 30 days of feeding, fish were challenged with *L. garvieae* and Kaplan-Meier analysis revealed a significant difference ($p<0.05$) in the cumulative survival between

175 treated and control groups (Fig. 3). Cumulative survivals were 75.0 and 52.5% in treated
176 and control groups, respectively.
177
178 **4. Discussion**
179 In the present study, we observed that dietary postbiotic supplementation may modify
180 bacterial community composition and structure within the fish intestinal ecosystem.
181 Previous studies have suggested that the manipulation of the host microbiota may
182 represent a valuable strategy to prevent and/or control pathological and physiological
183 disorders (Pérez et al. 2010). Although there was no significant difference in the final
184 weight between treated and control groups, possibly due to the short period of the study,
185 we observed that dietary postbiotic supplementation conferred significantly improved
186 protection against *L. garvieae* infection. Postbiotics are nonviable bacterial products or
187 metabolic byproducts from probiotic microorganisms that have biological activity in the
188 host (Patel and Denning 2013). In fact, postbiotics aim to mimic the beneficial effects of
189 probiotics while avoiding the risk of administering live microorganisms. Although the
190 mechanisms underlying their effects seem to be mediated through an interaction between
191 the host and microbial products, there is limited information on their effects on fish
192 intestinal microbiota. In our study, the proportion of sequences affiliated to Tenericutes,
193 and to a lesser extent, Spirochaetes and Bacteroidetes was increased in the intestinal
194 samples of fish treated with the postbiotic as compared to those of untreated fish, whereas
195 the abundance of Fusobacteria was higher in untreated fish. Interestingly, we observed
196 that all sequences affiliated to the phylum Tenericutes were classified as belonging to the
197 genus *Mycoplasma*, which were dominant in treated fish. A recent study demonstrated
198 that abundance of potential pathogenic *Vibrio* species appeared to be inversely correlated
199 with the presence of *Mycoplasma* species within the mid-intestinal microbiota of farmed

Chinook salmon (Ciric et al. 2019). Likewise, a recent study demonstrated that *Mycoplasma* species was the dominant taxon in the gut microbiota of both resistant and susceptible lines of rainbow trout, although it was more abundant in the resistant line (Brown et al. 2019). It is therefore reasonable to assume that disease resistance may be associated with dietary postbiotic supplementation, which increased *Mycoplasma* species levels within the fish intestinal ecosystem. However, further studies are needed to explore the ability of *Mycoplasma* species to prevent bacterial infections.

In conclusion, the ability of a novel postbiotic from lactic acid bacteria to modify the intestinal microbiota and confer disease resistance was elucidated in this study. These findings, together with evidence from previous studies in other species (Kareem et al. 2016; Izuddin et al. 2019), suggest that dietary postbiotic supplementation may represent a suitable alternative to the use of probiotics, thereby avoiding potential risks of administering live microorganisms.

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Data availability

All data are available on request from the authors.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Research involving human participants or animals

The study was conducted considering the 3Rs principle (reduction, replacement and refinement). The care and use of fish were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

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Figure captions

Fig. 1. Relative abundance of dominant bacterial lineages found in the intestinal samples from rainbow trout fed a diet with or without postbiotic for 30 days.

Fig. 2. 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 Yue & Clayton measure of dissimilarity. The first and second axes represent 45.6% and 30.2% of the variation, respectively.

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 ($p<0.05$) than the control group according to the Kaplan–Meier method.