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PROBIOTIC INGESTION PREVENTS TAIL-ROT IN sENEGALESE SOLE EARLY CULTURE

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Introduction

The current work focuses on an unexpected observation during the execution of a long-term experiment in which the effects of probiotic supplementation on *Solea senegalensis* culture was being tested. The initial main goal of the experimental design was to evaluate the long-term effects of prolonged intake of probiotics from the first stages of larval development to the juvenile stage. In a periodic routine sampling of the culture on day 45 after hatching, the presence of tail-rotting in some sampled animals was observed. Here, we present the results of the evaluation carried out at this experimental point after this observation, focusing on the potential beneficial effect of the tested probiotic on Senegalese sole early culture to prevent tail rot.

Material and methods

All protocols involving animals were approved (authorization number 2021-02) by the Spanish and institutional bioethical guidelines of the Animal Welfare Service following European Union Directive 2010/63/EU for the protection of animals for experimental uses and Spanish regulations (RD/2013). The initial batch of embryos for the experiment was obtained by in vitro fertilization (IVF) from F1 broodstock following the protocol described by Rasines (Rasines et al, 2013). To ensure the genetic origin of the larvae, maximizing uniformity of the experimental replicates, gametes from only one female and one male were used for IVF. The progeny was split at 1 day post hatching (dph) into six 200 L round tanks (3 per experimental condition) at a 50 larvae/L density. General culture protocol and feeding regime were based on Cañavate and Fernández-Díaz, 1999 with some modifications. Two experimental groups were created: the control group (CTRL) in which live food was enriched with a commercial product (Easy Dry SELCO®, Inve Aquaculture) and the experimental group (PROBIO) in which live food was enriched (10^{11} CFU/mL) with *Pediococcus acidilactici* MA 15/5M (Bactocell®, Lallemand). Immediately after the observation of tail rot in the 45 dph sampling, a prophylactic treatment with hydrogen peroxide was provided to all tanks to avoid deterioration of the specimens.

Cumulative mortality was evaluated daily along the experiment. Thirty fish per experimental group (10 fish/replicate) were sampled for weight at 45 dph. SGR $((\ln W_f - \ln W_0)*100/T_f-T_o)$ was used as parameter to monitor growth. After biometrical analysis, each animal was placed under a stereomicroscope and dorsal images focusing on the tail were captured. Tail areas were measured using Adobe Photoshop CC 2020. In addition to the quantitative approach, the tails were evaluated subjectively by five different people. Evaluators were given an illustrated scale with examples of severity of tail rot starting from 0 (no lesion), 1 (low; a reduction of 25% of fin tissue), 2 (moderate; 50% of tissue reduction) to 3 (high incidence, practically absence of tail fin rays). The evaluators blindly assigned each image a level of affection based on the scale with a margin of 0.5 points for each of the 60 images. The mean of the five data for each image was considered as the tail rot incidence value for the specimen.

In order to analyse the immune response generated in the fish, total RNA was extracted from 9 fish of each group (3 per tank). Each fish body was homogenized (T25 Ultra Turrax ®, IKA) in an initial volume of 1 mL of TRI Reagent® (Merck). After cDNA synthesis (2 µg; High-Capacity RNA-to-cDNA™ Kit, Applied Biosystems), the following genes were evaluated by qPCR using SYBR green master mix (Applied Biosystems): hepcidin (HAMP1), complement c3 (C3), leucocyte cell-derived chemotaxin 2 (LECT2), non-specific cytotoxic cell receptor (NCCRP1), tumor necrosis factor a (TNFA) and interleukin 1 beta (IL1B). Three technical replicates were used in the qPCR analysis. The levels of the expression of these genes were normalized to eukaryotic elongation factor 1 alpha (eEF1A) levels using the formula $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001).

Statistical analysis was performed using SPSS 21.0. Non-parametric variables were analyzed using Mann-Whitney test. Normally distributed variables were analyzed using the student's t-test. Values with $p < 0.05$ were considered to be statistically significant. Data are expressed as mean ± SEM.

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Results and Discussion

Mortality data were globally very low at 45 dph. In both experimental groups for this time point, survival was similar and above 98%. Taking into account specific growth rate values, the statistical analysis revealed significant difference in SGR ($p = 0.0161$) indicating a slightly higher growth rate in PROBIO group. Regarding the tails, the statistical analysis showed a strong significant difference ($p < 0.0001$) between the fin area of both groups. While the CTRL group presented a mean area of 0.1893 ± 0.0518 cm^2, the PROBIO group showed a mean value of 0.3240 ± 0.0090 cm^2. These quantitative values were in line with the severity differences ($p < 0.0001$) issued by the evaluators. While in the CTRL group, the mean value of the incidence was scored with 1.8530 ± 0.0993 a.u.; in the PROBIO group the mean was 0.2500 ± 0.0528 a.u., since almost any fish showed signs of tissue reduction. Gene expression analysis corroborated that the animals in the CTRL group were undergoing an activation of their immune system with an overexpression of C3 ($p = 0.0034$) and LECT2 ($p = 0.0012$). Taking these data together, the observations recorded in this experiment indicate that the bioencapsulation of the probiotic strain *Pediococcus acidilactici* MA 15/5M in rotifers and artemia metanauplii may be a useful biotechnological tool to prevent the appearance of tail rot in the early culture of Senegalese sole.

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