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Dietary administration of the probiotic *Shewanella putrefaciens* Pdp11 promotes transcriptional changes of genes involved in growth and immunity in *Solea senegalensis* larvae

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ABSTRACT

Senegalese sole (*Solea senegalensis*) has been proposed as a high-potential species for aquaculture diversification in Southern Europe. It has been demonstrated that a proper feeding regimen during the first life stages influences larval growth and survival, as well as fry and juvenile quality. The bacterial strain *Shewanella putrefaciens* Pdp11 (SpPdp11) has shown very good probiotic properties in Senegalese sole, but information is scarce about its effect in the earliest stages of sole development. Thus, the aim of this study was to investigate the effect of SpPdp11, bioencapsulated in live diet, administered during metamorphosis (10–21 dph) or from the first exogenous feeding of Senegalese sole (2–21 dph). To evaluate the persistence of the probiotic effect, we sampled sole specimens from metamorphosis until the end of weaning (from 23 to 73 dph). This study demonstrated that probiotic administration from the first exogenous feeding produced beneficial effects on Senegalese sole larval development, given that specimens fed this diet exhibited higher and less dispersed weight, as well as increases in both total protein concentration and alkaline phosphatase activity, and in non-specific immune response. Moreover, real-time PCR documented changes in the expression of a set of genes involved in central metabolic functions including genes related to growth, genes coding for proteases (including several digestive enzymes), and genes implicated in the response to stress and in immunity. Overall, these results support the application of SpPdp11 in the first life stages of *S. senegalensis* as an effective tool with the clear potential to benefit sole aquaculture.

1. Introduction

In the past five decades, aquaculture has become one of the fastest-growing food industries. It is estimated that, currently, more than half of the total food of aquatic origin consumed by the world population comes from this sector [1]. Senegalese sole (*Solea senegalensis*) is a flatfish considered one of the most interesting species for European aquaculture diversification owing to its nutritional properties, high commercial value and growing market demand [2]. Although the Senegalese sole production cycle can be successfully completed in fish farms, the consolidation of its industrial production is hampered by several factors including high larval mortality rates related to nutrition, growth dispersion, difficulty in establishing optimal weaning conditions, elevated incidence of skeletal anomalies, and high vulnerability to infectious diseases [2]. As in all flatfish, sole metamorphosis is characterized by a dramatic anatomical transformation that involves a remodelling of the head and a change to an asymmetric shape. Such transformation is associated with a change from a pelagic to a benthic habitat that implies important changes in food habits and digestive physiology. As a result, the early stages of larval development and particularly metamorphosis are critical periods of sole rearing, determining further growth features in later production steps [2]. Along these lines, it is worth noting that bacterial colonization of the fish gut occurs during the larval stage, when the gastrointestinal tract is not yet fully developed, and that the composition of the larval microbiota resembles the microflora of the first ingested live feed rather than that of

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the surrounding environment [3]. In fish, the intestinal microbiota plays a pivotal role, being involved in the stimulation of epithelial proliferation, in nutrition and digestion through the production of vitamins and enzymes, and in the immune system development of the host (reviewed in Ref. [4]). Therefore, it is of utmost importance to provide a proper feeding regimen during the first life stages to produce juvenile sole of high quality.

In the search for increased productivity and economic benefits, aquaculture practices can sometimes produce a degree of stress in fish, decreasing their immune competence and making animals more susceptible to disease, especially to infections ([5] and references within). Control of pathogenic bacteria in aquaculture has traditionally been carried out by administration of antibiotics or chemotherapeutic agents. However, the massive use of antimicrobials has a negative environmental impact and promotes increased resistance, jeopardizing the viability of the sector and affecting animal health and welfare, food safety and environmental protection [6]. Therefore, viable alternatives to stimulate the natural mechanisms of animal immune defence are essential. In this context, probiotics constitute a promising ecologically and economically sustainable option to improve the health status of cultured fish while reducing the use of antibiotics and other chemotherapeutic agents [7]. In fish aquaculture, probiotic-supplemented diets favourably affect growth performance and feed efficiency in addition to improving animal welfare, stress tolerance and resistance to diseases, particularly through the modulation of the intestinal microbiota and the immune system [7]. *Shewanella putrefaciens* Pdp11 (SpPdp11) is a bacterial strain initially isolated from the skin mucus of healthy gilthead seabream (*Sparus aurata*) that exerts remarkable probiotic characteristics in gilthead seabream and Senegalese sole farming [8]. Several studies have demonstrated that juvenile specimens of *S. senegalensis* that received SpPdp11 in the diet displayed a modulation of the intestinal microbiota and liver fatty acid composition, enhanced growth, and heightened pathogen resistance [8,9]. Moreover, the dietary administration of this probiotic to juvenile sole can improve stress tolerance to high stocking densities by modulating the expression of important immune genes and the intestinal conditions [5]. Furthermore, when administered jointly with the antibiotic oxytetracycline, SpPdp11 compensates the apoptotic effect of this drug [10]. More recently, it has been established, also in juvenile sole, that the administration of SpPdp11 in combination with the prebiotic alginate resulted in an increase of the transcription of genes related to antioxidative defences, and, more importantly, these effects were maintained after the cessation of the probiotic treatment [11]. To our knowledge, only two studies have investigated the effects of this probiotic when administered during the larval development of *S. senegalensis* [4,12]. These studies have employed probiotic pulses of different durations but always beginning at metamorphosis (10 days post-hatching, dph) and they have focussed on the modulation of the gut microbiota composition. Therefore, there is no information concerning the effect of SpPdp11 when administered to *S. senegalensis* larvae from the first exogenous feeding, and little is known about the molecular mechanisms underlying the effect of this probiotic in the early life stages of the Senegalese sole.

In an attempt to answer these questions, we herein compared the effects of two different probiotic administration protocols during Senegalese sole larval development, one from the first exogenous feeding until the completion of metamorphosis (2–21 dph) and the other during metamorphosis (10–21 dph). We examined the transcriptional expression of 18 genes encoding proteases (including digestive enzymes) or coding for relevant proteins involved in major biological functions such as growth, stress response and immunity. To assess the persistence of the stimulatory effects of the probiotic during sole larval development and after the suspension of the treatment, we evaluated early and late responses (at 23 and 73 dph). Effects on weight, total protein content, alkaline phosphatase activity, intestinal microbiota composition and innate immune parameters were also investigated to support the biological interpretation of the gene expression results.

2. Materials and methods

2.1. Fish rearing conditions and experimental design

Animals were cultured according to the European Union Guidelines (2010/63/EU) and Spanish legislation (RD 1201/2005 and law 32/2007) at the Spanish Institute of Oceanography (Santander, Spain), and all experiments were approved by the Ethics and Animal Welfare Committee of this institution. Embryos were obtained from wild, naturally spawning captive broodstock held under natural condition. Eggs were incubated at 19.0 ± 0.5 °C in 70 l cylinder-conical incubating tanks with gentle aeration and a continuous water flow of 0.5 l min^{-1}. After hatching, larvae were distributed into 280 l circular polyester resin tanks (40 larvae l^{-1}), with constant aeration and water renewal. Temperature was maintained at 18.3 ± 0.8 °C and salinity at 35.4 g l^{-1} throughout the experiment. Continuous illumination of 1000 lux at the surface of the water was provided until 10 dph, and then a 12:12 L:D cycle was established until day 21, whereas a 0:24 L:D cycle was used after the pelagic stage [13]. Continuous water inflow was maintained at 5–80% exchange (2–21 dph), increasing from weaning until 400%, to supply appropriate oxygen and nitrite levels for sole larval and post-larval culture [14,15]. The feeding regimen was as described by Cañavate and Fernández-Díaz [13]. From 2 to 10 dph, larvae were fed twice a day with rotifers enriched with the marine microalgae *Nannochloropsis gaditana* and *Isochrysis galbana*. From 10 to 60 dph, a co-feeding consisting of *Artemia* and the commercial pellet diet Gemma Micro (total lipids 15%, crude protein 55%, Skretting, Burgos, Spain) was introduced. *Artemia* nauplii (AF strain INVE Aquaculture, Ghent, Belgium) were supplied from 10 to 12 dph and *Artemia* metanauplii (EG strain, INVE Aquaculture, Ghent, Belgium) thereafter. Both *Artemia* stages were previously enriched with a commercial emulsion (DHA Super Selco, INVE Aquaculture, Ghent Belgium) and then added to the tanks four times a day, whereas dry feed was supplied eight times a day (four of them at night). *Artemia* doses were increased (from 2 to 14 metanauplii ml^{-1}) with larval age. Weaning started at 48 dph and finished at 66 dph, when larvae were fed with dry feed only (total lipids 17%, crude protein 58%, GemmaWean, Skretting, Burgos, Spain). During this period, the amount of dry feed was gradually increased (from 11.2 g m^{-2}, 45.5% of total feed to 39.2 g m^{-2}), while *Artemia* doses were progressively reduced (from 14 metanauplii ml^{-1}). At the end of the experiment, the fry were fed 7% of total tank biomass.

The experimental design of this study is shown in Supplementary Fig. 1. Three groups on feeding regimens were compared: two groups receiving the SpPdp11 probiotic bacterial strain (CP and PP) and the control group (CC). The group CP group received the probiotic through the living vector of *Artemia*, throughout metamorphosis, from 10 to 21 dph. On the other hand, the PP group was given the probiotic, bioencapsulated in rotifers or *Artemia*, from the first exogenous feeding to the end of metamorphosis (2–21 dph). The control group, CC, was fed a standard diet without probiotics. Each diet was evaluated in triplicate.

Sampling times were selected on the basis of changes in morphogenesis in order to cover metamorphosis, weaning and post-weaning, given that those are the most important stages in Senegalese sole development. Thus, at least ten specimens from each group were collected once metamorphosis was completed (23 dph), at the beginning and at the end of weaning (48 and 66 dph), and at 73 dph. Fish were anaesthetized with tricaine methanesulphonate (MS-222), rinsed with distilled water, weighed, immediately frozen in liquid nitrogen and stored at −80 °C for later use.

2.2. Total protein content and alkaline phosphatase activity

Total protein content and alkaline phosphatase activity were