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Comparison of the effects of the dietary addition of two lactic acid bacteria on the development and conformation of sea bass larvae, *Dicentrarchus labrax*, and the influence on associated microbiota

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ABSTRACT

Probiotics may have many effects on health and development of fish larvae. One of the most promising is related to spinal conformation, though the mode of action is not clearly understood. The present study attempted to investigate the effects of two strains of lactic acid bacteria on associated microbiota, histological development and gene expression. Sea bass larvae were fed since 5 dph (day post hatch) with either a standard control diet (Diet C), or the same diet supplemented with *Pediococcus acidilactici* MA18/5M (Diet P), or with an autochthonous strain of *Lactobacillus casei* (*X2*; Diet L). The two lactic acid bacteria were incorporated in the diets at the levels of 10^6 and 10^7 CFU (colony forming units) g^{-1} in two consecutive experiments, respectively. The experimental treatments maintained the lactic acid bacteria above the detection threshold in the larvae. In the second experiment, the Bray–Curtis indices revealed the dissimilarity between the Bacterial Community Profiles (BCPs) associated with Diet P and those of the two other dietary groups. The two lactic acid bacteria promoted growth, especially by 20–22 dph, but the development seemed affected differently in the two groups. The osteocalcin gene was overexpressed at 20–22 dph in group L, suggesting a difference in the early bone development compared with Group P. A possible consequence was the highest incidence of spinal deformities in Group L. At day 62 dph, the ossification was achieved and normal in 60% of the larvae in Group P, whereas this rate was only 13 and 19% in Groups C and L, respectively. The evaluation of probiotics should not be therefore limited to growth measurements, and should take into account ontogenetic chronology for improving larval quality with such treatments.

1. Introduction

Developmental conformation is a critical issue for fish hatcheries. Spinal deformities are particularly frequent, with many suspected causes, either physical, chemical, environmental, infectious, genetic or nutritional (Brown and Núñez, 1998). Consequently, many ways to limit these deformities have been proposed. Probiotics are applied to fish with positive effects on health and nutritional status, while antagonizing pathogenic bacteria. These treatments may be therefore useful to reduce some causes of skeletal deformation, and this was confirmed with several strains of probiotic lactic acid bacteria in rainbow trout, sea bass, and clownfish (Aubin et al., 2005; Autin et al., 2012; Avella et al., 2010; Frouël et al., 2008).

The interaction between dietary lactic acid bacteria and bone conformation is not clearly understood, and probably indirect. It may be related to the inhibition of pathogenic bacteria (Villamil et al., 2010). Probiotics may also stimulate the immune system, and reduce inflammation in fish larvae (Picchietti et al., 2009). Gil-martens (2010) suggested that local inflammation could affect the integrity of the spine, which would be thus exposed to a risk of deformation. Besides these pathological aspects, probiotics may improve many health factors that are essential for fish development in relation to either welfare (Avella et al., 2010; Rollo et al., 2006), digestion (Frouël et al., 2008; Tovar-Ramírez et al., 2004) or anti-oxidative status (Tovar-Ramírez et al., 2010).

Walter (2008) studied the role of lactobacilli in the mammalian intestinal tract, and concluded that allochthonous probiotic strains are particularly efficient for activating the immune system, even

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though they can hardly persist in the gut. A similar observation may apply to the intestine of fish, where allochthonous lactobacilli that modulate the immune response disappear soon after withdrawal from the diet (Panigrahi et al., 2005; Son et al., 2009). The selection of candidate probiotics from the host or from the local environment was however recommended to improve the potential for colonization and protection against pathogens (Verschuere et al., 2000). In the present experiment, two strains of lactic acid bacteria were incorporated in the dry diet that was used to feed sea bass from mouth opening onwards. The commercial strain of *Pediococcus acidilactici* was already tested with several fish species (Ferguson et al., 2010; Gatesoupe, 2002; Merrifield et al., 2009, 2010), suggesting in particular some potential to alleviate vertebral deformities (Aubin et al., 2005; Autin et al., 2012). A strain of *Lactobacillus casei* was isolated from the local hatchery, and selected as candidate probiotic after characteristics of antagonism to pathogens, biofilm colonization, and gnotobiotic tests (Lamari et al., in preparation, Lamari et al., 2012). The effects of the two strains were compared in regard to larval development and associated microbiota.

2. Materials and methods

2.1. Larval rearing

Two batches of sea bass larvae were allotted in two successive experiments at 1 day post hatch (dph) in 9 tanks of 35 L (3000 larvae/tank). The running water was not recycled in the rearing unit, and the water flow rate was progressively increased from 15 L h^{-1} at 5 dph to 35 L h^{-1} at 40 dph. The temperature was progressively increased from 17 °C at 1 dph to 20 °C at 25 dph. The salinity was dropped to 28 practical salinity units (psu) during the start feeding period (7–13 dph), and then brought to the normal (35 psu). The larvae were kept in the dark until 4 dph, and then photoperiod was maintained at 18–6 light/dark from 4 dph onwards. The light intensity was progressively increased from 10 to 200 lx between 4 and 25 dph, and then kept constant.

The diet was composed of 55% fish meal, 12% fish protein hydrolysate (CPSP G), 20% soy lecithin, 8% vitamin mix (Gisbert et al., 2005), 4% mineral mix (Gisbert et al., 2005), and 1% betaine, on a dry matter basis. This basic composition was used for the control group C. *Pediococcus acidilactici* MA 18/5M was added to diet P in its dry commercial form (Bactocell® PA10, Lallemand Inc.; Barreau et al., 2012). *Lactobacillus casei X2*, which was used for diet L, was cultivated on MRS broth, and harvested as pasty pellet after centrifuging at 4500*g* for 30 min. Each bacterial preparation was mixed with the dry components of diet P or L, after re-suspension in tap water. The same amount of water without bacterial suspension was used for control diet C. The final concentrations of probiotics were adjusted at 10^6 and 10^7 CFU (colony forming units) g^{-1} of the diet in Experiments 1 and 2, respectively, after air-drying at 45 °C. The three diets were ground and pelleted to a set of granulometric sizes suitable for each developmental stage (Cahu and Zambonino Infante, 2001). The pellets were stored at 4 °C until use, and then distributed continuously during the day light periods with automatic feeders. The amounts of *P. acidilactici* averaged 1.1×10^6 and 1.8×10^7 CFU g^{-1} in diet P, whereas diet L contained *L. casei* at the rate of 1.4×10^6 and 3.4×10^7 CFU g^{-1} in the two consecutive experiments, respectively, after counts on MRS-agar plates. The three diets were tested in triplicates in both experiments. In Experiment 1, the experimental diets were distributed from 10 dph to the end (45 dph). In Experiment 2, the larvae were fed the experimental diets from mouth opening (5 dph) to 41 dph, and then all the tanks were fed the control diet till the end (62 dph).

2.2. Histological observations

In Experiment 2, the skeletal and chondro-osseous development was assessed on a total of 103 fish, with 15 or 16 individuals sampled per dietary group at 41 and 62 dph. The cartilaginous and bony tissue structures were distinguished by using the alcian blue-alizarin red double staining technique (Darias et al., 2010). Briefly, whole fish carcasses were fixed in 4% formalin for at least 24 h. Alcian blue (Sigma) was used to stain the cartilage of 41 and 62 dph old larvae for 60 min and 24 h, respectively, before neutralization for 3 min with a solution of 1% KOH in ethanol. After rehydration, 1 volume 3% H_2O_2 and 9 volumes 1% KOH were used to bleach the 41 and 62 dph old larvae for 30 and 60 min, respectively. After clearing in 30% sodium borate with 1 g trypsin (Sigma) for 20 h, bone tissue was stained with alizarine red (Sigma) for 20 h. The larvae were dehydrated and preserved in 100% glycerol.

Histopathological features, including inflammation, were evaluated on 20 fish sampled in each group at 20, 41 and 62 dph (162 fish in total). The tissue preparation and step sectioning were performed according to Spitsbergen et al. (2000). Briefly, whole fish carcasses were fixed in 4% formalin, after ventral incision of the abdomen of large individuals. Scales and fins were carefully removed, and the samples were dehydrated and embedded in paraffin. Serial sagittal step sections were cut from the left side of the fish. Four step sections from 62 dph old fish were mounted on glass slides, 1 from eye anterior chamber level, 1 from eye posterior chamber level, 1 just medial to the eye, and 1 at midline. Sections were routinely stained with Hematoxylin–Eosin–Saffron (HES).

2.3. Osteocalcin gene expression

The method was described by Darias et al. (2010). The larvae were sampled at 20–22 dph for RNA extraction, as the most significant differences in gene expression were obtained at 22–23 dph in previous experiments. Larvae were also sampled at 41 dph in Experiment 2. Between 200 and 350 mg of fresh weight of larvae were collected from each tank for RNA extraction. Total RNA was reverse-transcribed (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA). Quantitative PCR were performed in triplicates in I-cycler with optical module (Bio-Rad), using a total volume of 15 μL that contained 5 μL cDNA (dilution, 10^{-2}), 0.5 μL primers (10 μmol L^{-1}), 7.5 μL iQ SYBR Green supermix 2× (Bio-Rad), and 2 μL sterile water. The PCR program consisted of an initial DNA denaturation of 94 °C for 90 s, followed by 45 cycles at 95 °C for 30 s, 60 °C for 60 s and 80 cycles at 95 °C for 10 s. The relative quantity of messenger was automatically normalized and measured with the software Bio-Rad IQ5, using Ef1 as housekeeping gene.

2.4. Microbiological data

The bacterial counts on Petrifilm aerobic count plates, TCBS and MRS agar were done as described previously (Gatesoupe, 2002) on sea bass

Table 1
Mean weights of sea bass larvae fed the three diets (mg ± standard error, SE). Superscript letters indicated significant differences between dietary groups at the same date and in the same experiment. The data were compared with one-way ANOVA followed by Tukey multiple comparison test, or with Kruskal–Wallis test (KW) followed by Dunn test. The type of test used depended on the normality and the equality of variance assumptions for the parametric tests. *P* levels are indicated with asterisks (n.s., not significant). For each comparison, the means without common superscript are significantly different.

Diet	C	P	L	ANOVA
Experiment 1				
22 dph	0.99 ± 0.10	1.11 ± 0.08	1.23 ± 0.05	n.s.§
30 dph	2.56 ± 0.21	3.29 ± 0.42	3.54 ± 0.46	n.s.§
45 dph	21.33 ± 1.69	24.17 ± 0.17	25.00 ± 0.76	n.s.§
Experiment 2				
20 dph	0.79^{b} ± 0.05	0.97^{a} ± 0.10	0.93^{a} ± 0.04	* (KW)
40 dph	17.19 ± 0.81	17.37 ± 0.67	17.68 ± 0.68	n.s. (KW)
62 dph	42.9 ± 3.7	53.1 ± 5.3	50.5 ± 1.4	n.s. (KW)

§ Two-way ANOVA after log transformation: significant differences between Ages*** and Diets* (C different from P and L), interaction not significant.