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Use of microalgae bioencapsulated in *Artemia* during the weaning of Senegalese sole (*Solea senegalensis* Kaup)

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ABSTRACT

The microalgae species *Chlorella minutissima* and *Tetraselmis chuii* were bioencapsulated in *Artemia* metanauplii and used during the weaning of Senegalese sole, *Solea senegalensis* post-larvae. We evaluated the effect of microalgae on: (i) survival and growth of the Senegalese sole post-larvae and juveniles, (ii) expression of genes related to the non-specific immune system, and (iii) the microbial load of sole juveniles. The survival of sole juveniles at the end of the experiment was significantly higher in the treatments added microalgae in their diet compared with the control treatment ($P<0.05$), while there was no effect on the final weight of sole juveniles. The total numbers of bacteria as determined by the counts on Zobell's medium were significantly lower in both treatments added microalgae compared with the control treatment ($P<0.05$), whereas no significant differences were shown among the different treatments in the numbers of presumptive *Vibrio* as determined by counts on TCBS. Four partial nucleotide sequences of genes related to the immune system were isolated from lymphoid tissues of *S. senegalensis*: natural resistance associated macrophage protein 1 (Nramp1), complement C3, transforming growth factor β1 (TGF-β1), and transferrin. The expression of these four genes was calculated at the end of the experiment in relation to the expression of *β-actin*, a housekeeping gene. No significant differences ($P>0.05$) were determined among the three treatments in relation to the expression of the four genes in homogenates of fish.

1. Introduction

Senegalese sole, *Solea senegalensis*, is a promising new fish species for the aquaculture industry in Southern Europe. It shows low mortalities during the larval stage and a high growth rate in culture (Dinis et al., 1999). Nevertheless, high mortalities have been observed during the weaning of post-larvae to an artificial diet (Fernandez-Diaz et al., 2001; Imsland et al., 2004). Nutritional, microbiological, and zootechnical factors, or a combination of these factors, could be critical for the improvement of the rearing process in order to increase survival rates and predictability of the production process during the weaning stage.

Microalgae have a known antibacterial and possible immunostimulant effect, as the incorporation of microalgae in the diet increased disease resistance in previous studies (Austin et al., 1992). The antimicrobial effect of microalgae could be caused by bacteria associated with microalgae or by the microalgae cells (Austin et al., 1992; Makridis et al., 2006; Tendencia and dela Pena, 2003). Bioencapsulation of microalgae in *Artemia* metanauplii after a short-term incubation results in decreased microbial load and a change of species-composition of the bacterial flora associated with the metanauplii (Makridis et al., 2006; Makridis et al., 2000). In addition, microalgae encapsulated in *Artemia* will eventually be digested by the larvae, provide some additional nutritional value to the *Artemia* metanauplii as food for the fish larvae, and may have some positive effect on the fish immune system.

Live food organisms, such as rotifers and *Artemia* are applied in the rearing of larvae of marine fish species during the first weeks of feeding, while fish larvae are adapted to be fed with artificial diet during the weaning stage. In the rearing of sole, the weaning may take place either by abrupt, or by gradual replacement of *Artemia* metanauplii with artificial diet (Canavate and Fernandez-Diaz, 1999; Engrola et al., 2007).

In most marine fish species, the specific immune system becomes functional several weeks after hatching, which means that defence against disease in first feeding larvae and post-larvae is based on the non-specific immune system (Falk-Pedersen, 2005; Magnadottir et al., 2005). A preliminary study on the development of the immune system in Senegalese sole has indicated that specific immunity mechanisms are poorly developed during the first weeks after hatching (Castro Cunha et al., 2008). The non-specific system, which includes innate immune

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system defences such as production of lysozyme, complement system, and production of iron-bounding proteins, provides the major protection against invading microorganisms during this developmental stage (Magnadottir et al., 2005). Cell surface components present on the surface of bacteria as well as microalgae cells may stimulate host cells that activate immune responses in the host organism. Stimulation of the immune system may be measured by quantification of the expression of genes related to the immune system compared with the expression of a housekeeping gene, such as *β-actin* (Panigrahi et al., 2007).

The aims of this study were to evaluate the effect of bioencapsulated microalgae on: (i) survival and growth of the Senegalese sole juveniles, (ii) expression of genes related to the non-specific immune system, and (iii) the microbial load of sole juveniles.

2. Materials and methods

2.1. Experimental procedures

Senegalese larvae were reared according to the protocol described by Engrola et al. (2007). Briefly, the larvae were fed rotifers (3–8 DAH), *Artemia* nauplii 5–10 DAH, enriched *Artemia* metanauplii (10–14 DAH) and frozen enriched metanauplii from 12 DAH and thereafter. Post-larvae of Senegalese sole (*Solea senegalensis*) were transferred to 12 four-litre flat-bottom trays 37 days after hatching (DAH). Each tray was stocked with 100 post-larvae. The larvae were fed an artificial diet (Aglonorse no. 1, EWOS, Bergen, Norway; approximately 5% of wet weight day^{-1}) and added daily a constant portion of *Artemia* metanauplii (approximately 500 metanauplii per larva). Three treatments were used, each treatment with four replicate tanks. *Artemia* were enriched in 0.6 g L^{-1} DC-DHA-Selco (INVE S/A, Belgium) according to manufacturer's instructions, rinsed, and thereafter incubated for a 20-minute period in cultures of *Tetraselmis chuii*, *Chlorella minutissima*, and in seawater in the case of *Tetraselmis*, *Chlorella*, and control treatment, respectively. After this short-term incubation, *Artemia* metanauplii were packed in ice-cube bags and stored at −20 °C for up to three days. Mortalities in each tank were recorded daily and at the end of the experiment 81 DAH all the fish were weighed. A recirculation system was used in the rearing system, which included a sand-filter, a skimmer, and a biological filter. Average water temperature and salinity (±SEM) during the experiment were 20±1 and 37 ppt, respectively.

2.2. Challenge of sole juveniles

Photobacterium damselae subsp. *piscicida*, CECT 5895, bacteria were cultured in tryptic soy broth added 2% (w/v) NaCl for 48 h, fixed in formalin, washed three times in PBS (phosphate buffered saline), and injected twice at two week interval in the peritoneum of two specimen of *Solea senegalensis* two years-old approximately. One week after the second immunization, the two immunized fish and one non-immunized fish were killed by a blow in the head. Samples from spleen, head kidney, and blood from the caudal vein were transferred to liquid nitrogen and stored at −80 °C.

2.3. Isolation of genes related to the immune system

Gene isolation was performed at the Institute for Molecular and Cell Biology in Porto. Several gene encoding for cytokines, genes involved in the innate immune system, and *β-actin* gene were searched through GenBank Database. The mRNA sequences from each gene were aligned using *ClustalW Multiple Sequence Alignment* tool to search for nucleotide homologies in order to develop primers. Primer design and testing was performed using the Basic Local Alignment Search Tool (BLAST). Four partial gene sequences related to the immune system were isolated from *S. senegalensis*: natural resistance associated macrophage protein 1 (Nramp1), complement C3, transforming growth factor β-1 (TGF β-1), and transferrin; whereas *b-actin* gene was also isolated and used as a housekeeping gene in this study. Primers designation, sequence and amplicon size used in this work are listed in Table 1.

2.4. RNA extraction and RT–PCR

Frozen samples were disrupted in liquid nitrogen with a mortar and pestle and homogenized with a 20G syringe. Total RNA was extracted by use of RNeasy Midi kit (QIAGEN, Hilden, Germany) in accordance to the manufacturer's recommendations. Total RNA concentrations were measured by spectrophotometer (GBC UV/VIS 918, Manchester, UK) at 260 and 280 nm, while RNA integrity was evaluated on a 1% agarose gel, and the samples were stored at −80 °C.

Complementary DNA (cDNA) was prepared by reverse transcription of 4 μg of total RNA from each sample using ThermoScript™ RT—PCR system (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Briefly, RNA was suspended in 10 μL of DEPC treated water, 1 μL of Oligo dT_{20} primer was added following incubation for 5 min at 65 °C and was finally cooled on ice, and 4 μL of cDNA synthesis buffer (5×), 1 μL of RNaseOUT and 1 μL of ThermoScript™ RT were added to the mixture and incubated for 1 h at 50 °C and additionally for 5 min at 85 °C. The vials with cDNA were stored at −20 °C for PCR amplification.

2.5. PCR amplification

PCR reactions were performed with the obtained cDNA using the primers previously described in a TPersonal Thermocycler Biometra (Goettingen, Germany). The PCR mixtures (final volume 25 μL) contained (per reaction) 2.5 μL of 1X PCR Buffer (Appligene, Watford, UK), 2.5 U of *Taq* polymerase (Appligene, Watford, UK), 0.6 mM dNTP mix (MBI Fermentas, Ontario, Canada), each primer at a concentration of 10 pmol μL^{-1} and 2 μL of cDNA. All amplifications were performed in all organs and consisted, for C3 and β-actin, of 30 cycles of 30 s at 94 °C for denaturation, 30 s at 55 °C for primer annealing and 1 min at 72 °C for elongation, for Nramp1 the amplification consisted of 35 cycles of 1 min at 94 °C, 30 s at 51 °C and 1 min at 72 °C. For TGF-1, the amplifications consisted of 30 cycles of 1 min at 92 °C, 30 s at 44 °C and 1 min at 72 °C, and for transferrin of 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C. All amplified fragments were run in a 1% agarose gel electrophoresis, visualized under a UV light transilluminator (UVP, CA, USA) and photographed with a Kodak *ds* 120 camera (Kodak).

2.6. Cloning and sequencing

The amplified DNA fragments of expected size were extracted from the agarose gel, purified using the QIAquick® Spin (QIAGEN, Hilden, Germany) following the manufacturer's recommendations, and inserted into competent *E. coli* cells for cloning. TOPO® TA Cloning kit (Invitrogen, Carlsbad, USA) was applied and the protocol was followed in accordance

Table 1
Primers based on the obtained partial gene sequences were designed and were used in the measurement of expression study of the five genes studied.

Primer	Nucleotide sequence	Fragment
C3 F	5'-TATAAGAACAAGGATCACGATG-3'	172 bp
C3 R	5'-GGTAGATGATCAATGAACCTC-3'	
Act F	5'-GGTGATGAAGCCCAGAGC-3'	177 bp
Act R	5'-TTTAGGGTTCAGGGGGG-3'	
Nrp1 F	5'-GACCTGCAGTCTGGAGCT-3'	292 bp
Nrp1 R	5'-TGGTGATGAGGACTCCGC-3'	
Tgf F	5'-CTGGGCTGGAAGTGGATAC-3'	238 bp
Tgf R	5'-CACAATCATATTGGACAGCTG-3'	
Trf F	5'-GAGCCTTACTATAATTATGATGG-3'	196 bp
Trf R	5'-TGACAACAGCATGAGCTGG-3'	