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Bio-Mos®: An effective inducer of dicentracin gene expression in European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

Concern over the use of dietary antibiotics in aquaculture has encouraged the industry to search for alternatives that both enhance performance and afford protection from disease. Bio-Mos®, derived from the outer cell wall of a specific strain of yeast *Saccharomyces cerevisiae* (Alltech Inc, USA) is a product that fits these criteria. Here, we present data on the impact of a Bio-Mos® supplemented diet on the mRNA copy number of the antimicrobial peptide dicentracin, whose transcript regulation has not yet been explored in fish. We analyzed Bio-Mos®-induced changes in the expression of sea bass (*Dicentrarchus labrax*) dicentracin, using a one-tube two-temperature real-time RT-PCR with which the gene expression can be absolutely quantified using the standard curve method. Our results revealed that 30 days of feeding fish with diets containing Bio-Mos® supplemented at either 3‰ or 5‰ significantly increased the dicentracin mRNA copy number in the head kidney. Furthermore, the mRNA copy number in fish fed at 3‰ was significantly higher than that of the group fed at 5‰ for the same period of feeding Bio-Mos®. A longer feeding period (60 days) did not further increase the dicentracin transcript levels as compared to the values recorded after 30 days of feeding either in the group fed at 3‰ or in the one fed at 5‰ diet. However, the transcript levels in fish fed at 3‰ proved to be significantly higher than those of the controls after 60 days of feeding. These findings offer new information about the response of antimicrobial peptides at the transcriptional level to diets supplemented with immune response modulators, and support a role of Bio-Mos® in promoting sea bass nonspecific immune system.

1. Introduction

Aquaculture is becoming a more concentrated industry of fewer, but much larger farms (Serrano, 2005). Under these circumstances, infectious diseases always represent a hazard and may cause major stock losses and problems for animal welfare. To control infectious diseases in aquaculture, the same strategies used in other areas of animal production are employed. As no antibiotics have been specifically designed for aquaculture, antibiotics that are effective and routinely used to treat human infections are also used for animals, for either therapy or prophylactic reasons (Serrano, 2005).

However, more recently, the use – and apparent over-use – of antibiotics in animal feed has been widely discussed. Scientific evidence has accumulated showing that in food-producing animals certain uses of antibiotics can promote antibiotic resistance in intestinal bacteria, and that this resistance can then be transmitted to the general population, causing treatment-resistant illness. The micro-organisms responsible for the most frequent fish infections treated with antibiotics belong to bacterial families that also produce infections in humans (FAO/NACA/WHO, 1997). Therefore, it is highly probably that antibiotic resistance is transferred (De Paola et al., 1995; Frost and Thwaites, 1998; Guardabassi et al., 2000; Angulo et al., 2000; Threlfall et al., 2000). The use of antibiotics can also create antibiotic resistance in nonpathogenic bacteria, the resistance genes of which can be transferred to disease-causing bacteria, resulting in antibiotic-resistant infections in humans (Cloeckaert et al., 2000).

Concern over the use of dietary antibiotics in aquaculture has encouraged the industry to search for alternatives that both enhance performance and afford protection from disease. Bio-Mos®, derived from the outer cell wall of a specific strain of yeast *Saccharomyces cerevisiae* by using a proprietary process developed by Alltech Inc, is a product that fits these criteria.

Bio-Mos® consists of a mannan and a glucan component, which have been shown to inhibit pathogen colonization by blocking type-1 fimbriae, filaments that allow pathogens to attach to the intestinal lining (Dawson and Pirvulescu, 1999), to serve as an immunomodulator, reducing intestinal microbial populations, and to improve the integrity and morphology of the intestinal mucosa.

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The theoretical role of Bio-Mos® in the gut has not been proven, but including this product in the diet has had a positive impact on animal performance. The efficacy of Bio-Mos® as a growth promoter and immune response modulator has been shown in pigs (Miguel et al., 2002) and in a broad number of poultry species (Sonmez and Eren, 1999; Iji et al., 2001). Few studies have examined the effect of this pronutrient on performance and the nonspecific (innate) immune system of fish. However, in these studies, feeding Bio-Mos® supplemented diets to common carp (*Cyprinus carpio*) enhanced growth and significantly improved feed utilization, antibody levels, bactericidal and lysozyme activity, and alternative complement pathway activity (Staykov et al., 2005). In sea bass (*Dicentrarchus labrax*) incorporating Bio-Mos® in the diet enhanced growth, activated the immune response, and increased resistance to bacterial infection, too (Torrecillas et al., 2007).

The endogenous antibacterial peptides (AMPs) are an important part of the innate immune system in various animal species. They are even more important in fish than in mammals, as fish rely more on their innate immune system (Hancock and Scott, 2000). AMPs are promptly synthesized at low metabolic cost, easily stored in large amounts, and readily available shortly after an infection (Oren and Shai, 1996). Several AMPs have been isolated in teleostean fish such as misgurin in loach (*Misgurnus anguillicaudatus*) (Park et al., 1997), epinecidin in grouper (*Epinephelus coioides*) (Yin et al., 2005), pleurocidin in winter flounder (*Pleuronectes americanus*) (Cole et al., 1997), moronecidin in hybrid striped bass (Lauth et al., 2002), pardaxin in sole (*Pardachirus marmoratus*) (Adermann et al., 1998), hepcidin in winter flounder (*P. americanus*), Atlantic salmon (*Salmo salar*), and perch (*Perca fluviatilis*) (Douglas et al., 2003; Rossi et al., 2007), parasin in catfish (*Parasilurus asotus*) (Park et al., 1998), and dicentracin in sea bass (*D. labrax*) (Salerno et al., 2007). Fish AMPs are principally located in circulating granulocytes and in a large panel of tissues, including the head kidney, which represents the main lymphoid tissue (Robert et al., 1999; Smith et al., 2000; Sarmento et al., 2004).

However, up to now, there are no studies in fish related to the expression of genes that encode for such immunologically relevant proteins. In particular, we know nothing about the response of AMP genes at the transcriptional level to diets supplemented with immune response modulators, although this knowledge could not only provide an accurate estimate of the function of these pro-nutrients in fish, but might also be relevant to understanding the mechanism by which the expression of AMPs is regulated. Accordingly, we present data here on the impact of a Bio-Mos® supplemented diet on the mRNA copy number of dicentracin in sea bass (*D. labrax*) head kidney, with the aim to relate these expression levels to the quantity of the immune response modulator incorporated in the diet.

2. Materials and methods

2.1. Experimental protocol

2.1.1. Diets

Three diets based in a commercial pellet (Hendrix-Skretting S.P.A.) were reformulated to contain 0‰ (Control), 3‰ (BM3), or 5‰ (BM5) Bio-Mos® (Alltech Inc., USA), replacing standard carbohydrates.

2.1.2. Animals and feeding trial

Sea bass (*D. labrax*) of mixed sexes were obtained from a local fish farm and kept at our department in Varese, Italy. Fish were stocked into long indoor tanks of 2500 L each and allowed to acclimate for 45 days before starting the trial. The tanks were connected to a water recirculation system where salinity (obtained by adding salt Oceanfish 600LT from Prodac Int® to dechlorinated tap water) was 22 g/L. Other water conditions were: temperature 21 ± 1 °C, pH 8.2, and total ammonia <0.2 mg/L; dissolved oxygen was maintained over 99% of the saturation by insufflating pure O_2 to the system.

A computerized multiprobe Rilheva® system (Xeo4, Italy) was used to continuously monitor dissolved oxygen, pH, and temperature in each tank. During the acclimation period, all fish were fed Hendrix-Skretting® Power Excel feed for marine fish.

After the acclimation period, 45 fish from the aforementioned population that had not suffered any apparent diseases were weighed and transferred into each of three experimental tanks (600 L) connected to the same recirculation system and were allowed to acclimate for five days. Then, fish in the first tank were fed with the control diet BM0 (0‰ Biomos), whereas fish in the other two tanks were fed with BM3 (3‰ Bio-Mos®) and BM5 (5‰ Bio-Mos®) diets, respectively. At the beginning of the experiment, the mean body mass of the sea bass was 276.3 ± 4.75 g. All fish were fed once a day at 1% body weight/day. Five fish from each of the three experimental groups were sampled after 30 and 60 days of feeding. Fish were sampled 15 min before the scheduled feeding time. For the molecular biology analysis, head kidneys were isolated, frozen immediately in liquid N_2, and stored at −80 °C.

2.1.3. Preparation of total RNA

Total RNA was extracted from sea bass head kidney using the PureYield RNA Midiprep System (Promega, Italy). The quantity of the RNA was calculated at an absorbance of 260 nm. The integrity and relative quantity of RNA was assessed by electrophoresis.

2.1.4. Generation of in vitro-transcribed dicentracin complementary RNAs (cRNAs) for standard curves

The absolute number of dicentracin gene transcript copies could be quantified by comparing them with a standard graph constructed using the known copy number of mRNA of this gene. For this, a forward and a reverse primer were designed based on the mRNA sequences of the *D. labrax* dicentracin (GenBank accession number AY303949) This primer pair was used to create templates for the *in vitro* transcription of cRNAs for dicentracin: The forward primer was engineered to contain a T7 phage polymerase promoter gene sequence to its 5′ end (*gtaatacgactcactatagggg*AGTGCGCCACGCTCTTTC) and used together with the reverse primer (5′-CTAGTCAAAAGCTGCGCGCT-3′) in a conventional RT-PCR of total sea bass head kidney RNA. RT-PCR products were then evaluated on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T cloning vector system (Promega), and subsequently sequenced in T7 and SP6 directions.

In vitro transcription was performed using T7 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

2.1.5. Generation of standard curves for dicentracin

The cRNAs produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples (Terova et al., 2008). Defined amounts of cRNAs at 10-fold dilutions were subjected in triplicate to real-time PCR using one-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy), including 1× TaqMan buffer, 3 mM $Mn(OAc)_2$, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units *rTH* DNA polymerase, and 0.5 units AmpErase® UNG enzyme in a 25 μL reaction volume.

AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For TaqMan® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50 μL reaction.