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Dietary β-glucan (MacroGard®) enhances survival of first feeding turbot (*Scophthalmus maximus*) larvae by altering immunity, metabolism and microbiota

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ABSTRACT

Reflecting the natural biology of mass spawning fish aquaculture production of fish larvae is often hampered by high and unpredictable mortality rates. The present study aimed to enhance larval performance and immunity via the oral administration of an immunomodulator, β-glucan (MacroGard®) in turbot (*Scophthalmus maximus*). Rotifers (*Brachionus plicatilis*) were incubated with or without yeast β-1,3/1,6-glucan in form of MacroGard® at a concentration of 0.5 g/L. Rotifers were fed to first feeding turbot larvae once a day. From day 13 dph onwards all tanks were additionally fed untreated *Artemia* sp. nauplii (1 nauplius ml/L). Daily mortality was monitored and larvae were sampled at 11 and 24 dph for expression of 30 genes, microbiota analysis, trypsin activity and size measurements. Along with the feeding of β-glucan daily mortality was significantly reduced by ca. 15% and an alteration of the larval microbiota was observed. At 11 dph gene expression of trypsin and chymotrypsin was elevated in the MacroGard® fed fish, which resulted in heightened tryptic enzyme activity. No effect on genes encoding antioxidative proteins was observed, whilst the immune response was clearly modulated by β-glucan. At 11 dph complement component *c3* was elevated whilst cytokines, antimicrobial peptides, toll like receptor 3 and heat shock protein 70 were not affected. At the later time point (24 dph) an anti-inflammatory effect in form of a down-regulation of *hsp 70*, *tnf-α* and *il-1β* was observed. We conclude that the administration of MacroGard® induced an immunomodulatory response and could be used as an effective measure to increase survival in rearing of turbot.

1. Introduction

Turbot (*Scophthalmus maximus*, *Psetta maxima*) aquaculture is a steadily growing industry with a production of approximately 77,000 t in 2013 [1]. However, intensive production of marine fish larvae is still hampered due to high and unpredictable survival rates [2]. These mortalities are often pathogen-associated [3] since the immune system of the larvae is not yet fully developed [4–7]. During these immune compromised early stages, which especially in marine larvae can comprise the first 2–3 months post hatch, the larvae rely solely on the innate immune system, whilst the adaptive arm is not fully established [7]. This limits the number of potential treatments as well as prophylactic methods against pathogens as vaccinations are not applicable until the acquired immune system is matured and antibiotics are problematic due to environmental aspects. However, the use of immunomodulators in larval culture could present a potential method to increase immunity and survival as they enhance the non-specific, innate immune system [8,9].

The application of immunomodulators has been widely studied in juvenile and adult fish (see Ref. [10] for review). A limited number of studies, however, have focused on early life stages [11–16]. Various routes of administration of immunomodulators to fish have been proposed, for example via feed, bath and injection. Due to the small size of fish larvae the latter method is not

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applicable, however, both oral and bath administration seem to be principally feasible. The disadvantage of bath treatments nevertheless lies in the large amount of substance needed due to high water volumes and water exchange rates. We therefore focused on dietary application in this study. During the early life stages most marine aquaculture fish species rely on life feed. Encapsulation of immunomodulators into rotifers has been suggested by Robles and colleagues [17] to be an effective method of administering medication to fish larvae.

Currently multiple substances are on the market that promise to have positive effects on fish health and survival. Among those, the carbohydrate β-glucan, especially β-1,3/1,6-glucan derived from yeast (*Saccharomyces cerevisiae*), is one of the most used immunomodulators.

The structure and immunostimulatory activity of β-glucan depends on its source, solubility, molecular mass, tertiary structure and the degree of branching (for review see Ref. [18]). In this study we focus on the commercially available β-glucan containing feed additive MacroGard® (Biorigin, Brazil). This product is an insoluble preparation of β-1,3/1,6-glucans from *S. cerevisiae* and it contains a minimum of 60% β-glucans plus lipids, protein, ash and moisture and no nucleotides.

As β-glucan has been shown to increase immunity and survival in various juvenile (i.e. stage from metamorphosis to sexual maturity) and adult fish (i.e. sexually mature) both in freshwater and marine aquaculture species as well as in marine fish larvae (i.e. the stage from hatching to metamorphosis) [12] it has been suggested to be one of the most potent immune system enhancers in aquaculture [19]. In mammals but as well in fish β-glucan is detected by multiple pattern recognition receptors including toll-like receptors and complement receptor C3 [20,21] but the main mammalian β-glucan receptor dectin-1 could not be identified in fish. In both mammals and fish β-glucan recognition results in the activation of macrophages, which induces phagocytosis, leukocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species, as well as the enhancement of complement activity [21–30]. Recent studies in juveniles and adult fishes have for example shown that yeast β-1,3/1,6-glucan in form of MacroGard® increases complement activity [31,32] and induced an anti-inflammatory effect [27] in carp (*Cyprinus carpio*) juveniles. In addition it was also shown that β-glucan can enhance growth and leucocyte infiltration into the epithelial layers of the gut of carp juveniles [33]. However, in juvenile turbot dietary MacroGard® did not increase protection against an infection with *Vibrio anguillarum* and complement and lysozyme activity were not influenced even though the white blood cell count was increased [34].

The immature immune system of fish larvae thus prevents inferences being made from studies focusing on juveniles to larval life stages. Nevertheless only a few studies were performed regarding earlier life stages. It was demonstrated that MacroGard® stimulates the classical complement pathway, lysozyme activity and α-2-macroglobulin in carp fry [11]. Skjermo et al. [12] used MacroGard® and β-glucan from *Chaetoceros mülleri* as dietary supplement in first feeding Atlantic cod (*Gadus morhua*) larvae. In contrast to MacroGard® β-glucan from *C. mülleri* increased survival whilst feeding of MacroGard® led to reduced dry weight of the larvae. Al-Gharabally and colleagues [15] investigated the effects of β-glucan and levamisole in blue-fin porgy (*Sparidentax hasta*) larvae and found reduced larval mortality and increased resistance against bacterial infection as well as enhanced lysozyme activity. In summary the data currently available demonstrates variable effects of β-glucan depending on the source of the immunomodulator, fish species tested and the age of the fish.

In the present study we hypothesized that yeast derived β-1,3/1,6-glucan (i.e. MacroGard®) has an immunomodulatory effect and can enhance survival of turbot larvae during the first stages of development and we aimed to elucidate some of the underlying mechanisms. To the best of our knowledge no data is yet available on the molecular effects of β-glucan on fish larvae. We therefore conducted an extensive analysis on the expression of genes involved in innate immunity as well as nutritional aspects. Expression of genes involved in growth, development, digestion, lipid metabolism, antioxidative activity as well as immune response were analysed. Additionally the microbiota of the larvae was analysed using denaturing gradient gel electrophoresis. This study therefore presents the first detailed analysis of the effects of MacroGard® on first feeding turbot larvae.

2. Material& methods

2.1. Animals

Turbot (*S. maximus*) larvae (1 day post hatch) were obtained from Stolt Sea Farm Norway and reared in the facilities of GEOMAR Kiel, Germany. From the start of the experiment larvae were kept in six green 75 L-tanks filled with filtered 30 L North Sea water (5 μm, UV-treated, 30 ± 1 PSU). The temperature was kept constant at 18 ± 1 °C and from 6 days post hatch (dph) the salinity was reduced stepwise to attain 17 ± 1 PSU at 20 dph by mixing with filtered Baltic Sea water. Larvae were kept in densities of approx. 40 larvae/L and greenwater technology (i.e. addition of *Nannochloropsis* spp.) was used in all tanks. 50% of the water was exchanged once a day with 5 μm-filtered, UV treated mix of North Sea and Baltic Sea water prior to feeding.

2.2. Cultivation of live feed

Rotifers (*Brachionus plicatilis*) were reared in sterile filtered Baltic Sea water (salinity = 17 ‰) in tanks with conical bottoms and fed with resuspended *Nannochloropsis* spp. concentrate (Blue-Biotech GmbH, Büsum, Germany). Prior to introducing individuals to the fish larval tanks, rotifers were harvested from the cultivation tank and transferred to two conical enrichment-tanks.

Artemia eggs (Premium Artemia, Sanders) were incubated for 24 h in filtered Baltic Sea water at 30 °C, harvested and the newly hatched nauplii were introduced into the fish larval tanks without any further treatment.

2.3. Experimental protocols

The larvae were fed once daily from 3 days post hatch (dph) with 3 *B. plicatilis*/ml. In the control treatment rotifers were enriched with 0.35 g/L Selco presso (INVE Aquaculture SA, Belgium) for 3 h. Yeast β-1,3/1,6-glucan in form of MacroGard® (batch number Q511156; kindly provided by Biorigin, Brazil) was used in the second treatment and 0.5 g/L MacroGard®, as used in Ref. [12], was added to the 0.35 g/L Selco enrichment and incubated with the rotifers for 3 h. In order to ensure that MacroGard® was taken up by *B. plicatilis* the suspension of MacroGard® in sterile sea water was sonicated for 4 × 30 s at power 6 (Sonifer® cell disruptor B-30, Branson Sonic Power Co.) prior to enrichment to ensure small enough particle size (≤20 μm) [35]. Sonicated MacroGard® was prepared fresh every day and particle size was verified by light microscopy. The treatments were run with three tank replicates each. From day 13 dph onwards all tanks were additionally fed untreated *Artemia* sp. nauplii (1 nauplius ml/L).

In order to assess mortality rates, dead larvae were removed and counted daily. For analysis of gene expression, RNA:DNA ratio, tryptic activity and size, larvae were anaesthetized with MS 222