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Proteomic analysis of rainbow trout (*Oncorhynchus mykiss*, Walbaum) serum after administration of probiotics in diets

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Abstract

The response of rainbow trout (*Oncorhynchus mykiss*, Walbaum) towards probiotics present in the feed was investigated by examining the proteome of serum as a measure of the acute phase response (APR). Proteomic analysis by two-dimensional electrophoresis (2D) concurrently with mass spectrometry was used to detect APR related proteins in rainbow trout serum following feeding with probiotics *Aeromonas sobria* GC2 and *Bacillus* sp. JB-1. Three candidate proteins increased following use of GC2, and were putatively identified as NADH dehydrogenase, dystrophin and mKIAA0350. Conversely, one of the proteins, which were induced following use of JB-1 was identified as transferrin.

Keywords: Probiotic; Fish disease; Acute phase response; Innate immunity; Proteomics

1. Introduction

There is concern over the use of antimicrobial compounds in aquaculture, and the increased emergence of antibiotic-resistance bacteria (Balcázar et al., 2006). Alternative strategies include the use of probiotic bacteria (Vine et al., 2006). To date, there has been a diverse range of organisms considered for use as probiotics (Irianto and Austin, 2002). Possible modes of action include inhibition of pathogenic bacteria by preventing their colonisation in the host (=competitive exclusion), the production of antimicrobial compounds, competition for nutrients chemicals and available energy, and stimulation or enhancement of the immune response (Gomez-Gil et al., 2000; Balcázar et al., 2006; Ringø and Gatesoupe, 1998; Vine et al., 2006; Sakata, 1990; Verschuere et al., 2000; Hong et al., 2005; Irianto and Austin, 2002, 2003).

The acute phase response (APR) has been defined as a rapid, orchestrated, physiologically induced response to tissue injury, infection, neoplasia, trauma and stress (Baumann and Gauldie, 1994; Jensen et al., 1997). This involves a large number of acute phase proteins (APP) and functions in a variety of defense-related activities, such as limiting the dispersal of infectious agents, repairing of tissue damage, the killing of micro-organisms and restoration of a healthy state (Larsen et al., 2001; Gerwick et al., 2000, 2002). Several of the mammalian positive APP has been identified, but it is anticipated that more are still unknown because the host response is complex, and has received only marginal attention. The proteins that have been identified include fibrinogen, C-reactive protein (CRP), α-2 macroglobulin, serum amyloid A (SAA), serum amyloid P (SAP) and mannose-binding protein A (also known as mannose binding lectin) (Sastry et al., 1991). In response to appropriate inflammatory stimuli, many

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of these defense-related molecules change in concentration within a few days; some proteins increase (positive APP), others decline (negative APP). Whereas reasons for a decline are debatable, there is a general consensus that positive APP probably all contribute to the individual's efforts to regain homeostasis as it repairs tissue damage and strives to contain and destroy potential pathogens (Gerwick et al., 2002).

Few APP are known in fish, and a better knowledge of them would provide a basis for more reliable methods to objectively assess fish health and welfare. Proteomic and genomic approaches have recently been applied to studies of the APR in some teleosts (Gerwick et al., 2000). The integration of traditional two-dimensional polyacrylamide gel electrophoresis (2D PAGE) with sensitive mass-spectrometric analysis methods and modern bioinformatics tools has greatly fostered the development and application of a new field of research called proteomics. The aim of the present study was to apply proteomic technology for the profile of alterations in serum APR-related proteins from rainbow trout after dietary administration of probiotics.

2. Materials and methods

2.1. Fish

Rainbow trout, *Oncorhynchus mykiss* (Walbaum) (average weight = 25 g) were obtained as separate stocks from commercial fish farms in the UK. The fish were maintained in aerated, free flowing, dechlorinated fresh water at 12 °C, and fed with commercial diet (Skretting) at 2% body weight every day. The health status was examined immediately upon arrival and at 14 days intervals thereafter for evidence of disease (after Austin and Austin, 1989). Prior to use, fish were starved for 24 h.

2.2. Probiotics

The two probiotic cultures, *Aeromonas sobria* GC2 and *Bacillus* sp. JB-1, which were obtained from ghost carp *Cyprinus* sp. and rainbow trout, respectively, have been described previously (Brunt and Austin, 2005). Identification was achieved using molecular analyses which included the sequence of genes encoding the subunit 16S rRNA for phylogenetic identification (Brunt and Austin, 2005). Stock cultures of bacterial isolates were stored in appropriate broth media containing 20% (v/v) glycerol at −70 °C. Cultures were routinely grown on tryptone soya agar (TSA; Oxoid) or broth (TSB; Oxoid) at 24 °C, and checked for purity by plating and re-plating on fresh media.

2.3. Treatment protocols

Two separate groups of rainbow trout were held in separate 125 l square fiberglass tanks in freshwater at 12 °C with a half water change every 24 h. On day zero (=control) all fish were anaesthetized (3-amino benzoic acid ethyl ester; Sigma–Aldrich), before bleeding (∼1 ml). Blood was allowed to clot at 4 °C for 2 h; sera were separated by centrifugation (4000 rpm for 25 min at 4 °C) and retained at −70 °C for subsequent analysis. Rainbow trout were returned to the tanks and treated with probiotics (GC2; JB-1) for 14 days (as described by Brunt and Austin, 2005), and then terminally bled.

2.4. Protein sample preparation

Total protein concentrations of control and probiotic treated serum were determined using the Bradford Assay and the appropriate dilutions calculated to allow 300 μg of protein per sample. To precipitate the protein, a 5× sample volume of ice-cold acetone was added and incubated overnight at −20 °C. After centrifugation (13,000 rpm, 30 min, 4 °C) the supernatant was discarded and the pellet allowed to air-dry. Protein pellets were rehydrated with 200 μl of rehydration sample buffer (8 M urea, 2% CHAPS; trace amount of bromophenol blue.) supplemented with 0.4% (w/v) dithiothreitol (DTT), 0.5% (v/v) IPG buffer (GE Healthcare) and incubated at room temperature for 2 h, or until the pellet was completely dissolved.

2.5. Two-dimensional PAGE (2D PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE), in which proteins are separated according to charge (p*I*) by isoelectric focusing (IEF) in the first dimension and according to size (MW) by SDS-PAGE in the second dimension, has a unique capacity for the resolution of complex mixtures of proteins (Görg et al., 1988).

Pre- and post-stimulus serum samples were subjected to simultaneous isoelectric focusing using immobilized pH gradient strips (IPG) (Immobiline DryStrip, pH 3–10 NL, 7 cm, GE Healthcare) using the Ettan IPGphor isoelectric focusing system (GE Healthcare). Rehydration samples were pipetted (125 μl) into the strip holders at a central point in the strip holder channel. Then, dry strip cover fluid (GE Healthcare) was applied to minimise