Available online at www.sciencedirect.com
ScienceDirect
Developmental and Comparative Immunology 31 (2007) 372–382
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding

A. Panigrahi[a], V. Kiron[a,*], S. Satoh[a], I. Hirono[b], T. Kobayashi[b], H. Sugita[c], J. Puangkaew[a], T. Aoki[b]

[a]*Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan*
[b]*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan*
[c]*Department of Marine Science and Resources, Nihon University, Fujisawa 252-8510, Japan*

Received 16 October 2005; received in revised form 27 June 2006; accepted 4 July 2006
Available online 7 August 2006

Abstract

This study elucidates the immune modulation including the expression of cytokine genes following dietary administration of three selected probiotic bacteria—*Lactobacillus rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* to fish, rainbow trout *Oncorhynchus mykiss*. They were fed for 45 days on either a basal control diet or one of the three probiotic diets containing the specific bacteria in freeze-dried form at a density of 10^9 CFU g feed^{-1}.

The non-specific immune parameters examined—superoxide anion production by the head kidney leukocytes and the alternate complement activity of serum was improved by probiotic feeding. Besides this, the relative gene expressions of interleukin-1β1, tumor necrosis factor 1 and 2 and transforming growth factor-β were up regulated in the spleen and the head kidney. The comparatively better performance of *E. faecium* could possibly be linked to their suitable ambient temperature conditions. Thus, probiotic bacteria delivered in feed exerts its influence on the immune system of fish, both at cellular and molecular levels.

Keywords: Rainbow trout; Probiotics; Non-specific immune responses; Cytokine genes; *Lactobacillus rhamnosus*; *Enterococcus faecium*; *Bacillus subtilis*; Temperature influence on probiotic efficacy

1. Introduction

Probiotics are defined as microbial cell preparations or cell components, which have a beneficial effect on the health and well being of the host [1]. The mechanisms by which such non-pathogenic microorganisms exert a positive influence upon ingestion largely remain unclear. Their inclusion in diet could exert beneficial physiological effects beyond the nutritional impact of food [2] by modulating specific functions in the gut and the immune system. Probiotics used for human and veterinary applications comprise a wide variety of microorganisms including members of the genus *Bacillus*, *Lactobacillus* and *Enterococcus* [3]. Lactobacilli have been proposed as a non-pathogenic bacteriotherapeutic means of modulating immune phenotype expression [4,5] and they may provide important regulatory signals to the immune system

*Corresponding author. Present address: Department of Fisheries and Natural Sciences, Bodø University College, 8049 Bodø, Norway. Tel.: +47 755 17399; fax: +47 755 17349.
E-mail address: kiron.viswanath@hibo.no (V. Kiron).

0145-305X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2006.07.004

[6] that influence systemic as well as local patterns of immunoreactivity [7]. *Lactobacillus rhamnosus* ATCC 53103 (GG) strain is reported to induce immune response and resistance against furunculosis when fed to rainbow trout [8]. *Bacillus subtilis* is a non-pathogenic aerobic gram-positive bacterium and oral administration of its spores to animals improved their intestinal bacterial flora [9,10], helped recovery from diarrhea [11] and improved survival [12]. *Enterococcus faecium* CH3 and *Lactobacillus salivarius* HA8, two lactic acid bacteria (LAB) of human origin, have the potential to inhibit the proliferation of myeloma cells [13]. *E. faecium* has also been found to enhance the immunity and growth in animals.

Cell surface components commonly present in gram positive and/or negative bacteria induce cytokine synthesis [14]. These soluble messenger molecules of the immune system interact with cells and receptors to generate mucosal antibody and cell-mediated immune responses. The strains of probiotic bacteria *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. bulgaricus* have been shown to induce production of interleukin (IL)-1, IL-2, IL-5, IL-6, tumor necrosis factor (TNF)-α and interferon [15–18]. Cytokines being stimulators as well as effectors of all immune and inflammatory responses, a better understanding of their normal operations is needed for realizing their therapeutic potential [19], fish being no exception.

Taking advantage of some of the known sequences of immune-related genes of rainbow trout [20], we compared the ability of three different probionts to induce the expression of cytokines IL-1β1 and β2, TNF1 and 2 (both TNF-α like) and transforming growth factor (TGF)-β, as well as certain non-specific immune responses.

2. Materials and methods

2.1. Bacterial strain: culture, harvest and storage

The bacterium *L. rhamnosus* ATCC 53103 (GG) was obtained from American Type Culture Collection (Rockville, USA) in freeze-dried form. The other two bacteria, *B. subtilis* and *E. faecium*, were obtained from laboratories in Japan. Their identity was confirmed biochemically using API 50 CH and API 20 E strip (BioMerieux, MarcyI'Etoile, France) respectively, and through sequencing of the 16S ribosomal RNA gene using DNA sequencer ABI PRISM 310 (Applied Biosystem, Foster City, USA). The reaction and inoculation of API strips was performed according to manufacturer's instructions. The culture was revived in Man, Rogosa and Sharpe agar (MRS; Merck, Darmstadt, Germany) [21] for *L. rhamnosus* GG, in Trypticase soy broth (TSB; BBL, Becton Dickinson Microbiology Systems, Cockeysville, USA) for *B. subtilis* and specific plates for *E. faecium*. A pure colony was inoculated in the seed culture, incubated at 30 °C for 24 h before mass culture in MRS broth or TSB media, harvested by centrifuging at 16,500*g* for 10 min and washed three times with sterile peptone water (0.85% NaCl and 0.1% polypeptone from Nihon Seiyaku, Tokyo, Japan). The harvested bacteria were brought into a uniform suspension that was freeze-dried (REL 206, Kyowa Vacuum Tech., Tokyo, Japan) by keeping the bacterial suspension for 60 h at −20 °C. The freeze-dried form was vacuum packed before storing at −20 °C until use.

2.2. Diet formulation and probiotic supplementation

Freeze-dried forms of bacteria were included (by weight to get the identical densities) in the diet during its preparation under possible sterile conditions. The diets were coded as LR, BS and EF for those containing *L. rhamnosus* GG, *B. subtilis* and *E. faecium*, respectively. The basal diet without probiont served as the control, termed CO. The diets contained fructo-oligosachharides (3%) as the prebiotic component, besides defatted fishmeal (protein source, 50%) and linseed oil (lipid source, 7%). The rest of the ingredients were included at nearly the same proportions as described in an earlier publication [22]. The diet preparation also followed the procedures in the aforementioned article. The pellets thus prepared were freeze-dried and stored at −20 °C before use. The viability of the incorporated LAB was assessed by vortexing 10 g of diet in 90 mL of peptone water and preparing serial dilutions, whereupon 0.1 mL portions were spread on to triplicate plates of Tryptic soy agar (TSA; BBL, Becton Dickinson, Cockeysville, USA), MRS and occasionally on blood liver agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). The bacterial counts were nearly 10^9 CFU g^{-1} for the probiotic diets. The daily rations were kept at 4 °C prior to feeding.

2.3. Fish feeding

The experiment was conducted following the guidelines for animal care existing at the Tokyo