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Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*

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ABSTRACT

Commercial gilthead sea bream feeds are highly energetic, fish oil traditionally being the main lipid source. But the decreased fish oil production together with the increased prices of this oil encourages its substitution by vegetable oils, imposing new nutritional habits to aquaculture species. Partial replacement of fish oil by vegetable oils in diets for marine species allows good feed utilization and growth but may affect fish health, since imbalances in dietary fatty acids may alter fish immunological status. The effect of dietary oils on different aspects of fish immune system has been reported for some species, but very little is known about the effect of dietary oils on immune-related genes expression in fish. Thus, the objective of this study was to elucidate the role of dietary oils on the expression of two pro-inflammatory cytokines, Tumor Necrosis Factor-α (TNF-α) and Interleukine 1β (IL-1β) on intestine and head kidney after exposure to the bacterial pathogen *Photobacterium damselae sp. piscicida*.

For that purpose, 5 iso-nitrogenous and iso-lipidic diets (45% crude protein, 22% crude lipid content) were formulated. Anchovy oil was the only lipid source used in the control diet (FO), but in the other diets, fish oil was totally (100%) or partially (70%) substituted by linseed (rich in n-3 fatty acids) or soybean (rich in n-6 fatty acids) (100L, 100S, 70L, 70S). Fish were fed experimental diets during 80 days and after this period were exposed to an experimental intestinal infection with the pathogen. Serum and tissue samples were obtained at pre-infection and after 1, 3 and 7 days of infection. RNA was extracted and cDNA was synthesized by reverse transcription from intestine and head kidney and the level expression of TNF-α and IL-1β were assayed by using quantitative real time PCR. The expression level of genes analysed was represented as relative value, using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Serum antibacterial activity was measured as serum bactericidal capacity and lysozyme activity.

Reduction of FO tends to reduce basal (pre-infection) genetic expression of both cytokines. However, complete FO replacement caused an over expression of both pro-inflammatory cytokines, particularly after 3 days of induced infection in fish fed soybean oil based diets. On the other hand, fish fed diets with low content of n-6 fatty acids showed better serum bactericidal capacity after infection, suggesting that the substitution of fish oil by vegetable oils containing high levels of n-6 fatty acids may induce imbalances on fish immune response, leading to a lower potential response against infections.

1. Introduction

The relationships between nutrition and health are complex and not limited to their critical role in resistance to infections, concerning most of the physiological functions related with health and also with welfare. In humans, both changes in nutritional habits and unbalanced diets, in terms of altered ratios between polyunsaturated fatty acids (PUFA), have been recognized as important factors that modulate and alter the complex relationship between immunity and nutrition, thus altering the capability to cope with infectious disease [1]. This type of alterations may also occur in aquaculture, since new nutritional strategies, such as

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inclusion of terrestrial vegetable oils (VOs) and meals in fish feeds, are being imposed as a consequence of the limited availability of fish oil (FO) and meal to cope with the increasing demand of these products [2].

Terrestrial VOs are rich in C18 fatty acids, mainly linoleic acid (LA; 18:2n-6), α-linolenic acid (ALA; 18:3n-3) and oleic acid (OA; 18:1n-9), but lack long-chain PUFAs (LC-PUFAs) such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) that have very important functions in fish [3,4]. Terrestrial VOs can be included in salmonids [3,4] or marine fish diets [5–10] at different levels of fish oil substitution without loss of growth performance. However, most of these species have adapted though evolution to the wild marine FO type lipid source (with a high n-3/n-6 fatty acid ratio of around 8:1), whereas inclusion of VOs with large quantities of n-6 PUFA (with a low n-3/n-6 ratio around 0.3–1) [11] will markedly change dietary fatty acids, and thus, new feeding strategies in aquaculture are changing the dietary n-3/n-6 ratio.

A well-balanced n-3/n-6 fatty acid ratio is a requirement for good health [12], since immune cells functioning and structure, cell signalling, eicosanoids production and even immune response as a whole, depend directly on the fatty acids functions and efficacy [1,13]. In fish nutrition, the use of vegetable oils has been shown to produce alterations in several parameters of the immune system in different aquaculture species, depending on the level of substitution, the type of vegetable oil used and the species studied [14]. Dietary vegetable oils can affect immunity at different levels, including effects on immune cells functionality (i.e., phagocytosis, macrophage respiratory burst activity) [10,15–19], immune cell fatty acid composition [17,20–23], humoral immunological processes such as serum lysozyme activity or alternative complement activity [17,19] or eicosanoid production [10,24,25]. Dietary induced alterations of immune system not always lead to a reduction in fish resistance to diseases, since infection depends on complex interactions between environment, pathogen and fish. In some studies changes in dietary n-3 PUFA have been reported to affect fish resistance to pathogens [11,15,16,26,27], whereas no effects have been found in other studies [28].

In spite of the increasing research regarding the role of dietary ingredients on fish health, very little is known about the role that dietary fatty acids have as modulator of the expression of certain genes involved in the immune response. Recently the first evidence of the effect of vegetable oils in expression of genes related with immune system in marine fish was described [29]. In those studies, complete substitution of anchovy oil by either single vegetable oils (soybean or linseed) or blends of them (50/50) induced a chronic hepatic expression of Mx protein (protein of resistance against mixovirus) gene in non-infected gilthead seabream (*Sparus aurata*). More recently [30], did not found differences in the expression of pro-inflammatory cytokines after stimulation with LPS in head kidney leukocytes from Atlantic salmon (*Salmo salar*) *ex-vivo* incubated in plasma from fish fed on different lipid sources (FO, rapeseed oil (RO) or their 1:1 blend (FO/RO)). However, the effect of vegetable oils on pro-inflammatory cytokines gene expression after challenging with a pathogen has not been yet studied in fish.

Thus, the objective of the present study was to elucidate how both total and partial substitutions of fish oil by different vegetable oils (soybean or linseed oils) affect gilthead sea bream immunological parameters and expression of pro-inflammatory cytokines after exposure to a sub-lethal concentration of a bacterial pathogen for this species: *Photobacterium damselae sub. piscicida*.

2. Material and methods

2.1. Feeding trial: experimental diets

Five iso-nitrogenous and iso-lipidic diets with a lipid content of 22% were produced by Biomar Iberia S.A. (Dueñas, Spain). Anchovy oil was the only source of lipid used for control diet (diet 100F), whereas fish oil was totally (100%) or partially (70%) replaced by soybean oil in diets 100S and 70S or linseed oil in diets 100L and 70L, respectively. Main ingredients of the diets and fatty acid composition of experimental diets is showed in Tables 1 and 2 respectively.

2.2. Fish

One thousand juvenile gilthead sea breams of 35 g initial body weight were obtained from a local farm (ADSA, S.A. Canary Islands, Spain). After two weeks of acclimation at the facilities held at the Canary Institute of Marine Science (ICCM), fish were randomly distributed into 20 fibreglass circular tanks of 500 l (50 fish/tank, quadruplicate for each diet).

Along the experimental period, fish were maintained in well-aerated running seawater at a water temperature and dissolved oxygen of 21.4–22.8 °C and 5–6.5 ppm, respectively and natural photoperiod (close to 12L:12D). The experimental diets were hand fed until apparent satiety three times a day, six days per week for 75 days. Feed intake was determined daily and all fish were individually weighted monthly. Both conversion index and specific growth rate were calculated according to the following formulae:

CI = Feed intake/weight gain,

SGR = ((ln final weight − ln initial weight)/t)*100, where t was experimental period in days.

At the end of the experiment, blood samples were collected from 4 fish per tank (16 per dietary treatment) to determine both serum lysozyme and bactericidal activities. Besides, head kidney and proximal intestine were removed from those fish to establish the basal expression of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1 β) genes. Intestine of 4 fish per tank (16 per treatment) were also sampled for fatty acid analysis.

2.3. Infection trial: exposure to a sub-lethal dose of pathogenic bacteria

At the end of feeding trial, 120 fish of about 120 g from each dietary treatment were randomly selected and distributed into 6 indoor cylindrical 500 l fibreglass tanks for a challenge test against bacterial pathogen as described later. Fish were reared under the same feeding experimental condition of oxygenation, photoperiod and water temperature, and fed the same experimental diets.

Infection trial was performed by triplicate assays for each dietary experimental group. Seventy two fish from each diet (24 for

Table 1
Main ingredients of the experimental diets.

	% dry weight
Oils (Fish[a]/linseed/soybean)	16.32
South-American fish meal	47.26
Soybean meal[b]	25.00
Sunflower meal[b]	3.67
Wheat	7.00
Vitamin Premix[c]	0.27
Mineral Premix[c]	0.48

[a] South American anchovy oil.
[b] No genetic modified organism.
[c] Premix according Proaqua commercial standards.