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Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (*Sparus aurata*) juveniles

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ABSTRACT

The use of plant ingredients in aquafeeds for piscivorous fish species is a reality that exposes fish to a number of antinutritional factors present in plants. The present study is the first to evaluate the effect of two purified antinutrients, saponins and phytosterols, in sea bream juveniles. For that purpose, seven diets were formulated: a control diet (fishmeal and fish oil based) and six experimental diets containing low (1 g kg^{-1}, SapL) or high (2 g kg^{-1}, SapH) levels of purified soya saponins, low (5 g kg^{-1}, PhytL) or high (10 g kg^{-1}, PhytH) levels of purified phytosterols or a combination of 1 g kg^{-1} saponins + 5 g kg^{-1} phytosterols (SapPhytL) or 2 g kg^{-1} saponins + 10 g kg^{-1} phytosterols (SapPhytH). Fish were fed for 48 days in order to evaluate growth performance, feed utilization, plasma cholesterol, and gut health as assessed by histomorphological evaluation and gene expression profiling of immune and functional markers. Fish fed the diets PhytH, SapPhytL and SapPhytH showed better feed utilization and PhytH and SapPhytH showed higher protein utilization than the other groups, although this was not reflected in improved growth performance. Histomorphological analysis of the distal intestine revealed increased variation in supranuclear vacuole sizes after 48 days of feeding diets SapH, SapPhytL and SapPhytH and increased number of intraepithelial leukocytes in response to all dietary treatments except SapL and SapPhytL. Although juvenile sea bream growth was not affected by dietary inclusion of saponins and phytosterols, the results indicated some disturbances of the intestinal mucosal structure that could compromise function and/or protection from potential dietary antigens or opportunistic pathogens.

1. Introduction

The aquaculture industry strives to improve its sustainability by shifting towards lower use of finite marine-harvested resources. During the past decade, a great deal of research has focused on reducing fish meal (FM) and fish oil (FO) in aquaculture feeds by introducing plant feedstuffs, which are now commonly used by the aquafeed industry (Rust et al., 2011). As awareness towards the importance of nutrition–health relationship gains ground, pressure to develop sustainable aquafeeds capable of eliciting proper growth while maintaining fish health and welfare increases (Kiron, 2012; Oliva-Teles, 2012). Partial replacement of FM and FO by plant sources has been shown to be feasible in several studies with gilthead sea bream (*Sparus aurata*) without affecting the zootechnical performance of the animals (Benedito-Palos et al., 2007; Benedito-Palos et al., 2008; Bonaldo et al., 2008; Dias et al., 2009; Gomez-Requeni et al., 2004; Kokou et al., 2012; Silva et al., 2010; Sitja-Bobadilla et al., 2005). Recently a study by Watson et al. (2013) reported the successful use of a 100% plant diet for sea bream without affecting growth performance; however, no information on fish intestinal physiology, histomorphology or immune parameters was provided. This may be significant because other studies with sea bream fed plant feedstuff-rich diets have demonstrated intestinal inflammation (Bonaldo et al., 2008) or impaired immune response (Kokou et al., 2012; Montero et al., 2010; Sitja-Bobadilla et al., 2005) without compromising fish growth.

Most plant-derived feedstuffs contain antinutritional factors, which are defined as substances that by themselves or through their metabolic products interfere with feed intake, nutrient digestibility, intestinal physiology, metabolism, growth, and/or health of the animal (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). The increasing use of plant feedstuffs in diets can expose fish to cumulative effects of antinutrients, which may result in a late manifestation of decreased

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growth, pathological conditions or less than optimal health (Krogdahl et al., 2010).

The development of a condition known as soybean meal-induced enteritis has been extensively described in Atlantic salmon (*Salmo salar*) (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007; Krogdahl et al., 2003; Marjara et al., 2012; Sahlmann et al., 2013; van den Ingh et al., 1991) and several other teleost species appear to react to soy in a similar way (Burrells et al., 1999; Hedrera et al., 2013; Uran et al., 2008). The specific agents causing this condition are not yet fully identified, but it is believed that one or more of the alcohol-soluble components of full fat soybean meal, such as saponins, are likely to be involved, as alcohol extracted soy protein concentrate does not cause pathological changes in the intestine of salmonids (Krogdahl et al., 2000; Van den Ingh et al., 1996).

Saponins are glycosides present in soybean meal and other plant feedstuffs that cannot be neutralized by heat during feed manufacturing. Several effects of saponins have been described in living organisms: impaired protein digestion, interference with cholesterol metabolism and entero-hepatic recirculation of bile salts, effects on the immune system, binding to cellular membranes with a consequential increase in cell permeability, and inhibition of active transport (Francis et al., 2002). In fish, decreased growth performance of rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis mossambicus*), Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*) has been attributed to the presence of saponins in the diets (Chikwati et al., 2012; Francis et al., 2001). Furthermore, feeding Atlantic salmon diets with sub-fractions of a soy extract containing saponins (Knudsen et al., 2007), soy saponin-supplemented diets containing lupin kernel meal (Knudsen et al., 2008), or soy saponin-supplemented diets containing pea protein concentrate (Chikwati et al., 2012) resulted in similar inflammatory changes as those induced by soybean meal.

Phytosterols are steroid alcohols naturally present in the lipidic portion of plants and may be present in fish feeds through the inclusion of plant ingredients. Data on the effects of these compounds in fish is scarce (Chikwati, 2007; Pelissero and Sumpter, 1992). In mammals, phytosterols are known to lower plasma cholesterol by inhibition of cholesterol uptake by the enterocytes, and by promoting fecal cholesterol and bile acid losses. As a consequence, hepatic conversion of cholesterol to bile acids increases, which further lowers plasma cholesterol levels (Ling and Jones, 1995; Ostlund, 2002). Studies evaluating the effects of replacing fish oil with plant oils in fish diets resulted in decreased growth at high inclusion levels of plant oil (Benedito-Palos et al., 2008; Dias et al., 2009; Izquierdo et al., 2005), and higher incidence of histopathological features in the intestinal mucosa, as well as decreased immune response at medium inclusion levels (Kokou et al., 2012; Montero et al., 2010; Sitja-Bobadilla et al., 2005). However, whether these effects are attributable to phytosterols is unknown.

The present work is, to our knowledge, the first to study the effects of inclusion of purified saponins and phytosterols, two antinutrients present in several plant feedstuffs that may be used as alternatives to fish-derived ingredients, in diets for gilthead sea bream, one of the most important cultured fish species in Europe. For that purpose, a 48 day feeding trial was conducted, and growth performance, feed utilization, plasma cholesterol, intestinal histomorphology and gene expression profiling of immune and functional markers in the distal intestine were evaluated.

2. Material and methods

2.1. Diets

Seven fish meal and fish oil based diets were formulated to contain 450 g kg^{-1} crude protein and 180 g kg^{-1} lipids (Table 1). The experimental diets comprised: a control diet, two diets containing saponins (1 g kg^{-1} and 2 g kg^{-1}; SapL and SapH, respectively), two diets containing phytosterols (5 g kg^{-1} and 10 g kg^{-1}; PhytL and PhytH, respectively) and two other diets with the antinutrient mixture (1 g kg^{-1} of saponins + 5 g kg^{-1} phytosterols; 2 g kg^{-1} saponins + 10 g kg^{-1} phytosterols; SapPhytL and SapPhytH, respectively). The levels of saponin used in the present study correspond to levels found in diets with about 200 and 400 g kg^{-1} soybean meal (Anderson and Wolf, 1995). These levels of saponins induce effects in Atlantic salmon (Chikwati, 2007; Chikwati et al., 2012). For the phytosterols, somewhat higher levels were chosen, corresponding to about 500 and 1000 g kg^{-1} inclusion level of soybean oil or 250 and 500 g kg^{-1} inclusion level of rapeseed oil in diets (Piironen et al., 2000). Based on results of preliminary studies with Atlantic salmon, these levels of phytosterols caused mild but distinct effects when supplemented alone (Chikwati, 2007). Soy saponins were commercially available at Organic Technologies (purity 95%; Coshocton, OH, USA). The phytosterol preparation was commercially available (purity >99%; Derive Resiniques et Terpenique, Dax, France), made from pine and produced to serve as functional additive in margarine for human consumption, purportedly for its cholesterol-lowering effects (Law, 2000). The dominating sterol was β-sitosterol, comprising 77%. As β-sitosterol is the main phytosterol also in soybean meal, the preparation was considered suitable as a model for soybean phytosterols. All dietary ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). The test diet was stored in hermetically closed plastic containers away from light and heat throughout the duration of the trial. Ingredient and proximate composition of the experimental diets are presented in Table 1.

2.2. Growth trial

The present experiment was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

The trial lasted 48 days and was conducted in a thermo-regulated recirculating water system equipped with 21 fiberglass tanks of 300 L water capacity, supplied with a continuous flow of filtered seawater. During the trial, a 12 h:12 h light:dark photoperiod was adopted, oxygen was maintained near saturation, water temperature was 25 ± 0.5 °C and salinity averaged 35 ± 1‰. Gilthead sea bream (*S. aurata*) juveniles were obtained from a commercial hatchery and kept in quarantine for four weeks during which they were fed a commercial diet (480 g kg^{-1} crude protein; 180 g kg^{-1} lipids; A. Coelho & Castro, Lda, Póvoa de Varzim, Portugal). After adaptation to the experimental conditions, groups of 30 fish with mean body weight of 12.5 ± 0.6 g (SD) were randomly distributed to each tank. Diets were randomly assigned to triplicate tanks. During the trial, fish were fed by hand to apparent satiation two times a day, six days per week. Fish were bulk weighed after two weeks (3 sampled specimens) and at the end of the trial.

2.3. Sampling

After two weeks, and at the end of the trial, three fish from each tank were randomly selected and euthanized by anesthetic overdose (ethylene glycol monophenyl ether, ref.: 8.07291, Merck, Whitehouse Station, USA) in ice water. The fish were dissected on chilled trays and the digestive tract was freed from the adjacent adipose and connective tissue. Two pyloric caeca and a section of the distal intestine were sampled for histological evaluation. The samples were rinsed in phosphate buffered saline (PBS), carefully blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%, pH 7.4) for 24 h and subsequently stored in ethanol (70%) until further processing.

At the end of the feeding trial, blood from three fish per tank was collected from the caudal vein using heparinized syringes and centrifuged at 1500 ×g for 10 min. Plasma samples were stored at −20 °C until analysis. After blood collection fish were euthanized, dissected and intestinal samples were collected for histological evaluation as described