Effect of antinutrients in European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) juveniles and on-growing fish

Philosophiae Doctor (PhD) Thesis

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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 European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) juveniles and on-growing fish.

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). Growth trials were undertaken to evaluate the effect of dietary antinutrients in growth performance and feed utilisation, gut physiology and health. Histomorphological studies of different sections of the intestine were analysed to evaluate the effect of antinutrient supplementation at the intestinal level. Additionally, the activity of brush border membrane enzymes was quantified in sea bass. The expression of genes considered markers of intestinal function was quantified in sea bream. Plasma cholesterol was also quantified to better understand the putative effects of the antinutrients in the entero-hepatic circulation of cholesterol and on lipid digestion. Fish immune status was evaluated by quantification of genes considered markers of inflammation.

In sea bass juveniles the antinutrients tested did not compromise growth performance. However histological alterations and gastrointestinal disturbances were observed, which may affect fish in the long term. On-growing sea bass showed high tolerance to the antinutrients tested, indicating that on-growing fish may be fed diets with higher substitution levels of fishmeal by plant products than juveniles without compromising growth, physiological function, and intestinal health. Sea bream juvenile's growth performance was also unaffected by dietary saponins and phytosterols at the levels tested. However, disturbances of the intestinal mucosal barrier that could compromise protection from potential dietary antigens or opportunistic pathogens were observed. On-growing sea bream showed high tolerance to dietary saponins and phytosterols either alone or in combination, thus indicating high ability to cope with dietary plant feedstuffs containing such antinutrients within the levels tested in the present study.

Although effects of dietary saponins and phytosterols were not very pronounced in both species at either development stages, juveniles showed to be more sensitive to the antinutrients than on-growing fish. Overall, it can be concluded that diets for sea bream and sea bass including relatively high amounts of the antinutrients tested can be used without affecting growth performance and with

only minor effects on animal well-being. However, the basal diet used in the present work was very simple and high quality, and the antinutrients effects may differ depending on basal diet / inclusion of plant ingredients with other antinutritional factors. Thus, utmost care should be taken to ensure that the diets will not compromise fish health as the cultivation conditions can induce animal stress and facilitate pathological episodes. Indeed, both species showed some immune reactions at the distal intestinal level, as well as some intestinal dysfunction, characterized either by decreased activity of the brush border enzymes (sea bass juveniles) or by down regulation of functional marker genes (sea bream juveniles). Long-term studies would be valuable to ensure that growth as well as fish health is maintained during the whole-rearing cycle.

Sumário

O uso de ingredientes de origem vegetal em dietas para espécies de peixes carnívoros é uma realidade que expõe os peixes a vários factores antinutricionais presentes em plantas. O presente estudo é o primeiro a avaliar o efeito de dois antinutrientes purificados, saponinas e fitoesteróis, em robalo europeu (*Dicentrarchus labrax*) e dourada (*Sparus aurata*), em duas fases do seu desenvolvimento: juvenis e fase de engorda.

Para tal, sete dietas foram formuladas: uma dieta control (à base de farinha de peixe e óleo de peixe) e seis dietas experimentais contendo níveis baixos (1 g kg^{-1}, SapL) ou altos (2 g kg^{-1}, SapH) de saponins de soja purificada, níveis baixos (5 g kg^{-1}, PhytL) ou altos (10 g kg^{-1}, PhytH) de fitoesteróis purificados ou uma combinação de 1 g kg^{-1} saponinas + 5 g kg^{-1} fitoesteróis (SapPhytL) or 2 g kg^{-1} saponinas + 10 g kg^{-1} fitoesteróis (SapPhytH). Foram realizados ensaios de crescimento para avaliar o efeito dos antinutrientes no crescimento e utilização do alimento bem como na fisiologia e saúde do intestino. De froma a avaliar o efeito dos antinutrientes a nível intestinal, realizaram-se estudos histomorfológicos de diferentes porções do intestino. Quantificou-se ainda a actividade de enzimas do prato estriado em robalo, bem como a expressão de genes considerados marcadores da função intestinal em dourada. Adicionalmente, o nível de colesterol plasmático foi quantificado de forma a tentar perceber de que forma os antinutrientes interferem na circulação entero-hepática do colesterol. O estado imunológico dos peixes foi avaliado através da quantificação da expressão de genes considerados marcadores de um estado inflamatório.

Em juvenis de robalo os antinutrientes não comprometeram o crescimento. No entanto, observaram-se alterações a nível histológico bem como perturbações gastrointestinais que podem afectar o peixe a longo-prazo. Em fase de engorda, o robalo demonstrou uma maior tolerância aos antinutrientes, o que pode indicar que nesta fase do seu ciclo de vida o robalo pode ser alimentado com dietas contendo maiores proporções de ingredientes de origem vegetal, em relação aos juvenis, sem comprometer o crescimento, função fisiológica ou saúde intestinal. O crescimento de juvenis de dourada também não foi afectado pelas saponinas ou fitoesteróis dentro dos níveis testados. Contudo, observaram-se alterações na barreira intestinal que podem afectar a sua função protectora contra potenciais antigenes alimentares ou patogenos oportunistas. A dourada em fase de engorda demonstrou uma maior tolerância a saponinas e fitoesteróis, quer quando suplementados isoladamente, quer quando combinados, indicando uma maior capacidade para lidar com dietas que incluam matérias-primas de origem vegetal contendo estes antinutrientes dentro dos níveis testados no presente estudo.

Embora os efeitos de saponinas e fitoesteróis alimentares não tenham sido muito pronunciados em qualquer das espécies em ambas as fases de desenvolvimento, os juvenis demonstraram maior sensibilidade aos antinutrientes do que os animais em fase de engorda. No geral, pode-se concluir que as dietas para dourada e robalo podem incluir quantidades relativamente altas dos antinutrientes testados sem afectar o crescimento e com apenas efeitos menores no bem-estar dos animais. No entanto, a dieta-base usada no presente estudo é muito simples e de alta qualidade e o efeito dos antinutrientes pode diferir com outra dieta-base / inclusão de ingredientes de origem vegetal com outros factores antinutricionais. Assim, deve garantir-se que as dietas não vão comprometer a saúde dos peixes, uma vez que as condições de cultivo podem induzir stress nos animais e facilitar episódios patológicos. Ambas as espécies mostraram reações imunológicas ao nível do intestino distal, assim como alguma disfunção intestinal, caracterizada tanto por diminuição da actividade das enzimas do prato estriado (juvenis robalo) como pela diminuição da expressão de genes considerados marcadores da função intestinal (juvenis de dourada). A realização de estudos de longa duração seria pertinente, de forma a garantir que o crescimento, bem como a saúde dos peixes se mantém durante todo o seu ciclo de vida em condições de cultivo.

Contents

Chapter 1

General Introduction

Aquafeeds: the green revolution

The global population is increasing and there is a pressing need to meet the food and nutrition requirements of a growing population. Additionally, the increasing awareness to the health promoting effects of aquatic products has increased the world consumption of fisheries products. Given that production from capture fisheries has leveled off and no additional supply will be provided until overexploited fisheries stocks are replenished, the pressure to provide aquatic products is placed on aquaculture (FAO, 2012). In the last three decades the aquaculture industry has expanded at an average annual rate of 8.8%. However, to maintain the present per capita consumption of aquatic food it has been estimated that by 2030 the world will require in addition to today's 148 million tonnes of fish at least another 23 million tonnes, which need to be provided by aquaculture (FAO, 2012). Accompanying the expansion of aquaculture is the aquafeeds industry, with a growth rate of 11% per year at present. Growth in fish feed production will continue as long as growth in aquaculture is maintained (Tacon et al., 2011). At present, compound aquafeeds for higher trophic level finfishes are still dependent upon capture fisheries for the supply of key dietary nutrient inputs, including fish meal (FM) and fish oil (FO). Nevertheless, the total use of FM by the aquaculture sector has gradually fallen since 2006 and is expected to decrease in the long term. This was due to the tighter rules concerning fishing quotas and unregulated fishing, as well as an increased use of more cost-effective and sustainable FM replacers (Tacon et al., 2011; FAO, 2012). With the increased awareness towards a possible FM scarcity, both industry and research have produced valuable knowledge on the digestive processes and nutritional requirements of many farmed species and on how to process raw materials in order to make them more suitable to use in aquafeeds. On the other hand, the search for appropriate FO replacers has proven more difficult since most alternatives have low contents of long chain poly-unsaturated fatty acids (LC-PUFA), which might have no deleterious effects for the cultured fish but would reduce the content of the attractive highly polyunsaturated fatty acids of the final products (Rust et al., 2011). Feedstuffs considered as FM and FO replacers can be generally classified as of terrestrial animal origin, marine origin, plant origin, and either terrestrial or marine single cell proteins. Despite the fact that production of terrestrial animal by-products is the most promising among the four, within the EU-countries their use was banned until 2012 and is still subject to tight regulation. Hence, within the EU, feedstuffs from plant origin are currently on the lead as FM and FO replacers in aquafeeds, being the most important alternative sources of protein and lipids for high trophic level fish species (Tacon et al., 2011).

Aquafeeds going green: pros and cons

During the last decade, sustained research into alternative ingredients is lowering rates of FM and FO inclusion in aquafeeds (Naylor et al., 2009). The most important plant feedstuffs currently used in aquafeeds comprise: cereals by-products meals and oils; oilseed meals and oils; pulses and protein concentrate meals (Tacon et al., 2011). Within the cereals, maize, wheat, rice and barley are the most produced worldwide, while soybean is the largest oilseed crop, followed by rapeseed, cottonseed and sunflower seed. Peas and lupins are the most available among the pulses, with protein concentrates being commercially available for use in compound feeds, including aquafeeds (Tacon et al., 2011). Despite the good nutritional characteristics of the aforementioned protein concentrates, their production processes result in high market prices not competitive with FM value.

Plant feedstuffs have a relatively constant nutritional composition and high availability on the world market, thus a great amount of research has focused on their evaluation as suitable ingredients for aquafeeds (Alexis, 1997; Tacon, 1997; Naylor et al., 2009; Welch et al., 2010). To be used in aquaculture feeds, plant ingredients should possess certain nutritional characteristics, such as low fibre content, high protein content, adequate amino acid profile, and high digestibility. Alternatives to FM and FO have been tested for many species of high-value cultivated fish, such as Atlantic salmon (*Salmo salar*), red drum (*Sciaenops ocellatus*), cobia (*Rachycentron canadum*), turbot (*Psetta maxima*), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), Japanese flounder (*Paralichthys olivaceus*), yellowtail (*Seriola quinqueradiata*) and rainbow trout (*Oncorhynchus mykiss*) (see reviews byGatlin et al., 2007; Rana and Hasan, 2009; Hardy, 2010; Welch et al., 2010). As a result of the sustained research into alternative feed ingredients during the last 10-15 years, FM inclusion levels in compound aquafeeds for marine fishes and salmon, for example, dropped from 50 to 20% and from 45 to 20%, respectively; while dietary FO inclusion levels dropped from 15 to 5% and 25 to 10% for marine fishes and salmon, respectively (Tacon et al., 2011). This downward trend is likely to continue, although the nutritional difficulties in replacing marine resources with alternative ingredients in aquafeeds have not been fully overcome.

Plant-derived feedstuffs have characteristics that place them at disadvantage to FM and FO, such as relatively low-protein content, amino acid imbalances and presence of endogenous compounds known as antinutritional factors or antinutrients (ANFs) (Simões-Nunes, 1987; Krogdahl, 1990; Tacon, 1997; Francis et al., 2001; Gatlin et al., 2007). Antinutrients have been defined as substances that by themselves or through their metabolic products in living systems interfere with food utilization and affect the health and production of the organisms (Makkar, 1993; Francis et al., 2001). Possible harmful effects of such compounds include: reduced palatability, reduced utilization

of food nutrients, altered nutrient balances of the diets, growth inhibition, immune modulation, intestinal dysfunction and liver damage, to name a few (Krogdahl et al., 2010). While some antinutrients are heat-labile and can be easily eliminated/neutralized during routine processing of the diets, others are heat resistant and more difficult to deal with (Francis et al., 2001; Gatlin et al., 2007). Plant feedstuffs generally contain more than one antinutrient. Therefore it is difficult to ascertain which individual factor is responsible for any adverse effects observed when using a specific plant ingredient in aquafeeds (Francis et al., 2001). Antinutritional factors are foreign to most cultivated fish species, particularly carnivorous species (Krogdahl et al., 2010). Each species may have different tolerance limits to the presence of antinutrients in their feed. Herbivorous and omnivorous species seem to tolerate plant antinutrients better than their carnivorous counterparts, which do not feed on plant feedstuffs in their natural habitat (Gatlin et al., 2007). Likewise, tolerance to antinutrients may also be dependent on the particular stage of fish life cycle (fry, juveniles, grow-out, broodstock) (Tacon, 1997). Another important factor to be considered is the interaction between antinutrients as they might lead to a modification of the toxic effects the antinutrient would exert individually (Francis et al., 2001).

With the evolution of the aquafeeds industry and the marked increase in the inclusion of plant-derived feedstuffs in aquaculture fish diets, the exposure to antinutrients has also increased. Although antinutritional factors may not cause an immediate violent reaction in living systems, there might be slow cumulative adverse effects ultimately resulting in disease or less than optimal health (Liener, 1980). Thus, it is plausible that antinutritional factors are involved in the manifestation of emergent diseases in aquaculture industry related to gut function and the immune response, such as low protein and lipid digestibility, enteritis and neoplasia (Krogdahl et al., 2010).

Enteritis model in fish

First described by van den Ingh et al. van den Ingh et al., 1991; Van den Ingh et al., 1996) and named by Baeverfjord and Krogdahl (1996), “non-infectious sub-acute enteritis” is a distal intestinal disorder that historically has been associated with the replacement of FM by full-fat or defatted (hexane extracted) soybean meal (SBM) in Atlantic salmon diets (Olli et al., 1994a; Olli et al., 1995; Refstie et al., 2001; Opstvedt et al., 2003). However salmon is not the only species developing enteritis when fed SBM (Burrells et al., 1999; Uran et al., 2008; Hedrera et al., 2013). There is also evidence that this pathology is not restricted to the inclusion of SBM in the diets, but is also observed when using other plant ingredients (Penn et al., 2011; Chikwati et al., 2012; Kortner et al., 2012). The characterization of the aforementioned intestinal inflammation has been

the subject of several studies and thus far histomorphological, functional and immunological alterations have been described.

Histologically, the distal intestinal tissue shows shortening of the primary and secondary mucosal folds, loss of the normal supranuclear vacuolization of the enterocytes, widening of the lamina propria and submucosa with concomitant infiltration by a mixed population of inflammatory cells identified as lymphocytes, macrophages, neutrophilic granulocytes and eosinophilic granular cells (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000b; Bakke-McKellep et al., 2007).

At the functional level the observations comprise reduction of the brush border enzymes activities (Bakke-McKellep et al., 2000b; Krogdahl et al., 2003), reduced endocytosis (Uran et al., 2008), increased membrane permeability (Nordrum et al., 2000; Knudsen et al., 2008) and increased fecal trypsin (Krogdahl et al., 2003). Hypocholesterolemia and decreased levels of bile acids due to impaired absorption (Kortner et al., 2013) and reduced lipid digestibility (Olli et al., 1994a; Krogdahl et al., 2003; Romarheim et al., 2008) have also been observed. Genomic studies reported changes in the expression of genes for transport proteins, ion channels and ion pumps, important for nutrient absorption and cell homeostasis maintenance (Gu et al., 2013; Sahlmann et al., 2013; Venold et al., 2013).

During the development of the diet-induced enteropathy, a number of immunological responses are also observed, namely enhanced recruitment and retention of macrophages, neutrophils and T-cells in the inflamed intestine and regulation of both pro- and anti-inflammatory responses (Bakke-McKellep et al., 2000b; Bakke-McKellep et al., 2007; Uran et al., 2008; Lilleeng et al., 2009; Kortner et al., 2012; Marjara et al., 2012; Sahlmann et al., 2013). Both innate and adaptive immune responses are observed in the onset of inflammation (Bakke-McKellep et al., 2000b). Immunohistochemical studies revealed increased staining of diffuse immunoglobulin M (IgM) in the lamina propria without a change in IgM-positive cells (Bakke-McKellep et al., 2000b) and increased detection of CD3ε-positive T-cells accompanied by an up-regulation of gene expression of T-cell-specific molecules: CD3, CD4, CD8α, CD8β, and TCRγ (Bakke-McKellep et al., 2007; Marjara et al., 2012; Sahlmann et al., 2013) confirming the involvement of T lymphocytes in the development of enteritis. Transcriptomic studies have revealed the involvement of several cytokines in the inflammation process such as IL-1β, TGFβ, IL-17, and IL-10 (Uran et al., 2008; Skugor et al., 2011; Marjara et al., 2012; Sahlmann et al., 2013).

The magnitude of diet-induced enteritis differs from species to species. In common carp, an omnivorous species, an inflammation was described during the first four weeks after feeding a diet containing 20% SBM. However, from the 4th to the 5th week (end of the trial) carp displayed signs

of recovery without changing the diet (Uran et al., 2008). In carp, immunological reactions similar to salmon were observed during the enteritis process: invasion of immune cells, higher activity of T-cells and also regulation of several pro- and anti-inflammatory cytokines. In both carp and salmon, the anti-inflammatory cytokine IL-10 gene expression was down-regulated during later stages of SBM-induced enteritis, whereas it was up-regulated during early stages of SBM-induced enteritis only in carp (Uran et al., 2008), possibly participating in the recovery process. This may explain the transient nature of the SBM-enteropathy in carp compared to Atlantic salmon (Marjara et al., 2012).

It is still unclear which specific factors are responsible for the diet-induced enteritis. Nevertheless the negative effects associated with the inclusion of SBM products in salmon diets have been attributed to the presence of one or a combination of ANFs present in the seeds. Soy protein concentrate (SPC) produced by aqueous alcohol extraction of the seeds showed a nutritional value comparable to FM in salmon diets (Olli et al., 1994b; Olli et al., 1995), whereas the alcoholic fraction resulting from the production of SPC caused morphological changes in the intestine of salmon similar to those caused by standard soy products (Van den Ingh et al., 1996). The processing techniques used during SPC production extract oligosaccharides, saponins, isoflavones, soluble polysaccharides and possibly other ANF yet unidentified (Van den Ingh et al., 1996; Refstie et al., 2005). During lactic acid fermentation, which has also shown to improve the nutritional value of de-hulled solvent extracted SBM for salmon, oligosaccharides and activity of trypsin inhibitor are lowered and factors affecting lipid metabolism and causing intestinal pathology in salmon are partly removed (Refstie et al., 2005). Thus, it appears that the factors causing the distal intestinal pathology and other negative effects are alcohol soluble and / or eliminated by lactic acid fermentation. Factors fitting this description include saponins, phytosterols, oligosaccharides and dietary fibre.

Antinutritional factors

Phytosterols

Chemistry and biological distribution

Phytosterol is a term applied to a large number of plant sterols, which are membrane constituents of all plants, and whose chemical structure and biological activity resemble that of cholesterol, the predominant sterol in animals (Piironen et al., 2000). Phytosterols are steroid alcohols that differ from cholesterol by having either an additional methyl or ethyl group on the carbon-24 position or

an additional double bond in the side chain (von Bergmann et al., 2005). Phytosterols can be hydrogenated and form phytostanols. The most common phytosterols and phytostanols are: β-sitosterol, sitostanol, campesterol, campestanol, stigmasterol and brassicasterol (Piironen et al., 2000).

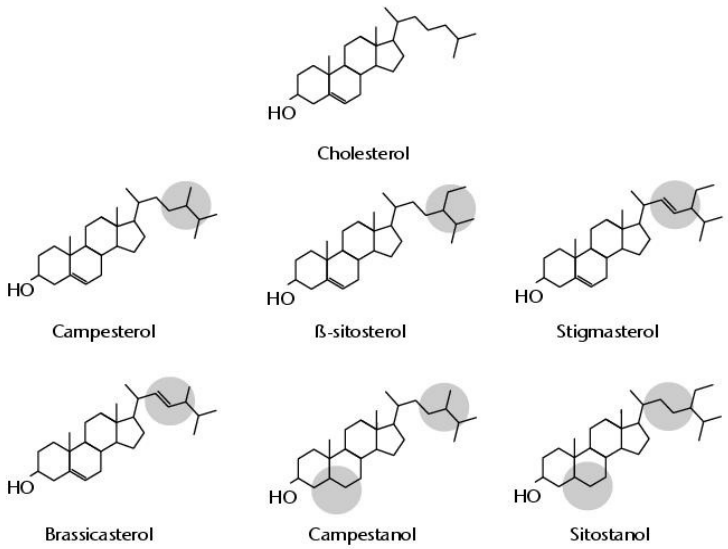

Figure 1 - Chemical structure of the most biologically relevant phytosterols (Trautwein and Demonty, 2007)

Vegetable oils are rich natural sources of phytosterols with the majority of the vegetable oils containing 1-5 g kg^{-1} of plant sterols. Total sterol content (g kg^{-1}) in soy and palm oils are 2.29 – 4.59 and 0.71 – 1.17, respectively while rapeseed and sunflower oils have higher total sterol content: 5.13 – 9.79 and 3.74 - 7.25, respectively. The predominant sterols in these oils are sitosterol followed by campesterol and stigmasterol (see details in Piironen et al., 2000).

Genetic factors as well as growing phase and storage conditions / duration may affect the sterol content of oil seeds and further variation can result from processing conditions (Piironen et al., 2000).

Effects in biological systems

Phytosterol intake by mammals is usually associated with hypocholesterolemia, thus being of major interest in prophylactic treatment against cardiovascular disease in humans (Vanstone et al., 2002; Skeaff et al., 2004). It is generally accepted that the mechanism by which phytosterols reduce plasma cholesterol in mammals is through the inhibition of cholesterol absorption in the intestine

(Ling and Jones, 1995). Although the mechanism by which inhibition of intestinal cholesterol absorption occurs is not totally comprehended, two models have been proposed. The first one proposes that plant sterols precipitate the marginally soluble cholesterol into a non-absorbable state and the second one suggests that plant sterols and stanols displace cholesterol from the micelles that assist its absorption into enterocytes (Hicks and Moreau, 2001). Phytosterols are taken up by the enterocytes but are generally poorly absorbed into the blood stream. A non-selective protein-mediated sterol uptake into the enterocytes from the intestine, followed by a reverse pumping of non-cholesterol sterols by selective transporters with an ability to discriminate between cholesterol and non-cholesterol sterols, is hypothesized to explain the low phytosterol absorption into the plasma (Chen, 2001; Igel et al., 2003). In any case, inhibition of cholesterol absorption increases fecal cholesterol losses and thus reduces plasma cholesterol levels. Phytosterols present in plasma also appear to promote hepatic conversion of endogenous cholesterol to bile acids, further lowering plasma cholesterol levels (Ling and Jones, 1995).

Effects in fish

Only limited information is available from research addressing effects of phytosterols in fish. In one study in Atlantic salmon, no effect on growth or intestinal histomorphology was seen at a dietary inclusion of 5 and 10 g Kg^{-1} (Chikwati, 2007). However, the phytosterols significantly lowered lipid digestibility, but without any effect on other macronutrient digestibilities or faecal dry matter. Moreover, a dose-dependent decrease of chymal bile salt concentration in the mid and distal intestines was also observed, as well as decreased plasma levels of bile salts, cholesterol, alanine aminotransferase and inorganic phosphate (Chikwati, 2007). Apart from the study by Chikwati (2007), most of the studies in fish nutrition focus on the effects of phytosterols as components of vegetable oils used in FO replacement on flesh quality from the consumer point of view (Izquierdo et al., 2003; Miller et al., 2008a; Pettersson et al., 2009a; Pettersson et al., 2009b).

Phytosterols are virtually absent in natural diets of carnivorous fish and few studies have been carried out in order to assess the effects of dietary phytosterols on fish health, as reviewed by Miller et al. (2008b).

Saponins

Chemistry and biological distribution

Saponins are a wide group of glycosides, largely distributed in higher plants, and considered to have a role in the plants defensive system (Francis et al., 2002). Saponins are constituted of a sugar

moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, linked to a hydrophobic aglycone (sapogenin). The aglycone may hold one or more unsaturated C-C bonds and can contain more than one sugar moiety attached. The great variety of known saponins results from the variability in the aglycone structure, the nature of the sugar moieties and their attachment position on the aglycone (Francis et al., 2002). According to the nature of the aglycone, saponins can be classified into steroidal (steroid aglycone), almost exclusively present in monocotyledonous angiosperms, and triterpenoid (triterpenoid aglycone), more common and occurring mainly in the dicotyledonous angiosperms (Sparg et al., 2004).

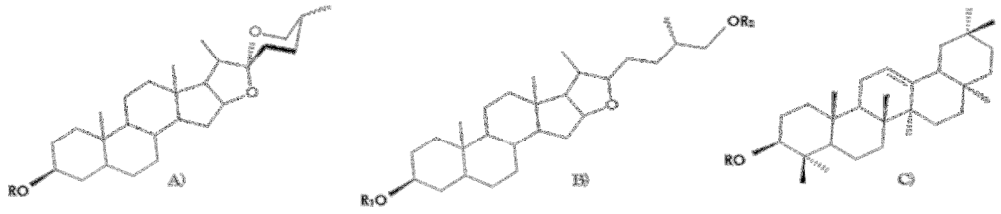

Figure 2 - Skeletons of aglycone: (A) steroidal spirostane, (B) steroidal furostane (C) triterpenoid. R = sugar residue (Cabrera-Orozco et al., 2013)

Saponin content in plant meals varies between 5 and 60 g kg^{-1} (dry weight) with, for example, soybean containing levels up to 56 g Kg^{-1} and lupin seed meal having 11 g Kg^{-1} (Fenwick et al., 1991; Francis et al., 2001). The saponin content of plant feedstuffs is affected by the plant species or cultivars, genetic origin, environmental and agronomic factors associated with growth of the plant, and post-harvest treatments, processing and storage conditions (Fenwick et al., 1991).

Effects in biological systems

Saponins are often used in cellular membrane permeation, allowing the passage of molecules that would normally not cross cell membranes (Sparg et al., 2004). Due to their amphipathic characteristics, saponins have the ability to interact with the membrane lipids altering their normal function (Francis et al., 2002). Saponins' lytic action on erythrocytes is believed to result from the high affinity of the aglycone moiety for membrane sterols with which it forms insoluble complexes, resulting in the formation of a pore (40-50 Å in diameter) in the erythrocyte's membrane (Seeman et al., 1973). Saponins may also alter the lipid environment of membrane proteins by interacting with the polar head of membrane phospholipids and cholesterol, forming micelle-like aggregates or

intercalating into the interior of the membrane bilayer (Francis et al., 2002). The lipid environment, particularly the membrane fluidity, has been recognized as playing an important role in the function of the embedded proteins, including ion channels, transporters and receptors, and in ion transport (Francis et al., 2002). Fluctuating membrane channels resulting from saponin action may also increase electrical conductance in planar lipid bi-layers or open large Ca-dependent K conductance channels causing membrane hyperpolarization. The ability of saponins to block membrane ion channels on neurons and human neutrophils has also been reported (Bei et al., 1998; Kai et al., 1998). Formation of a saponin – cholesterol complex, alterations in membrane phospholipid organization, formation of phospholipid breakdown products and saponin's three-dimensional orientation all seem to be involved in the actions of saponins on membranes (Francis et al., 2002).

Several biological actions have been attributed to saponin action in living systems, including hypoglycemic activity, effects on animal reproduction, inhibition of cancer cell growth, molluscidal, antifungal and virucidal activities, as well as being detrimental to protozoans, to name a few. Additionally, effects on feed intake and growth, on protein digestion, on cholesterol metabolism and on the immune system have also been reported (Francis et al., 2002).

In the intestine, saponins were found to increase enterocyte permeability in vitro, and to inhibit active transport and facilitate the uptake of substances that are normally not absorbed, like allergens (Johnson et al., 1986). The presence of Gypsophila saponins enhanced the uptake of β-lactoglobulin, a milk allergen, in the jejunum of brown Norway rats and damaged the mucosal epithelium (Gee et al., 1997). On the other hand, hindrance of micronutrients (minerals, vitamins) absorption by saponins has been reported in rats, pigs and chicks (Southon et al., 1988; Jenkins and Atwal, 1994).

Dietary saponins are generally considered as having deleterious effects in animals; however, both positive and negative effects have been reported in a variety of species (Sen et al., 1998). The effect of saponins on growth and feed intake seems to be largely dependent on the source of saponins and animals species. No effect on growth of chickens, rats and mice were observed, while improved growth and feed efficiency were reported for ruminants and pigs. On the other hand, dietary saponins depressed growth and feed consumption in gerbils and decreased egg production in chicken (reviewed by Francis et al., 2002). The negative effects of saponins have been ascribed to its astringent taste, which decreases feed intake, reduction of intestinal motility, reduction of protein digestibility and damage to the intestinal membrane with a concomitant inhibition of nutrient transport (Francis et al., 2002).

Reduced protein digestibility has been attributed to the formation of saponin-protein complexes (Potter et al., 1993). Saponins of various botanical origins have shown to lower blood cholesterol in several animal species, including humans (Al-Habori and Raman, 1998). Possible explanations to such phenomenon are formation of large mixed micelles with bile acids, impeding their return to the liver and consequently accelerated cholesterol metabolism to synthesize new bile acids, or formation of saponin-cholesterol complexes leading to decreased cholesterol absorption in the intestine (Francis et al., 2002).

Immunostimulatory activity of saponins is well established, purportedly mediated through stimulation of the cell mediated immune system, as well as enhanced antibody production (Oda et al., 2000). Saponins induced specific cytotoxic T-lymphocyte responses, strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens (Rajput et al., 2007). Saponins not only have stimulatory effects on specific immunity components but also induce non-specific immune reactions, such as inflammation and monocyte proliferation (Rajput et al., 2007). There is also evidence showing that saponins may stimulate the immune response by increasing the uptake of antigens from the gut due to enterocyte permeation (Francis et al., 2002).

Dietary saponins are exposed to many ligands in the intestinal lumen such as bile salts, dietary cholesterol and membrane sterols of the mucosal cells, and nutrients or antinutrients in food, all of which may reduce or enhance their activity (Francis et al., 2002) and making it hard to predict their effects in living systems.

Effects in fish

Knowledge on the effects of saponins in fish is fragmentary and sometimes conflicting. Saponins are present in many traditional fish poisons and saponins in the water can be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy et al., 1990). Bureau et al. (1998) observed that Quillaja saponins (3 g kg^{-1} diet) reduced feed intake and growth of Chinook salmon and depressed growth of rainbow trout. In the same study, dietary levels above 1.5 g kg^{-1} saponins damaged the intestinal mucosa in both species. Francis et al. (2005) concluded that 1.5 g kg^{-1} of Quillaja saponins in the diet potentiated growth and nutrient utilization in common carp and tilapia. Soysaponins included in salmon diets (1 and 2 g Kg^{-1}) had no significant effect on growth and intestinal histomorphology (Chikwati, 2007). Soysaponins did not affect macronutrient apparent digestibility (lipids, protein or starch) but reduced activities of the mucosal enzymes leucine aminopeptidase and maltase in the distal intestine at the higher inclusion level of 2 g kg^{-1} diet. Soysaponin inclusion had a negative dose-dependent effect on bile salt concentration in the mid and distal intestine while trypsin activity significantly increased with increased dietary soysaponin

levels. Soysaponin also lowered plasma cholesterol and bile salt concentration, and alanine aminotransferase activity levels compared to the control group (Chikwati, 2007). According to Iwashita et al. (2009), saponin supplementation (3.8 g kg^{-1}) to a casein-based semi-synthetic diet induced poor development of microvilli and pinocytotic vacuoles, and accumulation of large vacuoles in the enterocytes of rainbow trout, without affecting growth. Japanese flounder fed diets containing graded levels of soy saponins (0, 0.8, 3.2 and 6.4 g kg^{-1}) showed decreased weight gain, feed efficiency and apparent digestibility coefficients of crude protein with increasing dietary soybean saponins. The histological structure of the distal intestine was also gradually impaired as soybean saponins levels increased (Chen et al., 2011). Similarly, in a dose-response study performed with Atlantic salmon fed a FM-based diet with soya saponin supplementation (0, 2, 4, 6, 10 g kg^{-1}), inflammation of the distal intestine of fish fed high (6-10 g kg^{-1}) saponin diets was observed, along with a dose-dependent reduction in plasma cholesterol and bile acids, and in the activity of brush border digestive enzymes. A growth promoting effect was observed in fish fed low (2 - 4 g Kg^{-1}) soy saponins inclusion levels (Penn et al., 2012).

The involvement of saponins in the distal intestinal enteritis induced by SBM in Atlantic salmon has been gradually gaining support. Morphological changes similar to those induced by SBM were observed in Atlantic salmon when soya saponins were added to a lupin kernel basal diet at inclusion levels of 1.7 and 2.6 g kg^{-1}, while when supplemented to a FM-based diet no morphological alterations were observed (Knudsen et al., 2007; Knudsen et al., 2008). In vitro studies with the lupin + saponin fed fish showed increased permeability of the gut wall. The authors concluded that soybean saponins increased the intestinal epithelial permeability but did not, per se, induce enteritis. Atlantic salmon fed a reference diet or a reference diet with added saponins (2 g kg^{-1}) showed slight histological changes and reduced activity of leucine aminopeptidase, a brush border membrane enzyme, in the distal intestine along with reduced lipid and fatty acid digestibility. Inflammation of the distal intestine was also observed with saponin supplementation to pea protein concentrate containing diets, together with reduced feed intake, reduced apparent digestibility of lipids, most amino acids and ash, decreased bile salt levels in intestinal chyme and decreased leucine aminopeptidase activity but increased trypsin activity in the DI (Chikwati et al., 2012).

Knowledge on the effects of dietary saponins in fish is still limited, and as Krogdahl et al. (2010) recommended in a recent review, “requires strengthening, particularly regarding interactions with other feed components, possible growth-promoting potentials and especially regarding effects in species other than salmonids”.

Fish digestive physiology

The main functions for the gastrointestinal tract (GI) are to acquire food, to process the ingested food, to absorb vital nutrients and to excrete waste. In vertebrates, the GI canal is constituted of several distinct regions that differ in morphology and histology as well as in physiological function. The functional characteristics of nutrient assimilation include mechanical breakdown of food to smaller parts, secretion of digestive enzymes and other components, digestion and absorption of nutrients. Other functions of the GI are osmoregulation, hormone secretion and protection of the organisms from alien substances and pathogens that may reach the GI with the food and water.

Following prey capture and manipulation by teeth or other parts of the oral cavity, the food is transported to the stomach through the esophagus. The stomach is absent in some vertebrates, such as some species of fish, in which the intestine directly follows the esophagus. Stomach secretions typically contain proteolytic enzymes as well as hydrochloric acid. After mixing and processing in the stomach, the bolus is emptied into the intestine where digestion continues and absorption occurs. There are large differences in morphology and physiology of the intestinal regions between vertebrate groups and between different species with differing feeding strategies within the same vertebrate group. Fish intestines vary in length from 0.4 to >38 times the body length. The percentage of plant material in the diet is a major factor determining intestinal length, with intestines of herbivorous fish generally being longer than those of carnivorous fish (Buddington et al., 1997; Clements and Raubenheimer, 2006). At the proximal end of the post-gastric intestinal region, many but not all fish species have numerous pyloric caeca which extend the intestinal surface area (Veillette et al., 2005; Clements and Raubenheimer, 2006). Functionally, the anterior region and the pyloric caeca are the primary sites for nutrient uptake (Nordrum et al., 2000). Following the pyloric caeca, the teleost intestines commonly have two regions, separated by a more or less distinct junction (Clements and Raubenheimer, 2006). The distal region has the lowest nutrient absorptive capacity and more endocytic activity (Ezeasor and Stokoe, 1981; Buddington and Diamond, 1987). Endocytosis of proteins in the distal intestine region has been suggested to have both nutritional and immunological importance (Clements and Raubenheimer, 2006).

Histomorphological organization

Despite the specialized regions of the GI tract, cross-sectional tissue organization remains fairly similar throughout the intestines of vertebrates. It consists of several histologically distinct tissue layers with correspondingly distinct functions. Lining the lumen is the epithelium, the barrier between the exterior and interior medium, which is attached to the connective tissue layer by the

basement membrane. Fish intestinal surface area is expanded through folding (mucosal folds) but lacks the typical crypts of the mammalian villi. The epithelium, together with the underlying lamina propria constitutes the mucosa. Adjacent to the lamina propria, a more or less distinct contractile muscularis mucosa separates the lamina propria from the submucosa. Within the submucosa layer, a submucosa nerve plexus is found. The stratum compactum, a layer of connective tissue, is often present below the submucosa and separates the submucosa from the circular muscle layer, followed by the myoenteric nerve plexus and the longitudinal muscle layer. The intestine perimeter is lined by the serosa, a connective tissue layer attached to mesenteric tissue.

The epithelial layer consists mainly of absorptive columnar cells, referred to as enterocytes, and mucus-secreting goblet cells. Adjacent enterocytes are joined together at the apical end of the lateral surface by junctional complexes. On the apical (luminal) surface of the enterocytes are found numerous extensions of the epithelial layer, called microvilli, and the whole apical surface of the epithelium is referred to as the brush border membrane (BBM). The microvilli greatly extend the surface area of the apical membrane, thus increasing the area for absorption and activity of membrane-bound digestive enzymes (Clements and Raubenheimer, 2006).

Digestion

Digestion consists of transforming food into molecules and elements suitable for transport across the intestinal wall, through processes of hydrolysis and solubilization of nutrients. Digestion comprises mechanical (grinding, tearing, mixing) and chemical processes (digestive enzyme breakdown). The digestive enzymes from the stomach – pepsin – and pancreas – numerous proteases, lipases and amylase – are of major importance for enzymatic hydrolysis of complex molecules – proteins, fats and carbohydrates – into smaller fragments which are further digested at the epithelium level by enzymes located in the BBM of the enterocytes.

In fish species with stomachs, protein digestion starts in the stomach, where the low pH from HCl secretion denatures most of the proteins as they are solubilized, opening their structure for easier attack by pepsin, a gastric proteolytic enzyme, which hydrolyses peptide bonds. The chyme then enters the intestine through the pyloric sphincter. Proteins and peptides entering the intestine, with or without prior processing in a stomach, are diluted and dissolved in alkaline secretions from the liver, pancreas and/or gut wall. In the intestinal lumen, protein digestion is carried out by alkaline proteolytic enzymes such as trypsin and chymotrypsin from the pancreatic tissue. The final steps of peptide hydrolysis take place at the brush border of the enterocytes by aminopeptidases or by intracellular peptidases following peptide transport across the membrane. However, some proteins

and peptides entering the intestine may resist proteolysis and reach the distal intestine more or less intact.

Lipids in fish food comprise mainly triglycerides, phospholipids, waxes, and free fatty acids (Leaver et al., 2008a). Marine fish have a pancreatic bile-acid-dependent carboxyl ester lipase capable of hydrolyzing a broad range of lipids including wax esters. Efficient lipid digestion requires emulsifiers, such as bile acids, that orient themselves on the surface of lipid droplets released during the physical, chemical and enzymatic degradation of the lipids. Bile acids are acidic steroids with powerful detergent properties, which are produced in the hepatocytes and secreted from the liver/gall bladder via the bile duct(s). They are formed from cholesterol and aid digestion by emulsifying dietary lipids and fat-soluble vitamins, allowing an efficient action of lipases and formation of micelles. If the emulsifying capacity is deficient, the digestion of released lipids may be hindered (Baeverfjord et al., 2006).

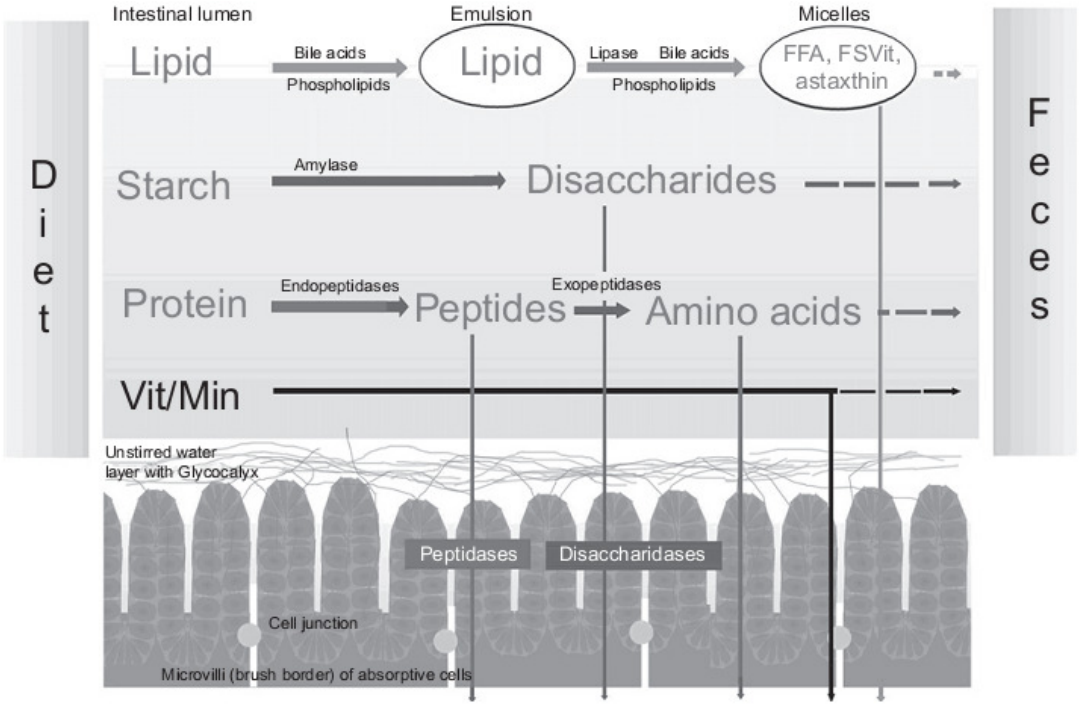

Figure 3 - Schematic drawing of the digestive processes along the digestive tract of fish. The location of various enzymes and other digestive components and the respective processes in the lumen as opposed to the intestinal mucosa are indicated. FFA: free fatty acids, FSVit: fat soluble vitamins. Design: F. Venold (Bakke et al., 2010a)

Fish have two categories of endogenous enzymes involved in carbohydrate digestion, pancreatic α-amylase and disaccharidases in the BBM of the enterocytes. α-amylase hydrolyzes α(1-4) glucoside-linkages in starch and similar molecules to shorter polymers, including maltose and branched oligosaccharides. Pancreatic α-amylase is present throughout the entire GI tract, including the distal intestine. The products of amylase and other carbohydrase activities are di- and oligo-saccharides. These low-molecular-weight carbohydrates are further hydrolyzed by saccharidases, such as maltase, located in the brush border membrane of enterocytes. The resulting monossaccharides are absorbed, metabolized or transported into the circulation.

Absorption

Nutrients released during digestion are transported across the BBM of the enterocytes lining the post-gastric alimentary tract, and if not metabolized in situ, exit across the basolateral membrane of the enterocytes and enter the circulatory system. Knowledge on the absorption mechanisms in fish gut is still scarce compared to that of mammals, but general mechanisms appear to be conserved in fishes. Nutrients can enter (or exit) absorptive cells following a concentration gradient by simple diffusion or via the paracellular route. Nutrients can also enter the cell by specialized protein transporters, which are more or less specific for different chemical classes of nutrients and carry the nutrient across the cell membrane independent of substrate concentration gradient. As for digestive enzymes, various nutrient transporters appear to have been conserved during evolution and have been found in fishes (Collie and Ferraris, 1995), such as the di- and tri-peptide transporter PepT1 (Verri et al., 2003).

Lipid absorption processes in fish occur mainly in the proximal region of the intestine and pyloric ceca (Diaz et al., 1997; Olsen et al., 1999; Rosjo et al., 2000; Denstadli et al., 2004; Hernandez-Blazquez et al., 2006). Lipid droplets accumulate in the supranuclear space of enterocytes (Sire et al., 1981; Fontagne et al., 1998; Olsen et al., 1999; Hernandez-Blazquez et al., 2006), are metabolized and packed into smaller lipoprotein particles (Caballero et al., 2003), which appear to exit the enterocytes by exocytosis (Hernandez-Blazquez et al., 2006). In most fish, lipids are assumed to be carried via the circulatory system to other organs. Lipid droplet accumulation in the enterocytes and number of lipoprotein particles in the intercellular spaces are affected by lipid sources. High levels of dietary neutral lipid appear to cause accumulation of large lipid droplets in the enterocytes of various species (Diaz et al., 1997; Fontagne et al., 1998; Olsen et al., 1999; Caballero et al., 2003). It has been suggested that this not only reduces intracellular metabolism, and consequently lipid transport across the basolateral membrane, but also limits further absorption of lipids across the brush border membrane, as has been demonstrated in mammals. This helps to

explain the lower lipid digestibility at high dietary lipid levels (Morais et al., 2005). In piscivorous species such as gilthead sea bream (*Sparus aurata*), intracellular lipid accumulation in fish fed plant oils increase dose-dependently compared to control fish fed fish oil as the sole lipid source (Caballero et al., 2003).

Nutrient absorption is influenced by a number of factors such as temperature, water salinity, intestinal region, differences due to natural dietary preferences, diet formulation, and diet availability (Ferraris and Ahearn, 1984; Collie, 1985; Buddington et al., 1987; Buddington and Hilton, 1987; Collie and Ferraris, 1995; Houpe et al., 1996; Lionetto et al., 1996; Buddington et al., 1997; Bakke-McKellep et al., 2000a; Nordrum et al., 2000; Berge et al., 2004; Jutfelt et al., 2007; Terova et al., 2009; Bakke et al., 2010b). The proximal region of the intestine contributes more to nutrient absorption than the more distal regions. However, in the intestine of many of the species studied so far, nearly the entire length of the post-gastric alimentary tract is capable of active nutrient transport (Ferraris and Ahearn, 1984; Collie, 1985; Bakke-McKellep et al., 2000a) and even the most distal region is equipped with a brush border membrane (Ezeasor and Stokoe, 1981; van den Ingh et al., 1991; Murray et al., 1996). The permeability of the distal region appears to be higher for small water-soluble molecules than more proximal regions (Collie, 1985; Schep et al., 1997; Bakke-McKellep et al., 2000a; Jutfelt et al., 2007). The absorptive cells of this region also have numerous supranuclear absorptive vacuoles, which indicate that the distal intestine of some fish is important in the uptake of intact macromolecules (McLean and Ash, 1987; McLean and Donaldson, 1990; Sire et al., 1992; Sire and Vernier, 1992). Absorption of large peptides and/or proteins has been demonstrated in distal intestinal regions of several fishes (McLean and Ash, 1987; McLean and Donaldson, 1990; Sire et al., 1992; Sire and Vernier, 1992; Concha et al., 2002). The relevance of protein absorption for fish is not entirely clear, but it was suggested to be a source of antigen presenting for mucosal immunological responses, as indicated by the successful use of some oral vaccines for fishes (Quentel et al., 2007).

Epithelial Barrier function

The intrinsic barrier function consists of the physical epithelial wall working with other mechanisms to prevent microorganisms from entering the host tissues. Starting from the luminal side, the epithelial monolayer with tight junctional complexes creates the primary physical barrier between the intestinal lumen and the lamina propria (Clayburgh et al., 2004). Beyond the direct physical barrier, mucus provides a physical and chemical barrier that act in a variety of ways to inhibit colonization or invasion by infectious agents, or contribute to innate and adaptive immune responses. Mucus is composed primarily of glycoproteins (Fletcher and Grant, 1968), but a number

of other substances have been identified, including cytokines (Lindenstrom et al., 2003), peptides (Cole et al., 1997; Fernandes et al., 2004b), lysozyme (Fernandes et al., 2004a), lipoprotein (Concha et al., 2003), complement (Dalmo et al., 1997), lectins (Itami et al., 1993; Tsutsui et al., 2003; Tsutsui et al., 2005), proteases (Aranishi and Mano, 2000), and antibodies (Cain et al., 2000; Hatten et al., 2001; LaFrentz et al., 2002).

The mucosal immune system (or gut-associated lymphoid tissue, GALT) comprises two functional groups, the innate and the adaptive immune system (Forchielli and Walker, 2005). The innate immune system creates the first line of defense with rapid response and clearance of pathogens (Collier-Hyams and Neish, 2005; Müller et al., 2005), whereas the adaptive immune system is slower but produces specific responses to the pathogen and maintains the specific response for a longer period of time (Cheroutre and Madakamutil, 2005). In fish, the innate immune system is supposed to have higher importance than in mammals, possibly because the specific defense response is slower in ectothermic vertebrates (Ellis, 2001; Magnadóttir, 2006).

The teleost mucosal immune system is more diffuse than that of mammals and consists of antigen processing macrophages in the epithelium and lamina propria. The distal intestine is considered to have more intraepithelial macrophages than the anterior region. These cells are supposed to function as antigen presenting cells (Buddington et al., 1997). Intestinal B-cells and T-cells have been found in most teleosts (Hart et al., 1988; Rombout and Joosten, 1998; Zapata et al., 2006; Bakke-McKellep et al., 2007) but, surprisingly, seem to be lacking in certain fish species (Wermenstam and Pilström, 2001). The higher phagocytotic activity of the distal region in comparison to the anterior region of fish intestines has been suggested to include an antigen sampling function by transferring antigen from the lumen to cells in the epithelium and the lamina propria (Hart et al., 1988; Rombout and Joosten, 1998).

Fish species

European sea bass (*Dicentrarchus labrax*)

Commonly known as European sea bass, *Dicentrarchus labrax* belongs to the class Osteichthyes, subclass Actinopterygii, order Perciformes and family Moronidae. It is an Atlantic coastal species normally found in the costal lines from Norway to Morocco, Canary Islands and Senegal, as well as in all the Mediterranean coasts and in the Black Sea. European sea bass was traditionally cultivated in coastal lagoons and tidal reservoirs before any reliable mass-production techniques were developed in the late 1960's, mainly in France and Italy. It is a fish very appreciated by consumers and it was the first marine non-salmonid species to be commercially produced in Europe.

Nowadays, it is exploited in most countries bordering the Mediterranean Sea with a total production of 74 768 tonnes in 2011 (FAO, 2013) with Greece, Turkey, Spain, Italy and France as the main producer countries. In Portugal, European sea bass is second to gilthead sea bream, with an estimated production of 461 tonnes in 2011 (FAO, 2013).

European sea bass is a eurythermic (2-32ºC) (Hidalgo and Alliot, 1988) and euryhaline species able to inhabit coastal inshore waters, occurring in estuaries, brackish water lagoons and occasionally in rivers. It is a dioecious (separate sex) species with only one breeding season per year, which takes place during the winter in the Mediterranean populations and up to June in the Atlantic populations. The eggs are small, pelagic and placed near to river mouths and estuaries or in littoral areas.

European sea bass is a carnivorous fish, whose juveniles feed on invertebrates, taking increasingly more fish with age until becoming piscivorous adults.

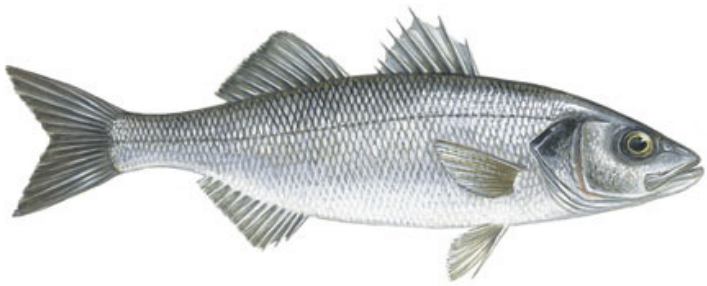

Figure 4 – European sea bass (*Dicentrarchus labrax*) (Fisheries-EU, 2014a)

Although sea bass is highly important to Mediterranean aquaculture, studies on its nutritional requirements are limited. Early studies with juveniles reported very high protein requirements ranging from 52 to 60% (Alliot et al., 1974; Metailler et al., 1981). Since then much fine-tuning has occurred, demonstrating that dietary protein levels can be decreased to values between 48-54% (Hidalgo and Alliot, 1988; Ballestrazzi et al., 1994; Peres and Oliva-Teles, 1999b). Other works reported that optimum growth of European sea bass can be achieved with even lower dietary protein values: 43-45% (Perez et al., 1997; Dias et al., 1998) given that digestible protein (DP) to digestible energy (DE) ratio is adequate.

Qualitative essential amino acids requirements (EAA) were shown to be the same as salmonids (arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophane and valine) (Metailler et al., 1973). Quantitative data on amino acid requirements based on dose-response studies is available for arginine, 3.9g/16g N (Tibaldi et al., 1994), lysine, 4.8g/16g N

(Tibaldi and Lanari, 1991), methionine, 2g/16g N (Thebault et al., 1985), tryptophane, 0.5g/16g N (Tibaldi et al., 1993) and threonine, 2.3-2.6g/16g N (Tibaldi and Tulli, 1999). Although there are no quantitative data for the other EAA, indirect estimations of requirements were made by the ideal protein method, based on the determined lysine requirements and whole body EAA profile (Kaushik, 1998). Thus, for histidine the estimated value was 1.6g/16g N, for isoleucine 2.6g/16g N, for leucine 4.3g/16g N, for valine 2.9g/16g N and for phenylalanine + tyrosine 2.6g/16g N.

Early studies reported better growth of European sea bass juveniles fed diets containing 12-24 % lipids as compared to 8% or 30% (Alliot et al., 1974) and no beneficial effects of lipid levels above 12% were reported (Metailler et al., 1981; Perez et al., 1997; Peres and Oliva-Teles, 1999a). Conversely, Dias et al. (1998) and Lanari et al. (1999) obtained better growth performances with increasing dietary lipids up to 18-19%, although much higher fat levels (30 percent of dry diet) appear to lead to a significant decrease in voluntary feed intake without affecting growth (Boujard et al., 2004).

Regarding essential fatty acids, the long chain polyunsaturated fatty acids of the ω-3 series (eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are essential for European seabass, but quantitative data on requirements is scarce. Early estimates suggest a requirement of about 1 percent of ω-3 PUFA (Coutteau et al., 1996). Recently, Skalli and Robin (2004) showed that the n-3 HUFA requirement for growth of juvenile European sea bass of 14g was 0.7% of a diet with a DHA:EPA ratio of 1.5:1 and 18% lipids.

High levels of starch (> 30 percent) appear to induce some growth and feed utilization depression. Digestibility of native starch is high (above 70%) but is further improved by processing starches (above 90%). At the post-absorptive level, a prolonged hyperglycemia has been reported in seabass (Peres et al., 1999; Enes et al., 2011).

Data on vitamin and mineral requirements of European sea bass are scarce. Kaushik et al. (1998) suggested that water-soluble vitamins allowances recommended for salmonids (N.R.C., 1993) can be applied in practical diets for European sea bass but not in purified diets. As to mineral requirements, phosphorous is the only one with an estimated requirement of around 0.65% (Oliva-Teles and Pimentel-Rodrigues, 2004).

Use of alternative plant ingredients in sea bass

Early studies to find alternatives to FM in European sea bass diets were undertaken by Alliot et al. (1979) and a significant amount of research has been performed since then, allowing the replacement of increasing amounts of FM and FO in this species diets. Almost total replacement of

FM protein (95%) by plant protein mixture in diets for sea bass (190g) was first achieved by Kaushik et al. (2004) with a mix of corn gluten, wheat gluten, soybean meal and rapeseed meal.

Sea bass (107g) fed diets containing 25 or 50% of either soybean meal, rapeseed meal, potato protein concentrate or a mixture of the three meals resulted in reduced energy, crude protein and crude fat digestibility as well as reduced growth and feed efficiency compared to the FM-based control diet (Lanari and D'Agaro, 2005). In the same study, inclusion of 25% SBM had no negative effect on sea bass performance. Tibaldi et al. (2006) found impaired growth performance and feed utilization, as well as reduced activity of the brush border enzymes γ-glutamyl transpeptidase and alkaline phosphatase in sea bass (188g) fed a diet where 50% of the FM protein was replaced by dehulled and toasted soy seeds subject to extrusion and mechanical oil extraction. Inclusion of pea protein concentrate and wheat gluten in sea bass (23 g) diets replacing up to 75% of FM resulted in increased feed intake with increasing inclusion of plant feedstuffs; however, no effects on growth or feed efficiency were observed (Tulli et al., 2007). Messina et al. (2013) showed that wheat gluten, soybean meal and extruded pea meal can replace up to 70% of the protein provided by FM without any effects on sea bass (24 g) growth, although decreased plasma cholesterol levels were observed in fish fed the diet with soybean meal and wheat gluten.

Sea bass (16g) fed a commercial diet with replacement of 0, 50 and 75% FO by soybean oil was successfully achieved without compromising growth, liver histology or plasma cholesterol levels (Figueiredo-Silva et al., 2005). Feeding sea bass (75 g) with diets where FO was partially replaced by either 60% rapeseed oil or 80% linseed oils resulted in lower growth, while replacement with 60% linseed or soybean oil did not affect growth performance (Montero et al., 2005). However, all test diets resulted in reduced LC-PUFA levels in fish flesh. Also in sea bass (5 g), dietary replacement of 60% FO by a blend of rapeseed, linseed and palm oils (10:35:15, w/w) resulted in reduced growth and reduced content of DHA and EPA in the flesh, together with increased number of absorptive vacuoles in the distal intestine enterocytes; the non-specific immune function, however, was reported not to be compromised (Mourente and Bell, 2006; Mourente et al., 2007). Bonaldo et al. (2008) showed that dietary incorporation of 30% soybean meal and 4.5% soybean oil had no effect on sea bass (18 g) growth, feed utilization, whole body composition and distal intestine histomorphology. Geay et al. (2011) tested in sea bass (180 g) the effect of a 100% plant diet, supplemented with amino acids and attractant, and observed reduced growth and DHA and EPA flesh content and decreased plasma lysozyme activity, as well as higher alternative complement activity.

European sea bass commercial diets contain plant feedstuffs, such as soybean meal, corn or wheat gluten, accounting for 30-40% of dietary protein, a number that is likely to increase as knowledge progresses (Tacon et al., 2011).

The increasing use of plant feedstuffs in diets can expose fish to cumulative effects of antinutritional factors, possibly affecting various physiological and metabolic processes in tissues and organs, and there may be late-onset or cumulative adverse effects resulting in a late manifestation of nutrient deficiencies, pathological conditions or less than optimal health in general (Krogdahl et al., 2010). Studies concerning the effects of antinutritional factors present in plant feedstuffs commonly used in aquafeeds is scarce and to our knowledge none have been performed in European sea bass.

Gilthead sea bream (*Sparus aurata*)

Commonly known as gilthead sea bream, *Sparus aurata*, belongs to the class Osteichthyes, subclass Actinopterygii, order Perciformes and family Sparidae. It is found in all the Mediterranean and along the eastern Atlantic from British Isles to Cape Verde and around the Canary Islands. It is rare in the Black Sea. Gilthead sea bream was cultivated extensively in coastal lagoons and saltwater ponds until the development of intensive rearing systems during the 1980's. The large-scale production started in Spain, Italy and Greece, countries that nowadays continue to be the main producers along with Turkey. Gilthead sea bream is highly adaptable to intensive rearing conditions, and total production in Europe was 97 999 tonnes in 2011 (FAO, 2013). In Portugal, gilthead sea bream is the main marine fish species produced in aquaculture, with an estimated production of 827 tonnes in 2011 (FAO, 2013).

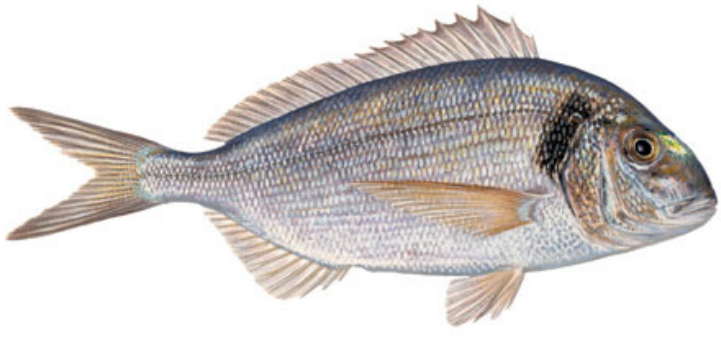

Figure 5 – Gilthead sea bream (*Sparus aurata*) (Fisheries-EU, 2014b)

Gilthead sea bream is a eurythermic (5-32ºC) and euryhaline species found in both marine and brackish waters. In the open sea, it inhabits rocky or sandy grounds as well as shallow areas up to depths of about 30 m. It is a protandrous hermaphrodite species that breeds in the open sea after late autumn, the juveniles returning to coastal waters in early spring. Mainly carnivorous, it feeds on shellfish (mussels and oysters), crustaceans, fish and, episodically, algae.

Regarding nutritional requirements of gilthead sea bream, initial studies using semi-purified diets pointed to a protein requirement of 40% for maximum growth of juveniles (Sabaut and Luquet, 1973). Later works using practical diets reported values of 45-46% protein requirement for juveniles (Santinha et al., 1996; Vergara et al., 1996b) and as high as 55% for fry (Vergara et al., 1996a) to achieve maximum growth.

Quantitative data on essential amino acids requirements based on dose-response studies is available for arginine, 2.6g/16g N (Luquet and Sabaut, 1974), lysine, 5g/16g N (Luquet and Sabaut, 1974; Marcouli et al., 2006), methionine + cysteine, 4g/16g N (Luquet and Sabaut, 1974) and thryptophan, 0.6g/16g N (Luquet and Sabaut, 1974). Indirect estimations of EAA requirements made by the ideal protein method, based on lysine requirements and whole-body EAA composition (Kaushik, 1998), estimated values of 1.7g/16g N for histidine, 2.6g/16g N for isoleucine, 4.5g/16g N for leucine, 3.0g/16g N for valine, 2.8g/16g N for threonine and 2.9g/16g N for phenylalanine + tyrosine. A more recent study based on the AA deletion method established the optimum dietary EAA requirements as follows: 5.55 g/16g N for arginine, 5.13 g/16g N for lysine, 2.98g/16g N for threonine, 1.89g/16g N for histidine, 2.55 g/16g N for isoleucine, 4.75 g/16g N for leucine, 2.60 g/16g N for methionine, 5.76 g/16g N for phenylalanine+tyrosine, 3.21 g/16g N for valine and 0.75 g/16g N for tryptophan (Peres and Oliva-Teles, 2009) .

Optimum dietary lipid levels have been estimated to be 15-16% (Vergara and Jauncey, 1993; Vergara et al., 1996b), while more recently Santinha et al. (1999) and Vergara et al. (1999) indicated as optimum dietary lipid levels 21 and 22%, respectively. An early study by Kalogeropoulos et al. (1992) estimated EFA requirements to be met with 0.9% LC-PUFA (ω-3) for 1 g gilthead sea bream fed a diet with 12% lipids (6% soybean oil+ 6% cod-liver oil). More recently it was reported that a dietary EPA + DHA level of 1 % was sufficient to meet EFA needs of gilthead sea bream (Benedito-Palos et al., 2009).

Available data suggest that diets for gilthead sea bream juveniles should include no more than 20% of digestible carbohydrates. At higher dietary inclusion levels, fish growth and feed utilization tend to be depressed. At the post-absorptive level, a prolonged hyperglycemia is also reported in sea

bream, with postprandial patterns comparable to those observed in European sea bass (Peres et al., 1999; Enes et al., 2011).

Estimations of quantitative requirements for some vitamins have been made and were reported as follows: between 63 and 83 mg/kg for niacin (Morris and Davies, 1995a), between 0.5 and 5 mg/kg feed for thiamine (vitamin B1) in juveniles (>60–200 g BW) (Morris and Davies, 1995b) and about 2 mg/kg of dry diet for pyridoxine (vitamin B6) (Kissil et al., 1981). Despite the lack of data on vitamin C requirements, in sea bream fed practical fishmeal-based diets lack of further supplementation with vitamin C did not affect growth performance (Henrique et al., 1998).

Regarding mineral requirements, available data is limited to phosphorus requirements, which were estimated to be 0.75 percent of the diet for juveniles (Pimentel Rodrigues and Oliva-Teles, 2001).

Use of alternative plant ingredients in sea bream

Several studies have been undertaken to evaluate the feasibility of FM and FO replacement in compound diets for gilthead sea bream. Pereira and Oliva-Teles (2004) showed that lupin seed meal can replace up to 30% of the FM protein in diets for juveniles (42 g) with no negative effects on growth performance. Corn gluten meal, without amino acid supplementation, was found to successfully replace up to 60% of FM protein in diets for juveniles (8 g) with no negative effects on fish performance, while 80% inclusion of corn gluten meal resulted in decreased growth and protein digestibility (Pereira and Oliva-Teles, 2003). Martinez-Llorens et al. (2007) concluded that dietary soybean meal might be included in the diets up to 30% for juveniles (15-80 g) and up to 50% for grow-out fish (80-350 g) without affecting animal's growth or feed efficiency. However, when sensory analysis and economic aspects were considered, the maximum inclusion level of soybean recommended in this study was only 20-22%. In 18 g juveniles, dietary inclusion of 30% soybean meal had no effect on fish performance, although histomorphological alterations were observed in the distal intestine (Bonaldo et al., 2008). Successful replacement of FM protein (100%) by a plant protein concentrate mixture (corn gluten, wheat gluten, soy protein concentrate) with amino acid supplementation in diets for gilthead sea bream (41 g) was first reported by Kissil and Lupatsch (2004). However, from an economic point of view, only 25% replacement of FM was economically practical due to the high cost of arginine supplementation. Sea bream fed diets with replacement of FM by a mixture of plant protein sources (corn gluten meal, wheat gluten, extruded peas, rapeseed meal) showed only a slight reduction in weight gain with diets including 50 or 75% plant ingredients, while in the 100% plant ingredient group weight gain was depressed, mainly as result of a marked reduction of feed intake; these fish also showed a lower fat gain along with a marked hypocholesterolemia (Gomez-Requeni et al., 2004). Partial or total replacement (50, 75, 100%) of

FM by a mixture of plant protein sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) with amino acid supplementation resulted in reduced growth and feed intake, increased liver fat deposition, increased number of lipidic vacuoles and/or deposition of protein droplets in the enterocytes and infiltration of eosinophilic granular cells in the intestinal submucosa of sea bream (16 g) fed the diet with 100% plant protein (Sitja-Bobadilla et al., 2005). In this study, plasma cholesterol levels decreased in a dose dependent manner with increasing plant protein inclusion and immunosuppression due to reduced complement activity was reported at inclusion levels above 75% (Sitja-Bobadilla et al., 2005). In a recent study by Kokou et al. (2012), the replacement of FM by bioprocessed soybean meal (20, 40 and 60%) resulted in reduced growth, reduced feed utilization and histomorphological alterations in the distal intestine of sea bream juveniles (16 g) fed the diet with 60% soybean meal, while all diets negatively affected some indicators of immune response.

Replacement of 60 % FO by soybean oil did not affect sea bream (10 g) growth; however, increased lipid accumulation in the hepatocytes was observed (Caballero et al., 2004). Replacement of 60% FO by either rapeseed, linseed or soybean oil also had no effect on growth or feed efficiency, while 80% FO replacement by plant oil resulted in significant reduced growth (Izquierdo et al., 2005). Wassef et al. (2007) reported no effects on growth when 60% FO was substituted by blends of plant oils (sunflower, cottonseed, linseed, soybean oil), although accumulation of lipid droplets in the hepatocytes was reported. Similarly, 60 and 80 % inclusion of linseed, rapeseed or soybean oils in diets for juveniles (79 g) resulted in increased accumulation of lipid droplets in the enterocytes (Montero et al., 2003).

Benedito-Palos (2007; 2008) studied the effect of replacing 0, 33, 66 and 100% FO by a blend of plant oils (rapeseed, linseed and palm oils) in low FM (20%) diets and reported decreased growth and feed intake in sea bream (16 g) fed the 100% vegetable oil diet over 11 weeks or 8 months, while up to 66% plant oil inclusion in the diets had no effect on fish performance. After 11 weeks of feeding on the experimental diets mentioned above, Santigosa et al. (2011) reported impaired digestive processes and accumulation of lipid droplets in the proximal intestine enterocytes of fish fed the 100% plant oil diet. On the other hand, after 8 months exposure to the test diets, no effects on the intestinal epithelium were reported (Benedito-Palos et al., 2008).

The concomitant replacement of FM and FO in a practical diet for gilthead sea bream during the grow-out phase (180 g) with a mixture of plant proteins (soybean, peas, corn and wheat; 40 and 60% inclusion levels) and oils (soybean, rapeseed; 65% inclusion level), resulted in a slight decrease in weight gain and a significant decrease in feed efficiency only when replacing 60% FM

and 65% FO simultaneously (Dias et al., 2009). Recently, a study by Watson et al. (2013) reported the successful use of a 100% plant-based diet for sea bream juveniles (11 g) without affecting animal growth performance or whole body composition.

Available data indicate that several alternative feedstuffs can replace significant amounts of FM and FO in sea bream diets, without negative effects on fish performance. Nonetheless, it must also be assured that animal health and welfare is maintained and that the resultant fish products are safe, nutritionally adequate and accepted by consumers.

Objectives

The overall objective of this thesis was to gain further knowledge on the effect of two antinutrients: saponins and phytosterols, present in plant feedstuffs commonly used in compound aquafeeds for two marine fish species of interest for Mediterranean aquaculture: the European sea bass and gilthead sea bream.

Specific objectives of this thesis was to have a comparative perspective of the effect of the two antinutrients, singly and in combination, in two life stages of fish, juveniles and on-growing, as developmental stage reportedly affects the tolerance threshold of the animals. The two species were chosen to better assess the potential effects of the antinutrients in fish of different feeding habits: a more carnivorous species (European sea bass) and a more omnivorous species (gilthead sea bream).

To avoid confounding effects of the target antinutrients due to the interaction with other antinutrients present in feedstuffs, purified antinutrients were used, and the diets were fishmeal-based. The antinutrients were tested at two inclusion levels, which were within the normal range of inclusion in practical diets, and potential interactions between them were also assessed. It was also aimed at establishing acceptable saponin and phytosterol limits in diets for sea bream and sea bass, which could be translated to inclusion limits of plant feedstuffs containing these antinutrients in practical diets for the two species.

The following aspects were given particular attention:

- In order to assess how the presence of antinutrients in the diets may affect fish performance, growth trials were performed and data on growth and feed utilization were collected.

- To understand the effects of the antinutrients on the intestinal structure and function, data on intestinal morphology, activity of brush border enzymes and gene expression of functional markers were obtained.

- Due to the intrinsic nature of the nutrition-health relationship, gene expression profiling of immune related genes was traced in the intestinal tissue.

Chapter 2

Saponins and phytosterols in diets for European sea bass (*Dicentrarchus labrax*) juveniles: effects on growth, intestinal morphology and physiology.

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Abstract

The present work aimed to elucidate the effects of two soy antinutrients, saponins and phytosterols, on growth, intestinal health and function of European sea bass juveniles. Seven fishmeal based-diets were formulated: a control diet without antinutrients and six experimental diets containing low or high levels of soyasaponins (SapL and SapH, respectively), low or high levels of phytosterols (PhytL and PhytH, respectively) and low or high levels of soyasaponins + phytosterols (SapPhytL and SapPhytH, respectively). A feeding trial was conducted for 59 days after which blood was collected for plasmatic cholesterol quantification and intestinal samples were collected for histology, digestive enzymes activity and gene expression analysis. Histology was also performed at day 15. PhytL resulted in high distal intestine (DI) relative weight and decreased plasma cholesterol while PhytH caused inflammatory changes in the DI. SapH depressed maltase and alkaline phosphatase activity in DI and the histological evaluation indicated some inflammatory changes. The SapPhytH resulted in decreased maltase activity in the DI and indications of inflammatory changes that were supported by the results on gene expression profiles. The antinutrients tested did not compromise growth, however caused some gastrointestinal disturbance that may affect fish in the long term.

Keywords: antinutrients; sea bass; saponins; phytosterols; growth; immune function; digestive enzymes; intestinal histology.

Introduction

Aquaculture is growing faster than any other animal food-production sector (FAO 2012) and this growth has been accompanied by a rapid increase of aquafeed production. For carnivorous species, aquafeeds have been largely based on fish meal and fish oil as the major protein and lipid sources, but as competition for these commodities increases, replacement by alternative sources is required for sustainable growth of the aquaculture industry.

Plant feedstuffs are currently the most abundant alternative to fish meal and fish oil due to worldwide availability and lower prices. Nonetheless, it is necessary to ensure that diets containing high plant feedstuff levels promote efficient growth and feed utilization, have minimal environmental impact, are safe for fish health, produce high quality flesh for human consumption, and are cost-effective (Gatlin *et al.* 2007).

A potential drawback of most plant-derived feedstuffs is the presence of a wide variety of antinutritional substances. Antinutrients are defined as substances which by themselves or through their metabolic products interfere with feed intake, nutrient digestibility, growth, physiology and/or health (Gatlin *et al.* 2007). Plant feedstuffs generally contain more than one antinutrient. Therefore it is difficult to ascertain which individual factor is responsible for any adverse effects observed when using a specific plant ingredient in aquafeeds (Francis *et al.* 2001). Indeed, several studies have been done on the effects of plant feedstuff inclusion in fish diets, but only few studies have been carried out to evaluate the effects of purified antinutrients (Hossain & Jauncey 1990; Pelissero *et al.* 1991; Krogdahl *et al.* 1994; Bureau *et al.* 1998; Becker & Makkar 1998, 1999).

Soy is one of the most commonly used ingredients for fish meal and fish oil replacement in aquafeeds, although its use in salmonids is limited due to the development of soy-induced enteritis (van den Ingh *et al.* 1991; Baeverfjord & Krogdahl 1996; Bakke-McKellep *et al.* 2007b). Although the specific agents causing this condition are not yet fully identified, it is believed that one or more of the alcohol-soluble components of full fat soybean meal such as saponins are likely involved, as alcohol extracted soy protein concentrate does not cause pathological changes in the intestine of salmonids (van den Ingh *et al.* 1996; Krogdahl *et al.* 2000). Soybean meal induced enteritis seems to involve T cell mobilization and has therefore been suggested to be a hypersensitivity reaction (Bakke-McKellep *et al.* 2007a; Marjara *et al.* 2012).

Saponins are heat-stable glycosides present in soybean meal and other plant feedstuffs and are characterized by their amphipathic nature and binding ability to membranes, cholesterol and bile salts (Francis *et al.* 2002). A large number of the biological effects of saponins have been ascribed to their action on membranes as they can increase intestinal mucosal cell permeability, inhibit active

mucosal transport, and facilitate the uptake of substances that are normally not absorbed. Effects on protein digestion, cholesterol metabolism and function of the immune and nervous systems have also been reported (Francis *et al.* 2002). In fish, decreased growth performance of rainbow trout, tilapia, Atlantic salmon and Chinook salmon have been attributed to the presence of saponins in the diets (Francis *et al.* 2001; Chikwati *et al.* 2012). The evidence for involvement of saponins in the soybean meal-induced enteritis of Atlantic salmon has been shown by several authors (Knudsen *et al.* 2007; Knudsen *et al.* 2008; Chikwati *et al.* 2012). Fish fed diets with subfractions of soy molasses containing saponins (Knudsen *et al.* 2007), a diet containing lupin kernel meal and partially purified soyasaponins (Knudsen *et al.* 2008), or a pea protein concentrate and purified saponins (Chikwati *et al.* 2012) resulted in similar lesions as those induced by soybean meal.

Phytosterols, also called plant sterols, are a group of steroid alcohols naturally occurring in plants. Data on the effects of these compounds when incorporated in fish diets is scarce (Pelissero & Sumpter 1992; Chikwati 2007). In mammals, phytosterols are known to lower plasma cholesterol by inhibition of cholesterol uptake by the enterocytes, thus increasing fecal cholesterol and bile acid loss. As a consequence, hepatic conversion of cholesterol to bile acids increases, which further lowers plasma cholesterol levels (Ling & Jones 1995; Ostlund 2002).

The *in vivo* effect of each antinutrient on the intestine is quite difficult to predict given the many potential ligands they are exposed to in the intestinal tract, such as bile acids, dietary cholesterol and enterocyte membrane sterols, nutrients and other antinutrients (Francis *et al.* 2002). All these potential interactions should be taken into consideration as they may reduce or enhance the effect of the antinutritional factors. Therefore, in this work we intended to evaluate the effect of two purified antinutrients, soy saponins and phytosterols provided in the diets either singly or in combination, in European sea bass (*Dicentrarchus labrax*). The rationale for choosing these antinutrients were the ability of both to interfere with cholesterol uptake and metabolism (Sidhu & Oakenfull 1986; Gee & Johnson 1988; Lasztity *et al.* 1998; Romarheim *et al.* 2008) and indications in the literature that soybean saponins play a key role in development of soybean induced enteritis in salmonids (Knudsen *et al.* 2008; Chikwati *et al.* 2012). A comprehensive approach assessing fish performance, intestinal morphology and physiology as well as health status was used.

Material and methods

Diets

Seven fish meal and fish oil based diets were formulated to contain 480 g kg^{-1} crude protein and 180 g kg^{-1} crude lipid (Table 1). The experimental diets included a control diet without supplementation,

and diets containing 1 g kg^{-1}or 2 g kg^{-1} soyasaponins (Organic Technologies, Coshocton, OH, USA, purity 95%) (diets SapL and SapH, respectively), 5 g kg^{-1} or 10 g kg^{-1} phytosterols (purity >99%; Derive Resiniques et Terpeniques, Dax, France) (diets PhytL and PhytH, respectively), or a mixture of 1 g kg^{-1} saponins + 5 g kg^{-1} phytosterols or 2 g kg^{-1} saponins + 10 g kg^{-1} phytosterols (diets SapPhytL and SapPhytH, respectively).

Table 1 – Composition and proximate analysis of the experimental diets.

	Experimental diets						
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**
Ingredients (g kg^{-1} dry weight)							
Fish meal[1]	590	590	590	590	590	590	590
Soluble fish protein concentrate[2]	50	50	50	50	50	50	50
Cod liver oil	111	111	111	111	111	111	111
Pre-gelatinized starch[3]	214	213	212	209	204	208	202
Vitamin premix[4]	10	10	10	10	10	10	10
Mineral premix[5]	10	10	10	10	10	10	10
Choline chloride (50%)	5	5	5	5	5	5	5
Binder[6]	10	10	10	10	10	10	10
Soy saponins[7]	-	1	2	-	-	1	2
Phytosterols[8]	-	-	-	5	10	5	10
Proximate analysis (g kg^{-1} dry matter)							
Dry matter	944	954	956	945	939	954	954
Crude protein	493	494	493	491	498	495	494
Crude fat	173	179	180	192	187	181	176
Starch	186	203	197	200	198	184	200
Ash	123	122	123	123	122	119	124
Gross energy (KJ g^{-1} DM)	22.4	22.5	21.5	21.8	22.2	22.1	22.0

[1] Vacuum Dried LT. Pesquera Diamante, S. A. Peru
[2] G-Special. Soropêche, France
[3] C-Gel Instant - 12016, Cerestar, Mechelen, Belgium
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU k^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet); dibasic calcium phosphate, 5.9 (g kg^{-1} diet).
[6] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England
[7] Soy saponins concentrate (95% purity) produced by Organic Technologies (Ohio, USA)
[8] Phytosterol extract produced from pine by les Derive Resiniques et Terpeniques (DRT) (France), Purity: >99% pure; including β-sitosterol: 77.3%, β-sitostanol 10.7%, campesterol: 8.8%, other: 2.2%.

All dietary ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). The levels of saponin used in the present study corresponds to levels found in diets with about 200 and 400 g kg^{-1} soybean meal (Anderson & Wolf 1995), which 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 meal or 250 and 500 g Kg^{-1} inclusion level of rapeseed meal in diets (Sarkar *et al.* 1996; Vlahakis & Hazebroek 2000), which based on results of preliminary studies with Atlantic salmon caused weak but distinct effects when supplemented alone (Chikwati 2007). The phytosterol preparation was commercially available, made from pine and produced to serve as functional additive in margarine for human consumption, purportedly for its cholesterol-lowering effects. 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.

Growth trial

The growth trial was performed at the Marine Zoological Station, Faculty of Sciences, Porto University. The trial lasted 59 days and was conducted in 21 fiberglass tanks of 100 L water capacity each connected to a thermo-regulated recirculating water system, supplying a continuous flow of filtered seawater. During the experiment the water temperature was maintained at 25±0.5ºC and salinity averaged 35±1 g L^{-1} Juvenile European sea bass (*Dicentrarchus labrax*) were obtained from a commercial hatchery and after arrival at the experimental facilities were kept in quarantine for two weeks. After acclimation, groups of 25 fish with an initial mean body weight of 27 g (±0.01 g) were randomly distributed to each tank. Diets were randomly assigned to triplicate tanks of fish. Fish were fed by hand to apparent visual satiety twice daily, six days per week. Fish were bulk weighed every two weeks after one day of food deprivation.

Sampling

After 15 and 59 days of feeding, two fish from each tank were sampled for histological evaluation. The fish were randomly selected and euthanized by anesthetic overdose (ethylene glycol monophenyl ether, ref.:8.07291, Merck, Whitehouse Station, USA) in ice water, and dissected on chilled trays. The digestive tract was freed from the adjacent adipose tissue and cut open longitudinally. Only fish with digestive contents throughout the intestinal tract were sampled. Two pyloric caeca and tissue pieces (ca. 5 x 5 mm) from the mid and distal intestine were rinsed in

phosphate buffered saline (PBS), blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%; pH 7.4) for 24h, and then transferred to alcohol (70% EtOH) until further processing.

At the end of the feeding trial, blood from three fish per tank (different from those sampled for histology) was collected from the caudal vein using heparinized syringes, centrifuged at 1500 x g for 10 minutes and the plasma stored at -20ºC until analysis. After blood collection, fish were euthanized as described above and the whole distal intestine collected, rinsed in PBS, blotted dry, placed in RNAlater (25 mM Sodium Citrate; 10 mM EDTA; 70 g ammonium sulfate/100 ml solution; pH 5.2; 1:10 w/v), left at 4ºC overnight, and then stored at -20ºC until analysis.

Six additional fish per tank were euthanized as described above for digestive enzyme analysis. The digestive tract was cut open after removal of the surrounding connective and adipose tissues, and the intestinal content was carefully discarded. The digestive tract was then divided into four sections: proximal intestine including pyloric caeca (PI), mid intestine 1 (the two thirds following the proximal intestine; MI1), mid intestine 2 (the last third proximal to the distal intestine; MI2) and distal intestine (DI). Each section was gently rinsed in PBS, blotted dry and immediately frozen in liquid nitrogen and stored at -80ºC until analysis. Prior to processing, samples were weighed to calculate the relative weight of the intestinal regions PI, MI and DI.

Chemical analysis

Proximate analysis of diets was performed as follows: dry matter after drying at 105ºC until constant weight; ash by incineration in a muffle furnace at 450ºC for 16 h; crude protein content (N x 6.25) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation units (model 1015 and 1026, respectively; Tecator Systems); lipid by petroleum ether extraction using a Soxtec HT System (extraction unit model 1043 and service unit model 1046; Tecator Systems; Hoganäs, Sweden); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 1261; PARR Instruments; Moline, IL, USA). Plasma cholesterol was determined using a kit (ref. 1001092; Spinreact, Girona, Spain).

Histology

Histology samples were processed and sectioned using standard histological techniques. Sections were stained with hematoxylin and eosin. Blind evaluation was performed with particular attention to inflammatory changes previously described in other species (Baeverfjord & Krogdahl 1996;

Krogdahl *et al.* 2003), namely: shortening, widening and fusion of intestinal folds, changes in enterocyte supranuclear absorptive vacuolization, connective tissue hyperplasia, and infiltration of inflammatory cells. A continuous scale scoring system was used as described by Penn *et al.* (2011) with the range of tissue scores set at 0-5. The overall value of the degree of enteritis was calculated by averaging the scores of the separate parameters.

Enzymatic activity

Activities of the brush border enzymes alkaline phosphatase (Alp) and maltase (Malt), chosen as indicators of the functional status of the gut wall (Krogdahl *et al.* 2003), were quantified in tissue homogenates of PI, MI1, MI2 and DI. Tissues were thawed and homogenized (1:20) in ice-cold 2mM Tris/50mM mannitol (pH7.1), containing phenyl-methyl-sulphonylfluoride (Sigma, P-7626; St. Louis, MO, USA) as serine protease inhibitor. Aliquots of homogenates were frozen in liquid nitrogen and stored at -80° C prior to analysis. The Alp and Malt activities were determined as previously described by Krogdahl *et al.* (2003). Briefly, Alp activity was measured colorimetrically with a kit (Sigma procedure no.104), using p-nitrophenyl phosphate as substrate. Malt activity was measured using maltose as substrate, followed by TRIS-glucose oxidase peroxidase (TGO) reagent addition for glucose detection. Samples were incubated at 37° C and absorbance read at 420nm. Enzyme activities were expressed as µmol substrate hydrolyzed $(h)^{-1}$ $(g \text{ of tissue})^{-1}$ for Alp and µmol substrate hydrolyzed $(min)^{-1}$ $(g \text{ of tissue})^{-1}$ for Malt.

Gene expression profiling

Quantitative real-time PCR (qPCR) was used to analyze the mRNA expression of the digestive enzymes alkaline phosphatase (Alp) and maltase (Malt). In addition, we analyzed expression profiles of several previously reported markers for intestinal inflammation including cluster of differentiation 4 (CD4; specific for putative T helper cells), interleukin 1β (IL-1β) and transforming growth factor β (TGF-β) (Marjara *et al.* 2012), caspase 3 (Casp3; Bakke-McKellep *et al.* 2007b), immunoglobulin M (IgM; Krogdahl *et al.* 2000) and tumor necrosis factor α (TNFα; Kortner *et al.* 2012). qPCR primers (Table 2) were designed from conserved regions of the studied genes using Primer3 software (http://frodo.wi.mit.edu/primer3). Prior to qPCR reactions, all primer pairs were used in gradient reactions in order to determine their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon of interest in all reactions. To verify correct amplification, PCR products were excised, purified and sequenced by Eurofins MWG Operon (Ebersberg, Germany). All

sequences were confirmed by alignments and by using NCBI nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov).

Table 2 - Primer pair sequences, efficiency, amplicon size and annealing temperature for genes used for real-time PCR. Grey shaded rows correspond to reference genes.

	5´- 3´ primer sequence					
Gene symbol	Forward	Reverse	**Amplicon size (BP)**	**Annealing temp. (°C)**	**Primer efficiency**	**Genbank accession nr.**
Alp	TTACCTCTGTGGGGTCAAGG	TAGCCCATTTGAGGATGGAG	116	60	1.96	[FJ860000]
Malt	ATGCCATGGAGGTGACTTTC	CAACCATTTCAGGCGTAGGT	105	60	2.00	[AM419039]
Casp3	TGATGTCGTCTCTGCCGTAG	ACCACCTCATACGCATCCTC	76	60	1.97	[DQ345773]
CD4	ACTTGTGATTTGGGCAGTCC	TGATGAGATGAGTGGGGTGA	92	60	1.89	[AM849812]
TGF-β	CATCTGGAACGCTGAAAACA	TGTTGCCTGCCCACATAGTA	138	58	1.96	[AM421619]
IL-1β	GTGGTGGACAAAGCCAGTCT	TCTCCTCTGCTGTGCTGATG	147	60	2.00	[AJ269472]
TNF-α	GAAAACGCCTCACACCTCTC	GTCCGCTTCTGTAGCTGTCC	132	62	1.90	[DQ070246]
IgM	AGAGGAGCTGCAGGAGGACA	GGACCTCCAGGCTGTGTGAC	132	60	2.00	[AJ400233]
ACTB	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	133	60	1.92	[AJ537421]
GAPDH	CAAGGTTGAGGGTGACAAGC	CAGCCTCACCCCATTTGATG	92	60	1.99	[AY863148]
EF1A	ACGTGTCCGTCAAGGAAATC	GGGTGGTTCAGGATGATGAC	109	58	1.94	[FM019753]
Tubb	AGCTGGTGGACTCTGTCCTG	ACCAGAGCCTGTACCACCAC	107	58	1.95	[FM003484]

Total RNA was purified from DI tissue samples (~50 mg) homogenized in 750µl of Trizol reagent (Life Technologies, Carlsbad, California, USA). RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).). RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Fisher Scientific, Hampton, New Hampshire, USA). Prior to reverse transcription, total RNA from all samples were subjected to DNase treatment using a TURBO DNA-free kit (Ambion, Life technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. First strand cDNA synthesis was performed in 20 µL reactions with 1.0 µg total RNA from all samples using Superscript III (Invitrogen, Life technologies, Carlsbad, CA, USA), and primed with Oligo$(dT)_{20}$ primers. Negative controls were performed in parallel by omitting RNA or enzyme. The obtained cDNA was diluted 1:10 and stored at -20 °C.The expressions of individual gene targets (n=9 fish/diet group) were analyzed using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland).

Each 10 µL DNA amplification reaction contained 2 µl of PCR-grade water, 5 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics), 2 µL of 1:10 diluted cDNA template and 0.5 µl (10 µM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control and an inter-run plate calibrator. The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (10 s), 58-62°C (depending on the primers used, see Table 2, 10 s) and 72°C (15 s). For determination of the quantification cycle (C_q) the "second derivative maximum method" measuring maximum increase rate of newly synthesized DNA per cycle was used on the basis of the LightCycler 480 software release 1.5.0 (Roche Diagnostics). To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and manual inspection of PCR products after each run by agarose gel electrophoresis. PCR reaction efficiency (E) for each gene assay was determined using 10-fold serial dilutions of randomly pooled cDNA. R^2 values of the standard curves were all >0.99. Beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1A (EF1A) and beta-tubulin (Tubb) were evaluated for use as reference genes by ranking relative gene expression according to their stability as described previously (Kortner *et al.* 2011). ACTB was found to be stably expressed and was therefore used as normalization factor. Mean normalized expression of the target genes were calculated from raw C_q values using a plate calibrator-normalized relative quantification (Pfaffl 2001) and expressed as fold changes of control group.

Statistics

Results were analyzed using IBM SPSS statistics 20 software package (SPSS® IBM, Chicago, IL, USA). Due to the non-orthogonal experimental design applied, normally distributed data were analyzed by one-way ANOVA, and Tukey's multiple range test was used for comparison of differences between means. For data which were not normally distributed even after transformation, (intestinal sections relative weight) the Kruskal-Wallis non-parametric analysis of variance was used followed by Mann-Whitney U test for pairwise comparison of the means. Differences were considered statistically significant at $P < 0.05$; when $0.5 < P < 0.1$ trends were discussed.

Results

Growth performance, feed intake, feed efficiency and protein efficiency ratio were not significantly affected by dietary antinutrient supplementation (Table 3).

Table 3 – Mean values (n=3) for growth parameters, feed intake, and feed utilization of fish fed the experimental diets for 59 days and results of the one-way ANOVA (*P*-values).

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Final body weight (g)	73	72	70	70	70	68	72	*2.02*	*0.727*
Weight gain	48	45	43	45	43	43	47	*1.94*	*0.782*
DGI[2]	2.0	1.9	1.9	1.9	1.9	1.8	2.0	*0.07*	*0.700*
Feed intake (g DM per fish)	52	49	49	46	48	48	49	*1.38*	*0.512*
FE[3]	0.90	0.91	0.86	0.93	0.89	0.88	0.92	*0.02*	*0.295*
PER[4]	1.83	1.85	1.75	1.89	1.79	1.78	1.87	*0.04*	*0.232*

[1] Standard Error of the Mean (pooled)
[2] Daily growth increment: $((IBW^{1/3} – FBW^{1/3}) / days) \times 100$
[3] Feed efficiency: weight gain: (g fresh weight) / feed intake (g dry matter)
[4] Protein efficiency ratio: weight gain (g fresh weight) / protein intake (g dry matter)

Regarding relative weight of intestinal sections (Table 4) the antinutrients did not affect the two most proximal sections, i.e. the pyloric (PI) and the mid (MI) intestine. The distal intestine (DI), however, was affected, with fish fed the diets with low level of phytosterols, i.e. PhytL and SapPhytL, showing higher relative weight of DI than fish fed the control diet. Fish fed the SapH diet also showed increased DI relative weight compared to the control fish.

Table 4 – Mean plasma cholesterol levels (n=3) and relative weights of different intestinal regions of fish fed the experimental diets for 59 days and results of the one-way ANOVA (*P*-values).

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Blood Plasma									
Cholesterol[2] (mmol l^{-1})	8.6^{a}	7.5^{ab}	7.7^{ab}	6.6^{b}	7.5^{ab}	8.3^{ab}	7.5^{ab}	*0.13*	*0.450*
Organ relative weights									
PI (%)	0.72	0.72	0.77	0.71	0.70	0.70	0.67	*0.04*	*0.939*
MI (%)	0.70	0.73	0.69	0.74	0.77	0.69	0.70	*0.03*	*0.530*
DI (%)[2]	0.17^{a}	0.16^{a}	0.32^{b}	0.53^{b}	0.17^{a}	0.37^{b}	0.22^{a}	*0.04*	*0.000*

[1] Standard Error of the Mean (pooled)
PI: Pyloric intestine; MI: mid intestine; DI: distal intestine.
[2] Differing superscript letters in the same line indicate significant differences between the dietary treatments ($P < 0.05$) as assessed by one-way ANOVA and Tukey's multiple range test.

Plasma cholesterol was significantly lower in fish fed the PhytL diet than fish fed the control diet (Table 4). It also tended to be lower in fish fed the other antinutrient-containing diets.

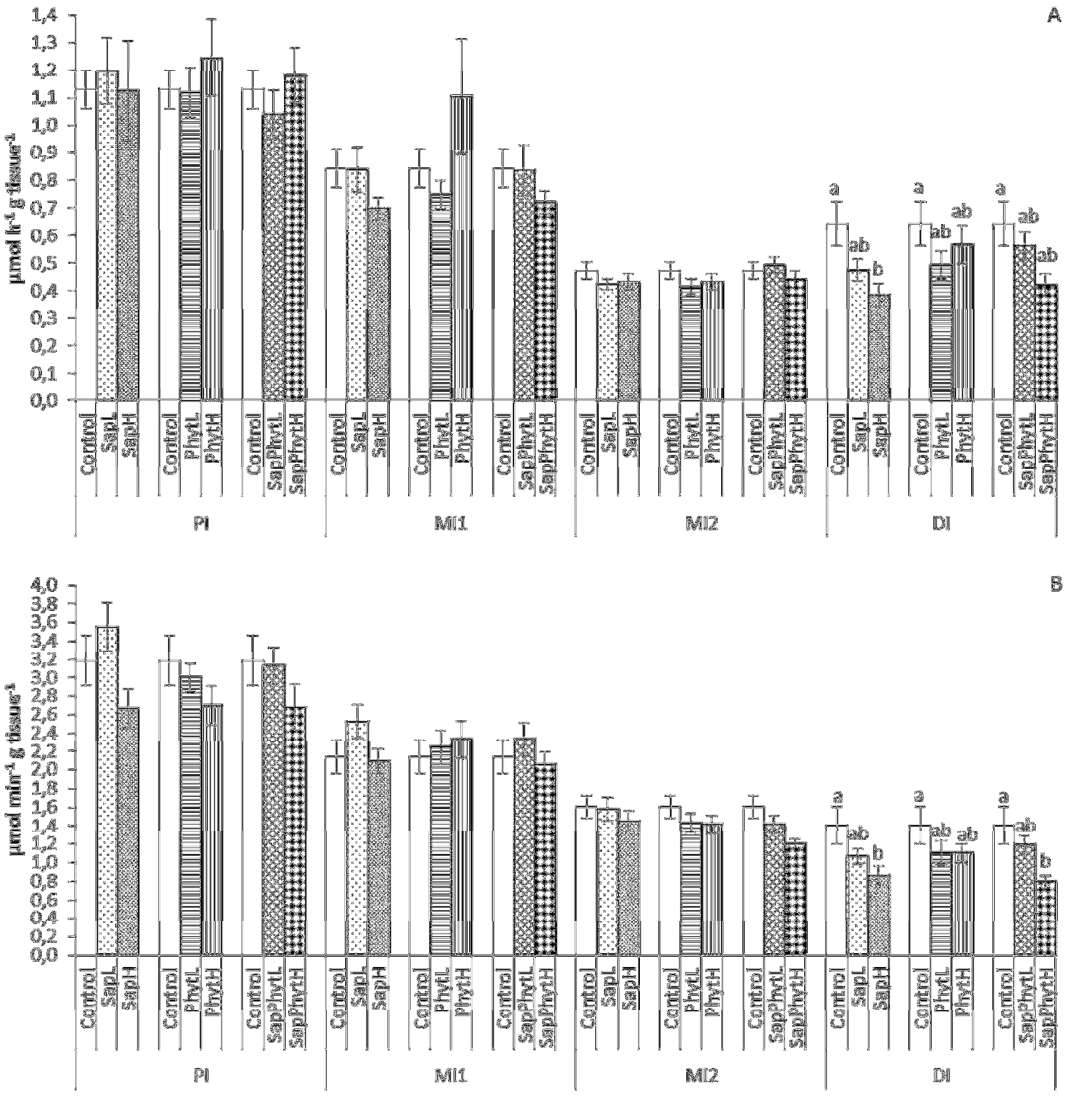

Figure 1 – Mean (n=3) alkaline phosphatase (A; in µU h^{-1} g of $tissue^{-1}$) and maltase (B; in µU min^{-1} g of $tissue^{-1}$) activities ± standard error of the mean (SEM) in four sections of the intestinal tract of sea bass fed the experimental diets for 59 days. The control group bar (blank) is the same within each intestinal section, and is repeated for comparison purposes only. Different letters within the same intestinal section and between treatment groups indicate significant differences ($P < 0.05$). P values obtained were as follows: A) 0.932, 0.099, 0.539 and 0.023, for PI, MI1, MI2 and DI, respectively; B) 0.058, 0.477, 0.161 and 0.011 for PI, MI1, MI2 and DI, respectively. PI: Pyloric intestine; MI: mid intestine; DI: distal intestine.

Saponins, phytosterols and their combination did not appear to affect Alp activity in the PI, MI1 or MI2 (Fig 1A). In the DI, however, SapH resulted in lower Alp activity when compared to the control diet. The same trend was seen for SapPhytH. Similarly, fish fed SapH and SapPhytH showed depressed Malt activity in the DI compared to fish fed the control diet (Fig 1B).

Table 5 – Average score of the different parameters used to assess the degree of enteritis developed by sea bass fed the experimental diets for 15 and 59 days and results of the one-way ANOVA (*P*-values).

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
15 days	1.5[a]	2.2[ab]	2.7[ab]	1.7[ab]	2.9[b]	2.4[ab]	2.6[ab]	*0.3*	*0.015*
59 days	1.4	2.8	2.2	2.2	2.9	2.7	2.8	*0.4*	*0.416*

[1] Standard error of the mean (pooled)
Differing superscript letters in the same line indicate significant differences between the dietary treatments ($P < 0.05$) as assessed by one-way ANOVA and Tukey's multiple range test.

Based on the histological observations (Table 5), diet PhytH caused morphological changes in the DI, but the results were significant only for the samples taken after 15 days of feeding. At the end of the feeding trial the pattern and magnitude of the responses were similar to those observed at 15 days, but due to higher individual variation at day 59, the differences between the groups fed antinutrients and the control group were not significant.

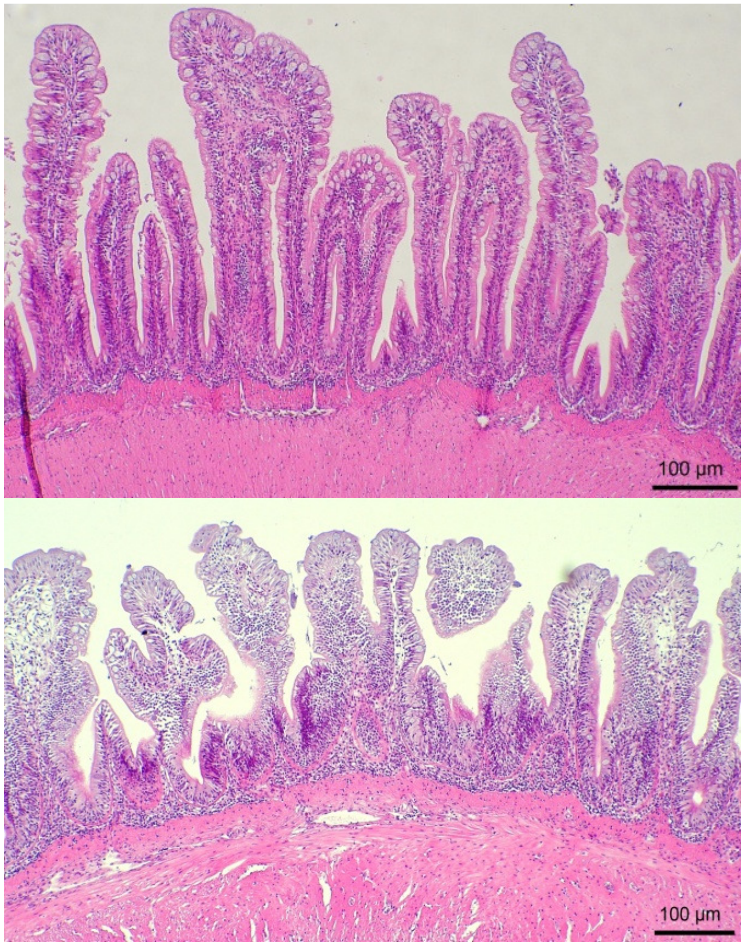

Figure 2 – Histomorphological appearance of the distal intestine of Sea bass fed the control diet (A) and the diet PhytH (B) for 15 days. In fish fed the PhytH diet, widening of the lamina propria and mucosal folds, loss of supranuclear vacuolization, increased numbers of intraepithelial leukocytes, and loss of normal basal enterocyte nucleus position are evident. H-E staining.

The histological alterations were as follows: widening of the lamina propria and thus the mucosal folds, increased numbers of intraepithelial leucocytes (Fig 2), loss of normal cellular architecture with nucleus migration to a more apical position, and high variation in the size and number of vacuoles (from total absence to hypervacuolization with multiple large supranuclear vacuoles; Fig 3A and 3B, respectively) in 4 of 6 sampled sea bass fed PhytH, and indicative of mild to moderate inflammatory changes. Similar but somewhat milder signs were observed in 3 of 6 sampled fish fed the SapH and 2 of 6 sampled fish fed SapPhytH diets.

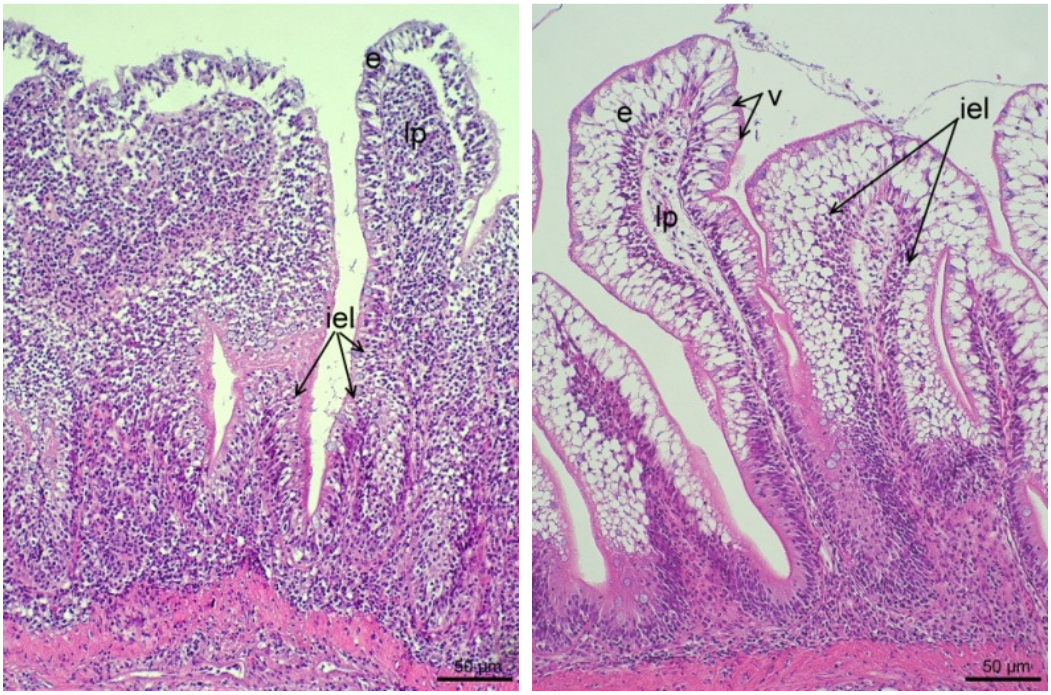

Figure 3 – Variation in vacuole size and number: (A) absence of supranuclear absorptive vacuolization in the enterocytes (diet PhytH); (B) supranuclear hypervacuolization with increased variation in vacuole size (diet PhytH). e: epithelium; v: vacuole; lp: lamina propria; iel: intraepithelial leukocytes (iel). H-E staining.

Inclusion of antinutrient did not significantly affect expression of genes encoding for AlP, Malt, CD4, Casp3, IL1β, TNFα and IgM in the DI (Table 6). Even so, it is noteworthy that IL1β and IgM showed the highest fold changes compared to the control, with up to a 10 fold up-regulation of IL1β in the PhytL group and 3.4 up-regulation of IgM in the PhytH group compared to the control group. The high variation within groups indicates that some fish reacted strongly whereas others did not. For IL1 β most of the difference in variation compared to the control was due to very high expression in one fish. Similarly, increased variation in expression of the other immune related genes was also due to expression in one or two fish. Fish fed the diet SapPhytH exhibited down-regulation of TGFβ compared to the groups fed diets SapH and PhytL. However, compared to the control group the TGFβ expression was not significantly altered (Table 6).

Table 6 –Gene expression of Alkaline Phosphatase (Alp), Maltase (Malt), Caspase 3 (Casp3), CD4, Transforming growth factor β (TGFβ), Interleukin 1β (IL1β), Tumor necrosis factor α (TNFα) and Immunoglobulin M (IgM) in the distal intestine of sea bass fed the experimental diets for 59 days.

Values represent fold changes relative to means expression levels observed in the control group. Results of the one-way ANOVA (*P*-values) are also provided.

Gene	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Alp	1.0	0.9	1.1	1.2	1.3	1.1	0.9	*0.16*	*0.669*
Malt	1.0	0.6	0.9	0.6	0.7	0.8	0.9	*0.10*	*0.292*
Casp3	1.0	1.1	1.2	1.1	1.2	1.4	1.1	*0.09*	*0.088*
CD4	1.0	0.9	0.9	1.3	1.0	1.2	0.9	*0.10*	*0.065*
TGFβ	1.0^{ab}	0.9^{ab}	1.1^{b}	1.1^{b}	1.0^{ab}	0.8^{ab}	0.8^{a}	*0.05*	*0.038*
IL1β	1	5	5	10	6	4	2	*2.99*	*0.780*
TNFα	1.0	0.7	0.9	1.0	1.3	1.2	0.8	*0.13*	*0.148*
IgM	1.0	1.2	0.6	0.7	3.4	2.2	1.3	*0.58*	*0.353*

[1] Standard Error of the Mean (pooled)
Differing superscript letters in the same line indicate significant differences between dietary treatment groups (P<0.05) as assessed by one-way ANOVA and Tukey's multiple range test.

Discussion

The finding that soybean saponins, phytosterols and a combination of both did not affect growth performance in European sea bass juveniles is in agreement with previous studies on the effects of saponins conducted with other species, such as rainbow trout (Penn 2005), channel catfish (Twibell & Wilson 2004) and Atlantic salmon (Krogdahl *et al.* 1995). According to Penn *et al.* (2012), supplementing a fishmeal based diet with either 2 or 4 g kg^{-1} soyasaponins resulted in higher growth performance in Atlantic salmon, while no effects on growth were observed when they were supplemented to a plant based diet. On the other hand, supplementation of 2 g kg^{-1} soyasaponins to a diet containing 330 g kg^{-1} protein from pea protein concentrate resulted in a decrease in Atlantic salmon growth (Chikwati *et al.* 2012). Also in common carp and tilapia fed diets containing purified *Quillaja saponaria* saponins, growth promotion was reported (Francis *et al.* 2005). However, Penn (2005), and later Gatlin *et al.* (2007), questioned the reliability of such results as the differences observed seemed to be related to uneven sex ratios rather than to the intake of saponins. Thus, the results reported in the present and in previous studies indicate that the putative effects of saponins may be linked to an interaction with other compounds present in some feed ingredients but not others, reinforcing the need to further investigate the effects of dietary soyasaponins in fish as the combination with other plant ingredients / antinutrients, fish species, and dosage appear to significantly affect responses.

Up to now there have been no published results on growth performance of fish fed diets supplemented with purified phytosterols. However, studies of effects of fish oil replacement by vegetable oils known to be rich in plant sterols, such as rapeseed oil, have shown no growth effects

in various fish species (Bell *et al.* 2001; Bell *et al.* 2003; Torstensen *et al.* 2004; Izquierdo *et al.* 2005; Pettersson *et al.* 2009). On the other hand, soy alcohol extract obtained as a byproduct of soy protein concentrate production, and considered to contain the saponin + phytosterol fraction of full fat soybean meal, has been reported to reduce feed intake and growth in Atlantic salmon, Chinook salmon and rainbow trout (van den Ingh *et al.* 1996; Bureau *et al.* 1998), although the authors suggested that soyasaponins and not phytosterol were responsible for this effect. Our results, however, suggest that the lower weight gain of fish fed soy-containing diets, including the alcohol-soluble fraction, cannot be attributed solely to saponins or phytosterols.

In the present study, saponins included in the diets up to 2 g kg^{-1} (SapH) had no significant effect on the intestinal morphology in sea bass, although some signs of mild morphological changes were observed. The lower supplementation level (1 g kg^{-1}; SapL) did not have such a marked effect following 15 days of feeding, but the higher score value following 59 days of feeding indicate a possible time-dependent increase in the severity of the changes for this saponin level, at least for some fish. These observations agree with a previous study in Atlantic salmon, which only reported clear signs of inflammation when soyasaponins were included at levels above 6 g kg^{-1} in the diets (Penn *et al.* 2012). Furthermore, signs of intestinal inflammation in Atlantic salmon fed plant based diets including 2 g kg^{-1} soyasaponins were observable only when combined with certain plant ingredients, such as pea protein concentrate (Chikwati *et al.* 2012; Kortner *et al.* 2012). The low dosage of soysaponins used in the present study together with the lack of antinutritional factors of fishmeal may explain the mild inflammation observed.

High levels of phytosterols (PhytH) induced more convincing signs of enteritis in the distal intestine of sea bass after 15 days of exposure to the antinutrient. The high variation observed in tissues sampled following 59 days of feeding may indicate recovery from the inflammatory state observed at the earlier sampling, with the timing of the recovery differing between individuals. Similar responses were reported by Urán *et al.* (2008) in the hindgut of soybean meal-fed common carp (*Cyprinus carpio* L.) where morphological anomalies were reported at weeks 1 and 3 after feeding was initiated, but some recovery was observed by the end of the trial at week 5 was observed.

Interestingly, some additive effects of the combination of antinutrients was indicated by the similar severity of the morphological changes observed in the sea bass fed the combination of the two antinutrients, at both low and high levels, compared to high levels of saponin and phytosterol alone. Additional studies to clarify the effects of saponins and phytosterols, and their combinations, in sea bass intestinal inflammation would be of interest.

Saponins have adjuvant properties, enhancing immune cell-mediated and antibody responses (Rajput *et al.* 2007). In trout, dietary inclusion of soyasaponins enhanced specific antibody responses in plasma after intraperitoneal injection with formalin fixed *Aeromonas salmonicida* bacterin (Penn 2005). Saponins also appear to stimulate non-specific immune reactions, such as monocyte proliferation and they induce cytokine production, such as interleukins and interferons, which may explain their immunostimulatory effects (Rajput *et al.* 2007). This may explain the involvement of T-cells in the soybean meal-induced intestinal inflammatory response of Atlantic salmon, as indicated by immunohistochemical detection of CD3ε-positive cells in the inflamed distal intestine with concomitant transcriptional up-regulation of T cell-specific molecules CD3, CD4, CD8α and CD8β in the same tissue (Bakke-McKellep *et al.* 2007a). Recent molecular studies based on transcriptomic approaches have supplied new information on the gut inflammatory response and dysfunction during diet-induced inflammation, and a number of potential molecular markers for enteritis have been identified, including inflammatory cytokines, genes related to intestinal barrier function and nutrient transporters (Skugor *et al.* 2011; Kortner *et al.* 2012; Marjara *et al.* 2012; Sahlmann *et al.* 2013). In the present study, the selected genes have all been proposed as potential diagnostic markers for intestinal inflammation (Krogdahl *et al.* 2000; Bakke-McKellep *et al.* 2007b; Kortner *et al.* 2012; Marjara *et al.* 2012). The gene expression profiles, observed only for samples taken at the end of the experiment, suggest that the molecular responses had peaked at some earlier time point and the lack of significant differences for IL1β expression may again be indicative of recovery by day 59 in some individuals. Nonetheless, the 10 and 6-fold increase in IL1β expression and 3.4 fold increase in IgM (PhytH group only) may confirm that the morphological changes observed in the PhytL and PhytH fed sea bass were of an inflammatory nature. Similar findings have been reported in the distal intestine of soybean meal-fed salmon (Bakke-McKellep *et al.* 2000; Krogdahl *et al.* 2000; Marjara *et al.* 2012). Most of the other genes displayed relatively stable expression levels irrespective of antinutrient supplementation. This corroborates the histological observations, which also showed that development of the pathological condition only occurred in some individuals, while others within the same experimental group presented no signs of morphological alterations.

High levels of dietary soyasaponins alone (SapH) or combined with high levels of phytosterols (SapPhytH) apparently caused some functional loss in the distal intestine, as suggested by the depressed Malt and Alp or Malt enzymatic activities, respectively. This corroborates the histological observations as an additional sign of an inflammatory response, which is always accompanied by tissue dysfunction. Our results are also in accordance with previous findings in Atlantic salmon fed diets containing either saponins or phytosterols at levels similar to those used in

the present work (Chikwati 2007). In the latter study, high dietary saponin levels lowered maltase activity in the distal intestine, while phytosterols exerted no effect. Previously, in European sea bass fed soybean meal-containing diets, reduced maltase and alkaline phosphatase activities in the whole digestive tract have also been reported (Tibaldi *et al.* 2006). A significant reduction in activities of brush border membrane enzymes has been shown in several studies in fish exhibiting intestinal inflammation, reflecting the functional loss accompanying inflammation (Krogdahl *et al.* 1995; Baeverfjord & Krogdahl 1996; Bureau *et al.* 1998; Bakke-McKellep *et al.* 2000; Krogdahl *et al.* 2003; Tibaldi *et al.* 2006). In the present study, while the decrease in enzymatic activity correlated well with the morphological changes in the DI, expression of the genes for these enzymes was not responsive. The unparallel responses between gene expression and enzymatic activity of digestive enzymes has been observed in many previous studies (Lehnert & Johnson 2002; Cahu *et al.* 2004; Wang *et al.* 2006), and may be attributed to post transcriptional, translational and protein degradation regulatory mechanisms. Indeed, it was recently reported that only ~40% of the variation in protein concentration can be explained by knowing the mRNA abundance (Vogel & Marcotte 2012).

In salmon with soybean meal-induced inflammatory changes, loss of physiological function is usually accompanied by a decrease of mucosal fold height and DI weight (Nordrum *et al.* 2000; Bakke-McKellep *et al.* 2007b; Kraugerud *et al.* 2007). In the current study, an increase of DI weight was observed in SapH, PhyL and SapPhyL-fed sea bass juveniles, but not in the other experimental groups. Whether this was caused by tissue changes could not be conclusively determined by histological methods. Channel catfish (*Ictalurus punctatus*) fed soybean meal that has not been heat treated have shown hypertrophy of the villous ridges in the distal intestine (Evans *et al.* 2005), which is consistent with an observed increase in visceral index (Peres *et al.* 2003). Thus the increased relative mass of the distal region of the gastrointestinal tract observed in the present work may be due to growth stimulation of the intestinal mucosa with increased cell proliferation. An additional explanation may be that the balance between cell proliferation and cell loss had been altered by the various antinutrients, which was also apparently affected by their inclusion level in the diet. Both cell proliferation and loss are apparently increased in Atlantic salmon fed SBM (Bakke-McKellep *et al.* 2007b; Venold *et al.* 2012; Kortner *et al.* 2013), but the inflammation leads to lower tissue weight in this species, suggesting more cell loss than proliferation. If cell proliferation exceeds cell loss, then increased tissue weight may result. Whether the increased relative DI weight observed was related to the inflammatory response and subsequent tissue changes, a result of unbalanced cell proliferation and cell loss, and/or a growth-stimulating effect of the antinutrients remains to be determined.

Phytosterols are known to lower plasma cholesterol levels in mammals, but the exact mechanism by which this occurs is not yet fully understood. Competition between phytosterols and cholesterol for intestinal absorption, inhibition of absorption due to loss of intestinal function, or impaired transport to the blood stream after uptake by the enterocytes, are potential explanations (Chikwati 2007). Similar to mammals, decreased plasma cholesterol levels have been reported in Atlantic salmon fed diets supplemented with 5 or 10 g kg^{-1} phytosterols (Chikwati 2007). Our results showed a hypocholesterolemic effect of phytosterols only at low dietary concentration. Similarly, in Artic charr fed diets with rapeseed oil replacing 25, 50 and 75% of fish oil (sitosterol levels of 0.4, 0.7 and 1.1 g Kg^{-1} lipids in the diets, respectively), cholesterol levels in white muscle were decreased only at 25 and 50% fish oil replacement levels, while the control and the diet with 75% rapeseed oil had similar cholesterol values (Pettersson *et al.* 2009). This may be due to compensation of the reduced cholesterol levels by enhanced endogenous synthesis. Indeed, Leaver *et al.* (2008) found an up-regulation of cholesterol synthesis-associated genes in Atlantic salmon when fish oil was replaced with vegetable oil in the diets, further supporting the compensation hypothesis.

Saponins have also been described as cholesterol lowering agents in several animals (Francis *et al.* 2002), including fish. Hypocholesterolaemia induced by saponins was observed in salmon fed diets supplemented with soyasaponins (Chikwati 2007; Penn *et al.* 2012), and formation of insoluble saponin-cholesterol complexes, resulting in reduced absorption and increased cholesterol loss in faeces was offered as an explanation (Chikwati 2007). In our study, soyasaponins did not lower plasma cholesterol at the levels tested. This may be explained by a response similar to that observed in chicks, for which there is evidence of increased cholesterol synthesis that compensates for the losses. (Jenkins & Atwal 1994). Another possible explanation is that the fishmeal in the diet provided enough cholesterol to conceal the saponin-induced excretion. However, further studies are required to confirm this.

In summary, the effects of phytosterol supplementation to juvenile sea bass diets had very different effects depending on inclusion level. Supplemented at 5 g kg^{-1} of the diet, phytosterols seemed to lower cholesterol plasma levels and increased the relative weight of the distal intestine without inducing overt inflammatory changes. In comparison, 10 g kg^{-1} phytosterols in the diet did not significantly lower plasma cholesterol, but caused inflammatory changes in the distal intestine, although possibly only temporarily, without changing the tissue's weight. Neither level reduced brush border digestive enzyme activities significantly. Saponins, on the other hand, reduced the activity of brush border membrane enzymes in the distal intestine when added at the higher (2 g kg^{-1}) inclusion level, which also appeared to cause some signs of inflammatory changes in the distal intestine and increased tissue weight. The lower saponin inclusion level did not appear to

significantly affect tissue responses, whereas some additive effects of the combination of the two antinutrients, also at lower inclusion levels, were indicated. Although growth was not compromised, the two antinutrients seemed to cause some gastrointestinal dysfunction that may stress the animals, rendering them more susceptible to other stressful factors. The high variability of responses within groups bears mentioning as some fish seemed to respond while others were unaffected by the antinutrients. Similarly, histological analysis showed the development of a pathological condition in some fish while others within the same experimental group showed no signs of morphological alterations. Additional studies to investigate the causes of the individual variation observed would be valuable.

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Chapter 3

Dietary saponins and phytosterols do not affect growth, intestinal morphology and immune response of on-growing European sea bass (*Dicentrarchus labrax*)

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Abstract

The current study is the first to investigate the effect of soy antinutrients, saponins and phytosterols, in on-growing sea bass. Seven diets were formulated: a control diet (fishmeal and fish oil based) and six diets containing 1g Kg^{-1} or 2g Kg^{-1} levels of soya saponins, 5g Kg^{-1} or 10g Kg^{-1} levels of phytosterols or a combination of 1g Kg^{-1} saponins + 5g Kg^{-1} phytosterols or 2g Kg^{-1} saponins + 10g Kg^{-1} phytosterols. After a 59 days feeding trial fish were weighed, faeces collected for digestibility studies and plasma sampled for cholesterol quantification. At day 15 and 59, distal intestine (DI) samples were collected for histological evaluation and quantification of maltase(Malt), alkaline phosphatase(AlP) and leucine aminopeptidase (LAP) activities. At day 59, DI was collected for gene expression of AlP, Malt and immune related genes.

Antinutrients had no effect on fish performance, digestibility, plasma cholesterol levels and digestive enzymes activities and gene expression. Only mild morphological changes were present in groups fed high levels of saponins+phytosterols. Expression of immune related genes showed no differences between groups.

On-growing sea bass showed high tolerance to the antinutrients tested, indicating that on-growing fish may be fed diets with higher substitution of fishmeal by plant products.

Keywords: antinutrients; sea bass; saponins; phytosterols; soya.

Introduction

Aquafeeds for carnivorous species have been largely dependent on fishmeal and fish oil as primary protein and lipid sources. However, world availability of these commodities is becoming limited (FAO 2012) and a wide variety of plant feedstuffs are being evaluated as alternative protein and lipid sources (soybeans, rapeseed, lupins, cottonseed, peas). However, most plant-derived feedstuffs contain antinutrients, which are defined as substances that by themselves or through their metabolic products interfere with feed intake, nutrient digestibility, growth, physiology and/or health of the animal (Francis *et al.* 2001; Krogdahl *et al.* 2010). Thus, it is of vital importance to ensure that diets containing high plant feedstuff levels still promote efficient growth and feed utilization, have minimal environmental impact, are safe to fish health, produce high quality flesh for human consumption, and are cost-effective. The animal response to antinutrients is however affected by factors such as species, age, size or gender. Several studies evaluating effects of antinutrients in fish diets have been conducted, mostly by varying dietary inclusion level of different feedstuffs. However, as more than one antinutrient are usually present in plant feedstuffs it is difficult to ascertain which individual factor is responsible for any adverse effects observed (Francis *et al.* 2001). For instance, both full-fat and solvent extracted soybean meal (SBM) induce inflammation in the distal intestine of salmonids whereas alcohol extracted soy protein concentrate does not cause pathological changes. Thus, although it is understood that one or more of the alcohol-soluble components of full fat soybean meal (such as saponins) are likely involved in the enteropathy, the specific antinutritional factor or factors are yet unknown (Van den Ingh *et al.* 1996; Krogdahl *et al.* 2010).

Saponins are ethanol soluble amphiphatic molecules present in soybeans and other legumes, characterized by their ability to bind cholesterol and bile salts and they often induce detrimental effects on cell membranes (Francis *et al.* 2002), increasing intestinal epithelial cell permeability, inhibiting active mucosal transport of nutrients, while possibly facilitating the uptake of substances that are normally not absorbed. Effects of saponins on protein digestion, cholesterol metabolism, immune and nervous system functions have also been reported (Francis *et al.* 2002). In fish, decreased growth performance of rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*) and Chinook salmon (*Oncorhynchus tshawytscha*) have been attributed to the presence of saponins in the diets (Francis *et al.* 2001; Chikwati *et al.* 2012). Fish fed diets with sub-fractions of soy molasses containing saponins (Knudsen *et al.* 2007), lupin kernel meal and partially purified soya saponins (Knudsen *et al.* 2008), or pea protein concentrate and purified saponins (Penn *et al.* 2011; Chikwati *et al.* 2012) resulted in similar lesions as those

induced by soybean meal. However the effects were probably not due to saponins alone, but to a combination of saponins and other constituents of the plant feedstuffs used.

Plant sterols, also called phytosterols, are a group of compounds naturally present in the lipidic portion of plants, and may therefore be introduced in fish feeds through replacement of fish oil by plant oils or when using full-fat seed meals. Phytosterols are structurally similar to cholesterol and can therefore induce hypocholesterolaemia in fish by competing with cholesterol for uptake by the enterocytes, resulting in increased cholesterol and bile acid losses in the faeces, and consequently, increased conversion of cholesterol to bile acids in the liver, further decreasing plasma cholesterol levels (Ling & Jones 1995). Data on the presence of phytosterols in fish diets and on their effects in fish is however scarce (Pelissero & Sumpter 1992; Chikwati 2007; Miller *et al.* 2008).

The increasing use of plant feedstuffs in diets can expose fish to cumulative effects of antinutritional factors, possibly affecting various physiological and metabolic processes in tissues and organs, and there may be late-onset or cumulative adverse effects resulting in a late manifestation of pathological conditions or less than optimal health (Krogdahl *et al.* 2010). Available data suggest that SBM can be included in sea bass diets at intermediate (Tibaldi *et al.* 2006) or high levels (Kaushik *et al.* 2004) without affecting zootechnical performance; however, there is still some concern that this may compromise fish health in the long term.

Besides a previous work from our team in juveniles (Couto *et al.* in press), there are no other available data on the effect of purified antinutrients in European sea bass (*Dicentrarchus labrax*). With the present study we aimed at studying the effect of soy saponins and phytosterols provided in the diets either singly or in combination in on-growing sea bass. These antinutrients were chosen due to their ability to interfere with cholesterol uptake and metabolism (Sidhu & Oakenfull, 1986; Lasztity *et al.* 1998; Romarheim *et al.* 2008), as well as the key role that soybean saponins play in the development of soybean meal-induced enteritis in salmonids (Knudsen *et al.* 2008; Chikwati *et al.* 2012). A comprehensive and exploratory approach was used, assessing fish zootechnical performance, diet digestibility, intestinal histomorphology and function. Gene profiling was also performed based on studies that identified several genes as potential markers for enteritis (Krogdahl *et al.* 2000; Bakke-McKellep *et al.* 2007;Kortner *et al.* 2012; Marjara *et al.* 2012).

Material and methods

The trial and sampling methods followed guidelines from the European Union directive 2010/63/EU on the protection of animals used for scientific purposes.

Table 1 – Composition and proximate analysis of the experimental diets.

	Experimental diets						
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**
Ingredients (g Kg^{-1} dry weight)							
Fishmeal[1]	604	604	604	604	604	604	604
Soluble fish protein concentrate[2]	50	50	50	50	50	50	50
Cod liver oil	118	118	118	118	118	118	118
Pre-gelatinized starch[3]	184	183	182	179	174	178	172
Vitamin premix[4]	10	10	10	10	10	10	10
Mineral premix[5]	10	10	10	10	10	10	10
Choline chloride (50%)	5	5	5	5	5	5	5
Binder[6]	10	10	10	10	10	10	10
Chromium Oxide	10	10	10	10	10	10	10
Soy saponins[7]	0	1	2	0	0	1	2
Phytosterols[8]	0	0	0	5	10	5	10
Proximate analysis (g Kg^{-1} dry matter)							
Dry matter (g Kg^{-1})	955	939	945	918	926	944	945
Crude protein	500	494	489	485	486	484	483
Crude fat	194	200	200	202	204	205	209
Starch	188	191	198	214	192	182	189
Ash	128	124	124	120	124	125	126
Chromium oxide	9	9	7	7	7	7	7
Gross energy (KJ g^{-1} DM)	22.2	22.9	21.9	22.5	22.6	22.4	22.3

[1] Vacuum Dried LT. Pesquera Diamante, S. A. Peru
[2] G-Special. Soropêche, France
[3] C-Gel Instant - 12018, Cerestar, Mechelen, Belgium
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet); dibasic calcium phosphate, 5.9 (g kg^{-1} diet).
[6] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England
[7] Soy saponins concentrate (95% purity) produced by Organic Technologies (Ohio, USA)
[8] Phytosterol extract predominantly beta-sitosterol (77.3%) produced by les Derive Resiniques et Terpeniques (DRT) (France)

Diets

Seven diets were formulated to contain 480 g Kg^{-1} crude protein and 180 g Kg^{-1} lipid (Table 1). The control diet was a fishmeal and fish oil based diet, while the experimental diets were similar to the control but contained low (1 g Kg^{-1}, diet SapL) or high (2 g Kg^{-1}, diet SapH) levels of purified soya saponins (purity 95%; Organic Technologies, Coshocton, OH, USA), low (5 g Kg^{-1}, diet PhytL) or high (10 g Kg^{-1}, diet PhytH) levels of purified phytosterols (purity >99%; Derive Resiniques et Terpenique, Dax, France), or a combination of low (1 g Kg^{-1} saponins + 5 g Kg^{-1} phytosterols, diet SapPhytL) or high (2 g Kg^{-1} saponins + 10 g Kg^{-1} phytosterols, diet SapPhytH) levels of both antinutrients. Chromium oxide was added to all diets as inert marker for digestibility quantification purposes. All dietary ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). The levels of saponins used in this study corresponds to levels found in diets with about 200 and 400 g kg^{-1} soybean meal (Anderson & Wolf 1995), which 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 meal or 250 and 500 g kg^{-1} inclusion level of rapeseed meal in diets (Piironen *et al.* 2000), which based on results of preliminary studies with Atlantic salmon caused weak but distinct effects when supplemented alone (Chikwati 2007). The phytosterol preparation was commercially available, made from pine and produced to serve as functional additive in margarine for human consumption, purportedly for its cholesterol-lowering effects. The dominating sterol was b-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.

Fish rearing conditions

The trial was performed at the Marine Zoological Station, Porto University. The trial lasted 59 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 12h:12h light:dark photoperiod was adopted, dissolved oxygen was maintained at 95%, water temperature was 25±0.5ºC and salinity averaged 35±1g L^{-1}. Sea bass (*Dicentrarchus labrax*) 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 12 fish with an initial mean body weight of 283 g were randomly distributed to each tank. Diets were randomly assigned to triplicate groups of these fish. During the trial, fish were fed by hand two times a day, 6

days a week; utmost care was taken to ensure all feed was consumed and no pellets were left on the tank floor or lost through the outlet. Fish were bulk weighed every 2 weeks after 1 day of food deprivation and feed intake of each tank was determined weekly.

Intermediate and final sampling

Sampling was conducted after 15 (intermediate) and 59 (final) days of feeding the experimental diets. Only fish containing digesta throughout the whole intestine length were sampled. Blood from four fish per tank was collected from the caudal vein using heparinized syringes, centrifuged at 1500 x g for 10 minutes and the plasma stored at -20ºC until analysis. After blood collection, fish were euthanized by anesthetic overdose (ethylene glycol monophenyl ether, ref.:8.07291, Merck, Whitehouse Station, USA) in ice water. Fish were then dissected on chilled trays, the digestive tract cut open longitudinally and a section (ca. 5 x 5 mm) of the distal intestine (DI; identified by the increased diameter, annular ring and darker mucosa) was sampled for histological evaluation. Samples were rinsed in phosphate buffered saline (PBS), gently blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%; pH 7.4) for 24h and then transferred to alcohol (70% EtOH) until further processing. Another section of the DI was sampled for enzyme activity. The section was excised, gently rinsed in PBS, blotted dry, immediately frozen in liquid nitrogen and stored at -80ºC until analysis. At the final sampling, an additional piece (50 mg) of the DI was collected, rinsed in PBS, blotted dry, placed in RNAlater® (25 mM Sodium Citrate, 10 mM EDTA, 700 g ammonium sulfate/1000 ml solution, pH 5.2; 1:10 w/v), left at 4ºC overnight and then stored at -20ºC until further processing. Also at the final sampling, the content of the DI (identified as previously described) was collected, pooled per tank and stored at -20ºC to analyze for nutrients digestibility.

Chemical Analysis

Diets were analysed for dry matter, ash, protein, lipids, energy and chromium oxide. Faeces were analysed for chromium oxide and lipids. The following procedures were used: dry matter after drying at 105ºC until constant weight; ash by incineration in a muffle furnace at 450ºC for 16 h; protein content (N x 6.25) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid by petroleum ether extraction using a Soxtec HT System (Tecator, Hoganas, Sweden); Starch according to Thivend *et al.* (1972); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA); chromic oxide was

measured by acid digestion according to Furukawa & Tsukahara (1966). Plasma cholesterol was determined using a cholesterol kit (ref. 1001092; Spinreact, Girona, Spain).

Histology

Histology samples were processed and sectioned using standard histological techniques. Sections were stained with hematoxylin and eosin. Blinded evaluation was performed with particular attention to inflammatory changes, as previously described in salmon (Baeverfjord & Krogdahl 1996; Krogdahl *et al.* 2003), namely: shortening, widening and fusion of intestinal folds, changes in enterocyte supranuclear absorptive vacuolization, connective tissue hyperplasia, and infiltration of inflammatory cells. A continuous scale scoring system was used as described by Penn *et al.* (2011) with the range of tissue scores set at 0-5. The overall value of enteritis degree was calculated by averaging scores of the separate parameters.

Brush border digestive enzyme activities

Activities of alkaline phosphatase (AlP), maltase (Malt) and leucine aminopeptidase (LAP) chosen as indicators of the functional status of the gut wall (Krogdahl *et al.* 2003), were quantified in tissue homogenates of DI. Tissues were thawed and homogenized (1:20) in ice-cold 2mM Tris/50mM mannitol (pH7.1), containing phenyl-methyl-sulphonylfluoride (P-7626; Sigma,St. Louis, MO, USA) as serine protease inhibitor. Aliquots of homogenates were frozen in liquid nitrogen and stored at -80° C prior to analysis. AlP and Malt activities were determined as previously described by Krogdahl *et al.* (2003). Briefly, AlP activity was measured with a kit (Sigma procedure no.104), using p-nitrophenyl phosphate as substrate. Malt activity was measured using maltose as substrate, followed by TRIS-glucose oxidase peroxidase (TGO) reagent addition for glucose detection. Samples were incubated at 37° C and absorbance read at 420nm. LAP activity was also determined colorimetrically using L-leucine-b-naphthylamide as substrate and the absorbance was read at 580nm. Enzyme activities were expressed as mol substrate hydrolyzed h^{-1} g of $tissue^{-1}$.

Gene expression

Table 2 - Primer pair sequences, efficiency, amplicon size and annealing temperature for genes used for real-time PCR.

	5´- 3´ primer sequence					
Gene symbol	Forward	Reverse	**Amplicon size (BP)**	**Annealing temp. (°C)**	**Primer efficiency**	**Genbank accession nr.**
AlP	TTACCTCTGTGGGGTCAAGG	TAGCCCATTTGAGGATGGAG	116	60	1.96	[FJ860000]
Malt	ATGCCATGGAGGTGACTTTC	CAACCATTTCAGGCGTAGGT	105	60	2.00	[AM419039]
Casp3	TGATGTCGTCTCTGCCGTAG	ACCACCTCATACGCATCCTC	76	60	1.97	[DQ345773]
CD4	ACTTGTGATTTGGGCAGTCC	TGATGAGATGAGTGGGGTGA	92	60	1.89	[AM849812]
TGF-β	CATCTGGAACGCTGAAAACA	TGTTGCCTGCCCACATAGTA	138	58	1.96	[AM421619]
IL-1β	GTGGTGGACAAAGCCAGTCT	TCTCCTCTGCTGTGCTGATG	147	60	2.00	[AJ269472]
TNF-α	GAAAACGCCTCACACCTCTC	GTCCGCTTCTGTAGCTGTCC	132	62	1.90	[DQ070246]
IgM	AGAGGAGCTGCAGGAGGACA	GGACCTCCAGGCTGTGTGAC	132	60	2.00	[AJ400233]
ACTB	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	133	60	1.92	[AJ537421]
GAPDH	CAAGGTTGAGGGTGACAAGC	CAGCCTCACCCCATTTGATG	92	60	1.99	[AY863148]
EF1A	ACGTGTCCGTCAAGGAAATC	GGGTGGTTCAGGATGATGAC	109	58	1.94	[FM019753]
Tubb	AGCTGGTGGACTCTGTCCTG	ACCAGAGCCTGTACCACCAC	107	58	1.95	[FM003484]

Quantitative real-time PCR (qPCR) was used to analyze the mRNA expression of the digestive enzymes alkaline phosphatase (Alp) and maltase (Malt). In addition, expression profiles of several previously reported markers for intestinal inflammation were analyzed, including cluster of differentiation 4 (CD4) specific for putative T helper cells, interleukin 1β (IL-1β) and transforming growth factor β (TGF-β) (Marjara *et al.* 2012), caspase 3 (Casp3) (Bakke-McKellep *et al.* 2007), immunoglobulin M (IgM) (Krogdahl *et al.* 2000), and tumor necrosis factor α (TNFα) (Kortner *et al.* 2012). qPCR primers (Table 2) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3). Optimal annealing temperature for each primer pair was established using gradient PCR. All chosen primers pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon of interest in all reactions. To verify correct amplification, PCR products were excised, purified and sequenced by Eurofins MWG Operon (Ebersberg, Germany). All sequences were confirmed by using NCBI nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov).

Total RNA was purified from DI tissue samples (~50 mg) homogenized in 750µl of Trizol reagent (Life Technologies, Carlsbad, CA, USA). Integrity of the RNA samples was evaluated by running an aliquot of the RNA sample on a 1% agarose gel. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Fisher Scientific, Hampton, NH, USA). Prior to reverse transcription, total RNA from all samples were subjected to DNase treatment using a TURBO DNA-free kit (Ambion, Life technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. First strand cDNA synthesis was performed in 20 µL reactions with 1.0 µg total RNA from all samples using Superscript III (Invitrogen, Life technologies, Carlsbad, CA, USA), and primed with Oligo$(dT)_{20}$ primers. Negative controls were performed in parallel by omitting RNA or enzyme. The obtained cDNA was diluted 1:10 and stored at -20 °C. The expressions of individual gene targets (n=9 fish/diet) were analyzed using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Each 10 µL DNA amplification reaction contained 2 µl of PCR-grade water, 5 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics), 2 µL of 1:10 diluted cDNA template and 0.5 µl (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control and an inter-run plate calibrator. The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (10 s), 58-62°C (depending on the primers used, see Table 2, 10 s) and 72°C (15 s). For determination of the quantification cycle (C_q) the "second derivative maximum method" measuring maximum increase rate of newly synthesized DNA per cycle was used on the basis of the LightCycler 480 software release 1.5.0 (Roche Diagnostics). To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and manual inspection of PCR products after each run by agarose gel electrophoresis. PCR reaction efficiency (E) for each gene assay was determined using 10-fold serial dilutions of randomly pooled cDNA. R^2 values of the standard curves were all >0.99. Beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1A (EF1A) and beta-tubulin (Tubb) were evaluated for use as reference genes, by ranking relative gene expression according to their stability as described previously (Kortner *et al.* 2011). ACTB was found to be stably expressed and was therefore used as normalization factor. Mean normalized expression of the target genes were calculated from raw C_q values using a plate calibrator-normalized relative quantification (Pfaffl, 2001) and expressed as fold changes relative to the control group.

Statistics

Results were analyzed using IBM SPSS 20 software package (SPSS® Inc; Chicago, IL, USA). The experimental unit considered was the tank (n=3) represented by the average of the 4 sampled fish

per tank. Data was tested for normality and homogeneity (Shapiro-Wilk and Levene tests, respectively) and when necessary it was transformed to achieve ANOVA assumptions. Results were analyzed by one-way ANOVA followed by Tukey`s multiple range test whenever statistical differences were found. Differences were considered statistically significant at $p < 0.05$.

Results

Fish promptly accepted all experimental diets and during the trial no mortality was recorded. Fish growth parameters and nutrient utilization are presented in Table 3.

Table 3 – Growth performance and nutrient utilization of sea bass (initial body weight: 282 ± 5g) fed the experimental diets for 59 days. The values are means calculated per diet (n=3) and pooled SEM and results of one-way ANOVA (P-values) are provided.

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Final body weight (g)	354	352	354	358	356	357	356	*2.61*	*0.998*
DGI[2]	0.87	0.98	0.92	0.96	0.92	0.96	0.91	*0.03*	*0.969*
Feed Intake (g DM fish^{-1})	129	109	132	117	136	127	123	*3.29*	*0.374*
FE[3]	0.75	0.62	0.65	0.79	0.68	0.70	0.65	*0.02*	*0.075*
PER[4]	1.51	1.26	1.33	1.62	1.40	1.46	1.34	*1.12*	*0.076*

[1] Standard Error of the Mean (pooled)
[2] Daily growth increment: $((IBW^{1/3} – FBW^{1/3}) / days) \times 100$
[3] Feed efficiency: weight gain: (g fresh weight) / feed intake (g dry matter)
[4] Protein efficiency ratio: weight gain (g fresh weight) / protein intake (g dry matter)

Daily growth Index (DGI) was within the normal range for on-growing sea bass and averaged 0.93 ± 0.12. The inclusion of saponins or phytosterols in sea bass diets had no effect on fish growth, feed intake or feed utilization, though a trend was observed for a decreased feed efficiency and protein efficiency ratio (PER) in fish fed with SapL, SapH, PhytH or SapPhytH. Saponins and phytosterols alone or in combination had no effect on dry matter or lipid digestibility or on plasma cholesterol levels (Table 4).

Table 4 – Plasma cholesterol levels, apparent digestibility coefficients (ADC) of dry matter and lipid, and activities (mol h^{-1} g $tissue^{-1}$) of the brush border enzymes alkaline phosphatase (AlP), maltase (Malt) and leucine aminopeptidase (LAP) in homogenates of the distal intestine of fish fed the experimental diets. The values are means of the scores (n=3) per diet and the pooled standard error of the mean (SEM) and results of one-way ANOVA (P-values) are given.

	Sampling ***day***	Experimental diets **Control**	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Cholesterol	*15*	7.3	6.3	6.6	7.3	6.5	6.6	6.9	*0.65*	*0.861*
(mmol L^{-1})	*59*	9	10	9	6	7	9	9	*1.33*	*0.401*
ADC dry matter[2]	*59*	65	63	62	68	66	64	63	*3.08*	*0.789*
ADC lipid[3]	*59*	85	88	82	86	81	78	80	*2.46*	*0.106*
AlP	*15*	1.5	1.5	1.4	1.4	1.4	1.5	1.4	*0.14*	*0.941*
Malt	*15*	2.2	2.1	2.3	2.1	21	2.3	2.2	*0.13*	*0.825*
LAP	*15*	1.8	2.0	1.8	1.7	1.8	1.8	1.7	*0.13*	*0.643*
AlP	*59*	1.7	1.6	1.5	1.3	1.4	1.6	1.5	*0.21*	*0.824*
Malt	*59*	2.5	2.2	2.5	2.1	2.1	2.5	2.2	*0.23*	*0.776*
LAP	*59*	1.8	1.7	1.8	1.6	1.7	1.8	1.7	*0.18*	*0.988*

[1] Pooled Standard Error of the Mean (SEM)
[2] ADC=100−100×(% chromium in food/% chromium in faeces).
[3] ADC=100−100×((% chromium in food/% chromium in faeces)×(% nutrient or energy in faeces /% nutrient or energy in food))

None of the diets had any effects on the activity of the distal intestinal brush border enzymes AlP, LAP and Malt (Table 4). Nor did exposure time to the antinutritional factors affect normal function of the selected enzymes as activity was similar to the control group.

Table 5 - Details of the score-based evaluation of the intestinal histology of fish fed the experimental diets for 15 and 59 days, based on changes observed in mucosal fold heights[1], width and cellularity of the lamina propria[2] and submucosa[3], the number of intraepithelial lymphocytes (IELs)[4] and eosinophilic granular cells (EGCs)[5], nucleus position within the enterocytes[6], and degree of enterocyte vacuolization[7]. A mean score was calculated from each of the above-mentioned characteristics. The values are means of the scores (n=3) per diet and the pooled standard error of the mean (SEM) and results of one-way ANOVA (P-values) are given.

Sampling day		Experimental diets								
		Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
15	Mucosal folds[1]	1.46	1.67	1.29	1.92	1.88	1.83	1.63	*0.09*	*0.501*
	Lamina propria[2]	1.21	1.17	1.17	1.33	1.50	1.17	1.42	*0.06*	*0.688*
	Submucosa[3]	2.25	1.67	1.58	2.13	2.04	2.25	2.17	*0.08*	*0.211*
	IELs[4]	3.00	2.75	3.13	3.50	3.38	3.75	3.67	*0.10*	*0.120*
	EGCs[5]	2.92	2.79	2.71	3.04	3.17	3.08	2.92	*0.08*	*0.741*
	Nucleus position[6]	2.08	1.92	2.00	2.88	2.71	2.58	2.67	*0.12*	*0.277*
	Vacuolization[7]	2.63	2.58	2.21	3.33	2.88	2.92	2.96	*0.17*	*0.716*
	Mean score	2.22	2.08	2.01	2.59	2.51	2.51	2.49	*0.08*	*0.348*
59	Mucosal folds[1]	1.38	2.00	1.63	1.82	2.00	1.63	2.29	*0.13*	*0.633*
	Lamina propria[2]	1.33	1.25	1.25	1.21	1.08	1.17	1.58	*0.06*	*0.472*
	Submucosa[3]	2.13	2.50	2.08	2.33	2.26	2.29	2.42	*0.09*	*0.876*
	IELs[4]	2.96	3.79	2.58	3.54	3.50	3.33	3.83	*0.10*	*0.333*
	EGCs[5]	2.58	3.25	2.92	2.71	3.00	2.63	2.58	*0.12*	*0.662*
	Nucleus position[6]	2.42	2.67	2.71	2.81	2.83	3.13	2.96	*0.10*	*0.810*
	Vacuolization[7]	2.38	3.04	2.88	3.38	2.75	3.42	3.25	*0.10*	*0.158*
	Mean score	2.17	2.64	2.43	2.54	2.49	2.51	2.70	*0.09*	*0.750*

[1] Pooled Standard Error of the Mean (SEM)

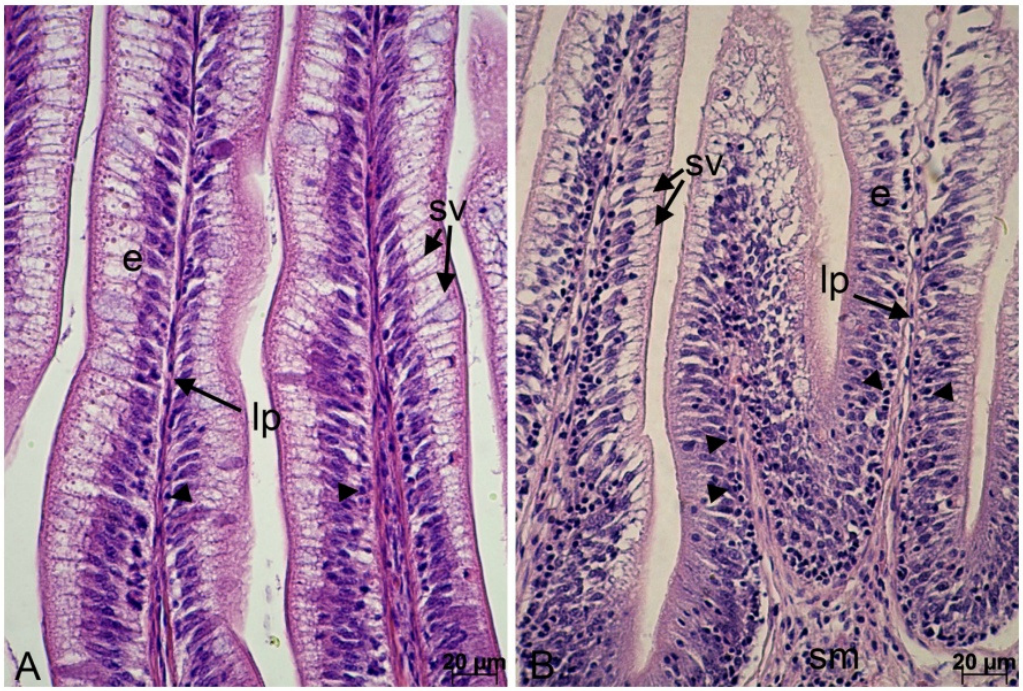

Figure 1 – Distal intestine histology of fish fed Control (A) or SapPhytH (B) diets for 15 days. e, epithelium; lp, lamina propria; sm, submucosa; sv, supranuclear vacuoles; arrowheads, intraepithelial leukocytes. H-E staining.

The distal intestine of sea bass showed no marked signs of inflammatory changes either after 15 or 59 days of feeding the experimental diets, and the histological scores were statistically similar to those of the control group (Table 5). Despite lacking significant differences, some qualitative differences were observed between the control group and fish exposed to antinutrients, mostly with regard to a variation in size of the supranuclear vacuoles present in the enterocytes,

Overall, the control group showed enterocytes with a more typical vacuolization and less variation in vacuole size than the groups fed diets with antinutritional factors for 59 days. Figures 1 and 2 represent the extremes observed between individuals fed the SapPhytH diet compared to controls, indicating individual variation in histomorphological changes due to antinutrient treatment.

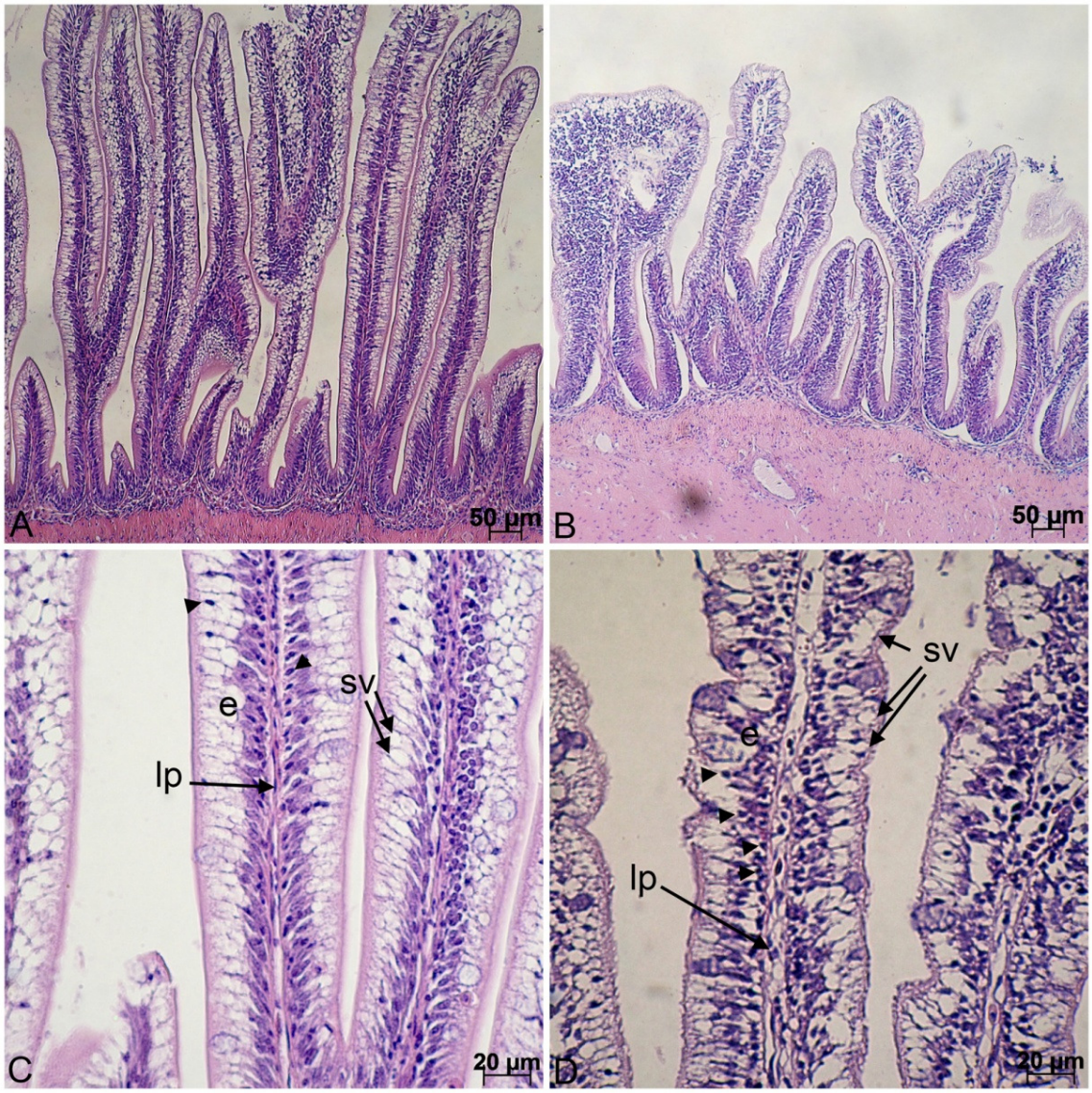

Figure 2 – Distal intestine histology of fish fed Control (A, C) or SapPhytH (B, D) diets for 59 days. The figure demonstrates changes in supranuclear vacuole sizes in the fish fed the SapPhytH diet (D) compared to the Control diet (C). e, epithelium; lp, lamina propria; sv, supranuclear vacuoles; arrowheads, intraepithelial leukocytes. H-E staining.

Table 6 – Mean (n=3) gene expression values for Alkaline Phosphatase (Alp), Maltase (Malt), Caspase 3 (Casp3), CD4, Transforming growth factor β (TGFβ), Interleukin 1β (IL1β), Tumor necrosis factor α (TNFα) and Immunoglobulin M (IgM) in the distal intestine of fish fed the experimental diets for 59 days. Values represent fold changes relative to the control group.

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
AlP	1.0	0.9	0.7	1.6	1.6	1.7	1.9	*0.15*	*0.295*
Malt	1.0	1.04	0.90	0.98	0.95	0.82	0.89	*0.04*	*0.748*
Casp3	1.0	0.62	1.05	1.20	0.79	0.97	1.16	*0.08*	*0.475*
CD4	1.0	1.01	0.94	0.95	1.09	1.13	0.81	*0.06*	*0.815*
TGFβ	1.0	0.92	0.99	1.10	1.16	1.18	1.01	*0.05*	*0.817*
IL1β	1.0	0.6	0.4	0.7	0.5	0.3	0.6	*0.10*	*0.393*
TNFα	1.0	0.92	0.73	1.31	0.93	0.97	0.76	*0.06*	*0.342*
IgM	1.0	1.60	3.92	6.38	7.74	2.41	1.50	*1.03*	*0.222*

[1] Pooled Standard Error of the Mean (SEM)

Similarly, there was no antinutrient effect on the distal intestinal expression of genes encoding the brush border enzymes AlP and Malt, nor were expression levels of Casp3, CD4, TGFβ and TNFα modulated (Table 6). Although not statistically different due to high variability, on average all diets containing antinutrients resulted in depressed IL1β gene expression compared to the control. On the other hand, IgM expression levels in fish fed diets PhytL and PhytH were 6 and 7 fold higher, respectively, than in control fish but differences were not statistically significant.

Discussion

The present study showed that sea bass in the grow-out phase tolerated well the presence of dietary saponins and/or phytosterols, thus indicating that older fish may have the capacity to cope with higher inclusion levels of plant ingredients in their diets. Sea bass growth was not affected by saponins or phytosterols (alone or in combination), which agrees with previous studies on the effects of saponins performed with rainbow trout (Penn 2005), channel catfish (Twibell & Wilson 2004) and salmon (Krogdahl *et al.* 1995). Penn *et al.* (2012) showed that soya saponins added to a plant-based diet (2 or 4 g Kg^{-1} inclusion level) and fed for 10 weeks had no effect on Atlantic salmon growth compared to an unsupplemented diet, while the same supplementation to a fishmeal

based diet resulted in enhanced growth. In Japanese flounder (*Paralichthys olivaceus*) fed a fishmeal-based diet containing 0.8 g Kg^{-1} soya saponins for 8 weeks the same growth promoting effect was observed while when dietary inclusion was 3.2 g Kg^{-1} or higher soya saponins had a negative effect on fish growth (Chen *et al.* 2011). Supplementation of 2 g Kg^{-1} soya saponins to a diet containing 330 g Kg^{-1} protein from pea protein concentrate also resulted in decreased growth in Atlantic salmon (Chikwati *et al.* 2012) after ~11 weeks of feeding. In our study, saponins seemed to somewhat decrease feed utilisation, albeit not significantly, which was not reflected regarding feed intake or growth, suggesting that potential negative effects might appear only after long-term exposure. The differing effects of dietary soya saponins reported in the different studies in fish reiterate the need to further study such effects as responses appear to vary greatly with combination with other plant feed ingredients / antinutrients, fish species, dosage and duration of exposure.

Studies addressing fish oil replacement by vegetable oils have often not demonstrated growth effects in various fish species (Torstensen *et al.* 2004; Izquierdo *et al.* 2005; Pettersson *et al.* 2009), including sea bass fed diets including soybean oil (250 or 500 g Kg^{-1}) for 12 weeks (Figueiredo-Silva *et al.* 2005). In contrast, sea bass fed a diet with 600 g Kg^{-1} fish oil replaced by either rapeseed oil for 21 weeks (Montero *et al.* 2005) or a blend of 100 g Kg^{-1} rapeseed, 350 g Kg^{-1} linseed and 150 g Kg^{-1} palm oils for 64 weeks (Mourente *et al.* 2007), resulted in reduced growth compared to fish fed a fish oil based diet. Although our findings from a feeding trial of relatively short duration (59 days ~ 8 weeks) indicated phytosterols did not depress sea bass growth, the longer lasting trials described in the mentioned studies by Montero *et al.* (2005) and Mourente *et al.* (2007) (21 and 64 weeks, respectively) investigating effects of plant oils do not allow to discard that it may contribute to the observed effects in the long term.

The hypocholesterolemic effects of dietary plant protein sources have been well demonstrated in terrestrial animals and in several fish species, such as rainbow trout (Kaushik *et al.* 1995), Atlantic salmon (Refstie *et al.* 1999), common carp (*Cyprinus carpio*; Hossain *et al.* 2001) and sea bass (Dias *et al.* 1997; Robaina *et al.* 1999). Saponins have been implicated in these effects in several animal species, including fish (Francis *et al.* 2002) and it has been observed in salmonids fed diets supplemented with soy saponins (Chikwati 2007; Penn *et al.* 2012). However, at the inclusion levels tested in the present study, soya saponins added to a fishmeal based diet did not lower plasma cholesterol in sea bass. Our findings agree with a previous work in Atlantic salmon that tested the effects of combining soya saponins (2 g Kg^{-1}) and plant ingredients in diets where no effect on plasma cholesterol levels was also found, irrespective of the basal diet composition (Chikwati *et al.* 2012).

The effect of phytosterols in lowering plasma cholesterol has been described in mammals (Vanstone *et al.* 2002; Skeaff *et al.* 2004) and was also found in Atlantic salmon fed diets with 5 and 10 g Kg^{-1} phytosterols (Chikwati 2007). In a recent study in our lab where sea bass juveniles were fed a fishmeal based diets including the same amounts of phytosterols as in the present trial only showed reduced cholesterol levels at the lower dietary phytosterols inclusion level (5 g Kg^{-1} phytosterols; Couto *et al.* in press). Interestingly, in a 12 week feeding trial in which sea bass juveniles were fed diets with fish oil partially replaced by soybean oil, there were no differences in plasma cholesterol levels (Figueiredo-Silva *et al.* 2005); the long period of exposure to the experimental diets could have played an important role in restoring levels. Pettersson *et al.* (2009) hypothesized that a compensatory mechanism triggered by a substantial drop in plasma cholesterol levels may enhance endogenous synthesis of cholesterol in Artic charr (*Salvelinus alpinus*) fed diets containing increasing levels of rapeseed oil and concurs with an up-regulation of genes associated with cholesterol synthesis when fish oil was replaced by vegetable oil in diets for Atlantic salmon (Leaver *et al.* 2008). Whether sea bass plasma cholesterol levels were maintained within normal levels due to the absence of antinutrients effect or to an enhanced synthesis to compensate faecal loss remains to be clarified. Cholesterol plays an important role in lipid digestibility as it is metabolised in the liver into bile salts which have implications in lipid digestion not only trough activation of bile salt activated lipase but also trough lipid emulsification and micelle formation. However, lipid digestion / absorption can be affected by many factors. Reduced lipid digestibility is a common finding for fish diets including high levels of plant feedstuffs in some (Olli *et al.* 1994, 1995; Penn *et al.* 2011), but not all studies (Kaushik *et al.* 2004; Hansen *et al.* 2007) and some of the components considered responsible for this are saponins, fibre, phytosterols and phytoestrogens (Krogdahl *et al.* 2010). The present study showed that dietary inclusion 1 or 2 g Kg^{-1} saponins had no effect on lipid digestibility, as it was also previously observed in Atlantic salmon (Chikwati 2007). However, when a plant protein source was present in the diet, saponin levels as low as 2 g Kg^{-1} affected lipid digestibility in Atlantic salmon (Chikwati *et al.* 2012). Also in salmon, lipid digestibility showed a dose-dependent decrease with dietary phytosterol levels (Chikwati 2007) whereas in the present study with sea bass no negative effect of dietary phytosterols on lipid digestibility was observed. Similarly, sea bass fed diets with fish oil replacement by either soybean or rapeseed oil also showed no differences in dry matter or lipid digestibility comparatively to a fish oil based control diet (Martins *et al.* 2006). Evidence shows that the negative effects on nutrient digestibility caused by plant ingredients and/or antinutrient supplementation can be attributed to intestinal dysfunction as a consequence of inflammatory changes caused by the diet (Olli *et al.* 1994, 1995; Penn *et al.* 2011; Chikwati *et al.* 2012). The present study apparently supports this, as

the absence of differences in lipid digestibility was consistent with the absence of overt intestinal inflammation.

Several studies have reported inflammation in the distal intestine of Atlantic salmon fed diets containing soybean meal (van den Ingh *et al.* 1991; Olli *et al.* 1994, 1995) and Knudsen *et al.* (2008) demonstrated that soya saponins (17 and 26 g Kg^{-1}), in combination with one or several unidentified components present in lupin meal, induced an inflammatory reaction in the distal intestine in Atlantic salmon. Recent studies also with Atlantic salmon demonstrated that signs of inflammation were present when soya saponins were included in the diets above 6 g Kg^{-1}, irrespective of whether the diet was plant or fishmeal-based (Penn *et al.* 2012). When Atlantic salmon was fed plant-based diets including soya saponins at levels as low as 2 g Kg^{-1}, signs of intestinal inflammation were observable only when combined with certain plant ingredients, such as pea protein concentrate (Chikwati *et al.* 2012; Kortner *et al.* 2012). Thus, saponins alone, when supplemented at levels corresponding to a ~300 g Kg^{-1} soybean meal diet, do not seem to have an adverse effect on the intestinal mucosa, as supported by our findings in sea bass. Phytosterols also had no effect on the distal intestine morphology of sea bass, as reported in Atlantic salmon as well (Chikwati 2007).

Previous works with sea bass also found no morphological alterations in the distal intestine when fish were fed diets containing ingredients of plant origin (Mourente *et al.* 2007; Bonaldo *et al.* 2008). Intestinal inflammation induced by soybean meal has often been associated with reduced activity of digestive brush border enzymes in Atlantic salmon (Baeverfjord & Krogdahl 1996; Krogdahl *et al.* 2003) and a similar effect has been reported when soya saponins were added to plant based diets (Penn *et al.* 2011; Chikwati *et al.* 2012). Our morphological data supports findings on distal intestine physiological function. Present results showed that neither enzyme activity nor enzyme gene expression in distal intestine were affected by diet composition, thus reinforcing the idea that on-growing sea bass tolerate the tested antinutrients without loss of physiological function.

The high occurrence of intraepithelial and mucosal leukocytes found in the distal intestine of all fish in the present study is in accordance with the findings of Abelli *et al.* (1997), who also observed increased numbers of IgM+ cells from the anterior and middle to the distal part of sea bass intestine, suggesting an immunological role of the distal segment (Salinas *et al.* 2011). An increase in diffuse IgM, but not IgM+ cells, were identified by immunohistochemical staining in the intestinal mucosa of soybean meal-fed (300 g Kg^{-1} inclusion level) Atlantic salmon (Bakke-McKellep *et al.* 2000). In the present work the up-regulation of IgM seems to be somehow associated with some oral tolerance to the antinutrients, as has been observed in other fish repeatedly exposed to antigenic

proteins (Rombout *et al.* 2011). Gene expression profiling in the present study is consistent with the lack of effect on the distal intestinal morphology of on-growing sea bass of dietary saponins and phytosterols.

Generally, our results indicated that on-growing sea bass have high tolerate the presence of dietary antinutrients and that high amounts of vegetable feedstuffs can be included in their feeds without affecting growth, intestinal function or immune status. Despite these promising findings, the negative trend observed in FE and PER and the observed histological alterations in the test groups should be further evaluated as prolonged exposure to the antinutrients could result in aggravation of the effects.

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Chapter 4

Effects of dietary soy saponins and phytosterols on gilthead sea bream (*Sparus aurata*) juveniles

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Submitted to Aquaculture

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 utilisation, 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.

Keywords: antinutrients; gilthead sea bream; saponins; phytosterols; soya.

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., 2008; Benedito-Palos et al., 2007; 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 were 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 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*) have 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.

Material and methods

Diets

Table 1 – Composition and proximate analysis of the experimental diets.

	Experimental diets						
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**
Ingredients (g kg^{-1} dry weight)							
Fish meal[1]	588	588	588	588	588	588	588
Soluble fish protein concentrate[2]	50	50	50	50	50	50	50
Cod liver oil	112	112	112	112	112	112	112
Pre-gelatinized starch[3]	215	214	213	210	205	209	203
Vitamin premix[4]	10	10	10	10	10	10	10
Mineral premix[5]	10	10	10	10	10	10	10
Choline chloride (50%)	5	5	5	5	5	5	5
Binder[6]	10	10	10	10	10	10	10
Soy saponins[7]	-	1	2	-	-	1	2
Phytosterols[8]	-	-	-	5	10	5	10
Proximate analysis (g kg^{-1} dry matter)							
Dry matter	918	900	914	911	923	916	918
Crude protein	456	458	458	456	453	458	456
Crude fat	172	170	177	177	182	178	183
Starch	186	203	197	200	198	184	200
Ash	134	134	133	134	134	135	134
Gross energy (KJ g^{-1} DM)	22.4	22.5	22.1	21.8	22.3	22.1	22.1

[1] Vacuum Dried LT. Pesquera Diamante, S. A. Peru
[2] G-Special. Soropêche, France
[3] C-Gel Instant - 12016, Cerestar, Mechelen, Belgium
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU k^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet); dibasic calcium phosphate, 5.9 (g kg^{-1} diet).
[6] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England
[7] Soy saponins concentrate (95% purity) produced by Organic Technologies (Ohio, USA)
[8] Phytosterol extract produced from pine by les Derive Resiniques et Terpeniques (DRT) (France), Purity: >99% pure; including β-sitosterol: 77.3%, β-sitostanol 10.7%, campesterol: 8.8%, other: 2.2%.

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 corresponds 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 meal or 250 and 500 g Kg^{-1} inclusion level of rapeseed meal 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). The phytosterol preparation was commercially available, made from pine and produced to serve as functional additive in margarine for human consumption, purportedly for its cholesterol-lowering effects. 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). Ingredient and proximate composition of the experimental diets are presented in Table 1.

Growth trial

The present experiment was directed by trained scientists (following FELASA category C recommendations) and 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 12h:12h light:dark photoperiod was adopted, oxygen was maintained near saturation, water temperature was 25±0.5ºC and salinity averaged 35±1‰. Gilthead sea bream (*Sparus 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 every two weeks after one day of food deprivation.

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 24h 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 x g for 10 minutes. Plasma samples were stored at -20ºC until analysis. After blood collection fish were euthanized, dissected and intestinal samples collected for histological evaluation as described above. Furthermore, a sample from the distal intestine was collected for gene expression profiling. The samples were gently rinsed in PBS, blotted dry, placed in RNAlater® (25 mM Sodium Citrate; 10 mM EDTA; 70 g ammonium sulfate/100 ml solution; pH 5.2; 1:10 w/v), left at 4ºC overnight and subsequently stored at -20ºC until analysis.

Chemical Analysis

Diets were analysed for dry matter, ash, protein, lipid and energy. The following procedures were used: dry matter after drying at 105ºC until constant weight; ash by incineration in a muffle furnace at 450ºC for 16 h; protein content was calculated from nitrogen analysis (N x 6.25) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid by petroleum ether extraction using a Soxtec HT System (Tecator, Höganäs, Sweden); starch according to Thivend et al. (1972); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA). Plasma cholesterol was determined using a cholesterol kit (ref. 1001092 Spinreact, Girona, Spain).

Histology

Fixed intestinal samples were processed and sectioned using standard histological techniques. Sections were stained with hematoxylin and eosin. Blinded evaluation was performed with particular attention to inflammatory changes (Baeverfjord and Krogdahl, 1996; Krogdahl et al.,

2003), namely: shortening, widening and fusion of intestinal folds, changes in enterocyte supranuclear absorptive vacuolization, connective tissue hyperplasia, and infiltration of inflammatory cells. A continuous scale scoring system was used as described by Penn et al. (2011) with the range of tissue scores set at 0-5. The overall enteritis score was calculated by averaging scores of the separate parameters described above.

Gene expression

Table 2 - Primer pair sequences, efficiency, amplicon size and annealing temperature for genes used for real-time PCR. Grey shaded rows correspond to reference genes.

	5´- 3´ primer sequence						
Gene symbol	Forward	Reverse	**Amplicon size (BP)**	**Annealing temp. (°C)**	**Primer efficiency**	**Genbank accession nr.**	**Reference**
CD8	GGACAAGTCTGGCATGGAAT	GTGGACGACGGAGACTTTGT	81	60	1.82	[AJ878605]	
TCRβ	AAGTGCATTGCCAGCTTCTT	TTGGCGGTCTGACTTCTCTT	130	60	2.00	[AM261210]	Cerezuela et al. 2012
MHC-I	CCCTCAGTGTCTCTCCTCCA	CAGACGAGTGAGGCTCTGTG	89	60	1.97	[DQ211540]	
MHC-II	CTGGACCAAGAACGGAAAGA	CATCCCAGATCCTGGTCAGT	176	62	1.86	[DQ019401]	Cerezuela et al. 2012
IgM	TCAGCGTCCTTCAGTGTTTATGATGCC	CAGCGTCGTCGTCAACAAGCCAAGC	131	62	1.93	[JQ811851]	Estensoro et al. 2012
IL1β	GCGACCTACCTGCCACCTACACC	TCGTCCACCGCCTCCAGATGC	133	60	1.80	[AJ277166]	Sitja-Bobadilla et al. 2008
TNFα	CAGGCGTCGTTCAGAGTCTC	GAGATCCTGTGGCTGAGAGG	84	62	1.96	[AJ413189]	
Casp3	CCAGTCAGTCGAGCAGATGA	GAACACACCCTCGTCTCCAT	113	60	1.89	[EU722334]	
TGFβ	AAACCAACTCGCTACCATGC	CTCCAGAGCCTGAGGAACAC	152	60	2.00	[AF424703]	
PCNA	GATGTGGAGCAGCTGGGTAT	TGTCTACGTTGCTGGTCTGG	205	60	1.92	[FG263675]	
FABP2	CGAGCACATTCCGCACCAAAG	CCCACGCACCCGAGACTTC	93	60	1.97	[AM957164]	Calduch-Giner et al. 2012
PepT1	CTACCCACTGATCGCCAAGT	GATGGGAACGTAGGCAGTGT	141	60	2.00	[GU733710]	
Aqp8	AGTTGGGACGTCTCTCGTGT	CTCGATGACCGACACACAAC	147	60	1.99	[DQ889225]	
EF1α	ACGTGTCCGTCAAGGAAATC	GGGTGGTTCAGGATGATGAC	109	60	2.00	[AF184170]	
GAPDH	CCAACGTGTCAGTGGTTGAC	AGCCTTGACGACCTTCTTGA	80	60	2.00	[DQ641630]	
Rps18	AGGGTGTTGGCAGACGTTAC	CGCTCAACCTCCTCATCAGT	97	60	1.96	[AM490061]	

Quantitative real-time PCR (qPCR) was used to analyze the mRNA expression of previously reported markers for intestinal inflammation and function, including immunoglobulin M heavy chain (IgM), cluster of differentiation 8 (CD8), T-cell receptor beta (TCRβ), interleukin 1β (IL1β), transforming growth factor β (TGFβ), major histocompatibility complex I (MHC-I), major

histocompatibility complex II (MHC-II), tumor necrosis factor α (TNFα), caspase 3 (Casp3), proliferating cell nuclear antigen (PCNA), fatty acid binding protein (intestinal; FABP2), intestinal peptide transporter (PepT1), and aquaporin 8 (Aqp8) (Kortner et al., 2012; Krogdahl et al., 2000; Marjara et al., 2012; Skugor et al., 2011; Terova et al., 2013; Venold et al., 2013). Previously validated and published gene-specific primers (Table 2) were used to quantify TCRβ, MHC-II (Cerezuela et al., 2012), IgM (Estensoro et al., 2012), IL1β (Sitja-Bobadilla et al., 2008), and FABP2 (Calduch-Giner et al., 2012). For the remaining genes, primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3). All chosen primer pair concentrations used at the optimal annealing temperatures gave a single band pattern for the expected amplicon of interest in all reactions.

Total RNA was extracted from DI tissue samples (~50 mg) using Trizol reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA) and purified with Direct-zol ™ RNA Miniprep (Zymo Research, Irvine, CA, USA) including an on-column DNase treatment according to the manufacturer's protocol. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Fisher Scientific, Hampton, NH, USA). Integrity of the RNA samples was evaluated by running an aliquot of the RNA sample on a 1% agarose gel. First strand cDNA synthesis was performed using 1.0 µg total RNA from all samples using Superscript III (Invitrogen™, Life Technologies, Carlsbad, CA, USA) in 20µL reactions, and primed with Oligo (dT)20 primers according to the manufacturer's protocol.

Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at −20°C. The expressions of individual gene targets (n=8 fish/diet) were analyzed using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Each 10 µL DNA amplification reaction contained 2 µl of PCR-grade water, 5 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics), 2 µL of 1:10 diluted cDNA template and 0.5 µl (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control and an inter-run plate calibrator. The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (10 s), 60-62°C (depending on the primers used, see Table 2, 10 s) and 72°C (15 s). For determination of the quantification cycle (C_q), the "second derivative maximum method" measuring maximum increase rate of newly synthesized DNA per cycle was used on the basis of the LightCycler 480 software release 1.5.0 (Roche Diagnostics). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of PCR products after each run by agarose gel electrophoresis. PCR reaction efficiency (E) for each gene assay was determined using 10-fold serial dilutions of randomly pooled cDNA. R^2 values of the standard

curves were all >0.99. Elongation factor 1α (EF1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein s18 (Rps18) were evaluated for use as reference genes, by ranking relative gene expression according to their stability as described previously (Kortner et al., 2011). Rps18 was found to be stably expressed and was therefore used as normalization factor. Mean normalized expression of the target genes were calculated from raw C_q values using a plate calibrator-normalized relative quantification (Pfaffl, 2001) and expressed as fold changes relative to the control group.

Statistics

Results were analyzed using IBM SPSS 21 software package (SPSS® Inc, Chicago, IL, USA). The experimental unit considered was the tank (n=3) represented by the average of three fish per tank. Data was tested for normality and homogeneity (Shapiro-Wilk and Levene tests, respectively) and when necessary data was transformed to achieve ANOVA assumptions. Results were analyzed by one-way ANOVA followed by Tukey`s multiple range test whenever statistical differences were found. When data could not be normalized, the Kruskal-Wallis non-parametric test and subsequent pairwise comparison was performed. Differences were considered statistically significant at $p < 0.05$.

Results

Table 3 – Mean values (n=3) for growth parameters, feed intake, feed utilization and plasma cholesterol levels of sea bream fed the experimental diets for 48 days and results of the one-way ANOVA (*P*-values).

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Final body weight (g)	40	42	41	38	41	43	40	*1.41*	*0.311*
DGI[2]	2.3	2.4	2.3	2.2	2.3	2.5	2.3	*0.08*	*0.320*
Feed intake (g DM per fish)	40	40	39	36	36	40	35	*1.36*	*0.054*
FE[3]	0.69^{a}	0.76^{ab}	0.75^{ab}	0.75^{ab}	0.81^{b}	0.79^{b}	0.79^{b}	*0.02*	*0.014*
PER[4]	1.52^{a}	1.66^{ab}	1.64^{ab}	1.64^{ab}	1.79^{b}	1.72^{ab}	1.74^{b}	*0.14*	*0.012*
Cholesterol (mmol L^{-1})	6.0	5.5	5.1	4.5	5.4	5.3	5.2	*0.47*	*0.466*

[1] Standard Error of the Mean (pooled)
[2] Daily growth increment: $((IBW^{1/3} – FBW^{1/3}) / days) \times 100$
[3] Feed efficiency: weight gain (g fresh weight) / feed intake (g dry matter)
[4] Protein efficiency ratio: weight gain (g fresh weight) / protein intake (g dry matter)

Fish promptly accepted all experimental diets and no mortality was recorded during the trial. Fish growth parameters and nutrient utilization are presented in Table 3. Daily growth index (DGI) was within the normal range for sea bream juveniles (2.2-2.5). The inclusion of saponins or phytosterols in sea bream diets had no effect on fish growth or feed intake. PhytH and SapPhytH-fed fish had higher feed efficiency (FE) and protein efficiency ratio (PER) compared to the control. The fish fed the SapPhytL diet exhibited higher FE than the control group.

All diets containing antinutrients resulted in decreased plasma cholesterol levels (Table 3) compared to the control, although the differences were not statistically significant.

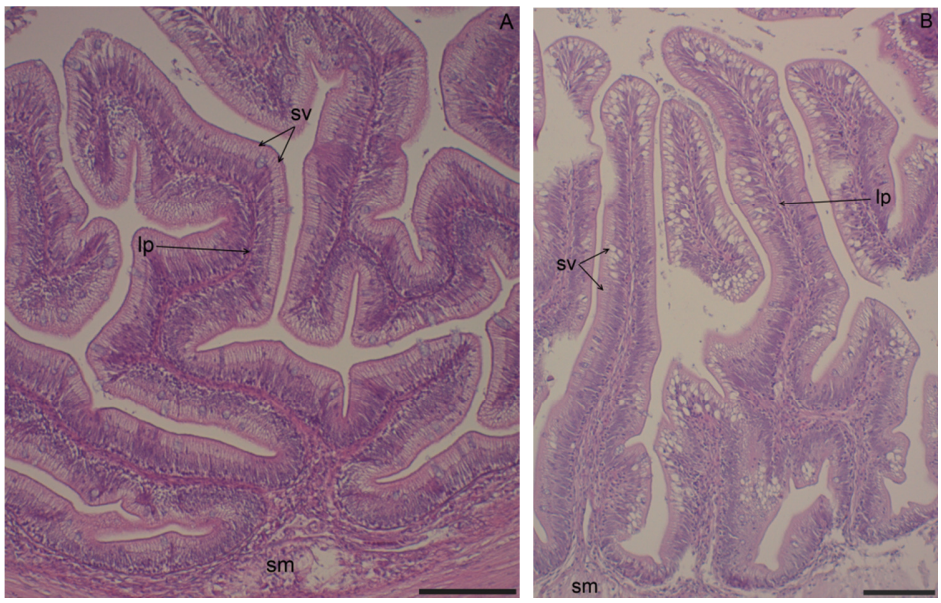

Figure 1 – Distal intestine histology of fish fed Control (A) or SapPhytH (B) diets for 48 days. The figure demonstrates the increased variation in supranuclear vacuole sizes in the fish fed the SapPhytH diet (B) compared to the Control diet (A). Scale bar = 100µm; lp, lamina propria; sm, submucosa; sv, supranuclear vacuoles.

After 15 days of feeding the experimental diets, fish showed no histomorphological alterations of either the pyloric caeca or the distal intestine (Table 4). At the end of the feeding trial, the pyloric caeca of fish fed PhytL diet had a higher number of intraepithelial leukocytes (IELs), although no other signs of inflammation were observed in this intestinal section.

Table 4 - Details of the score-based evaluation of the intestinal histology of sea bream fed the experimental diets for 15 and 48 days, based on changes observed in mucosal fold heights[1], width and cellularity of the lamina propria[2] and submucosa[3], the number of intraepithelial lymphocytes (IELs)[4] and eosinophilic granular cells (EGCs)[5], nucleus position within the enterocytes[6], and variation of enterocyte vacuolization[7]. A mean score was calculated from each of the above-mentioned characteristics. The values are means of the scores (n=3) per diet and the pooled standard error of the mean (SEM) and results of Kruskal-Wallis (P-values) are given.

			Experimental diets								
Time	Section		**Control**	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[*]	*P-values*
15 days	PC	Mucosal folds[1]	1.0	1.1	1.1	1.1	1.2	1.2	1.1	*0.10*	*0.733*
		Lamina propria[2]	1.0	1.0	1.1	1.0	1.1	1.1	1.0	*0.05*	*0.298*
		Submucosa[3]	1.0	1.0	1.0	1.1	1.1	1.2	1.1	*0.08*	*0.656*
		IELs[4]	2.4	2.8	2.4	2.8	2.9	2.8	2.8	*0.15*	*0.156*
		EGCs[5]	2.50	2.50	2.50	2.50	2.56	2.50	2.56	*0.03*	*0.511*
		Nucleus position[6]	1.50	1.50	1.50	1.50	1.50	1.50	1.50	*0.00*	*1.000*
		Vacuolization[7]	1.00	1.00	1.06	1.00	1.00	1.00	1.00	*0.02*	*0.423*
		Mean score	1.50	1.53	1.53	1.57	1.60	1.60	1.57	*0.04*	*0.617*
	DI	Mucosal folds[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.11	*0.04*	*0.423*
		Lamina propria[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.00*	*1.000*
		Submucosa[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.00*	*1.000*
		IELs[4]	2.4	3.1	2.7	3.1	3.2	2.9	2.8	*0.16*	*0.069*
		EGCs[5]	2.5	2.67	3.06	3.06	3.06	2.72	3.00	*0.02*	*0.423*
		Nucleus position[6]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.04*	*0.423*
		Vacuolization[7]	1.0	1.0	1.2	1.0	1.1	1.2	1.2	*0.12*	*0.166*
		Mean score	1.43	1.53	1.53	1.53	1.53	1.53	1.53	*0.03*	*0.421*
48 days	PC	Mucosal folds[1]	1.0	1.2	1.1	1.3	1.2	1.1	1.2	*0.10*	*0.295*
		Lamina propria[2]	1.0	1.0	1.0	1.2	1.2	1.1	1.2	*0.10*	*0.236*
		Submucosa[3]	1.00	1.00	1.06	1.00	1.06	1.00	1.00	*0.03*	*0.511*
		IELs[4]	2.4 ^{a}	2.8 ^{ab}	2.8 ^{ab}	3.7 ^{b}	2.7 ^{ab}	3.0 ^{ab}	2.5 ^{ab}	*0.17*	*0.042*
		EGCs[5]	2.50	2.50	2.56	2.50	2.56	2.50	2.56	*0.04*	*0.617*
		Nucleus position[6]	1.50	1.50	1.50	1.50	1.56	1.50	1.61	*0.03*	*0.141*
		Vacuolization[7]	1.0	1.0	1.0	1.0	1.1	1.1	1.0	*0.05*	*0.510*
		Mean score	1.50	1.57	1.57	1.73	1.60	1.57	1.57	*0.04*	*0.134*
	DI	Mucosal folds[1]	1.00 ^{a}	1.00 ^{a}	1.00 ^{a}	1.00 ^{a}	1.00 ^{a}	1.03 ^{b}	1.00 ^{a}	*0.00*	*0.003*
		Lamina propria[2]	1.0	1.1	1.0	1.0	1.0	1.0	1.0	*0.09*	*0.423*
		Submucosa[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.02*	*1.000*
		IELs[4]	2.5 ^{a}	2.7 ^{ab}	3.1 ^{b}	3.1 ^{b}	3.1 ^{b}	2.7 ^{ab}	3.0 ^{b}	*0.09*	*0.034*
		EGCs[5]	2.50	2.50	2.50	2.50	2.50	2.50	2.50	*0.00*	*1.000*
		Nucleus position[6]	1.0	1.1	1.1	1.0	1.2	1.0	1.0	*0.05*	*0.051*
		Vacuolization[7]	1.0 ^{a}	1.4 ^{abc}	1.9 ^{c}	1.1 ^{ab}	1.4 ^{abc}	1.6 ^{bc}	1.9 ^{c}	*0.20*	*0.036*
		Mean score	1.40 ^{a}	1.50 ^{abc}	1.67 ^{c}	1.50 ^{ab}	1.60 ^{bc}	1.60 ^{bc}	1.63 ^{bc}	*0.04*	*0.036*

[*] Standard error of the mean (pooled)

Differing superscript letters in the same line indicate significant differences between the dietary treatments ($P < 0.05$) as assessed by Kruskal-Wallis allpairwise test.

The distal intestine showed more significant histological alterations. The statistically significant difference in the height of the mucosal folds identified in fish fed SapPhytL diet should be interpreted carefully, however, as the score difference was subtle and not considered of biological significance. The groups fed SapH, PhytL, PhytH and SapPhytH showed increased number of IELs compared to the control. SapH, SapPhytL and SapPhytH treatment resulted in higher degree of variation in vacuole size when compared to the control group, as illustrated by Figure 1. Relative to the control group, the fish fed SapH, PhytH, SapPhytL and SapPhytH showed a significantly higher overall mean score. In general, however, the degree of histomorphological alterations was low and considered to be minor variations from the normal histological appearance of the distal intestinal epithelium.

Table 5 –Gene expression of cluster of differentiation 8 (CD8), T-cell receptor beta (TCRβ), major histocompatibility complex I (MHC-I), major histocompatibility complex II (MHC-II), Immunoglobulin M heavy chain (IgM), Interleukin 1β (IL1β), tumor necrosis factor α (TNFα), caspase 3 (Casp3), transforming growth factor β (TGFβ), proliferating cell nuclear antigen (PCNA), fatty acid binding protein (intestinal) (FABP2), intestinal peptide transporter (PepT1) and Aquaporin 8 (Aqp8) in the distal intestine of sea bream fed the experimental diets for 48 days. Values represent mean fold changes (n=3) relative to means expression levels observed in the control group. Results of the one-way ANOVA (*P*-values) are also provided.

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
CD8	1.0	1.1	0.9	1.0	1.2	0.9	0.8	*0.28*	*0.937*
TCRβ	1.0	1.3	1.1	1.0	1.1	1.2	1.0	*0.22*	*0.949*
MHC-I	1.0	1.1	0.9	1.0	1.3	0.7	0.9	*0.23*	*0.714*
MHC-II	1.0	1.1	1.1	0.6	1.2	1.6	1.1	*0.23*	*0.199*
IgM	1.0	1.1	1.2	1.1	0.9	1.0	0.9	*0.18*	*0.854*
IL1β	1.0	1.1	0.9	0.9	1.0	1.0	0.9	*0.10*	*0.831*
TNFα	1.0	1.3	1.0	0.7	1.3	0.9	0.6	*0.42*	*0.869*
Casp3	1.0	1.0	0.9	0.9	1.2	0.8	0.7	*0.25*	*0.946*
TGFβ	1.0	1.0	0.9	0.9	1.0	1.0	0.8	*0.26*	*0.993*
PCNA	1.0	1.2	1.1	1.0	1.0	1.0	1.1	*0.12*	*0.913*
FABP2	1.0	0.3	0.2	0.1	0.3	0.6	0.2	*0.27*	*0.583*
PepT1	1.0	0.2	0.1	0.2	0.3	1.8	0.2	*0.64*	*0.846*
Aqp8	1.0	1.3	1.0	0.7	1.4	0.7	0.5	*0.50*	*0.994*

[1] Standard Error of the Mean (pooled)

No significant differences between the dietary groups were found in the transcript levels (Table 5) of the immune related genes CD8, TCRβ, MHC-I, MHC-II, IgM, IL1β, TNFα, Casp3 and TGFβ. Although the individual variation was high, the mean values were very similar between groups. No significant differences were found in the gene expression of PCNA, FABP2, PepT1 and Aqp8, markers of intestinal function. However, consistently lower transcript levels of FABP2 and PepT1 were observed in all experimental groups, except SapPhytL, compared to the control (not statistically significant).

Discussion

The finding in the present study that 1-2 g kg^{-1} soy saponins supplemented to a fishmeal-based diet had little effect on juvenile sea bream growth is in accordance with some of the previous works in channel catfish (Twibell and Wilson, 2004), rainbow trout (Penn, 2005) and Atlantic salmon (Chikwati, 2007; Pelissero and Sumpter, 1992; Penn et al., 2012). Based on results of other experiments, soy saponins have been suggested to be responsible for decreased growth in fish fed soybean meal-containing diets (Bureau et al., 1998; Van den Ingh et al., 1996). However, the growth depression effects have been observed only at high levels of saponin inclusion (Chen et al., 2011; Penn et al., 2012) or when combined with other plant ingredients (Chikwati et al., 2012). On the other hand, a growth promoting effect was reported for Atlantic salmon and Japanese flounder fed fish meal or non-legume-based diets with inclusion of 2 - 4 g kg^{-1} (Chikwati et al., 2012; Penn et al., 2012) and 0.8 g kg^{-1} soy saponins (Chen et al., 2011), respectively. In the latter study, the authors suggested that saponins may have exerted an increase in permeability of intestinal cell membranes, thus increasing nutrient absorption. A similar effect could be the explanation for the increased FE observed in the present study in fish fed diets with both antinutrients combined.

Effects of dietary phytosterols in fish have so far not been subject to extensive research despite their presence in plant oils used as fish oil substitutes. Only two reports have been found (Chikwati, 2007; Couto et al., in press). The use of plant oils in gilthead sea bream feeds has often been associated with depressed growth and feed utilization when included at high levels (Benedito-Palos et al., 2008; Dias et al., 2009; Izquierdo et al., 2005; Santigosa et al., 2008). Thus, phytosterols may contribute to this negative effect. However, in the present study, in which the inclusion level of phytosterols (5 and 10 g kg^{-1}) corresponded to a 100% replacement of fish oil by soybean oil in the diets, no negative effect on sea bream growth compared to the control group was evident.

Moreover, the recent study by Watson et al. (2013) reported increased weight gain and feed efficiency in sea bream fed a fish meal-free diet with soybean oil as the lipid source compared to a

group fed a fish meal-free diet with fish oil as the lipid source. In the present trial, improved feed efficiency and protein efficiency ratios in fish fed the diets PhytH, SapPhytL and SapPhytH were observed, and higher growth might have resulted had the trial lasted longer, which appeared to be in line with the results by Watson et al. (2013). The exact phenomenon that may lead to such observations are yet to be scrutinized. It is our conclusion that the antinutrients had little effect on the growth of juvenile sea bream following 48 days of feeding at the levels employed in this study.

The hypocholesterolemic effect of plant ingredients in fish diets has been well described (Dias et al., 2005; Kaushik et al., 1995; Kortner et al., 2013; Kraugerud et al., 2007), also in sea bream (Sitja-Bobadilla et al., 2005). Although the causative agents are yet to be identified, both saponins and phytosterols are candidate antinutrients with the potential to interfere with luminal cholesterol uptake. In the present study, fish exposed to the dietary antinutrients showed a trend towards lower plasma cholesterol levels. Hypocholesterolemia has been described in Japanese flounder (Deng et al., 2012) and Atlantic salmon fed diets supplemented with saponins (Chikwati, 2007; Gu et al., 2013b; Penn et al., 2012). However, a study by Chikwati et al. (2012) demonstrated that addition of saponins to a pea protein concentrate-containing diet resulted in lower growth, lipid digestibility and occurrence of histological alterations in the distal intestine in salmon, but had no effect on plasma cholesterol levels. A cholesterol lowering effect of phytosterols is well described for mammals (Skeaff et al., 2004; Vanstone et al., 2002) and has also been observed in Atlantic salmon fed 5 and 10 g kg^{-1} phytosterols (Chikwati, 2007) or plant oils with high phytosterol levels (Liland et al., 2013), as well as in sea bass fed 5 g kg^{-1} phytosterols (Couto et al., in press). Interestingly in the latter study the high phytosterol dose (10 g kg^{-1}) seemed to have less impact on plasma cholesterol levels, which could be due to a compensatory mechanism, as previously described in Artic charr (Pettersson et al., 2009). These findings are supported by a study with salmon in which genes associated with cholesterol synthesis were up-regulated in fish fed diets containing plant oil (Leaver et al., 2008). The lack of significant differences in plasma cholesterol in the present should be considered in light of the high variation for these results. The observations deserves further investigation as plasma cholesterol levels might have been maintained due to absence of impaired dietary cholesterol absorption or by the triggering of a compensatory mechanism.

The histomorphological changes observed in the present study were very mild and, although statistically significant, the differences were judged to be minor and to represent normal adaptation to changes in diet composition. In previous studies from our laboratories, a fish meal-based diet supplemented with phytosterols (5 or 10 g kg^{-1}) showed no effect in the pyloric caeca either in sea bass (Couto et al., in press) or Atlantic salmon (Chikwati, 2007). Similarly, soy saponin supplementation (up to 10 g kg^{-1}) to both fish and plant meal diets did not produce

histomorphological changes in the pyloric caeca of Atlantic salmon (Gu et al., 2013b). While the increased number of IELs in the pyloric caeca of fish fed 5 g kg^{-1} phytosterols from the present study suggests an immune reaction towards the antinutrients in the diet, the results do not allow convincing conclusions to be made.

In Atlantic salmon, soy saponins have been associated with the onset of morphological changes in the distal intestine known as soybean meal-induced enteritis (Chikwati et al., 2012; Knudsen et al., 2008; Knudsen et al., 2007; Penn et al., 2012). When supplemented to a fish meal based diet in levels up to 4 g kg^{-1}, no histomorphological changes were observed in Atlantic salmon (Knudsen et al., 2008; Penn et al., 2012; Penn et al., 2011). However, 6 - 10 g kg^{-1} of saponins supplemented to a fishmeal-based diet (Penn et al., 2012), 1.07 and 1.63 g kg^{-1} saponins supplemented to a diet containing 250 g kg^{-1} lupin kernel meal (Knudsen et al., 2008), and 2 g kg^{-1} saponins supplemented to a diet containing 310 g kg^{-1} pea protein concentrate (Chikwati et al., 2012) resulted in at least some signs of intestinal inflammation. These studies suggest that soy saponins alone at high levels (>6 g kg^{-1}) can induce inflammatory changes, whereas lower levels (<4 g kg^{-1}) do not, unless combined with saponins or other components present in other plant ingredients. In the present study, the supplementation of saponins to the fish meal-based diet induced higher vacuole size variation in the enterocytes, which is not indicative of any specific event, but constitutes a deviation from the normal cell architecture and can have consequences at a functional level. Similar abnormal enterocyte vacuolization was previously observed in rainbow trout juveniles (Iwashita et al., 2009; Iwashita et al., 2008) fed diets containing 3.8 g kg^{-1} saponins. The ability of saponins to interfere with cellular membranes and increase permeability has been shown in several mammals (Alvarez and Torres-Pinedo, 1982; Gee et al., 1997), as well as fish (Knudsen et al., 2008; Nordrum et al., 2000). The increased membrane permeability may result in impaired barrier function allowing the uptake of substances or microbes that would not normally be absorbed, making the underlying mucosa more exposed to foreign antigens from the gut lumen (Knudsen et al., 2008). The increased number of IELs observed in the distal intestine of sea bream fed high levels of saponins could therefore be a secondary response to disruption of the cell membrane, resulting in decreased barrier function by the enterocytes.

Phytosterols are naturally absorbed by enterocytes but are poorly transported to the blood stream (Chen, 2001; Igel et al., 2003), which could lead to their accumulation inside the cell. Sea bream fed diets with plant oils as lipid sources exhibited lipid droplet accumulation in the enterocytes of the anterior intestine (Caballero et al., 2003; Santigosa et al., 2011) and an impaired lipid transit capacity seems to be responsible for the observed effect. The histological results of the present study did not indicate that phytosterols alone caused accumulation of lipid droplets, however the

increased number of IELs may suggest that the putative dietary load of phytosterols inside the enterocytes were recognized by the tissue as foreign substances, inducing an immune reaction in this intestinal region of sea bream. The same reaction was observed in sea bass juveniles fed diets containing phytosterols at similar levels (Couto et al., in press). When combined with saponins, and possibly helped by their aforementioned membrane permeating action, increased vacuole size was observed, suggesting that phytosterols were more efficiently absorbed in these groups and accumulated to a greater extent inside the absorptive vacuoles. In accordance with the present study, sea bream fed diets with plant protein and oil exhibited increased lipid accumulation in the enterocytes, higher number of immune cells in the mucosa and decreased immune function (Sitja-Bobadilla et al., 2005). These effects could be attributed to the presence of saponins, phytosterols and possibly other compounds present in the complex plant ingredients used.

The genes selected for expression profiling in the present study have been proposed as potential diagnostic markers for intestinal inflammation and associated physiological and metabolic disorders (Bakke-McKellep et al., 2007; Kortner et al., 2012; Krogdahl et al., 2000; Marjara et al., 2012). The finding that sea bream did not develop intestinal inflammation during the period of exposure to the antinutrients is supported by the molecular studies undertaken that showed similar levels of immune related gene transcripts across all dietary groups. The expression of PepT1 and FABP2, on the other hand, although not statistically significant may indicate a loss of function. Low expression levels of PepT1 (solute carrier family 15 member 1) have been reported previously in salmon fed soybean meal (Gu et al., 2013a), as well as in salmon (Kortner et al., 2012) and sea bream (Terova et al., 2013) fed diets with pea protein concentrate, which also contains high levels of saponins. Similarly, low levels of FABP2 expression in the distal intestine were observed in all experimental groups. The decreased expression of these important nutrient transporters could be attributed to a loss of intestinal integrity and metabolic function, and is in line with the decreased carrier-mediated transport observed after soybean meal feeding in salmon (Nordrum et al., 2000). Venold et al. (2013) observed a reduction of FABP2 gene and protein level during the development of soybean meal-induced enteritis in salmon and attributed the findings to a rapid proliferation of the intestinal epithelial cells, as indicated by a concomitant up-regulation of the PCNA coding gene, possibly resulting in larger proportion of immature enterocytes that did not have full functionality. However, in the present study, PCNA was similar in all groups. Therefore, the low levels of PepT1 and FABP2 transcripts seems not to be attributable solely to an immature epithelium, but rather to a different mechanism, possibly a more direct disrupting effect of saponins and phytosterols (alone or in combination) on the intestinal function. The mechanisms by which saponins or/and phytosterols possibly affect the expression PepT1 and FABP2 cannot be interpreted from the present study and

merit further research as they are implied in peptide transport across the enterocyte membrane (PepT1) and fatty acid shuttling in the enterocytes (FABP2) and can, therefore, affect normal nutrient flow and metabolism.

In summary, the results suggested that dietary saponins and phytosterols, alone or in combination, and at the levels tested did not affect sea bream growth performance but may improve feed utilization as shown for fish fed the diets PhytH, SapPhytL and SapPhytH. The histological alterations of the distal intestinal mucosa observed were not characterized as inflammatory, but did suggest some changes in accumulation of substances in the absorptive vacuoles and leukocyte mobilization to the intraepithelial space. However, the mechanisms behind these events could not be understood from the present study and deserve further investigation. The lack of inflammation was supported by gene expression data. Overall, there was some indication of a compromised mucosal barrier, which did not seem to cause any deleterious effect to sea bream in this study, but could potentially render fish more susceptible to reactions towards antigens provided by feed sources or to pathogenic or commensal microbes.

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Chapter 5

Effects of dietary soy saponins and phytosterols on gilthead sea bream (*Sparus aurata*) during the on-growing period

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Abstract

Plant ingredients contain antinutritional factors that may affect fish growth performance and health when included in their diets. The present work aimed at studying the effect of two soy purified antinutrients, saponins and phytosterols, on growth, intestinal morphology and immune response of on-growing sea bream. Fish were fed one of seven diets for 48 days: control diet (fishmeal and fish oil based) and six experimental diets containing low (0.1%) or high (0.2%) levels of soya saponins, low (0.5%) or high (1.0%) levels of phytosterols or a combination of 0.1% saponins +0.5% phytosterols or 0.2% saponins +1.0% phytosterols. At day 15 and at the end of the trial pyloric caeca (PC) and distal intestine (DI) samples were collected for histological evaluation. At the end of the trial plasma was sampled for cholesterol analysis and DI was sampled for gene expression of immune and functional genes. Saponins and phytosterols did not affect fish growth, feed utilisation or plasma cholesterol levels. Histologically only a mild increase in the number of eosinophilic granular cells in the PC after 15 and 48 days of feeding in fish fed 0.1% saponins and 0.1% saponins + 0.5% phytosterols, respectively. Gene expression of immune related genes was not affected by the presence of dietary antinutrients. Sea bream in grow-out phase showed high tolerance for saponins and phytosterols either alone or in combination, thus indicating an ability to cope with dietary plant feedstuffs containing such antinutrients, at least within the concentration range used in the present study.

Keywords: antinutrients; gilthead sea bream; saponins; phytosterols; soya.

Introduction

Cultivation of carnivorous fish species relies heavily on compound feeds, which have traditionally been based on fish meal and fish oil as protein and lipid sources. However, with the paucity of and high competition for such commodities on the global market, the search for alternative protein and lipid sources has become a high priority (FAO, 2012). At present, plant feedstuffs such as soybeans, rapeseed, lupins or peas, are common ingredients in most dietary formulations for cultured fish species. However, most plant feedstuffs contain antinutritional factors (ANFs) that can interfere with the growth, physiology and/or health of the animals (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). Hence, it is vital to ensure that diets containing high plant feedstuff levels still promote efficient growth and feed utilization, have minimal environmental impact, are safe for fish health, produce high quality flesh for human consumption, and are cost-effective. The animal's responses to antinutrients are neither random nor linear, and may depend on several factors such as species, age, size or gender, to name a few.

Plant feedstuffs usually present more than one antinutrient and it is difficult to determine which individual factor is responsible for any adverse effects observed in fish fed diets containing plant-derived ingredients (Francis et al., 2001). For instance, both full-fat and solvent extracted soybean meal (SBM) induced inflammation in the distal intestine of Atlantic salmon (*Salmo salar*) whereas alcohol extracted soy protein concentrate does not cause pathological changes (Van den Ingh et al., 1996; Krogdahl et al., 2010). These studies pointed towards the involvement of one or more of the alcohol-soluble components of full fat soybean meal (such as saponins) in the enteropathy. However, the specific antinutritional factor or factors responsible for the intestinal disorder are yet unknown (Van den Ingh et al., 1996; Krogdahl et al., 2010).

Saponins are a wide group of glycosides, soluble in alcohol and present in soybeans and other legumes. Their amphipathic nature cause effects on cell membranes, which may increase cell permeability, inhibit active transport of nutrients and interfere with membrane homeostasis, allowing the passage of molecules normally not absorbed (Francis et al., 2002). Saponins have high affinity with cholesterol and bile salts and may therefore interfere with lipid digestion and sterol metabolism. Effects of saponins on protein digestion, as well as immune and nervous system functions have also been reported (Francis et al., 2002). In rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*) decreased growth performance has been attributed to the presence of saponins in the diets (de la Higuera et al., 1988; Bureau et al., 1998; Chikwati et al., 2012). Fish fed diets with sub-fractions of soy molasses-containing saponins (Knudsen et al., 2007), lupin kernel meal supplemented with partially purified soya saponins

(Knudsen et al., 2008), or pea protein concentrate supplemented with purified saponins (Chikwati et al., 2012) all resulted in similar intestinal lesions as those induced by soybean meal. The authors suggested that the observed effects were probably due to a combination of saponins and other ANFs present in the plant ingredients rather than to the saponins alone.

Phytosterols are a group of compounds present in the lipidic portion of plants, and may therefore be introduced in fish feeds through replacement of fish oil by plant oils or when using full-fat seed meals. Phytosterols present a sterol-like chemical structure and can compete with cholesterol for enterocyte uptake resulting in increased cholesterol and bile acid losses in the feces, thus increasing conversion of cholesterol to bile acids in the liver and further decreasing plasma cholesterol levels (Ling and Jones, 1995). Data on the effects of dietary phytosterols in fish diets is scarce (Pelissero and Sumpter, 1992; Chikwati, 2007; Miller et al., 2008) and their effects in combination with other antinutrients have not yet been studied. Due to their chemical structure, the interaction between saponins and phytosterols in the intestinal lumen is likely, but responses in fish are not known.

Several studies demonstrated the possibility of replacing fish meal and fish oil with plant feedstuffs in gilthead sea bream (*Sparus aurata*) diets without affecting fish performance (Gomez-Requeni et al., 2004; Sitja-Bobadilla et al., 2005; Benedito-Palos et al., 2007; Benedito-Palos et al., 2008; Bonaldo et al., 2008; Dias et al., 2009; Silva et al., 2010; Kokou et al., 2012). The inclusion levels tolerated by the animals seem to be, among others, dependent on the life stage Martinez-Lorenz et al. (2007) concluded that dietary soybean meal might be included in the diets up to 30% in juveniles and up to 50% in grow-out fish without affecting animal's performance. However, even without compromising fish growth, inclusion of plant feedstuffs can affect immune defense mechanisms and gut integrity (Sitja-Bobadilla et al., 2005; Bonaldo et al., 2008; Montero et al., 2010; Kokou et al., 2012), which may result in late manifestation of pathological conditions or less than optimal health (Krogdahl et al., 2010).

In the present study, we aimed to study the effect of dietary soy saponins and phytosterols (alone or in combination) in on-growing sea bream. These ANFs were selected due to their ability to interfere with cholesterol uptake and metabolism (Sidhu and Oakenfull, 1986; Gee and Johnson, 1988; Lasztity et al., 1998; Romarheim et al., 2008), the role that soybean saponins apparently play in the development of soybean meal-induced enteritis in salmonids (Knudsen et al., 2008; Chikwati et al., 2012), as well as their ability to bind to each other. A comprehensive approach was used, assessing fish zootechnical performance, plasma cholesterol levels and intestinal histomorphology. Gene expression profiling was also performed based on recent studies that identified several potential markers for diet-induced enteropathy, including genes related to inflammatory cytokines, intestinal

barrier function and nutrient transporters (Skugor et al., 2011; Kortner et al., 2012; Marjara et al., 2012; Sahlmann et al., 2013).

Material and methods

Diets

Seven fish meal and fish oil based diets were formulated to contain 450 g kg^{-1} crude protein and 180 g kg^{-1} crude lipid (Table 1). The experimental diets included a control diet without supplementation, and diets containing 1 g kg^{-1}or 2 g kg^{-1} soyasaponins (Organic Technologies, Coshocton, OH, USA, purity 95%) (diets SapL and SapH, respectively), 5 g kg^{-1} or 10 g kg^{-1} phytosterols (Derive Resiniques et Terpeniques, Dax, France, purity >99%) (diets PhytL and PhytH, respectively), or a mixture of 1 g kg^{-1} saponins + 5 g kg^{-1} phytosterols or 2 g kg^{-1} saponins + 10 g kg^{-1} phytosterols (diets SapPhytL and SapPhytH, respectively). All dietary ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). 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), which induce effects in Atlantic salmon (Chikwati, 2007; Chikwati et al., 2012). For the phytosterols, somewhat higher levels were chosen, corresponding to dietary inclusion levels of about 500 and 1000 g kg^{-1} of soybean oil or 250 and about 500 g Kg^{-1} of rapeseed oil (Piironen et al., 2000), levels which, based on results of preliminary studies with Atlantic salmon, were expected to cause weak but distinct effects when supplemented alone (Chikwati, 2007). The phytosterol preparation was commercially available, made from pine and produced to serve as functional additive in margarine for human consumption, purportedly for its cholesterol-lowering effects. 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.

Growth trial

The present experiment was directed by trained scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Table 1 – Composition and proximate analysis of the experimental diets.

	Experimental diets						
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**
Ingredients (g kg^{-1} dry weight)							
Fish meal[1]	588	588	588	588	588	588	588
Soluble fish protein concentrate[2]	50	50	50	50	50	50	50
Cod liver oil	112	112	112	112	112	112	112
Pre-gelatinized starch[3]	210	209	208	205	200	204	198
Vitamin premix[4]	10	10	10	10	10	10	10
Mineral premix[5]	10	10	10	10	10	10	10
Choline chloride (50%)	5	5	5	5	5	5	5
Binder[6]	10	10	10	10	10	10	10
Chromium oxide	5	5	5	5	5	5	5
Soy saponins[7]	-	1	2	-	-	1	2
Phytosterols[8]	-	-	-	5	10	5	10
Proximate analysis (g kg^{-1} dry matter)							
Dry matter	926.2	922.3	917.2	927.1	922.2	920.5	916.1
Crude protein	450.5	449.9	438.6	453.7	452.5	454.9	456.6
Crude fat	174.8	176.0	171.0	185.1	192.0	180.4	187.0
Starch	188.9	192.6	198.4	170.2	160.2	173.4	166.7
Ash	139.2	141.4	134.9	140.0	138.6	137.3	138.4
Gross energy (KJ g^{-1} DM)	21.2	21.2	21.0	21.9	22.3	21.7	21.8

[1] Vacuum Dried LT. Pesquera Diamante, S. A. Peru
[2] G-Special. Sopropêche, France
[3] C-Gel Instant - 12016, Cerestar, Mechelen, Belgium
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU k^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet); dibasic calcium phosphate, 5.9 (g kg^{-1} diet).
[6] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England
[7] Soy saponins concentrate (95% purity) produced by Organic Technologies (Ohio, USA)
[8] Phytosterol extract produced from pine by les Derive Resiniques et Terpeniques (DRT) (France), Purity: >99% pure; including β-sitosterol: 77.3%, β-sitostanol 10.7%, campesterol: 8.8%, other: 2.2%.

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 12h:12h light:dark photoperiod was adopted, dissolved oxygen was maintained at 95%, water temperature was 25±0.5ºC and salinity averaged 35±1‰. Gilthead sea bream (*Sparus aurata*) 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 15 fish with mean body weight of 112 ±5 g were randomly distributed to each tank. Diets were randomly assigned to triplicate groups of these fish. During the trial, fish were fed by hand to apparent satiation two times a day, six days a week. Fish were bulk weighed every two weeks after one day of food deprivation.

Sampling

After two weeks of feeding the experimental diets, 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 24h and subsequently transferred to 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 x g for 10 minutes. Plasma samples were stored at -20ºC until analysis. After blood collection, fish were euthanized, dissected and intestinal samples collected for histological evaluation as described above. Furthermore, a section of distal intestine was also collected for gene expression profiling. These samples were gently rinsed in PBS, blotted dry, placed in RNAlater® (25 mM Sodium Citrate; 10 mM EDTA; 70 g ammonium sulfate/100 ml solution; pH 5.2; 1:10 w/v), left at 4ºC overnight and subsequently stored at -20ºC until analyses.

Chemical Analysis

Diets were analysed for dry matter, ash, protein, lipids and energy. The following procedures were used: dry matter after drying at 105ºC until constant weight; ash by incineration in a muffle furnace at 450ºC for 16 h; protein content (N x 6.25) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid by petroleum ether extraction using a Soxtec HT System (Tecator, Hoganas, Sweden); Starch according to Thivend et al. (1972); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA). Plasma cholesterol was determined using a cholesterol kit (ref. 1001092 Spinreact, Girona, Spain).

Histology

Intestinal samples were processed and sectioned using standard histological techniques. Sections were stained with hematoxylin and eosin. Blinded evaluation was performed with particular attention to any inflammatory changes (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003), namely shortening, widening and fusion of intestinal folds, changes in enterocyte supranuclear absorptive vacuolization, connective tissue hyperplasia, and infiltration of inflammatory cells. A continuous scale scoring system was used as described by Penn et al. (2011) with the range of tissue scores set at 0-5. The overall value of enteritis degree was calculated by averaging scores of the separate parameters described above.

Gene expression

Quantitative real-time PCR (qPCR) was used to analyze the mRNA expression of previously reported markers for intestinal inflammation and function, including immunoglobulin M heavy chain (IgM), cluster of differentiation 8 (CD8), T-cell receptor beta (TCRβ), Interleukin 1β (IL1β), transforming growth factor β (TGFβ), major histocompatibility complex I (MHC-I), major histocompatibility complex II (MHC-II), tumor necrosis factor α (TNFα), caspase 3 (Casp3), proliferating cell nuclear antigen (PCNA), fatty acid binding protein (intestinal; FABP2), intestinal peptide transporter (PepT1) and Aquaporin 8 (Aqp8) (Krogdahl et al., 2000; Skugor et al., 2011; Kortner et al., 2012; Marjara et al., 2012; Terova et al., 2013; Venold et al., 2013). Previously validated and published gene-specific primers (Table 2) were used to quantify TCRβ, MHC-II (Cerezuela et al., 2012), IgM (Estensoro et al., 2012), IL1β (Sitja-Bobadilla et al., 2008), FABP2 (Calduch-Giner et al., 2012), for the remaining genes primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3).

Table 2 - Primer pair sequences, efficiency, amplicon size and annealing temperature for genes used for real-time PCR. Grey shaded rows correspond to reference genes.

	5´- 3´ primer sequence						
Gene symbol	Forward	Reverse	**Amplicon size (BP)**	**Annealing temp. (°C)**	**Primer efficiency**	**Genbank accession nr.**	**Reference**
CD8	GGACAAGTCTGGCATGGAAT	GTGGACGACGGAGACTTTGT	81	60	1.82	[AJ878605]	
TCRβ	AAGTGCATTGCCAGCTTCTT	TTGGCGGTCTGACTTCTCTT	130	60	2.00	[AM261210]	Cerezuela et al 2012
MHC-I	CCCTCAGTGTCTCTCCTCCA	CAGACGAGTGAGGCTCTGTG	89	60	1.97	[DQ211540]	
MHC-II	CTGGACCAAGAACGGAAAGA	CATCCCAGATCCTGGTCAGT	176	62	1.86	[DQ019401]	Cerezuela et al 2012
IgM	TCAGCGTCCTTCAGTGTTTATGATGCC	CAGCGTCGTCGTCAACAAGCCAAGC	131	62	1.93	[JQ811851]	Itziar-Estensoro et al 2012
IL1β	GCGACCTACCTGCCACCTACACC	TCGTCCACCGCCTCCAGATGC	133	60	1.80	[AJ277166]	Sitja-Bobadilla et al 2008
TNFα	CAGGCGTCGTTCAGAGTCTC	GAGATCCTGTGGCTGAGAGG	84	62	1.96	[AJ413189]	
Casp3	CCAGTCAGTCGAGCAGATGA	GAACACACCCTCGTCTCCAT	113	60	1.89	[EU722334]	
TGFβ	AAACCAACTCGCTACCATGC	CTCCAGAGCCTGAGGAACAC	152	60	2.00	[AF424703]	
PCNA	GATGTGGAGCAGCTGGGTAT	TGTCTACGTTGCTGGTCTGG	205	60	1.92	[FG263675]	
FABP2	CGAGCACATTCCGCACCAAAG	CCCACGCACCCGAGACTTC	93	60	1.97	[AM957164]	Calduch-Giner et al 2012
PepT1	CTACCCACTGATCGCCAAGT	GATGGGAACGTAGGCAGTGT	141	60	2.00	[GU733710]	
Aqp8	AGTTGGGACGTCTCTCGTGT	CTCGATGACCGACACACAAC	147	60	1.99	[DQ889225]	
EF1α	ACGTGTCCGTCAAGGAAATC	GGGTGGTTCAGGATGATGAC	109	60	2.00	[AF184170]	
GAPDH	CCAACGTGTCAGTGGTTGAC	AGCCTTGACGACCTTCTTGA	80	60	2.00	[DQ641630]	
Rps18	AGGGTGTTGGCAGACGTTAC	CGCTCAACCTCCTCATCAGT	97	60	1.96	[AM490061]	

All chosen primers pair concentrations used at the optimal annealing temperatures gave a single band pattern for the expected amplicon of interest in all reactions. Total RNA was extracted from DI tissue samples (~50 mg) using Trizol reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA) and purified with Direct-zol ™ RNA Miniprep (Zymo Research, Irvine, CA, USA) including an on-column DNase treatment according to the manufacturer's protocol. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Fisher Scientific, Hampton, NH, USA). Integrity of the RNA samples was evaluated by running an aliquot of the RNA sample on a 1% agarose gel. First strand cDNA synthesis was performed using 1.0 µg total RNA from all samples using Superscript III (Invitrogen™, Life Technologies, Carlsbad, CA, USA) in 20µL reactions, and primed with Oligo $(dT)_{20}$ primers

according to the manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at −20°C.

The expressions of individual gene targets (n=8 fish/diet) were analyzed using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Each 10 µL DNA amplification reaction contained 2 µl of PCR-grade water, 5 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics), 2 µL of 1:10 diluted cDNA template and 0.5 µl (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control and an inter-run plate calibrator. The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (10 s), 60-62°C (depending on the primers used, see Table 2, 10 s) and 72°C (15 s). For determination of the quantification cycle (C_q) the "second derivative maximum method" measuring maximum increase rate of newly synthesized DNA per cycle was used on the basis of the LightCycler 480 software release 1.5.0 (Roche Diagnostics). To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of PCR products after each run by agarose gel electrophoresis. PCR reaction efficiency (E) for each gene assay was determined using 10-fold serial dilutions of randomly pooled cDNA. R^2 values of the standard curves were all >0.99. Elongation factor 1α (EF1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein s18 (Rps18) were evaluated for use as reference genes, by ranking relative gene expression according to their stability as described previously (Kortner et al., 2011). Rps18 was found to be stably expressed and was therefore used as normalization factor. Mean normalized expression of the target genes were calculated from raw C_q values using a plate calibrator-normalized relative quantification (Pfaffl, 2001) and expressed as fold changes relative to the control group.

Statistics

Results were analyzed using IBM SPSS 21 software package (SPSS® Inc, Chicago, IL, USA). The experimental unit considered was the tank (n=3) represented by the average of 3 fish per tank. Data was tested for normality and homogeneity (Shapiro-Wilk and Levene tests, respectively) and when necessary, data was transformed to achieve ANOVA assumptions. Results were analyzed by one-way ANOVA followed by orthogonal contrasts as follows: the control was compared to each of the experimental diets, SapL was compared to SapPhytL, and SapH was compared to SapPhytH (to assess possible cumulative effects of mixing antinutrients) whenever statistical differences were found. Histological data could not be normalized, thus the Kruskal-Wallis non-parametric test and

specific pairwise comparison (as described above) was performed. Differences were considered statistically significant at $p < 0.05$.

Results

Fish promptly accepted all experimental diets and during the trial no mortality was recorded. Fish growth parameters and nutrient utilization are presented in Table 3. Daily growth index (DGI) was within the normal range for on-growing sea bream and averaged 1.94 ± 0.03 (SEM). The inclusion of saponins or phytosterols in sea bream diets had no significant effect on fish growth, feed intake or feed utilization. Saponins and phytosterols alone or in combination had no effect on plasma cholesterol levels (Table 3).

Table 3 – Mean values (n=3) for growth parameters, feed intake, feed utilization and plasma cholesterol levels of sea bream (initial body weight = 112 ± 5g) fed the experimental diets for 48 days and results of the one-way ANOVA (*P*-values).

	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
Final body weight (g)	197	187	189	186	195	193	194	*5.37*	*0.722*
DGI[2]	2.04	1.82	1.89	1.86	2.06	1.95	1.96	*0.08*	*0.311*
Feed intake (g DM per fish)	117	106	114	116	119	121	121	*4.40*	*0.501*
FE[3]	0.85	0.85	0.82	0.81	0.81	0.75	0.82	*0.04*	*0.258*
PER[4]	1.91	1.90	1.86	1.78	1.79	1.64	1.79	*0.08*	*0.373*
Cholesterol (mmol L^{-1})	8.25	8.33	9.17	9.29	7.27	7.84	9.17	*0.66*	*0.292*

[1] Standard Error of the Mean (pooled)
[2] Daily growth index: $((IBW^{1/3} - FBW^{1/3})/days) \times 100$
[3] Feed efficiency: weight gain: (g fresh weight)/feed intake (g dry matter)
[4] Protein efficiency ratio: weight gain (g fresh weight)/protein intake (g dry matter)

On day 15, pyloric ceca (PC) showed increased number of eosinophilic granular cells (EGCs; Table 4) when sea bream were fed the SapL diet (2.89) compared to the control group (2.50). The overall mean score of the histomorphological alterations was higher in the PhytL group (1.68) compared to fish fed the control diet (1.48).

Table 4 - Score-based evaluation of the intestinal histology (pyloric caeca, PC and distal intestine, DI) of sea bream fed the experimental diets for 15 and 48 days, based on changes observed in mucosal fold heights[1], width and cellularity of the lamina propria[2] and submucosa[3], the number of intraepithelial lymphocytes (IELs)[4] and eosinophilic granular cells (EGCs)[5], nucleus position within the enterocytes[6], and variation of enterocyte vacuolization[7]. A mean score was calculated from each of the above-mentioned characteristics. The values are means of the scores (n=3) per diet. Pooled standard error of the mean (SEM) and results of Kruskal-Wallis (P-values) are given.

			Experimental diets								
Time	Section		**Control**	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhyt**	**SapPhyt**	*SEM*[1]	*P-values*
15 days	PC	Mucosal folds[1]	1.06	1.00	1.00	1.06	1.00	1.00	1.00	*0.03*	*0.511*
		Lamina propria[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.00*	*1.000*
		Submucosa[3]	1.00	1.22	1.00	1.42	1.00	1.67	1.00	*0.11*	*0.058*
		IELs[4]	2.78	3.22	2.94	3.33	2.83	3.11	2.78	*0.05*	*0.090*
		EGCs[5]	2.50[a]	2.89[b]	2.67[ab]	2.86[ab]	2.72[ab]	2.86[ab]	2.72[ab]	*0.05*	*0.046*
		Nucleus position[6]	1.00	1.00	1.00	1.11	1.00	1.00	1.00	*0.04*	*0.423*
		Vacuolization[7]	1.0	1.0	1.7	1.0	1.0	1.0	1.2	*0.15*	*0.120*
		Mean score	1.48[a]	1.62[ab]	1.61[ab]	1.68[b]	1.51[ab]	1.59[ab]	1.53[ab]	*0.03*	*0.035*
	DI	Mucosal folds[1]	1.00	1.00	1.11	1.06	1.11	1.06	1.19	*0.08*	*0.572*
		Lamina propria[2]	1.00	1.11	1.11	1.06	1.17	1.08	1.08	*0.07*	*0.709*
		Submucosa[3]	1.00	1.11	1.11	1.06	1.11	1.06	1.08	*0.06*	*0.702*
		IELs[4]	2.50	2.44	2.56	2.59	2.56	2.56	2.53	*0.11*	*0.211*
		EGCs[5]	2.50	2.50	2.50	2.44	2.61	2.56	2.64	*0.07*	*0.416*
		Nucleus position[6]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.00*	*1.000*
		Vacuolization[7]	1.0	1.2	1.6	1.4	1.8	1.2	1.4	*0.24*	*0.318*
		Mean score	1.43	1.48	1.56	1.56	1.62	1.50	1.57	*0.04*	*0.108*
48 days	PC	Mucosal folds[1]	1.00	1.04	1.00	1.00	1.00	1.12	1.00	*0.05*	*0.533*
		Lamina propria[2]	1.00	1.00	1.00	1.12	1.67	1.00	1.12	*0.10*	*0.089*
		Submucosa[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.04	*0.02*	*0.462*
		IELs[4]	2.7	3.2	2.9	3.2	2.7	3.1	2.7	*0.13*	*0.112*
		EGCs[5]	2.50[a]	2.79[ab]	2.68[ab]	2.87[ab]	2.71[ab]	2.83[b]	2.71[ab]	*0.04*	*0.047*
		Nucleus position[6]	1.00	1.00	1.00	1.00	1.00	1.00	1.04	*0.02*	*0.462*
		Vacuolization[7]	1.1	1.3	1.5	1.1	1.3	1.4	1.6	*0.22*	*0.611*
		Mean score	1.48	1.61	1.57	1.62	1.63	1.64	1.62	*0.04*	*0.203*
	DI	Mucosal folds[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.08	*0.03*	*0.462*
		Lamina propria[2]	1.04	1.12	1.08	1.19	1.08	1.04	1.12	*0.06*	*0.665*
		Submucosa[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.04	*0.02*	*0.462*
		IELs[4]	2.5	2.6	2.6	2.9	2.9	2.8	2.7	*0.14*	*0.595*
		EGCs[5]	2.37	2.54	2.58	2.62	2.62	2.58	2.67	*0.04*	*0.076*
		Nucleus position[6]	1.00	1.00	1.08	1.00	1.00	1.00	1.17	*0.04*	*0.170*
		Vacuolization[7]	1.00	1.9	1.2	1.2	1.1	1.6	1.7	*0.16*	*0.108*
		Mean score	1.42	1.59	1.5	1.56	1.52	1.57	1.64	*0.04*	*0.066*

[1] Standard error of the mean (pooled)

Differing superscript letters in the same line indicate significant differences between the dietary treatments ($P < 0.05$) as assessed by Kruskal-Wallis allpairwise test.

At the end of the trial, the number of EGCs was higher in the PC of fish fed the SapPhytL diet (2.83) compared to the control group (2.50). The overall mean score, however, showed no significant differences between dietary treatments. Overall, the degree of histomorphological alterations was low and reflected only minor variations from the normal histological appearance of the PC epithelium. Although statistically significant in a few cases, the differences found were carefully interpreted as the score difference was subtle and seemed to lack true biological significance. Thus dietary saponins and phytosterols, either alone or in combination, were not considered to have significant histological effects on the PC or distal intestine of the fish, and were considered similar to the control group (Table 4) on both day 15 and at the end of the feeding trial.

Table 5 –Gene expression of cluster of differentiation 8 (CD8), T-cell receptor beta (TCRβ), major histocompatibility complex I (MHC-I), major histocompatibility complex II (MHC-II), Immunoglobulin M heavy chain (IgM), Interleukin 1β (IL1β), tumor necrosis factor α (TNFα), caspase 3 (Casp3), transforming growth factor β (TGFβ), proliferating cell nuclear antigen (PCNA), fatty acid binding protein (intestinal) (FABP2), intestinal peptide transporter (PepT1) and Aquaporin 8 (Aqp8) in the distal intestine of sea bream fed the experimental diets for 48 days. Values represent fold changes relative to means expression levels observed in the control group. Results of the one-way ANOVA (*P*-values) are also provided.

Gene	Experimental diets								
	Control	**SapL**	**SapH**	**PhytL**	**PhytH**	**SapPhytL**	**SapPhytH**	*SEM*[1]	*P-values*
CD8	1.0	1.0	0.8	1.0	1.1	0.8	0.8	*0.17*	*0.782*
TCRβ	1.0	0.8	0.7	1.2	0.9	0.9	1.0	*0.18*	*0.646*
MHC-I	1.0	1.1	1.3	1.5	1.3	1.1	0.7	*0.16*	*0.081*
MHC-II	1.0^{ab}	1.1^{ab}	0.8^{ab}	1.7^{b}	1.1^{ab}	1.3^{ab}	0.4^{a}	*0.21*	*0.024*
IgM	1.0	0.6	0.9	0.9	0.8	0.7	0.5	*0.18*	*0.210*
IL1β	1.0^{ab}	0.8^{a}	1.7^{b}	1.2^{ab}	1.4^{ab}	1.2^{ab}	0.9^{ab}	*0.13*	*0.044*
TNFα	1.0	1.2	1.0	1.3	1.1	1.2	0.7	*0.26*	*0.784*
Casp3	1.0	1.1	1.2	1.2	1.0	0.9	0.8	*0.09*	*0.086*
TGFβ	1.0	1.1	1.3	1.2	0.9	0.8	1.0	*0.11*	*0.120*
PCNA	1.0	0.9	1.2	1.2	1.1	1.0	1.0	*0.15*	*0.735*
FABP2	1.0	0.6	0.5	0.5	0.2	0.4	0.5	*0.33*	*0.404*
PepT1	1.0^{a}	0.9^{a}	2.1^{b}	1.4^{ab}	1.0^{a}	1.3^{ab}	1.0^{a}	*0.15*	*0.019*
Aqp8	1.0	0.9	0.5	0.9	1.2	1.5	0.3	*0.89*	*0.892*

[1] Standard Error of the Mean (pooled)

No differences were found on the transcript levels (Table 5) of the immune related genes CD8, TCRβ, MHC-I, IgM, TNFα, Casp3 or TGFβ, or on the functional genes PCNA, FABP2 and Aqp8. Fish from the PhytL group, however, showed higher MHC-II expression when compared to the control group. The SapH diet promoted higher IL1β gene expression than the control and SapPhytH

diets. Nevertheless, when comparing to the control group, supplementation of saponins or phytosterols had no effect on the transcript levels of the investigated immune-related genes.

The gene PepT1, encoding the intestinal peptide transporter 1 and considered a functional intestinal marker, showed higher expression levels in fish fed the SapH diet than the control and SapPhytH fed groups.

Discussion

Results of the present study indicate that on-growing phase tolerate the presence of saponins and / or phytosterols in the diets within the expected range of values in practical diets including vegetable feedstuffs.

Dietary saponins and phytosterols had no clear effect on sea bream zootechnical performance either in terms of growth or feed utilization. These observations are in agreement with results of similar studies in other species such as rainbow trout (Penn, 2005), channel catfish (Twibell and Wilson, 2004) and salmon (Krogdahl et al., 1995) fed diets containing saponins. However, the effects of saponins on fish growth are yet to be fully understood as diverging results are often found in the literature. For instance, Japanese flounder (*Paralichthys olivaceus*) fed a FM-based diet supplemented with 0.32% or higher levels of soya saponins showed reduced growth while supplementation of 0.08% saponins resulted in higher growth compared to a saponin-free diet (Chen et al., 2011). In Atlantic salmon fed a FM-based diet with 0.2 or 0.4% inclusion of saponins, a similar growth promoting effect was observed whereas the same saponin supplementation to a plant-based diet, did not affect growth (Penn et al., 2012). Also in salmon, addition of 0.2% soya saponins to a diet containing high levels of pea protein concentrate resulted in decreased growth (Chikwati et al., 2012). These studies suggest that the diverging effects of saponin supplementation seem to be related to variable doses of saponins in the diets. Additionally, cumulative effects of saponins and/or interaction with other antinutrients present in the complex diets is a possible scenario. In the present study saponins were added to a simple diet with protein provided only by fish meal and in that matrix saponins did not have a pronounced effect in sea bream performance.

Vegetable oils (VO) have variable amounts of phytosterols (Piironen et al., 2000), which are known to affect cholesterol metabolism (Hicks and Moreau, 2001) and interfere with lipid digestion, thus having the potential to affect fish growth. However, although several studies aiming to partially replace fish oil (FO) by VO in sea bream diets reported decreased growth and feed efficiency when high VO inclusion levels were used (Izquierdo et al., 2005; Benedito-Palos et al., 2008; Santigosa et

al., 2008; Dias et al., 2009) this was most likely related to essential fatty acids deficiencies rather than dietary phytosterol levels. Indeed, dietary phytosterols were shown not to affect growth of Atlantic salmon and sea bass (Chikwati, 2007; Couto et al., in press). Similarly, in the present study, the inclusion of phytosterols at levels corresponding to 100% replacement of FO by soybean oil had no effect on sea bream growth, thus reinforcing the assumption that phytosterols are not responsible for the decreased zootechnical performance observed in the mentioned fish oil replacement studies. A grate degree of variation was observed in the present work and it could be responsible for masking effects of the antinutrients as diets SapL, SapH and PhytL resulted in reduced growth although not statistically significant. To proper understand the effect of saponins and phytosterols in sea bream, longer studies using more complex diets would be of interest.

Inclusion of plant protein sources have been described to lower plasma cholesterol levels in several fish species such as Chinook salmon (Kennish et al., 1992), rainbow trout (Kaushik et al., 1995), Atlantic salmon (Refstie et al., 1999), common carp (Hossain et al., 2001), sea bass (Dias et al., 1997; Robaina et al., 1999) as well as sea bream (Sitja-Bobadilla et al., 2005). Both saponins and phytosterols have the potential to interfere with luminal cholesterol uptake and are therefore liable candidates for the observed effects.

Hypocholesterolemia has been observed in response to soy saponin supplementation in salmonid diets (Chikwati, 2007; Penn et al., 2012; Gu et al., 2013b). The ability of saponins to interact with cholesterol and thus increase faecal loss is the putative cause for the hypocholesterolemia associated with saponins. However, fish have been shown to have the ability to regulate cholesterol metabolism to compensate for the excessive loss, thus regulating cholesterol levels in the plasma (Leaver et al., 2008). Such mechanisms may explain why saponins *per se* did not lower plasma cholesterol levels in the present study. Similarly, in Atlantic salmon soya saponins (0.2%) included in diets containing plant protein sources also had no hypocholesterolemic effect, irrespective of diet composition (Chikwati et al., 2012).

The hypocholesterolemic effect of phytosterols has been described in mammals (Vanstone et al., 2002; Skeaff et al., 2004). In fish hypocholesterolemia was observed in Atlantic salmon fed diets with 0.5 and 1.0% phytosterols (Chikwati, 2007) or VO with high phytosterol levels (Liland et al., 2013) and in sea bass juveniles fed a FM based diet with 0.5% phytosterols supplementation (Couto et al., in press). In on-growing sea bream from the present study plasma cholesterol levels in fish fed the ANF-suplemmented diets was not significantly different from the control group, which may suggest either a higher tolerance of the animals to the deleterious effects of the ANFs or more efficient cholesterol regulatory mechanisms. The putative interaction between phytosterols and

saponins was also not reflected in plama cholesterol values. However, the low cholesterol levels observed in groups PhytH and SapPhytL, althoug not statistically significant should be taken in consideration as the high variation may have masked potential effects. Thus, from the present data set it is not possible to conclude whether on-growing sea bream have high tolerance to the ANFs or are able to regulate cholesterol metabolism to compensate for increased cholesterol losses.

Replacement of fish meal by soybean meal in Atlantic salmon diets has been associated with the occurrence of intestinal inflammation mainly in the distal segment of the gut (van den Ingh et al., 1991; Olli et al., 1994; Olli et al., 1995; Baeverfjord and Krogdahl, 1996; Refstie et al., 2001; Opstvedt et al., 2003). This intestinal disorder has also been observed when using other plant ingredients (Penn et al., 2011; Chikwati et al., 2012; Kortner et al., 2012). Other species may also develop enteritis when fed soy-containing diets (Burrells et al., 1999; Uran et al., 2008; Hedrera et al., 2013). Sea bream fed a diet containing 30% soybean meal showed widening of the lamina propria due to cell infiltration (Bonaldo et al., 2008), a feature also described in the enteritis model in salmon (Krogdahl et al., 2003). The causative agents of such distal intestinal alterations have not yet been fully identified; however saponins seem to play an important role in the onset of this inflammation. Knudsen et al. (2008) verified that soya saponins (1.7 and 2.6%) in combination with one or several unidentified components present in lupin meal, induced an inflammatory reaction in the distal intestine in Atlantic salmon. Additional studies in salmon have reported the appearance of inflammatory signs when fish were fed plant-based diets including soya saponins at levels as low as 0.2%, but only when combined with certain plant ingredients such as pea protein concentrate (Chikwati et al., 2012; Kortner et al., 2012), and when soya saponins were included in the diets above 0.6%, irrespective of whether the diet was plant or fish meal-based (Penn et al., 2012). The present study further supports that saponins alone, when present in levels corresponding to ~ 30% dietary soybean meal, do not seem to be responsible for any histomorphological alterations previously reported by Bonaldo et al. (2008), but may have contributed to the onset / progression of the disorder. Phytosterols, on the other hand, did not produce any adverse effects on the intestinal morphology of Atlantic salmon (Chikwati, 2007).

Moreover, in sea bream fed diets containing both protein and lipids of plant origin, increased lipid accumulation in the enterocytes, increased leukocytes numbers and decreased immune function has been documented (Sitja-Bobadilla et al., 2005). In the present study, although sometimes statistically significant, histological alterations were minor and did not allow clear conclusions. However, the increased number of eosinophilic granular cells (EGCs) in the pyloric intestine of fish fed SapL diet at day 15 and of fish fed the SapPhytL diet at the end of the trial, may indicate an immune reaction. EGCs present in the intestinal tissue may compraise mast cells (MCs) or

acidophilic granulocytes (ACs) recruited from circulation, particularly in the presence of an inflammatory event, as in sea bream both MC and AC contain eosinophilic granules in their cytoplasm (Lui et al., 2013). ACs are believed to be involved in the regulation of the inflammatory response (Sepulcre et al., 2007; Lui et al., 2013) by production of IL1β upon degranulation (Sepulcre et al., 2007). Additionally, a study by Cuesta et al. (2006) found that sea bream ACs expressed MHCII, suggesting a role as antigen-presenting cells. In the present study, the expression levels of MHCII and IL1β were higher in fish fed PhytL and SapH diets, respectively. The development of soybean-induced enteritis is characterized by a number of events occuring at various levels of biological organization. The involvement of T-cells in the intestinal inflammatory response of Atlantic salmon was indicated by immunohistochemical detection of CD3ε-positive cells in the inflamed distal intestine with concomitant transcriptional up-regulation of T cell-specific molecules CD3, CD4, CD8α and CD8β in the same tissue (Bakke-McKellep et al., 2007). Transcriptomic approaches have further identified a number of potential molecular markers of gut inflammatory response and dysfunction during diet-induced inflammation, including genes related to pro- and anti-inflammatory cytokines (e. g. IL-1β, TGFβ, IL-17, IL-10), intestinal barrier function, and nutrient transporters (e. g. aquaporin, PepT1) (Uran et al., 2008; Skugor et al., 2011; Kortner et al., 2012; Marjara et al., 2012; Sahlmann et al., 2013). In common carp, an omnivorous species, recovery from the inflammatory process after 5 weeks of feeding soybean meal containing diets (20%) was described at the histomorphological level and characterized by an up-regulation of IL1β and TNFα (Uran et al., 2008). In the present study, most of the immune genes displayed relatively stable expression levels, which is in line with the general lack of histopathological changes.

Down regulation of PepT1 (solute carrier family 15 member 1) has been reported previously in salmon fed diets with soybean meal (Gu et al., 2013a) or pea protein concentrate and saponins (Kortner et al., 2012), and in both studies, fish presented marked intestinal inflammation. Such observations were associated with the apparent functional loss, which accompanies the inflammatory response. In our study PepT1 was up- regulated in fish fed the SapH diet compared to the control and SapPhytH groups. A recent study by Terova et al. (2013) described abundant expression of PepT1 in the pyloric caeca and proximal intestine whereas low expression levels were found in the stomach and in the distal intestine. The same low expression levels were found in the present study, which indicates that PepT1 is more functionally important in the proximal parts of the sea bream intestine.

In conclusion, this work indicated that sea bream in grow out phase seemed to tolerate well dietary soy saponins and phytosterols, alone or in combination, and little effects were observed at the

zootechnical, histomorphological or molecular level. The immune gene expression profiles from fish fed the ANF-containing diets were similar to the control, further supporting maintenance of the animal's health and immune condition. Due to the short duration of the study it is not prudent to affirm that saponins and phytosterols have no effect in sea bream and that high levels of plant ingredients can be included in this species diets. The diversity of effects of dietary soya saponins and phytosterols reported in fish advice that further studies are carried out as responses appear to vary greatly depending on combination of plant ingredients or antinutrients, fish species, dosage and duration of exposure.

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Chapter 6

General discussion and final considerations

Overall, the present thesis allowed the following conclusions:

Within the levels tested, growth of sea bass juveniles or on-growing fish was not compromised by the dietary inclusion of phytosterol and saponins, either alone or in combination, although the two antinutrients seemed to cause some gastrointestinal dysfunction that may stress the animals, possibly rendering them more susceptible to other stressors.

In sea bass juveniles, dietary phytosterol had different effects depending on inclusion level. At 5 g kg^{-1} in the diet, phytosterols seemed to lower cholesterol plasma levels and to increase the relative weight of the distal intestine without inducing overt inflammatory changes. In contrast, at 10 g kg^{-1} in the diet, phytosterols did not affect plasma cholesterol levels, but caused mild inflammatory changes in the distal intestine.

Also in sea bass juveniles, saponins at the higher (2 g kg^{-1}) dietary inclusion level reduced the activity of brush border membrane enzymes in the distal intestine, and also appeared to cause some signs of mild inflammatory changes in the distal intestine.

On growing sea bass fed the dietary antinutrients did not exhibit significant alterations of histomorphology, immune status or digestive function. However, the increased variability observed in sampled fish at the end of the feeding trial suggested that the response to the dietary stimuli was subject to great individual variation.

Sea bream juvenile's performance was also not affected by dietary saponins and / or phytosterols at the levels tested. Interestingly, improved feed utilization was observed in fish fed the diets PhytH, SapPhytL and SapPhytH and such effect deserves to be further evaluated.

In sea bream juveniles, some alterations of the distal intestinal mucosa due to the ANFs present in the diets were observed, which suggested abnormal accumulation of substances in the absorptive vacuoles and concomitant leukocyte mobilization to the intraepithelial space. Saponins and phytosterols did not, however, induce distal intestine enteritis, as supported by gene expression and histological data. Overall, there were some indications of a compromised mucosal barrier, which per se did not seem to cause deleterious effect to sea bream, but could render the fish more susceptible towards antigens provided by feed or by commensal microbes.

Sea bream during the on-growing phase seemed to tolerate the levels of ANFs tested and negligible effects were observed at the zootechnical, histomorphological or molecular level. The immune gene expression profiles of fish fed the ANF-containing diets were similar to the control, further supporting the maintenance of animal's health and immune condition. However, due to the short duration of the trials it is not prudent to conclude that saponins and phytosterols have no effects in sea bream.

Up to now, most studies on this issue have been dedicated to salmonids. Salmon fed a fishmeal diet supplemented with low levels of soy saponins presented increased growth (Penn et al., 2012), while when higher supplementation levels were tested growth depression was observed. The study by Penn et al. (2012) suggest that fish have a tolerance threshold for ANF levels and above that level negative effects on growth performance are observed. Thus, just considering zootechnical

performance, our results seem to indicate that sea bream have a higher tolerance for the ANFs tested than sea bass.

Plasma cholesterol levels have often been found depleted in sea bream and sea bass fed diets containing plant ingredients (Gomez-Requeni et al., 2004; Sitja-Bobadilla et al., 2005). Both saponins and phytosterols have the ability to interfere with cholesterol entero-hepatic circulation and metabolism (Ling and Jones, 1995; Francis et al., 2002), as well as to bind to each other. In the present study, it was shown that dietary phytosterols had a more pronounced effect on plasma cholesterol levels in sea bass than in sea bream. Sea bass juveniles fed the PhytL diet showed significant lower cholesterol levels than the control (chapter 2), while only a tendency towards lower plasma cholesterol levels was observed in on-growing fish, irrespective of the phytosterol inclusion level (chapter 3). In sea bream, on the other hand, only juveniles exhibited a tendency towards lower cholesterol levels in all experimental groups (chapter 4).

From these results it seems that when cholesterol levels drop to a certain level, a regulatory mechanism is triggered, since low dietary phytosterols levels seem to decrease plasma cholesterol levels, while at the higher dietary phytosterol level such effect was not observed, suggesting that the lowering plasma cholesterol effect of phytosterols was counterbalanced by a homeostatic feedback mechanism. Though the ability of fish to regulate cholesterol levels was previously demonstrated (Leaver et al., 2008b), the present study does not allow conclusions on whether plasma cholesterol levels where maintained due to internal regulation or if the fish were not affected by the presence of antinutrients in the diets. Overall, dietary antinutrient effects on plasma cholesterol were more pronounced in sea bass than in sea bream, and juveniles seemed to be more sensitive than on-growing fish.

Histomorphological alterations of the intestinal mucosa are findings in fish fed some diets containing plant ingredients (Burrells et al., 1999; Krogdahl et al., 2003; Bonaldo et al., 2008; Uran et al., 2008; Hedrera et al., 2013). A pathological condition known as enteritis (Baeverfjord and Krogdahl, 1996) is characterized by a number of histological alterations that have not been observed in either sea bass or sea bream. However, in the present study some mild histological alterations were observed in some fish fed the diets containing antinutritional factors. After 15 days of feeding the PhyH diet, sea bass juveniles showed significant increase of histological alterations in the distal intestine (chapter 2). By the end of the trial, however, the differences were no longer statistically significant compared to the control group. Although a recovery process has been described in common carp (Uran et al., 2008), that does not seemed to be case, at least not for all the sampled fish. Our evaluation showed that some fish could indeed have recovered from the intestinal lesions, but it could also happen that they did not suffer any alterations to begin with. Additionally, some fish sampled at the final sampling showed a worsening of the alterations found at 15 days. These observations resulted in high variability and consequently a loss of statistical significance. In any case, the findings suggest individual variation in sea bass' sensitivity toward the ANFs studied in these trials.

Sea bream juveniles also showed some histomorphological alterations in the distal intestine, but only at the end of the feeding trial in fish fed diets SapH and SapPhytH (chapter 4). The alterations comprised increased variation in size of the enterocytes' supranuclear vacuoles and a concomitant increase in the number of intraepithelial leukocytes. Although the processes that lead to the

alterations in the intestinal mucosa cannot be clearly explained, it suggests an accumulation of abnormal substances in the absorptive vacuoles and consequent mobilization of leukocytes to the intraepithelial space. Despite sea bream juveniles having shown mild histomorphological alterations in the distal intestine only at the end of the trial, thus appearing to be more resistant to the ANFs effects than sea bass, juveniles of both species showed similar histological alterations. On-growing fish, on the other hand, did not show any relevant histomorphpological alterations in the distal intestine (chapter 5), further confirming that older fish are more tolerant to dietary saponins and phytosterols.

The findings that expression of immune-related genes were stable in all studies performed concurs with the absence of convincing inflammation related to intake of the ANFs tested, as confirmed by the histological analysis. Although sea bass juveniles showed a trend towards an up-regulation of IL1B in group PhytL (chapter 2), which suggests that the morphological changes observed could be of an inflammatory nature, additional studies would be necessary to further investigate saponin and / or phytosterol effects on sea bass and sea bream immune status over time.

At the functional level, juveniles were also more affected by the dietary ANFs than on-growing fish. Sea bass juveniles showed decreased brush border membrane enzyme activity in the distal intestine when fed high levels of saponins (chapter 2), while sea bream juveniles showed a trend towards down-regulation of genes FABP2 and PepT1 (chapter 4), considered markers of intestinal function. In both cases, there were indications of some disruption of normal intestinal function and although it was not reflected in decreased growth, the potential long-term consequences of such effects should be taken into consideration and merits further research.

Saponins and phytosterols, either alone or in combination, showed to be well tolerated by both sea bream and sea bass in the two life stages studied, at least when exposed for relatively short periods (7-8 weeks). Fish fed the experimental diets performed well in terms of growth and feed utilization at both life stages. However, there was a tendency for FE to decrease in sea bass during the on-growing phase (chapter 3) and to increase in sea bream juveniles (chapter 4). These results suggest long –term exposure to the tested antinutrients may have effects not detected in the relatively short term exposures, and this should be further investigated.

Despite the lack of negative effects in sea bream and sea bass growth, both species showed some histological alterations at the distal intestinal level, as well as some intestinal dysfunction, characterized either by the decreased activity of the brush border enzymes (sea bass juveniles) or by down regulation of functional marker genes (sea bream juveniles).

Although dietary saponin and phytosterol effects were not very pronounced in either developmental stage of both sea bass and sea bream, juveniles were generally more sensitive than on-growing fish. This suggests that plant ingredients containing the ANFs tested can be included in higher amounts in diets for sea bream and sea bass in the grow-out phase. However, utmost care should be taken to ensure that the diets will not compromise fish health, as the rearing conditions can induce animal stress and facilitate pathological episodes.

This thesis represents the first study to address the effects of purified antinutrients often present in diets containing plant feedstuffs in the two most important produced fish species in the Mediterranean region, European sea bass and gilthead sea bream.

The results presented herein suggest the potential of both species to cope with high inclusion levels of plant feedstuffs in their diets, but also generate a great number of new questions which require additional research. There has been persistent advice towards the importance of studying the effects of individual antinutrients in fish diets with the purpose of clearly identifying the responsible agents of any negative effects observed when high inclusion levels of plant feedstuffs are added to the diets. The need to continue this research line is undeniable, if the replacement of fish meal and fish oil by plant ingredients in fish diets is to continue. Knowledge on the effects of antinutrients in fish is still rudimentary and has been gathered slowly but steadily. Although there is already a great deal of research performed in Atlantic salmon the effects are not transferable to other species, as demonstrated by the present work.

The present study indicates that regarding ANF concerns, there is reason to be optimistic regarding high inclusion levels of plant ingredients in sea bream and sea bass diets. Further studies should, however, be undertaken to study other ANFs and to ensure that growth as well as fish health is maintained through compound diets containing high levels of plant feedstuffs used in aquaculture in the long term and that the final product is beneficial for human health.

The high variability of responses within groups merits mention, as while some fish seemed to be affected by the antinutrients others were clearly unaffected. Selective breeding of the more tolerant individuals could increase the species' potential to cope with higher inclusion levels of dietary plant ingredients. Additional studies to investigate the causes of the individual variation observed and whether the aquaculture industry can benefit from any potential genetic variation would be valuable.

The diversity of effects of dietary soya saponins and phytosterols reported in fish in this and other studies indicate that further investigations should be carried out as responses appear to vary greatly depending on combination of plant ingredients or antinutrients, fish species, dose, and duration of exposure.

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