Norwegian University
of Life Sciences

Master's Thesis 2020 30 ECTS

Department of Animal and Aquacultural Sciences (IHA)
Faculty of Biosciences

Effects of the soybean antinutrients saponin, isoflavonoid, and phytosterol on morphology and immune responses in the distal intestine of Atlantic salmon

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Acknowledgements

I would like to express my gratitude to professor Trond Storebakken who invited me to join this project when the original plan was canceled due to the COVID-19 pandemic, and thanks for his support and advices throughout writing of my thesis. For the new project, I had the great opportunity to work with professor Åshild Krogdahl who was the leader of the project and my main supervisor during the accomplishment of current work. I am sincerely grateful to her for allowing me to participate in the feeding experiment of Atlantic salmon, sampling of intestinal tissue, preparation of the samples for histological analysis as well as for providing me the raw data and giving me guidance and comments.

In addition, I would like to give special thanks to Trond M. Kortner, Ellen Hage, Elin Valen and Kristi Præsteng at Department of Paraclinical Sciences (PARAFAG), Bjørn Hansen and Ricardo Benicio at fish lab, Frank Sundby and Hanne Hustoft at LabTek, Ismet Nikqi and Fahimeh Rajabi at center for feed technology (FôrTek), for giving me help and advices.

Finally, I would like to thank my love Kristoffer Vinje for always being understanding, supportive and making the days wonderful!

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Abbreviations

AAP	Alanine aminopeptidase
ADC	Apparent digestibility coefficient
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANFs	Anti-nutritional factors
AST	Aspartate aminotransferase
APCs	Antigen-presenting cells
BBM	Brush border membrane
CF	Condition factor
Cho	Cholesterol
CP	Crude protein
DI	Distal intestine
DISI	Distal intestine somatic index
DM	Dry matter
FBW	Final body weight
FFA	Free fatty acid
GALT	Gut-associated lymphoid tissue
GC	Goblet cell
Glu	Glucose
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HSI	Hepatosomatic index
IBW	Initial body weight
IECs	Intestinal epithelial cells
Iso	Isoflavonoids
LAP	Leucine aminopeptidase
LP	Lamina propria
MI	Mid intestine
MISI	Somatic index of mid intestine
NSE	Non-specific esterase
NSPs	Non-starch polysaccharides
PI	Pyloric intestine
PISI	Somatic index of proximal intestine
Phy	Phytosterol
PPC	Pea protein concentrate
qPCR	Quantitative polymerase chain reaction
RAS	Recirculation aquaculture system
RelCW	Relative carcass weight
SBM	Soybean meal
SBMX	Processed soybean meal
SBMIE	Soybean meal-induced enteritis
SC	Stratum compactum
SG	Stratum granulosum
SGR	Specific growth rate
SNV	Supranuclear vacuolization
SPC	Soy protein concentrate
TG	Triglyceride

Abstract

Two experiments were included in this study. The first experiment was designed to investigate the effects of the soybean antinutrients saponins, isoflavonoids, phytosterols and the mix of them on the distal intestine morphology of Atlantic salmon. The fish were fed six different diets: positive control with soybean meal (SBM), negative control with soy protein concentrate (SPC), SPC added one of the following (level given as pure compound): 0.5% saponins (SPC+Sap), 1.5% isoflavonoids (SPC+Iso), 1% phytosterols (SPC+Phy), and a diet with SPC added a mix of the three antinutrients (SPC+Mix) in the same amounts as in the diet with the single inclusions.

Fish fed SBM diets showed the typical characteristic of SBM induced enteritis (SBMIE) in the distal intestine, followed by mild signs of enteritis in fish fed SPC+Sap and SPC+mix. Fish with SPC, SPC+Iso and SPC+Phy showed normal intestinal morphology. Compared to fish fed SPC, somatic index of the distal intestine (DISI) was significantly lowered in fish fed SBM and SPC+Mix. Apparent digestibility of dry matter (DM), crude protein (CP) and lipid in fish fed SBM was significantly higher than in fish fed SPC-based diets. However, capacity of brush border membrane enzyme leucine aminopeptidase (LAP, mmol/kg body weight) in distal intestine (DI) and bile salt concentration of DI content were significantly decreased in fish fed SBM diet compared to fish fed SPC. Antinutrient supplementation tended to decrease bile salt concentration in DI chyme and plasma triglycerides (TG).

The analyses of gene expression in DI tissue in fish fed SBM, showed upregulation of proinflammatory cytokine IL-17A, proliferating cell nuclear antigen PCNA, apoptosis gene caspase CASP6, heat shock protein 70 HSP70, tissue modeling gene collagenase3 MMP13 and down-regulation of proinflammatory cytokine IL-8 indicated activation of immune responses, increased cell proliferation, as well as increased apoptosis, stress and cell repair during the development of SBMIE. In addition, upregulation of tight junction protein genes CLDN25B and CDH1 and down-regulation of CLDN15, antioxidant gene catalase CAT, and water channel gene aquaporin AQP8AB suggested that SBM interfered with barrier functions and fluid permeability of distal intestine. For fish fed SPC with saponin and the mixed antinutrients, the expression of genes IL-8, AQP8AB, CLDN15, CLDN25B and CAT showed similar regulation as SBM-fed fish, indicating similar dysfunctions of DI.

The second experiment was a follow-up study of the first, for which only the results of the feed quality recordings are presented in this thesis. The diets were the following: a negative control

with soy protein concentrate as the main protein source (SPC), the SPC supplemented with saponins (SPC+sap) or with a crude saponin extract from SBM (SPC+imp), and a fourth diet in which SPC was replaced with a processed soybean meal (SBMX). The processed soybean meal was provided by the company Evonik. The aim of the processing was to reduce the level of antinutrients. In this study, only the physical quality of these four diets were analyzed and evaluated. Although lower expansion ratio was showed in SBMX and higher fat leakage in SPC+Sap, the overall variations between the diets were small and would most likely not affect the nutritional value of the diets as eaten by the fish.

Key words: soybean meal, soy protein concentrate, soybean meal induced enteritis, saponin, isoflavonoid, phytosterol.

1 Introduction

According to the Food and Agriculture Organization of the United Nations (2020), world aquaculture fish production reached 82 million tonnes in 2018, which accounts for 46% of global fish production. The amount has been increased twenty percent compared to the production in 2000, which was 26% of global fish production. Global fish consumption by humans has increased at an average annual rate of 3.1 percent in past sixty years, higher than that of all other animal protein rich foods which increased by 2.1 percent per year (FAO, 2020). This high rate of increasing fish consumption will promote aquaculture industry to expand even more intensively in the future, with a corresponding increase in demands for fish feed. Previously, fish meal was used as the main protein source in the fish feed. However, the inclusion level has decreased gradually over the last decades owing to limited supply and therefore high price source and been replaced by plant-based protein sources, mainly obtained from oilseed, legumes and cereal grains.

Soybean (*Glycine max* L. Merrill) is the major legume used for protein ingredients in aquafeed today due to their high level of protein, balanced amino acids profile, high availability and lower price compared to fishmeal. Soybean is primarily produced for its oil content, supplying oil for human consumption. Soybean meal (SBM) is a by-product of soybean oil production. It is mainly solvent extracted (Heuzé et al., 2016) and contains 47-49% protein (Heuzé et al., 2020). Numerous studies have focused on the nutritional and health effects of replacement of fishmeal by SBM in salmonids. Presence of antinutritional factors (ANFs) has been a main consideration when high level of dietary SBM is added in the fish feed because such components are believed to be the factors responsible for causing the negative effects on fish health and growth. In Atlantic salmon, the most pronounced effect of SBM-based diet is the induction of inflammation in the distal intestine, usually referred to as SBM induced enteritis (Baeverfjord & Krogdahl, 1996; Bakke-McKellep et al., 2000; Chikwati et al., 2013a; Chikwati et al., 2013b; Krogdahl et al., 2003; Sahlmann et al., 2013; Van den Ingh et al., 1991). Studies have shown that saponin, one of the most important ANFs in soybean, is involved in SBM induced enteritis (Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015b). However, whether the enteritis is an effect of saponins alone, or if other ANFs are involved, is not clear (Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015b).

Saponin is heat-stable and thus it remains in SBM based feed regardless of the precooking of soybean and high temperature extrusion processing of feed. Compared to SBM, soy protein

concentrate (SPC) with protein content of 62% to 72% (Lusas & Rhee, 1995) contains lowered level of antinutrients such as saponins and soluble carbohydrate which were removed during the aqueous-alcohol extraction of SPC production (Lusas & Rhee, 1995). In a study with Atlantic salmon, the presence of SPC in the diet did not induce enteritis (Van den Ingh et al., 1991). As a result, SPC is preferred over SBM in the feed for Atlantic salmon, at least in Norway it is the case. A report from Nofima (an institute for applied research within the fields of fisheries, aquaculture and food research in Norway) shows that SPC was the main plant protein source used in Norwegian salmon farming in 2016, constituting 19% of the feed (Aas et al., 2019). Soybean meal was not used according to the data analyzed in the report, which was based on information from the four large Norwegian feed companies, BioMar AS, Cargill, MOWI ASA and Skretting AS (Aas et al., 2019).

Most fish feed in Norway is processed by extrusion technology. Extrusion increases the physical and nutritional quality of pellets by cooking the ingredients within the extrusion barrel under high temperature, high pressure and with moisture addition (Forte & Young, 2016). Starch and protein components undergo gelatinization and denaturation, respectively, which improves the binding properties of molecules and increase the digestibility of nutrients and inactivates heat-labile antinutrients such as protease inhibitors and lectins. Furthermore, expansion of the extrudate is important for the process of oil coating and for obtaining the optimal sinking rate of the pellet. Physical quality of the final extruded pellets is not only influenced by diet formulation, but also by extrusion parameters and screw configuration.

In the present study, the effects of antinutritional factors saponins, isoflavonoids, phytosterols and mix of these three components on histological changes in Atlantic salmon were investigated. The effects on growth performance, nutrient digestibility, enzyme activities and immune responses were evaluated in the first experiment whereas physical quality of the diets was analyzed and compared in the second experiment.

2 Literature review

2.1 Antinutritional factors in soybean

Antinutritional factors (ANFs) are naturally occurring substances in foods and feedstuffs, which may adversely affect health and nutrient utilization when ingested by animals or human (NRC, 2011). The main ANFs present in soybeans are proteinase inhibitors, lectin, non-starch

polysaccharides (NSPs), oligosaccharides, phytic acids, saponin, isoflavonoids and phytosterols. In nature, these components have biological functions and protect the plants from insects and herbivores. In short-term feeding, lower level of ANFs in the diets of animals may be beneficial in terms of antioxidative, immunostimulatory or prebiotic aspects as summarised by Krogdahl (2010). However, at higher levels, harmful effects may occur such as reduced palatability and nutrient digestibility, inhibition of growth and intestinal dysfunction. Furthermore, the antinutrients might also cause immune modulation, pancreatic hypertrophy, hypoglycaemia and liver damage (Reviewed by Krogdahl et al., 2010). Some of the ANFs, for example proteinase inhibitors and lectins, are heat labile and can be inactivated by heat treatments, whereas the rest of the antinutrients in soybean are rather heat stable. They can be reduced or removed by supplementation with enzymes which inactivates them, by fermentation or extraction (Reviewed by Krogdahl et al., 2010).

2.2.1 Proteinase inhibitors

Protease inhibitors interfere with the proteolytic activity of enzymes in gastrointestinal tract of animals (Liener, 1994). The two main families of protease inhibitors found in soybean are Kunitz trypsin inhibitor (Kunitz, 1947) and Bowman-Birk inhibitor (Bruneton, 1995). These two inhibitors are soluble proteins and accounts for 0.2 to 2% of total soluble protein (Shewry & Casey, 1999). Kunitz inhibitors have one specific site to inhibit one molecule of trypsin at a time. Bowman-Birk inhibitors have two separate sites and can inhibit one molecule of trypsin and one of chymotrypsin at the same time (Cabrera-Orozco et al., 2013). The inhibitors contain disulphide-bonds, which stabilizes the structure. The Kunitz inhibitor soybean trypsin inhibitor (KTI) has two, whereas Bowman-Birk inhibitors have seven. Therefore, the latter is more stable to heat, acids and bases (Cabrera-Orozco et al., 2013). In the gastrointestinal tract of animals, these inhibitors form inactive complexes with trypsin or chymotrypsin, inhibiting proteolysis and reduce protein digestibility (Cabrera-Orozco et al., 2013). In a short-term feeding with lower inhibitor levels, the enzyme activity is not affected importantly because the proteinase inhibitors stimulate pancreas to secrete more enzymes, increasing the trypsin and chymotrypsin level in the intestinal content (Krogdahl et al., 2003). However, after longer-term feeding the pancreas can no longer manage to compensate for decreased enzyme activity by increasing secretion (Krogdahl et al., 2003). Furthermore, these inhibitors also cause hypertrophy of the pancreas due to chronic hypersecretion of pancreatic enzymes, which leads to deviation of the sulfur amino acids those are used for synthesis of trypsin (Matthews, 1989). Thus these protease

inhibitors inhibit protein synthesis and animal growth (Carbonaro et al., 2000; Friedman & Brandon, 2001; Grant, 1989; Hedemann et al., 1999).

In studies with salmonids, proteinase inhibitors have been found to reduce both protein and lipid digestibility (Krogdahl et al., 1994). Inactivation of protease inhibitors can be achieved by heat treatment during the processing of ingredients and feeds such as pre-cooking prior to solvent-extraction, toasting after extraction and extrusion process of feed. The efficiency of inactivation is related to the temperature, moisture content and heating time. The optimal inactivation of inhibitors, without severely affecting the protein quality, might leave small amounts of them in the feedstuff (Gatlin et al., 2007).

2.2.2 Lectins

Lectins are highly specific carbohydrate-binding proteins and can be found in all organisms. They are also known as agglutinins or hemagglutinins for their ability to bind to the cell surface and cause agglutination (Gatlin et al., 2007). Legume seed contains high level of lectins, approximately 1 - 10% of the total soluble protein (Damme et al., 1998). Soybean lectin is usually referred to as soybean agglutinins (SBA). Lectins are stable proteins that are resistant to digestive enzymes and easily pass into the intestine. In the small intestine, lectins can bind glycoproteins on the surface of microvilli and disrupt the intestinal membrane integrity (Liener, 1994). Thus, lectins induce changes in digestive, absorptive, protective or secretory functions of the digestive system (Cabrera-Orozco et al., 2013). Hendriks et al (1990) studied binding of soybean lectins to enterocytes of intestine from Atlantic salmon. The results showed that tissues from both the proximal and the distal intestine can bind this lectin. The binding appeared to be stronger for the cells from the distal than the proximal intestine. Lectins are soluble and heat labile and thus application of proper moisture and heat can inactivate the biological activity of lectins (Sathe & Venkatachalam, 2004).

2.2.3 Oligosaccharides and non-starch polysaccharides

Carbohydrate fraction in soybeans contains predominantly non-starch polysaccharides (NSPs) from 20 to 30%, followed by 5% oligosaccharides, 5% sucrose and less than 1% of starch (Choct et al., 2010). Of the NSPs, 8% are insoluble polymers cellulose and the rest are soluble polymers arabinoxylans and beta-glucans (Choct, 1997; Choct et al., 2010). The carbohydrate content can be lowered by dehulling. SMB are available, with or without hulls. Hull accounts

for 8% of the whole beans, in which 86% is carbohydrate (Lusas & Rhee, 1995). In monogastric animals and fish, sucrose and gelatinized starch can be digested and absorbed, but cellulose and other, non-starch polysaccharides (NSPs) are not digestible and leave the body undigested.

Arabinoxylans, one of the NSPs in SBM, consist of a linear backbone of β-(1,4) xylose residues with arabinose substitution (Butardo & Sreenivasulu, 2016). They are abundant in wheat and rye (Butardo & Sreenivasulu, 2016). On the other hand, β-glucans are abundant in oats and barley, in which glucose monomers are linked via β-(1,4) and β-(1,3) glycosidic bonds (Mudgil, 2017). These soluble NSPs are non-digestible for monogastric animals and fish due to the lack of enzymes to break down the glycoside linkages (Choct et al., 2010; Hansen & Storebakken, 2007; Refstie et al., 1998). Some of the negative effects are associated with their viscous nature (Sinha et al., 2011). The soluble NSPs increase the viscosity of intestinal content and may decrease nutrient digestion and absorption, as shown for both poultry and pigs (Choct, 2015). Very few similar studies have focused on the effects in fish (Sinha et al., 2011). In one study with Atlantic salmon, inclusion of soybean NSP in the diet increased the viscosity of chyme in gastrointestinal tract, resulting in the reduction of amino acid and lipid absorption (Refstie et al., 1999). Improvement in the digestibility of NSPs can be achieved by supplementation of the diets with NSP-degrading enzymes. For example β-glucanases and xylanases have been successfully used in the diets of pigs and chickens (Sinha et al., 2011).

The main low molecular weight oligosaccharides in soybean are stachyose and raffinose in a ratio of about 4:1 (Choct et al., 2010). In monogastric animals, high levels of soy oligosaccharides inclusion in the diet can cause intestinal disorder, reduced growth rate, flatulence and diarrhea (Veldman et al., 1993; Zhang et al., 2001). In salmonids species, oligosaccharides might be related to the reduced growth performance (Refstie et al., 1998; Refstie et al., 2005). To reduce the negative effects of soybean oligosaccharides in animals and fish, reduction can be obtained by fermentation with bacterial or fungal organisms (Gatlin et al., 2007; Refstie et al., 2005).

2.2.4 Phytic acids

Phytic acid is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol with molecular formula $C_6H_{18}O_{24}P_6$, abbreviated as IP6 (Ahmed et al., 2014). Phosphorus bound to inositol, predominantly in the form of phytate, constitutes 60 to 80% of total phosphorus in cereals, legumes, nuts, and oilseeds (Skoglund et al., 2009). The amount of phytate in SBM is

around 4%, and SPC contains approximately 7-10% (Gatlin et al., 2007). The bioavailability of phosphorous in phytate form is very low in monogastric animals and fish because they lack the enzyme phytase to hydrolyze phytate. Furthermore, phytate can also chelate with divalent cations such as calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), and iron (Fe), and the insoluble complexes are not utilized by monogastric animals, including many fish species, reducing the digestion and absorption of these minerals (Kumar & Sinha, 2018). It was also reported that phytate can bind proteins, limiting the enzymatic activity and digestibility of protein (Cheryan & Rackis, 1980). Besides, if the unutilized phosphorous is excreted into the environment, it accumulates in the soil and water, and can cause eutrophication in water system (Cheryan & Rackis, 1980). Moreover, important amounts of a nutrient, for which the world's resources, are emptied very fast, is wasted.

Phytate is heat stable and hence the bioavailability of phosphorous is not improved by heat treatment. Phytase is added in the feeds for monogastrics, especially for swine and poultry, to degrade phytate and increase bioavailability of P and other minerals. But for cold-water fish dietary supplementation with phytase has little effects, as the enzymes have low activity at low temperatures. Low-phytate cultivars of soybean have been developed but they are not utilized widely in fish feeds (Gatlin et al., 2007).

2.2.5 Isoflavonoids

The isoflavonoid group includes isoflavones, isoflavonones, isoflavans, pterocarpans, rotenoids. Among them, isoflavones are the most abundant in soybeans, up to 4 g/kg (Hammond et al., 2004), predominantly genistein and daidzein (Figure 1B) (Farooqui & Farooqui, 2018; Nemitz et al., 2016). Isoflavones possess a similar structure to the hormone estrogen 17-β-estradiol, therefore they are classified as phytoestrogen (Figure 1A) (Zuiter, 2014). Due to this structural similarity, they can bind to estrogen receptor and mimic or antagonize the effects of estrogen in human and mammals (Setchell, 1998; Wang, 2002; Zaheer & Humayoun Akhtar, 2017). The effects of isoflavones on the health of human both positive and negative have been studied extensively (Munro et al., 2003). Anti-estrogenic effects in reproductive tissue could help reduce the risk of hormone-associated cancers such as breast, uterine, and prostate (Wang, 2002). Estrogenic effects can be also beneficial like cholesterol-lowering by upregulating the expression of cholesterol 7-α-hydroxylase enzyme and low density lipoprotein (LDL) receptor genes, to enhance bile acid synthesis and excretion, and to modulate the endocrine system (Munro et al., 2003). Phytoestrogens are also considered as endocrine disruptors which interfere

with regulation of reproduction, feed intake, lipid metabolism etc. as reviewed by Krogdahl & Bakke (2015a).

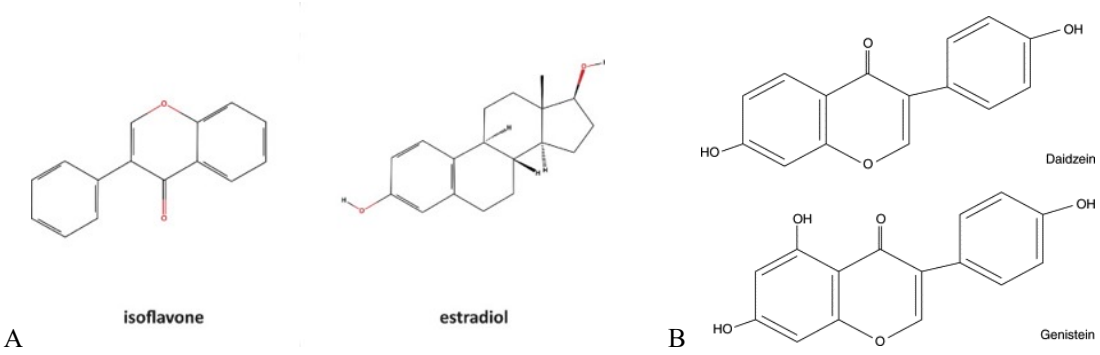

Figure 1 A: The structure of isoflavone and estradiol. (Zuiter, 2014). B: Chemical structure of genistein and daidzein (Fehily, 2004).

In fish estrogen is suggested to interfere with the immune system (Milla et al., 2011) and increases pathogen susceptibility (Casanova-Nakayama et al., 2011). Under estrogen exposure, the expression of immune-related genes has been observed to be down-regulated when trout were fed a diet supplemented with 20 mg kg^{-1} 17β-estradiol (Cabrera-Orozco et al., 2013). The effects of estrogen during smoltification of Atlantic salmon may lead to inhibition of smoltification (Lerner et al., 2012). However, present information on the effects of isoflavones on fish health, immune system and growth is limited. Further investigations need to be conducted to be able to conclude regarding the effects of isoflavones in Atlantic salmon.

2.2.6 Phytosterols

Phytosterols are naturally occurring steroid alcohols with structural similarity to cholesterol, with an extra methyl or ethyl group (Salgado et al., 2019). The comparison of chemical structures of phytosterols and cholesterol is shown in Figure 2. The most abundant phytosterols in plants are sitosterol, campesterol and stigmasterol (Turpeinen & Merimaa, 2011). Phytosterols can be found in legume seed oils at levels between 1-10 g/kg (Bot, 2019). Like isoflavones, phytosterol also possess anticancer properties (Shi et al., 2010; Woyengo et al., 2009). They are also known to lower the level of blood cholesterol in human (Salgado et al., 2019). In Atlantic salmon diets, dosage levels of 5 and 10 g kg^{-1} phytosterols were found to decrease lipid digestibility, especially digestibility of saturated fatty acids (Chikwati, 2007).

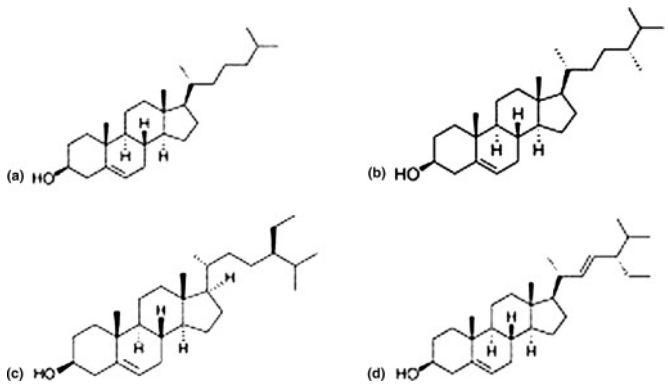

Figure 2 Chemical structures: (a) cholesterol, (b) campestrol, (c) sitosterol, and (d) stigmasterol (Temelli et al., 2012).

2.2.7 Saponins

Saponins are naturally occurring bioactive compounds with an aglycone unit linked to one or more sugar chains (El Aziz et al., 2019). The aglycone or sapogenin unit is usually either a sterol or a triterpene unit. Figure 3 shows the structure of a typical triterpenoid saponins from soybean (Savage, 2003). The carbohydrate portion of the molecule is water-soluble, whereas the sapogenin is fat-soluble (Savage, 2003). Therefore, they can form stable soap-like foam in aqueous solution, the origin for the name *sapo* meaning soap in Latin (Faizal & Geelen, 2013). The physiological role of saponins in plants is known to be antimicrobial, to inhibit mould and protect the plants against herbivores or insects (Francis et al., 2002; Mugford & Osbourn, 2013). They are usually present in legumes in the range of 1–5g kg^{-1}, but the level in soybean is generally higher than in other common plant feedstuffs (Anderson & Wolf, 1995). Francis et al (2001) reviewing available literature, concluded that saponin exhibit positive effects of antifungal, antiviral, anticancer, adjuvant and antioxidant properties. On the other hand, they also inhibit protein digestion and vitamin absorption (Francis et al., 2001; Francis et al., 2002).

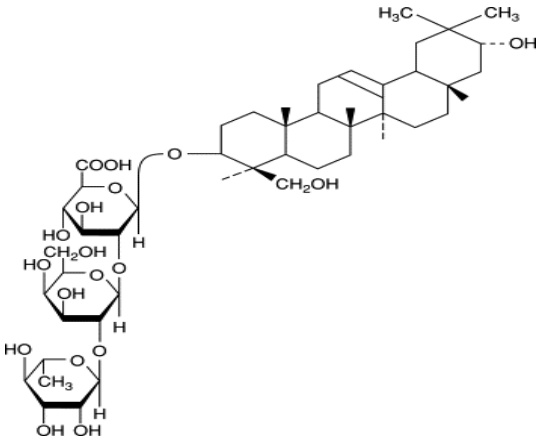

Figure 3 Chemical structure of triterpenoid saponins from soybean (Savage, 2003).

Saponins are heat-stable, alcohol-soluble amphipathic molecules. The amphipathic property of saponins enables them to bind to cell membrane and form holes. The mechanism of saponin activities on membranes is shown in Figure 4. In animals, it is reported that saponin increases the permeability of intestinal mucosal cells, facilitating uptake of antigens and potential toxins that are normally not absorbed by the enterocytes (Summarized by Krogdahl and Bakke 2015a). Of particular interest in recent studies of salmonids is that saponins seem to be involved in the inflammatory changes of distal intestine (Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015b), and the effects are more severe when supplemented with lupin kernel meal or pea protein concentrate (PPC) (Chikwati et al., 2012; Knudsen et al., 2008; Krogdahl et al., 2015b).

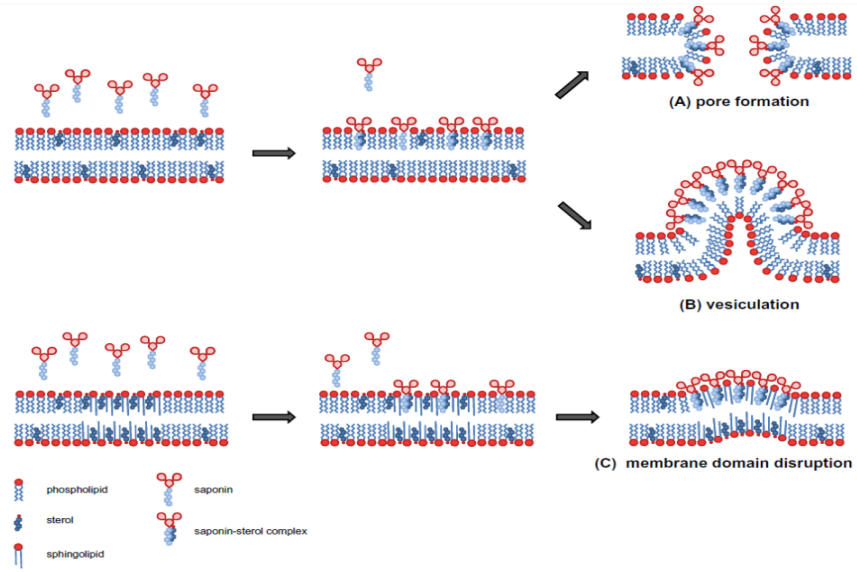

Figure 4 Schematic models of the molecular mechanisms of saponin activities towards membranes (Augustin et al., 2011). Saponins integrate with membrane sterols, leading to A: pore formation in the membrane, B: vesiculation or C: sphingolipid/sterol enriched membrane domain.

2.3 Soybean meal induced enteritis (SBMIE) in the distal intestine of Atlantic salmon

Dietary SBM causes induction of enteritis (SBMIE) in the distal intestine of Atlantic salmon (Baeverfjord & Krogdahl, 1996; Bakke-McKellep et al., 2000; Chikwati et al., 2013a; Chikwati et al., 2013b; Krogdahl et al., 2003; Sahlmann et al., 2013; Van den Ingh et al., 1991). A 10% SBM inclusion in salmon feed resulted in moderate histological changes in the distal intestine, increasing in severity with increasing inclusion level (Krogdahl et al., 2003). Histological signs of SBMIE can be seen as early as two days after introduction of SBM-containing diet (Baeverfjord & Krogdahl, 1996). The study of Chikwati et al observed signs of inflammation after five days (Chikwati et al., 2013b; Sahlmann et al., 2013) and all signs seem to be fully developed within seven days (Baeverfjord & Krogdahl, 1996). The key component that causes the intestinal enteropathy has been shown to be the antinutrient saponin, however if other antinutrients are present, the effect might become more severe (Chikwati et al., 2012; Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015b). In the study by Penn et al (2011) a diet with high level of pea protein concentrates (350 g kg-1) and low fishmeal (100 g kg− 1) showed inflammation in the distal intestine similar to those described for soy enteritis. Peas are also known to contain saponins, but at lower levels than in soybean meal (Mohan et al., 2016).

2.3.1 Structure and functions of gastrointestinal tract of Atlantic salmon

The gastrointestinal tract (GIT) of Atlantic salmon starts with a mouth, followed by esophagus, stomach (ST), proximal intestine (PI) with adjoining pyloric caeca, mid intestine (MI) and the distal intestine (DI) to anal. Figure 5 shows the tract from ST to DI. The GIT is the main site of digestion and absorption of nutrients in fish. However, for fish in seawater, the requirement of certain minerals can be supplied via the gills. Ingested food pass from the mouth through the esophagus to the stomach where the mechanical and initial enzymatic digestions of nutrients occur. The proenzyme pepsinogen and hydrochloric acid (HCl) are secreted from the epithelial layer of stomach. The latter activate pepsinogen to pepsin which, by splitting certain peptide bonds denatures and opens up the structure of the feed proteins. Once the acidic chyme is fed into the PI where the majority of nutrients are digested and absorbed, to a lesser extent in MI and DI. Secretion of bicarbonate from pancreas increases the pH and neutralize the digesta. Many pancreatic enzymes, in particular proteolytic, are secreted in inactive forms. Proenzymes of trypsin is activated by enterokinase from mucosal cells and chymotrypsin and carboxypeptidase are then activated by the trypsin (Bjørgen et al., 2020). These enzymes aid in

the hydrolysis of proteins, and other pancreatic enzymes lipase for hydrolysis of lipids and α-amylase for hydrolysis of starch (Bakke et al., 2010). Bile salts, produced in the liver and stored in the gall bladder, are released to the intestine for participation in lipid digestion. They help emulsification of lipid and assist the digestion and absorption of these. The hydrolyzed nutrient components pass from the intestinal lumen through the brush border membrane (BBM) of the intestinal epithelium, where some are further digested with the assistance of BBM enzymes such as aminopeptidases, maltase, sucrases, alkaline phosphatases and monoglyceride lipases (Bjørgen et al., 2020).

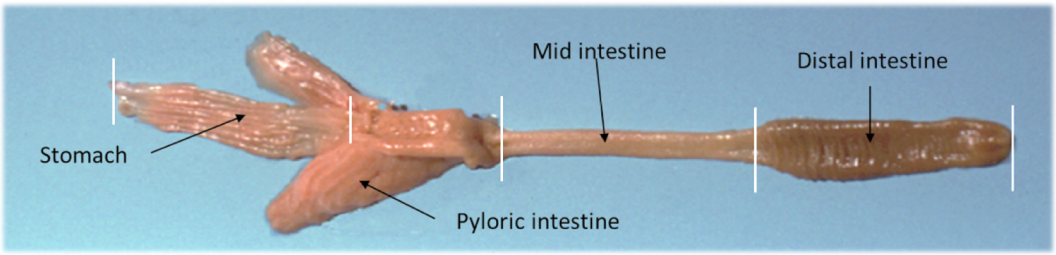

Figure 5 Four sections of the gastrointestinal tract from left to right: stomach, pyloric intestine, mid intestine and distal intestine. Photo by Krogdahl.

The histological structure of intestinal wall of Atlantic salmon is generally divided into four layers, starting from the most internal to the most external, the mucosa, submucosa, muscularis, and serosa (Roberts, 2003). As illustrated in Figure 6, the mucosa is composed of a layer of columnar epithelial cells and the connective tissue of lamina propria (LP), forming mucosal folds (villi). The lamina propria constitute both the center of the mucosal folds and a layer beneath these (Løkka et al., 2013). Submucosa comprises stratum compactum (SC) and stratum granulosum (SG) (Løkka et al., 2013). The muscularis consists of two layers, the inner circular and the outer longitudinal muscle fiber, which aid in the movement of the contents in the lumen. At last, the serosa is a layer of mesothelial cells with connective tissue (Roberts, 2003), separating the tract from the abdominal cavity (Løkka et al., 2013).

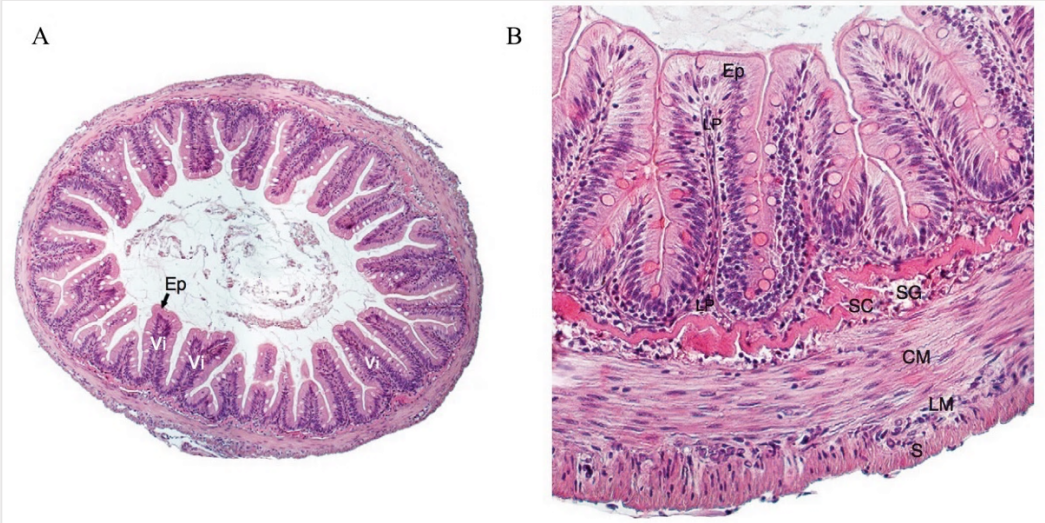

Figure 6 Cross section of the intestine in Atlantic salmon. A) Ep: epithelium, Vi: villi. B) LP: lamina propria, SC: stratum compactum, SG: stratum granulosum, CM: circular muscle, LM: longitudinal muscle, S: serosa. Modified from (Jutfelt, 2011).

The epithelium of the intestine is a monolayer of epithelial cells, mainly enterocytes with an elongated nucleus and large absorptive supranuclear vacuoles (SNVs). The extension of the epithelium membrane is shaped into finger-like microvilli, lining up the brush border. The epithelium also contains goblet cells, mucus-producing cells. In addition to digestive functions, the gastrointestinal tract is involved in immune system. Both the innate (non-specific) immune system and the adaptive (specific) immune system are present in the intestinal wall of fish (Jutfelt, 2011). The innate immune system is an antigen-independent defense mechanism with rapid response to a wide range of pathogens and has no immunologic memory to recognize the same pathogen again (Marshall et al., 2018). The function of the mucus layer as a physical barrier is included in this immune system, which physically removes non-specific harmful agents from the epithelium (Jutfelt, 2011). Furthermore, antimicrobial factors, such as antimicrobial peptides, reactive oxygen species, and hydrogen are secreted with the mucus together to protect the epithelium (Jutfelt, 2011). In the epithelium and the lamina propria, phagocytotic cells such as macrophages and neutrophils can be found, which phagocytose and digest pathogens (Jutfelt, 2011). Phagocytic action of the innate immune response also activates the adaptive immune response which is an antigen-specific defense mechanism, recognizing non-self-antigen from self-antigens (Marshall et al., 2018). The cells involved in the adaptive immune system are T-cells and B-cells. T-cells have specific antigen-binding receptors, known as T-cell receptors (TCR) and with the action of antigen-presenting cells (APCs) they can recognize a specific antigen. On the other hand, B-cells do not require APCs, but produce

antibodies to foreign antigens (Marshall et al., 2018). Although Teleost fish lack gut-associated lymphoid tissue (GALT) such as the Peyer's patches or lymph nodes of mammals, the most important cells related to immune defense are present in the gastrointestinal tract also in fish (Georgopoulou & Vernier, 1986; Press & Evensen, 1999).

2.3.2 Histological signs and immune responses during the development of SBMIE

As described previously, the main morphological changes of SBMIE is characterized by 1) reduction in mucosal fold height, 2) widening of submucosa and lamina propria with infiltration of inflammatory cells, 3) decrease or absence of supranuclear vacuoles in the absorptive enterocytes (Baeverfjord & Krogdahl, 1996; Bakke-McKellep et al., 2000; Van den Ingh et al., 1991; Van den Ingh et al., 1996), as well as 4) increased number of goblet cells (GC) and a shortening of the microvilli (Van den Ingh et al., 1991). Figure 7 presents representative images of normal intestinal morphology and the typical histological changes of soybean-induced enteritis of samples from the first experiment.

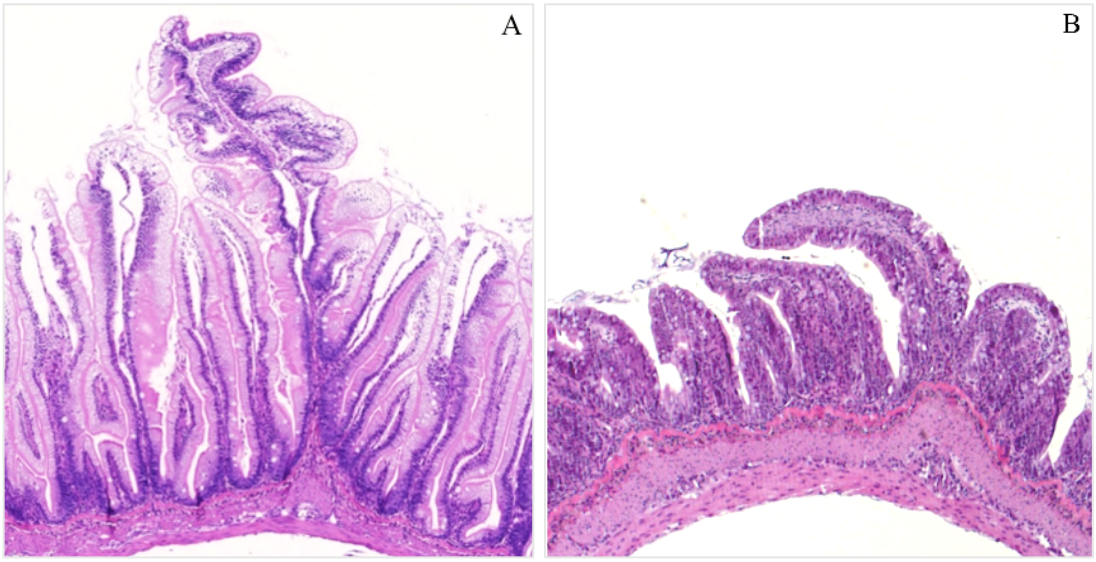

Figure 7 A: Normal histological appearance of distal intestine in fish fed SPC diet from the first experiment of current study. B: Inflammation of distal intestine in fish fed on SPM diet showing the most inflammatory changes. Photo by Elvis Chikwati.

The inflammation of the distal intestine caused by SBM has been proven to be related to impairment of macronutrients digestion and growth performance of the fish (Baeverfjord & Krogdahl, 1996; Krogdahl et al., 2003; Olli & Krogdahl, 1994). This can be explained by the finding by Bakke-McKellep et al. (2000) who observed the significantly reduced enzyme

activities in the distal intestinal epithelial cells, both in the brush border and in the intracellular structures. Analyzed enzymes in brush border included, 57-nucleotidase (57N), Mg2 + -ATPase, alkaline phosphatase (ALP) and leucine aminopeptidase (LAP), and enzymes in the intracellular structures were alkaline and acid phosphatase, non-specific esterase (NSE) and alanine aminopeptidase (AAP) (Bakke-McKellep et al., 2000). These enzymes are responsible for the final breakdown of nutrients into the smaller molecules which can be absorbed by enterocytes. Furthermore, in the transcriptional profiling of early responses during the development of SBMIE investigated by Sahlmann et al. (2013), impairment of digestive and metabolic functions was indicated by down regulation of genes related to endocytosis, exocytosis, detoxification, transporters and metabolic processes from day 5.

Chikwati et al (2013b) studied the intestinal epithelial cell (IEC) turnover in the DI of Atlantic salmon fed SBM and found that inflammation caused by dietary SBM markedly decreased enterocyte turnover time. The intestinal epithelial cell (IEC) turnover comprises proliferation, migration, differentiation and shedding of damaged or senescent epithelial cells by apoptosis (Chikwati et al., 2013b). The fast proliferation and migration rate and reduction in fold height, resulted in a dominance of immature cells along the mucosal fold in fish fed SBM (Krogdahl et al., 2003).

The inflammatory reaction to SBM in the distal intestine of the salmon was characterized by the increase of leukocytes in the lamina propria such as neutrophils and macrophages (Bakke-McKellep et al., 2000). Up-regulation of immune-related genes was observed during the first 5 days, including GTPase IMAP family members, NF-kB-related genes and regulators of T cell and B cell function (Sahlmann et al., 2013). T-cell-like responses were suggested to be involved in SBMIE by Bakke-MacKellep et al. in 2007. In their study they found that the expression of a complex polypeptide (CD3pp), CD4 and CD8b in the distal intestine of SBM-fed fish was significantly increased compared to fish meal-fed reference fish.

2.4 Extrusion processing of aquafeed

Extruders used in fish feed are either single or twin-screw extruder. The extruder used for processing of the experimental feeds of this study is a twin-screw extruder (Bülher, BCTG 62/60 D, Uzwil, Switzerland). It consists of a barrel, two co-rotating shafts with screw elements, die and cutter connected at the outlet of the barrel. The extruder is equipped with a control system where the application of thermal energy and screw speed can be adjusted and also the

profile of processing conditions in the extruder can be read from the screen (Figure 8). Prior to extrusion, the mixed ingredients are transported from the mixer to preconditioner where thermal energy is added in either liquid or steam form. The overall goal of the preconditioning is to ensure uniform hydration of ingredients (Forte & Young, 2016). The ingredients are heated in the conditioner to 80-90°C and this reduces the energy input in the extrusion. Preheated, mixed and hydrated ingredients are turned into a melt in extrusion under high temperature (120-130°C), high pressure (20-30 bar) and shear forces (Sørensen, 2012). The residence time of extrudate within the extruder is typically less than one minute (Forte & Young, 2016). The feed mash is shaped into pellets with certain length and expansion through the die in combination of the rotating knives and afterwards the pellets are dried, cooled, coated and packed (Sørensen, 2012).

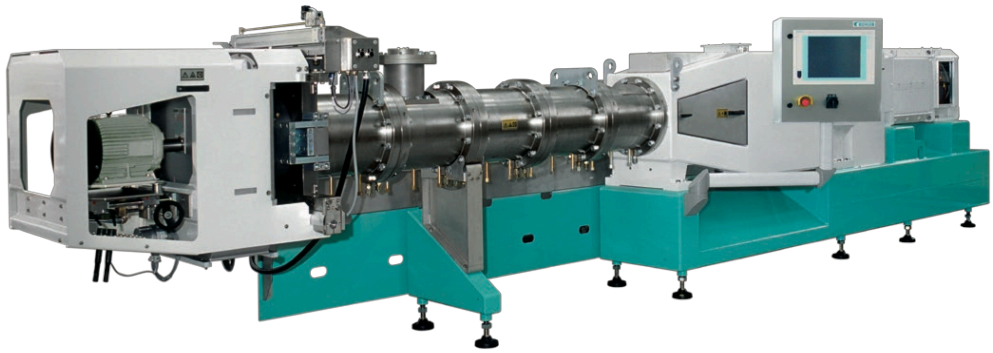

Figure 8 Co-rotating twin-screw extruder (Bülher, BCTG 62/60 D, Uzwil, Switzerland). Photo from Bülher group.

2.4.1 Expansion mechanism of the extrudate

The viscoelastic melt produced as a result of the chemical and physical transformations within extruder will expand rapidly once it leaves the die (Forte & Young, 2016). The expansion is driven by the vaporization of moisture content at the condition of high temperature and lowered pressure (Dethlefsen, 2017). Although the temperature inside the extruder exceeds 100°C, water still presents in liquid form because of the pressure. As the extrudate leaves the die, pressure drops significantly and causes the water to boil, evaporate and produce porous structure in the pellets. This step is usually called swelling or puffing as shown in Figure 9. Further evaporation of water and expansion of the pellets increase the pressure within the bubbles and lead to collapse of those bubbles. At this point, the elasticity of the ingredients mix allows the pellet materials to recoil, which is called contraction or setting (Dethlefsen, 2017;

Forte & Young, 2016). Since the water content and temperature drop fast, recoil stops after a short time. The starch and protein components harden or "freeze" the pellet structure (Forte & Young, 2016).

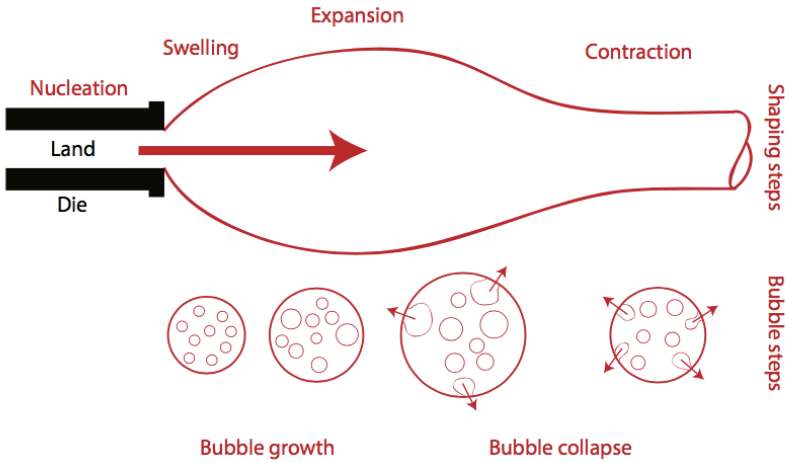

Figure 9 The development of expansion process (Dethlefsen, 2017).

Expansion ratio is one of the most important physical quality parameters for salmon feed. The porous structure of the pellets produced during the expansion eases the oil coating process. Feed for Atlantic salmon are lipid rich, containing 30-35% of lipids. The lipids cannot be added directly in the extruder with other ingredients, because fat acts as lubricant and limits pressure development and expansion ratio (Forte & Young, 2016). Therefore, the lipids are added into the pellets after extrusion by vacuum coating.

2.4.2 Physical quality of extruded fish feed

Fish feed represents a major cost in intensive aquaculture. The formulation and processing of the feed aims, not only to secure a well-balanced nutrient and energy supply to the fish, at the lowest possible cost, but also high physical quality of the pellets in order to minimize feed wastage and maximize feed intake and utilization (Sørensen, 2015). Physical quality of feed is usually defined as the ability of processed feed to withstand handling without creating excessive amount of fines (Sørensen, 2012; Sørensen, 2015). In general, the parameters measured to evaluate physical quality of pellets include particle size, bulk density, expansion ratio, hardness, durability, water stability index and oil leakage rate.

Hardness and durability, the most important characteristics of mechanical quality for the feed, should be at a level which can keep the pellet integrity during the transportation and pneumatic

feeding system, not affect the feed intake and utilization due to too hard or too soft pellets (Sørensen, 2015). Expansion ratio affects the oil holding capacity and the bulk density of feed. Bulk density in turns influences the sinking velocity, usually the bulk density of 320-400 g l^{-1} required for floating feed and 450-550 g l^{-1} for sinking feed (Rokey & Huber, 1994). Water stability index (WSI) is important for the slow sinking feed in water before capture by the fish (Sørensen, 2012). The feed should be able to be intact as long as it is in the water with and nutrient leakage should be minimal. Fat leakage from fish feed is correlated with the microstructure of the pellets (Sørensen, 2012). Leakage of oil reduces the energy contents of the fish feed. Moreover, the oil left on the pipe wall in the pneumatic system can lead to accumulation of dust and fines, eventually causing blockage (Sørensen, 2012).

3 Experimental design

This study was commissioned by the company Evonik with two successive experiments which were conducted and analyzed by the research group "Nutrition and health in Domestic Animals" at NMBU, Faculty of veterinary medicine. The first of these experiments was conducted to validated the experimental facilities and experimental design regarding suitability for evaluation of improvement of nutritional value in SBM which had been subject to new processing methods. These results are open for publication. The second experiment compared the ability to induce SMBIE of soybean meal, soybean protein concentrate (SPC), soy protein isolate (SPI) and a soybean meal which had been processed by a new method to reduce antinutrient level (SBMX). It had a main part and a short follow-up. The main part of the results of this latter study is the intellectual property of EVONIK and cannot yet be disclosed. However, the results of the evaluation of the technical quality of the feed, which was evaluated by me with equipment available at LabTek, are included in this thesis. The feed mixtures from the first study were not available when my thesis work started. The present thesis is based on results from the first experiment, as well as the results regarding analyses of feed samples, which was from the follow-up of the second experiment.

3.1 Fish trial

The feeding trials with Atlantic salmon which was conducted in accordance with the regulations given by the Norwegian Food Safety Authorities, took place at the Center of fish research at Norwegian University of Life Sciences (NMBU) Campus Ås, managed by the Department of

Animal and Aquacultural Sciences at the Faculty of Biosciences. Average weight of the fish was 28 g at start. The fish were randomly selected and evenly distributed in 24 cylindrical fiberglass tanks, 30 fish per tank. The water capacity of the tanks is 250 l, with diameter of 80 cm and water depth 40 cm. The tanks are connected to RAS facilities (recirculating aquaculture system), supplied with freshwater at an average rate of 8 L/min. They were divided into six groups corresponding to six different experimental diets and divided in four replicates for each group. During the grading and transferring of fish, AQUI-S® aquatic anesthetic was used. The fish were starved the last 48h before the feeding with experimental diets started.

The rearing conditions such as water temperature, water flow and oxygen saturation were kept constant throughout the experiment period with daily measurements. Average of water temperature was 14.4 °C and oxygen saturation 86%. The water flow rate was 7-8 l/min during first period and adjusted to 9-10 l/min towards the end of the feeding period. Ammonia nitrogen, nitrite levels and pH level in the outlet water of each tank were measured once every second week. Average ammonia nitrogen and nitrite levels remained below 0.1 mg/l and pH averaged 7.8. A twenty-four hours light regime was employed during the experiment.

The main feeding period lasted 52 days and was followed by an additional 28 day feeding period to obtain sufficient feces for digestibility assessments. The fish were supplied with feed 10% in excess based on anticipated growth, three times a day, 45 minutes per feeding. The feeding was conducted using an automatic system with a container fixed on the top of each tank, where the conveying belt drops the feed to the tank automatically according to the system settings. The uneaten pellets were collected by a retch wire screen and counted. The amount of uneaten feed was estimated by multiplying the numbers of pellets by average weight of one dry pellet. Daily feed intake in each tank was calculated as described by Helland et al (1996).

3.2 Formulation and chemical composition of diets

In the first experiment, there were six diets, a positive control with soybean meal (SBM), a negative control with soy protein concentrate (SPC), and three diets supplemented with antinutrients, i.e., the SPC added one of the following (level given as pure compound): 0.5% saponins, 1.5% isoflavonoids, 1% phytosterols, and a diet with SPC added a mix of the three antinutrients in the same amounts as in the diet with the single inclusions. The formulation and chemical composition are shown in Table 1.

Table 1 Formulation and chemical composition of the first experimental diets.

	SBM PositiveC	SPC NegativeC	SPC Sap	SPC Iso	SPC Phy	SPC Mix
Ingredients %						
Fish meal[a]	15	15	15	15	15	15
Soybean meal[b]	25	0	0	0	0	0
SPC[c]	0	25	25	25	25	25
Corn gluten meal[d]	1	9	9	9	9	9
Vital wheat gluten[e]	13.9	4.9	4.9	4.9	4.9	4.9
Wheat[f]	15	15	15	15	15	15
Fish oil[g]	24	25	25	25	25	25
MCP[i]	1.8	1.8	1.8	1.8	1.8	1.8
Limestone[j]	0.4	0.4	0.4	0.4	0.4	0.4
L-lys[k]	1.2	1.2	1.2	1.2	1.2	1.2
DL met[l]	0.9	0.9	0.9	0.9	0.9	0.9
L Trp[m]	0.1	0	0	0	0	0
L Arg[n]	0.5	0.5	0.5	0.5	0.5	0.5
L Thr[o]	0.2	0.2	0.2	0.2	0.2	0.2
L Val[p]	0.4	0.4	0.4	0.4	0.4	0.4
Stay C 35%[q]	0.1	0.1	0.1	0.1	0.1	0.1
Yttrium oxide[r]	0.01	0.01	0.01	0.01	0.01	0.01
Premix[s]	0.5	0.5	0.5	0.5	0.5	0.5
Supplements %						
Saponin[t]			0.5			
Isoflavonoid[u]				1.5		
Phytosterol[v]					1.00	
Mix						3.00
Proximate compoxition %						
Dry matter	92.6	94.4	95.0	92.4	91.5	94.1
Crude protein	38.9	41.7	41.7	40.6	40.0	41.5
Crude fat	22.2	23.8	23.0	22.1	22.4	23.8
Starch	15.1	13.7	14.0	13.2	12.9	13.5
Ash	6.1	6.2	6.2	6.0	5.9	6.0

[a]NorsECO-LT, Norsildmel, Fyllingsdalen, Norway. [b]DeNoFa, Fredrikstad, Norway. [c]68% protein, phytase treated, Becej, Serbia. [d]AGRANA Starke GmbH; Raiffeisenweg 2-6, A-4082 Aschach; [e]Gluvital 21000, Cargill, Barby, Germany. [f]Feed grade, Felleskjøpet, Kambo, Norway. [g]NorSalmOil, Norsildmel, Fyllingsdalen, Norway. [h]Choline Chloride-70%, Indukern, S.A., Spain. [i]Mono calcium phosphate monohydrate-Feed Grade, Yara Animal Nutrition, Oslo, Norway. [j]Franzefoss Miljøkalk AS, Rud, Norway. [k]Lysine monoHCl, 99% feed grade, Cheil Jedang, Indonesia. [l]Rhodimet® NP 99, *DL*-methionine, 99% feed grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo,

Brazil. [m]98% feed grade, PT. Cheil Jedang, Indonesia. [n]L-Arginine, 98.5%, Sigma-Aldrich Logistik GmbH, Steinheim, Germany. [o]98.5% feed grade, CJ (Shenyang) Biotech Co. LTD, Liaoning, China. [p]96.5% feed grade, Ajinomoto Eurolysine, Paris, France. [q]ROVIMIX, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland. [r]Metal Rare Earth Limited, Jiaxing, China. [s]Vilomix Norway AS, Hønefoss, Norway; provides per kg feed, Vitamin A 2500 IU; Vitamin D3 2400 IU; Vitamin E 0.2 IU; Vitamin K3 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 mg; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.20 g; Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; total Ca: 0.915 g; total K: 1.38 g; total Na 0.001 g; total Cl 1.25 g. [t,u,v] Three antinutrients were provided by EVONIK company.

The formulation and the chemical composition of the diets from the follow-up studies which were subject to characterization of technical quality are shown in Table 2. Four diets were made, three of them with 25% soy protein concentrate (SPC). The first of these contained SPC, the second and third with SPC plus 0.5% impurities from the processing of SBMX, and 0.5% saponins, respectively. The fourth diet contained the processed soybean meal SBMX from which antinutrients have been removed. To adjust for differences in protein content of the soybean products, diets based on SPC contain 9% corn gluten meal and 4.9% vital wheat gluten while SBMX diet contains 1% corn gluten meal and 13.86% vital wheat gluten.

Table 2 Formulation and chemical composition of the second experimental diets.

	SPC	SPC+Imp	SPC+Sap	SBMX
Ingredients %				
Fish meal, LT	15	15	15	15
Soy protein concentrate	25	25	25	
Soybean Impurities[a]		0.5		
Soybean Saponin[b]			0.5	
Soybean meal-X[c]				25
Corn gluten meal 65%	9	9	9	1
Vital wheat gluten	4.9	4.9	4.9	13.86
Wheat	15	15	15	15
Fish oil	25	25	25	24
Choline chloride[d]	0.06	0.06	0.06	0.06
MCP	1.82	1.82	1.82	1.82
Limestone	0.4	0.4	0.4	0.4
L-lys	1.2	1.2	1.2	1.21
DL met	0.9	0.9	0.9	0.9
L Trp	0.03	0.03	0.03	0.06
L Arg	0.5	0.5	0.5	0.5
L Thr	0.18	0.18	0.18	0.18
L Val	0.4	0.4	0.4	0.4
Stay C 35%	0.1	0.1	0.1	0.1
Y2O3	0.01	0.01	0.01	0.01
Premix	0.5	0.5	0.5	0.5
Proximate composition %				
Dry matter	95.4	95.3	93.5	94.6
Crude protein	42.8	42.6	41	43.3
Crude fat	21	22.4	22.2	20.0
Starch	14	14	14	15.6

[a]Impurities from saponin extraction procedure. [b]Soybean saponin same as in the first experiment. [c]New soybean meal from which antinutrients have been reduced. [d]Choline chloride 70%, C5H14ClNO, 139.6g/mol, Vilomix, Hønefoss, Norway. Other ingredients were same as in the first experimental diets.

3.3 Feed processing and extrusion parameters

All experimental feeds were processed at FôrTek (the center for feed technology), NMBU. The processing machines and procedure were same for the first and second experiment, except for the extrusion parameters and screw configurations which were displayed separately in tables and figures below. Prior to mixing, some of the ingredients were ground. These included fish meal, corn gluten meal, MCP, limestone and L-lys. Grinding was done in the machine Alpine

Pin Mill (160 UPZ, DOM:1988, Serial number: 5896.1) with 18 RPM speed and 7.5 HP motor. The screen size was 0.5 mm. Then these and the rest ingredients were mixed in ISDECA mixer (60-liter paddle-mixer, prototype, Fôrtek, Forberg, Norway). The mixed ingredients were directly sent to the extruder through a feeder which is connected to the extruder on the top. The feeding rate was 32 kg/h for all diets. The extruder used was a co-rotating twin-screw extruder with five-section (Bühler, BCTG 62/60 D, Uzwil, Switzerland). The extrusion parameters and screw configurations of the feed processing in first experiment are shown in Table 3 and Figure 10, respectively.

Table 3 Extrusion parameter of the first experiment diets.

	SBM PositiveC	**SPC** NegativeC	**SPC** Sap	**SPC** Iso	**SPC** Phy	**SPC** Mix
Parameter						
Die size	2	2	2	2	2	2
Number of dies	4	4	4	4	4	4
Calibration (Hz)	5	5	5	5	5	5
Feeder (kg/h)	32	32	32	32	32	32
Section 1 (°C)	40.1	39.6	40.2	41	42	41.6
Section 2 (°C)	82.4	84.5	84.8	84.5	86	85.6
Section 3 (°C)	92.7	97.1	97.5	98	98.4	98.2
Section 4 (°C)	106.6	108.2	108	108	108	108
Section 5 (°C)	97.6	96	95.1	94	93	93
Die temperature (°C)	98	98	98	98	98	97
Die pressure (bar)	32	32	32.6	28	27	27
SME (Wh/kg)	610	615	648	590	618	627
Drive power (kW)	6.2	6.5	6.7	6.2	6.5	6.6
Torque (Relative, %)	59	67	70	65	67	68
Screw speed (rpm)	240	215	215	215	215	215
Extr. water (kg/h)	10.5	10.5	10.5	10.5	10.5	10.5
Knife speed (rpm)	2600	2600	2600	2600	2600	2600
Number of knifes	6	6	6	6	6	6

Outlet	R							L	R							Inlet		R
	40	60	60	60	60	80	80	20	120*	100	80	60	40	60*	80	100	80	80

Figure 10 Screw configuration for experimental diets of first experiment.

* Polygon. R (right), L (left): Flow direction of each screw element.

Numbers are the length in cm of each screw element.
The crew at the inlet are undercut conveying elements with right flow direction. They have larger channel depth than the other elements.
Red arrow: 5mm spacer ring and 90° offset between the screw elements.

The extrusion parameters of the feed processing for the second experiment are shown in Table 4. Extrusion screw configuration for SPC, SPC+Imp and SPC+Sap diets is shown in Figure 12. The screw configuration for SBMX diet was the same with the configuration for the first experiment. For some reason, the system was not able to read the temperature in section 3 of the extruder during processing of SPC diets. Generally, the temperature is increasing gradually from section 1 to 5 in extrusion process, therefore the temperature at section 3 most likely drops between the temperature of section 2 and 4. For SBMX two 2mm dies were used with knife speed of 2950rmp. This was not the same for SPCs. High temperature profile, higher die temperature and pressure in the extruder sections were recorded and to prevent the potential damage of high-speed knife to the extrusion outlet within such short distance, the number of dies was changed from two (Figure 11A) to four for SPCs diets, with lower knife speed at 1800rpm. Calculated SME, drive power and torque were also lower in SBMX.

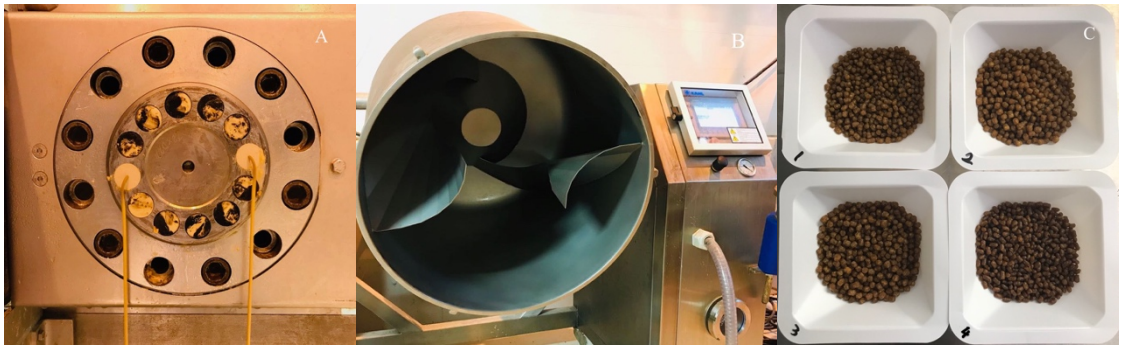

Figure 11 A: Feed is extruded through 2mm dies from the twin-screw extruder. B: Vacuum coater. C: Four experimental feed pellets 1-SPC, 2-SPC+Imp, 3-SPC+Sap and 4-SBMX. Photos were taken during the processing of second experiment diets.

The extruded feed pellets were dried for 50 minutes to reduce the moisture content to 8-9%. An IR machine (MB25 moisture analyser, Ohaus, Nänikon, Switzerland) was used to measure the moisture content. Drying was done in batch-driers with fan heaters (15KW, Inelco heaters, Dania-heater 15kW, Fjerritslev, Denmark). After drying, feed went through oil coating process in a vacuum coater (Gentle Vacuum Coater (GVC) - 80 prototype, Fôrtek, Amandus-Kahl) Figure 11B. At each coating process, 15 kg feed were weighed and added to the coater. At the same time, 5 kg fish oil which is preheated to 40 °C and transferred into a small pressurized

tank (30 liters) with a hose and nozzle (nozzle type: 6508). When the coating machine started, fish oil was sprayed from the nozzle inserted from the hole at the coater top. Air was cleared out to create vacuum and the pressure gradually increased to 2 bar. Then, air was released again into the coater and meanwhile fish oil was forced into the porous structure of the pellets. The coated feed pellets (Figure 11C) were packed in 20 kg bags.

Table 4 Extrusion parameters of diets in the second experiment.

Parameter	SPC	SPC+Imp	SPC+Sap	SBMX
Die size	2	2	2	2
Number of dies	4	4	4	2
Calibration (Hz)	5	5	5	5
Feeder (kg/h)	32	32	32	32
Section 1 (°C)	36	37	37	42.7
Section 2 (°C)	90	68	80	81.3
Section 3 (°C)	*	*	*	105.2
Section 4 (°C)	113	115	115	104.7
Section 5 (°C)	114	123	123	93.9
Die temperature (°C)	105	120	122	92
Die pressure (bar)	19.1	28.3	30	5.6
SME (Wh/kg)	544	711	670	245
Drive power (kW)	5.6	7.4	7.1	4.6
Torque (Relative, %)	58	79	77	40
Screw speed (rpm)	215	200	200	245
Extr. water (kg/h)	10.5	10.5	10.5	9.9
Knife speed (rpm)	1800	1800	1800	2950
Number of knifes	6	6	6	6

*The system did not read the temperature at section 3 during the processing of SPC diets.

Outlet	R							L	R								Inlet		R
	40	20	60	60	80	80	100	20	120*	60	60	60	40	60*	80	80	80	80	80

Figure 12 Extrusion screw configuration for SPC, SPC+Imp and SPC+Sap diets. The screw configuration for SBMX diet was the same with the configuration for the first experiment.

3.4 Sampling

In the first feeding experiment the fish were fed for 52 days after which four fish were randomly selected from each tank and anesthetized with MS-222. The fish were then weighed and measured for fork length individually. Blood samples from the caudal vein were collected in 1ml Eppendorf tubes using 1.2 ml heparinized vacutainers and stored in dry ice until analyzed. Fish were then killed with sharp blow to the head and cut open to collect intestinal samples. The viscera fat and connective tissue around the gastrointestinal tract were removed, and the tract was sectioned into four parts as shown in Figure 5. Stomach was removed and not sampled. The intestinal parts were cut opened longitudinally, the gut contents were collected in 8ml Eppendorf tubes and the tissues of the intestines were weighed and sampled. The content of pyloric intestine was divided in two from first half (PI1) and second half (PI2), so were the content of the distal intestine (DI1 and DI2). The content of the mid intestines was sampled as whole. The pyloric caeca were cleared of external fat, weighed and frozen in liquid nitrogen. The tissue of the distal intestine was divided and longitudinally and transverse in four parts, DI1 and DI2. One of the two proximal halves were cut in two and one part sampled in 20 ml miniature vials with formalin and stored at 4 °C for histological assessment, the other for gene expression, stored in RNA later. The other half of the proximal intestine was collected for enzyme analyses and preserved by freezing in liquid nitrogen and stored at -80 °C. Similar samples were collected from the distal half of the distal intestine. For gene expression, distal intestine samples were washed in PBS and collected in 8ml Eppendorf tubes with RNAlater and stored at 4 °C for 24 h then at -20 °C. At last, the livers were removed and weighed. The remaining 26 fish in each tank were batch weighed and fed as earlier for an additional 28 days during which four times of stripping for feces collection were performed. Once every week fish was anaesthetized by MS-222 and stripped for feces by the method of Austreng (1979). The fecal samples were pooled by tank and stored at -20 °C prior to analysis.

4 Analysis of samples and statistics

4.1 Physical quality analysis of feed samples

The analysis of pellet physical quality was carried out in LabTek (feed analysis laboratory) at the Animal Science Department, NMBU. Pellets diameter and length were measured by using electronic digital caliper. 30 pellets from each diet were randomly taken as samples. Based on

the measured size, expansion ratio was calculated as expansion ratio % = [(pellet width-die diameter) × die diameter^{-1}] ×100. Bulk density of the coated pellets was measured in triplicate for each diet by weighing the mass of the samples in a volume of 1 l measuring cup and marked as g/l. Texture analyzer (Tinius Olsen H5KT Benchtop Tester) was used for hardness analysis. Sample size for hardness was 10 pellets from each diet. Sinking velocity was measured by dropping 30 randomly selected pellets one by one into the center of a transparent tube with a diameter of 30 mm and a height of 150 cm. The tube was filled with tap water of drinking quality and 15 °C as the same temperature of the water in the fish tank at lab. The time for one pellet traveling through over 100 cm between two fixed points, one close to the top and another one close to bottom, was recorded on a stopwatch. Sinking velocity was measured as the distance per second cm/s.

To test fat leakage of the pellets, coffee filters (Bleached Size 4, 200 pcs, Rema 1000) were used. Samples were measured 10 g with three parallels and spread one layer on one side of the filter using sample spoon (Figure 13). Then the filters with the samples were put in zipper seal sample bags carefully and placed on the flat surface for three days. The weight difference of the filter before and after three days was considered as the total fat loss. Fat leakage was calculated as following formula: Fat leakage = [(FW-IW) /(SW × fat% in samples)] × 100. Where FW is the final weight of filter; IW is the initial filter weight; SW is sample weight.

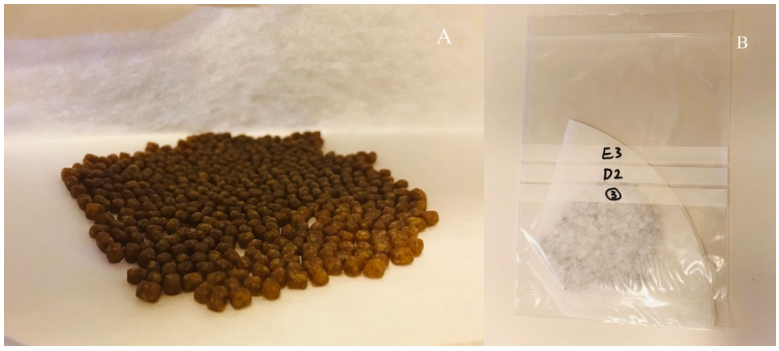

Figure 13 A: Feed pellets layered evenly in the coffee filter. B: Filter with samples placed in a zipper sample bag.

For water stability index, 20 g sample of each diet in three parallels were poured in a cylindrical wire-mesh basket (20 cm in length and 8cm in diameter) which then inserted in 600 ml beaker glass with 300 ml water of room temperature. The baskets were pre-weighed and recorded. Thereafter, the beakers with the samples were placed in a shaking water bath (Julabo SW22)

with shaking frequency of 120 rpm for 120 minutes at 25 °C. After 2 hours, the samples were taken out and dried in an oven at 104 °C for 16 hours. The dry weight of sample was recorded and used to calculate the water stability index, by the formula: WSI = [FW/ (IW×DM% in samples] ×100. Where FW is the final weight of samples and IW is the initial sample weight.

4.2 Chemical analysis of feed and faecal samples

Pooled fecal samples were lyophilized and homogenized, and dry matter was estimated by difference. The dry matter contents of feed were measured by oven drying at 103 °C ± 2 °C. Ash content was analyzed by combustion at 550 °C to constant weight. Nitrogen content was determined by the Kjeldahl method (Kjeltec-Auto System Tecator, Höganäs, Sweden) and crude protein was calculated using conversion factor 6.25. Crude fat was analyzed by accelerated solvent extraction, ASE (ASE® 350 Accelerated Solvent Extractor, Nerliens Mezanski). Starch was analyzed by adding α–amylase and amyloglucosidase to broken down starch into glucose units which is then was determined spectrophotometrically (RX4041 Randox Daytona+, England). Yttrium was measured by inductivity coupled plasma (ICP) mass-spectroscopy as described by Refstie et al. (1997).

4.3 Histological analysis of distal intestine

The histological structure of distal intestine was analyzed according to standard method at the Section for Pathology of the Veterinary School (Oslo, Norway). The DI samples were taken out from the formalin and sectioned longitudinally in length of approximately 3-4 mm. Afterwards the sections were dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin. The DI samples with paraffin were sectioned in 3µm thickness and stained with haematoxylin and eosin (H&E). The examination was performed under a light microscope (Carl Zeiss microscopy AX10, Germany). The tissue parameters of distal intestine such as enterocyte vacuolization, lamina propria width and infiltration, submucosa width and infiltration and mucosal fold height were evaluated by using a semi-quantitative scoring system. The degree of morphological change was assigned to five categories: normal, mild, moderate, marked and severe, 1 to <3 represents normal; 3 to <5, mild changes; 5 to <7, moderate changes; >7 to <9, marked changes, and 9-10, severe changes.

4.4 Analysis of enzyme activities and bile salt concentration

Activity of the brush border membrane enzyme leucine aminopeptidase (LAP) was measured in the homogenates of all intestinal sections tissue. The homogenates were prepared from tissues thawed in ice-cold tris-mannitol buffer (1:20 w/v) containing the serine proteinase inhibitor 4-[2-Aminoethyl] benzenesulfonylfluoride HCl (Pefabloc® SC; Pentapharm Limited). The activity of LAP was determined colorimetrically using L-leucine-β-naphthylamide as the substrate described by Krogdahl et al. (2003).

Trypsin activity in intestinal chyme were analyzed on pooled freeze-dried gastrointestinal contents from PI, MI and DI. Trypsin activity in intestinal chyme was determined colorimetrically according to Kakade et al. (1973) on pooled freeze-dried samples, using the substrate benzoyl-arginine–p-nitroanilide (BAPNA) (Sigma no. B-4875; Sigma Chemical Co., St. Louis, MO, USA) and a curve derived from a standardized bovine trypsin solution.

Bile salt concentration in intestinal chyme was determined using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory at the Faculty of Veterinary Medicine, NMBU.

4.5 Analysis of plasma metabolites

The levels of plasma metabolites: free fatty acids (FFA), glucose, cholesterol, triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by standard protocols (Advia 1800, Siemens Healthcare Diagnostics, Erlangen, Germany) at the Central Laboratory of the NMBU School of Veterinary Medicine (Oslo, Norway).

4.6 Quantitative Real Time PCR (qPCR)

Real-time qPCR assays were performed following the MIQE guidelines (Bustin et al., 2009) on 4 pooled samples per diet group. Each pooled sample consisted of equal amounts of total RNA from each of n=4 animals sampled from the same tank replicates. Total RNA was extracted manually in randomized order using Trizol® reagent and further purified using a PureLink column-based kit (Thermo Fisher Scientific). The RNA extraction included a DNase treatment according to the manufacturer's protocol. RNA integrity was evaluated by the 2100

Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies). RNA purity and concentration were measured using Take3 micro-volume plates and Epoch microplate spectrophotometer (BioTek Instruments). All samples had RNA integrity numbers (RIN) >8, with a mean RIN value of 9.3, indicative of excellent RNA quality. Total RNA was stored at -80 °C until use. First strand cDNA was synthesized using 250 ng total RNA from all samples to obtain tank pools of n=4 individuals each (i.e., 1µg total RNA per cDNA reaction) using Superscript IV VILO Mastermix (Thermo Fisher Scientific) in 20 µl reactions and primed with a mixture of Oligo(dT)20 and random hexamer 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. A panel consisting of 25 target genes with key roles in intestinal immune, metabolic and stress/antioxidant function were profiled. See Table 5 for details.

Expression of individual gene targets was analyzed using the LightCycler 96 (Roche Diagnostics). Each 10 µl DNA amplification reaction contained 2 µl PCR-grade water, 2µL of 1:10 diluted cDNA template (corresponding to 8ng total RNA), 5 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µl (final concentration 500nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC). The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95 °C (10 s), 60 °C (10 s) and 72 °C (15 s). 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. For target gene normalization, gapdh, rnapolii and hprt1 were evaluated for use as reference genes by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variance, and hprt1 was used as the internal normalization factor. Mean normalized levels of target genes were calculated from raw quantification cycle (Cq) values.

Table 5 Target gene names, category and function.

Gene name	Acronym	Category	Function
Interleukin 1β	IL1β	Immune	Pro-inflammatory
Interleukin 4	IL4	Immune	Pro-inflammatory
Interleukin 8	IL8	Immune	Pro-inflammatory
Interleukin 10	IL10	Immune	Anti-inflammatory
Interleukin 17a	IL17A	immune	T cell marker
Interferon γ	IFNγ	Immune	Pro-inflammatory
Transforming growth factor β	TGFβ	Immune	Anti-inflammatory
Cluster of differentiation 3 γδ	CD3γδ	Immune	T-cell marker
Cluster of differentiation 8 β	CD8β	Immune	T-cell marker
Fork-head box P3	FOXP3	Immune	T-cell signalling
Major histocompatibility complex, class 1	MHC1	Immune	Antigen presentation
Myeloid differentiation primary response gene 88	MYD88	Immune	NF-κB activation
Collagenase 3	MMP13	Immune	Tissue remodeling
Proliferating cell nuclear antigen	PCNA	Immune	Cell proliferation
Fatty acid binding protein 2b	FABP2B	Metabolism	Fatty acid transporter
Aquaporin 8ab	AQP8AB	Metabolism	Water channel
Superoxide dismutase 1	SOD1	Stress/Antioxidant	Antioxidant defense
Catalase	CAT	Stress/Antioxidant	Antioxidant defense
Heat shock protein 70	HSP70	Stress/Antioxidant	Stress response
Claudin-15	CLDN15	Barrier function	Tight junction proteins
Claudin-25b	CLDN25B	Barrier function	Tight junction proteins
E-Cadherin	CDH1	Barrier function	Tight junction proteins
Zonula occludens 1	ZO1	Barrier function	Tight junction proteins
Mucin-2	MUC2	Barrier function	Mucus protein
Caspase 6	CASP6	Immune	Apoptosis

4.7 Calculations

Average weight gains for per fish (WG) was calculated as the difference of final body weight and the initial weight. Specific growth rate (SGR) = [(ln FBW − ln IBW)/number of days] × 100, where FBW and IBW are final body weight and initial body weight, respectively. Apparent digestibility coefficient (ADC) =100 – [100 × (nutrient in faeces/yttrium oxide in faeces) × (yttrium oxide in feed/nutrient in feed)]. Condition factor (CF) = (body weight/fork length^3) ×100. Relative carcass weight (RelCW) = (CW/Body weight) × 100, where CW was the weight of the fish after organs were removed. The relative weight of liver and intestine to body weight were calculated as: Somatic Index (SI) = Organ weight /Body weight × 100. Somatic index of liver was marked as hepatosomatic Index (HSI) and the indices of intestines were marked as pyloric intestine somatic index (PISI), mid intestine somatic index (MISI) and distal intestine somatic index (DISI).

4.8 Statistical analyses

Differences in histological scores for the various evaluated morphological characteristics of the distal intestine tissue were analysed for statistical significance using the Fisher exact test. Post hoc analysis for significant Fisher exact test results was conducted using the Chisq.post.hoc test in the Fisfer package in the R statistical package (version 3.6.2; 2019).

Biological data and physical quality of pellets were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) for post hoc comparison on R Studio (Version 1.3.959 © 2009-2020 RStudio, PBC). The level of significance for all analysis was set at $P < 0.05$.

5 Results

5.1 Histological changes of distal intestine

Figure 14 displayed the proportions of sampled individuals that were scored as "normal", "mild", "moderate", "marked", or "severe" for enterocyte supranuclear vacuolization, mucosal fold height, lamina propria and submucosal cellularity in the distal intestine. Positive control SBM fed fish showed the typical characteristics for soybean meal induced enteritis (SBMIE) such as reduced supranuclear vacuolation, shorting of mucosal fold height, and increased width and cell infiltration of both lamina propria and submucosa. Fish fed SPC diet, as a negative control, did not show any abnormalities of intestine. Supplementation of SPC with saponins altered the histological structure towards inflammation for all four observed inflammation biomarkers. The observed effects were milder than for SBM and significant only for supranuclear vacuolization and width of submucosa. The intestinal structure of the fish groups with the supplementation of isoflavonoids and physterols showed the same appearance with the negative group SPC in most parameters, except for a non-significant mild increase of submucosa cellularity. However, the mix of antinutrients caused the mildest changes in toward inflammation for all these biomarkers, only significant for supranuclear vacuolization.

Figure 14 Proportions of sampled individuals that were scored as "normal", "mild", "moderate", "marked", or "severe" for enterocyte supranuclear vacuolization, mucosal fold height, lamina propria and submucosal cellularity in the distal intestine. Different letters on the top of the columns indicate significant differences among diets ($p<0.05$).

5.2 Growth performance and nutrient digestibility

After 52 days of feeding, mortality was zero. Fish fed on the six different diets did not show significant differences in weight gain (WG) or specific growth rate (SGR) as shown in Table 6. For the sampled fish, the condition factor (CF) and the relative carcass weight (RelCW) differed significantly among the diets. CF were significantly higher in SPC group than SPC with antinutrients saponin, isoflavonoids and the mix antinutrients, but not different from SBM and SPC with phytosterol groups. Lower RelCW were observed in fish fed on SPC diet compared to fish fed on SBM and SPC+Iso diets.

Table 6 Growth performance, condition factors and relative carcass weight (Mean ± S.E.M*).

	SBM	SPC	SPC+Sap	SPC+Iso	SPC+Phy	SPC+Mix	P value
IBW	28 ± 0.3	28 ± 0.3	28 ± 0.1	29 ± 0.3	28 ± 0.3	28 ± 0.3	0.728
FBW	89 ± 4	87 ± 2	82 ± 7	84 ± 4	87 ± 1	83 ± 1	0.526
WG	61 ± 4	59 ± 2	54 ± 4	55 ± 4	59 ± 1	55 ± 2	0.501
SGR	2.2 ± 0.08	2.2 ± 0.04	2.1 ± 0.07	2.1 ± 0.09	2.2 ± 0.05	2.1 ± 0.05	0.543
CF	1.3 ± 0.02^{ab}	1.4 ± 0.01^{a}	1.3 ± 0.03^{b}	1.2 ± 0.03^{b}	1.3 ± 0.01^{ab}	1.3 ± 0.01^{b}	**0.002**
RelCW	86 ± 0.3^{a}	84 ± 0.2^{b}	86 ± 0.1^{ab}	86 ± 0.5^{a}	86 ± 0.3^{ab}	85 ± 0.3^{ab}	**0.006**

*S.E.M: standard error of the mean. IBW: initial body weight. FBW: final body weight. WG: weight gain. SGR: specific growth rate. FCR: feed conversion ratio. CF: condition factor. RelCW: relative carcass weight.

Apparent digestibility coefficient (ADC) of dry matter (DM), crude protein (CP), starch and lipid of fish fed the six experimental diets are presented in Figure 15. Diet effects were shown in the ADC of all nutrients. Overall, fish fed SBM diet had higher nutrients digestibility than SPCs. Digestibility of DM, CP and lipids in SPC based salmon were significantly lower than those of SBM diet. Supplementation with saponin, isoflavonoids, phytosterols and the mix to the SPC diet did not change the nutrient digestibilities markedly compared to the SPC diet. The only significant changes were slightly higher ADC of DM for SPC+Iso and higher ADC of CP in SPC+Mix group than SPC.

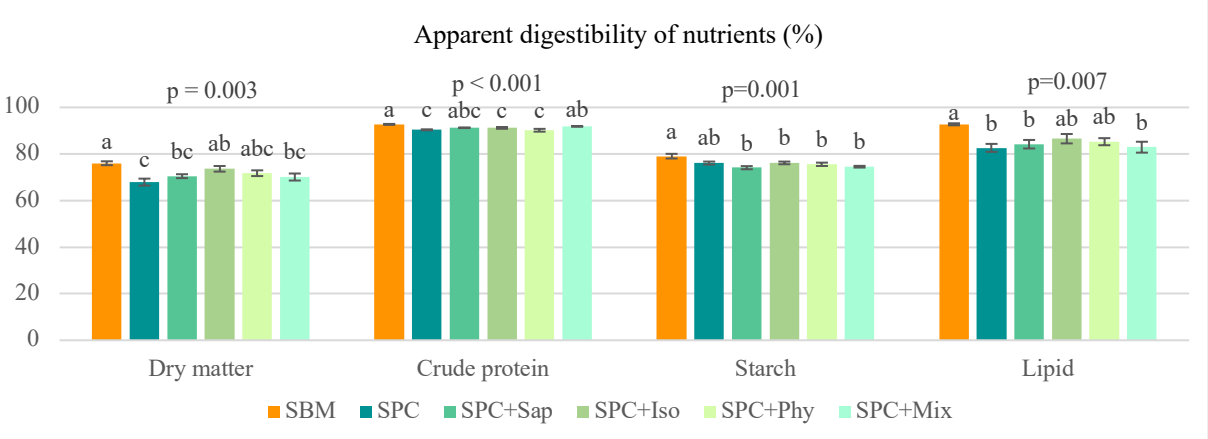

Figure 15 The result of one-way ANOVA for nutrient digestibility. Error bars show standard error of the mean (S.E.M.). Significant differences between diets are denoted with letters.

5.3 Organ somatic indices

In figure 16, the bar chart shows the results regarding hepatosomatic index (HSI), and somatic indices of pyloric intestine (PISI), mid intestine (MISI) and distal intestine (DISI). Fish fed on SBM diet had higher HSI but showed lower PISI and MISI compared to all SPC diets. On the other hand, the value of MISI in SBM fed fish was significantly lower than SPCs. Among SPCs, the supplements of saponin, isoflavonoids and phytosterols in the diet did not affect HSI, PISI and MISI of the fish compared to those of SPC diet. For DISI value, fish fed SBM was significantly lower than fish with SPC diet, and saponin and the mix in the diets showed the same effects on DISI as the SBM-fed fish.

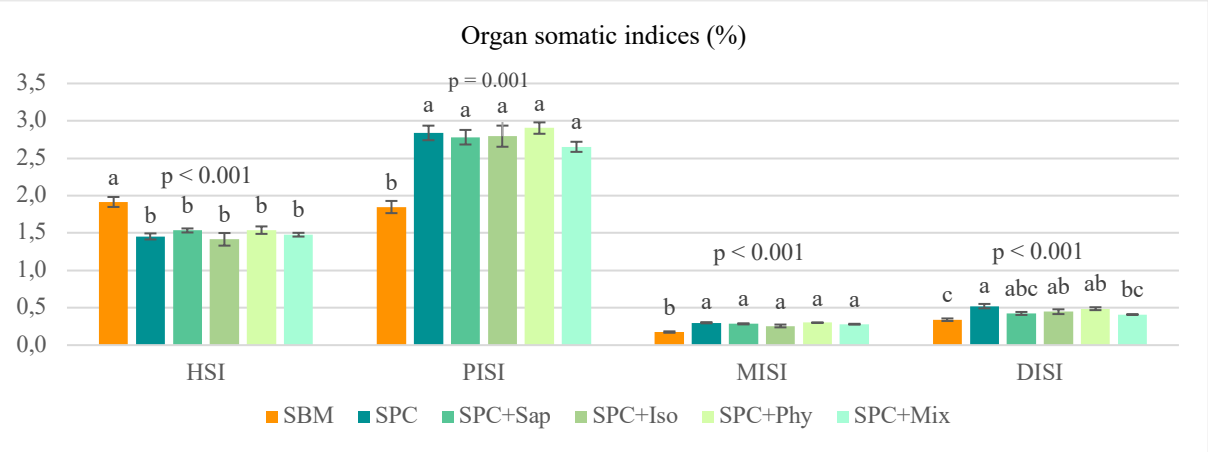

Figure 16 The result of one-way ANOVA for organ somatic indices. Error bars show standard error of the mean (S.E.M.). Significant differences between diets are denoted with letters.

5.4 Brush border membrane enzyme LAP activity

Figure 17 shows the activity of the brush border membrane enzyme LAP (mmol/h/g tissue) in PI, MI and DI. Fish fed SBM diet showed a trend of lowered LAP capacity in DI compared to fish fed SPC. The supplementation of saponin alone and together with other antinutrients displayed same trend as for the SBM diet in number but not statistically significant. Isoflavonoids and phytosterols did not change the LAP capacity in the DI of salmon compared to SPC group fish. There were no significant differences LAP capacity in MI and PI between diets.

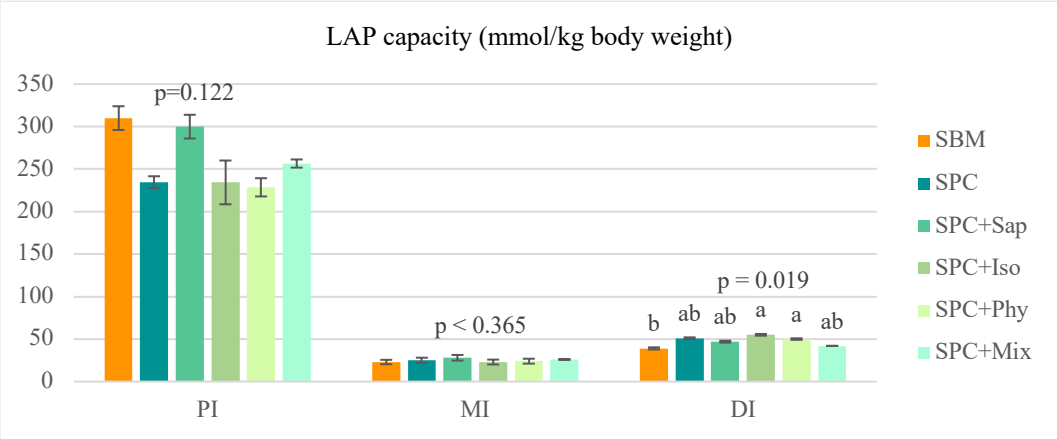

Figure 17 LAP capacity (mmol/kg body weight) in PI, MI and DI. Error bars show standard error of the mean (S.E.M.). Significant differences between diets are denoted with letters

5.5 Bile salt concentration and trypsin activity in intestinal chyme

As shown in Table 7, the bile salt concentration in DI content of fish fed SBM was significantly lower than in fish fed SPC, while the concentration in MI of the fish was not different between these two diets. The antinutrient supplementations tended to decrease the bile salt concentration in DI chyme. Saponin group had the same level of bile salts in DI chyme as the SBM diet group. Isoflavonoids, phytosterols and the mixture decreased the concentration numerically, but not significant compared to the SPC group. No diet effects were observed on bile salt concentration in the chyme from intestinal sections PI1 and PI2. The same was the case for trypsin activity of the chyme in all intestinal sections of the salmon.

Table 7 Bile salt concentration and trypsin activity in intestinal chyme (Mean ± S.E.M*).

	SBM	SPC	SPC+Sap	SPC+Iso	SPC+Phy	SPC+Mix	P value
Bile salt (U mg^{-1})							
PI1	218 ± 11	205 ± 16	209 ± 16	229 ± 9	241 ± 3	202 ± 19	0.307
PI2	164 ± 6	156 ± 11	140 ± 9	153 ± 9	170 ± 7	144 ± 12	0.228
MI	147 ± 9^{a}	137 ± 3^{ab}	107 ± 7^{b}	132 ± 11^{ab}	146 ± 11^{a}	114 ± 4^{ab}	**0.009**
DI	29 ± 7^{b}	71 ± 1^{a}	26 ± 3^{b}	45 ± 12^{ab}	52 ± 10^{ab}	38 ± 6^{ab}	**0.005**
Trypsin activity (U mg^{-1})							
PI1	176 ± 29	117 ± 15	129 ± 5	151 ± 15	115 ± 8	152 ± 17	0.125
PI2	156 ± 16	110 ± 7	104 ± 22	107 ± 15	111 ± 6	124 ± 12	0.132
MI	102 ± 26	78 ± 18	53 ± 17	71 ± 21	77 ± 13	75 ± 23	0.689
DI	45 ± 6	43 ± 4	23 ± 4	29 ± 5	27 ± 7	29 ± 13	0.225

*S.E.M: standard error of the mean.

5.6 Plasma metabolites

The level of the blood plasma metabolites free fatty acids (FFA), glucose (Glu), cholesterol (Cho), triglycerides (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are illustrated in Table 8. Glucose level in fish fed the SBM diet was higher than in those fed the SPC diet, whereas the groups fed saponins, alone or in a mixture with the other antinutrients were not significantly different from either the SBM or the SPC group. Cholesterol level was significantly higher in the fish from SBM group than all SPC diets. For the level of triglycerides in the fish blood, SBM and SPC did not show differences. The antinutrients isoflavonoids and phytosterols lowered level of TG in the fish compared to SPC.

Table 8 The level of blood plasma metabolites (Mean ± S.E.M*).

	SBM	SPC	SPC+Sap	SPC+Iso	SPC+Phy	SPC+Mix	P value
FFS	0.6 ± 0.02	1.1 ± 0.24	0.8 ± 0.07	0.8 ± 0.07	1.1 ± 0.32	0.8 ± 0.03	0.228
Glu	8.6 ± 0.45^{a}	6.9 ± 0.16^{b}	7.7 ± 0.34^{ab}	7.1 ± 0.21^{b}	7.3 ± 0.18^{b}	7.6 ± 0.27^{ab}	**0.007**
Cho	9.0 ± 0.35^{a}	6.4 ± 0.25^{b}	6.6 ± 0.11^{b}	6.5 ± 0.19^{b}	6.6 ± 0.22^{b}	5.8 ± 0.04^{b}	**<0.001**
TAG	3.8 ± 0.29^{ab}	4.8 ± 0.59^{a}	3.9 ± 0.21^{ab}	3.5 ± 0.19^{b}	3.5 ± 0.16^{b}	5.0 ± 0.59^{a}	**0.041**
ALT	51 ± 2	64 ± 6	69 ± 9	66 ± 7	60 ± 4	54 ± 7	0.260
AST	779 ± 38	954 ± 38	999 ± 34	959 ± 139	929 ± 37	901 ± 97	0.424

*Abbreviations FFA: free fatty acids, Glu: glucose, Cho: cholesterol, TG: triglycerides, ALT: alanine aminotransferase and AST: aspartate aminotransferase. S.E.M: standard error of the mean. P-value in bold indicates significant <0.05. ^{a,b} Significant differences between diets.

5.7 Gene expression

As shown in Table 9, Real-time quantitative PCR analyses of DI tissue of SBM-fed fish, compared to SPC-fed fish, displayed up-regulated expression of proinflammatory cytokine IL-17A, proliferating cell nuclear antigen PCNA, apoptosis gene caspase CASP6, heat shock protein 70 HSP70, tissue modeling gene collagenase3 MMP13, tight junction protein genes CLDN25B and CDH1. The expression of proinflammatory cytokine IL8, water channel gene aquaporin AQP8AB, tight junction protein gene CLDN15 and antioxidant gene catalase CAT were down regulated. For fish fed SPC with saponin and the mixed antinutrients, the expression of genes IL-8, AQP8AB, CLDN15, CLDN25B and CAT showed similar regulation with SBM-fed fish. The addition of isoflavonoids and phytosterols in the diet increased AQP8AB expression. And, diet with phytosterols upregulated the expression of IL-1β compared to the SPC-fed fish.

Table 9 Gene expression of distal intestine tissue.

	SBM	SPC	SPC+Sap	SPC+Iso	SPC+Phy	SPC+Mix	S.E.M.*	P value
IL1β	0.003^{ab}	0.002^{b}	0.002^{b}	0.002^{b}	0.006^{a}	0.002^{b}	0.0003	**0.001**
IL4	0.01	0.01	0.01	0.01	0.01	0.01	0.001	0.837
IL8	0.008^{b}	0.013^{a}	0.007^{b}	0.009^{ab}	0.010^{ab}	0.012^{ab}	0.0005	**0.012**
IL10	0.0004	0.0005	0.0004	0.0005	0.0005	0.0005	0.00003	0.911
IL17A	0.0008^{a}	0.0005^{ab}	0.0004^{b}	0.0004^{b}	0.0006^{ab}	0.0005^{ab}	0.00004	**0.029**
IFNγ	0.001	0.001	0.001	0.001	0.001	0.001	0.0001	0.794
TGFβ	0.02	0.02	0.01	0.02	0.02	0.02	0.001	0.456
CD3γδ	0.18	0.18	0.18	0.22	0.29	0.17	0.015	0.122
CD8β	0.05	0.04	0.04	0.05	0.05	0.05	0.002	0.788
FOXP3	0.007	0.007	0.005	0.005	0.009	0.006	0.0005	0.163
MHC1	0.52^{ab}	0.49^{ab}	0.51^{ab}	0.53^{ab}	0.71^{a}	0.44^{b}	0.026	**0.034**
MYD88	0.08	0.08	0.06	0.06	0.07	0.06	0.003	0.137
FABP2B	6.5	8.1	9.2	10.3	8.8	8.2	0.46	0.287
MMP13	0.19^{a}	0.02^{b}	0.05^{b}	0.03^{b}	0.03^{b}	0.06^{b}	0.013	**<0.001**
AQP8AB	0.04^{c}	0.45^{b}	0.33^{bc}	0.85^{a}	0.88^{a}	0.21^{bc}	0.073	**<0.001**
CLDN15	2.2^{b}	4.3^{a}	2.8^{ab}	4.3^{a}	4.5^{a}	3.06^{ab}	0.234	**0.004**
CLDN25B	1.69^{a}	0.98^{b}	1.04^{ab}	1.19^{ab}	1.22^{ab}	1.23^{ab}	0.072	**0.043**
CDH1	5.6^{a}	2.8^{b}	3.4^{b}	3.2^{b}	3.3^{b}	3.4^{b}	0.23	**<0.001**
ZO1	0.37	0.30	0.30	0.27	0.31	0.32	0.016	0.559
MUC2	7.68	8.13	7.73	9.47	11.02	8.34	0.528	0.442
HSP70	17.7^{a}	9.5^{b}	9.7^{b}	10.3^{b}	12.0^{b}	10.9^{b}	0.66	**<0.001**
PCNA	0.60^{a}	0.22^{b}	0.29^{b}	0.30^{b}	0.27^{b}	0.33^{b}	0.031	**<0.001**
CASP6	0.77^{a}	0.45^{b}	0.47^{b}	0.42^{b}	0.56^{ab}	0.45^{b}	0.034	**0.008**
SOD1	2.47	2.15	1.92	1.92	1.93	2.13	0.074	0.205
CAT	0.09^{c}	0.33^{a}	0.18^{bc}	0.34^{a}	0.41^{a}	0.21^{b}	0.024	**<0.001**

*Pooled standard error of the mean. P-value in bold indicates significant <0.05. ^{a,b} Significant differences between diets.

5.8 Physical quality of feed pellets

The result of the physical quality of the diets in the second experiment is presented in Table 10. The diet with SBMX showed significantly lower value on diameter, expansion ratio and water stability than SPC and higher value for length and bulk density. Among three SPC based diets, SPC with saponins has the highest value for diameter, expansion ratio and fat leakage. Diet with SPC has the highest bulk density and sinking velocity.

Table 10 Pellet physical quality of the diets in second experiment (Mean ± S.E.M.)*.

	SPC	SPC+Imp	SPC+Sap	SBMX	P value
Diameter (mm)	2.8 ± 0.02^{c}	3.1 ± 0.03^{b}	3.2 ± 0.03^{a}	2.5 ± 0.02^{d}	**<0.001**
Length (mm)	3.5 ± 0.05^{b}	3.4 ± 0.05^{b}	3.5 ± 0.04^{b}	4.2 ± 0.05^{a}	**<0.001**
Expansion (%)	40 ± 1^{c}	56 ± 1^{b}	60 ± 1^{a}	25 ± 1^{d}	**<0.001**
Bulk density (g/l)	677 ± 3^{b}	641 ± 2^{c}	635 ± 3^{c}	706 ± 3^{a}	**<0.001**
Hardness (N)	25 ± 0.8	26 ± 0.6	29 ± 0.9	27 ± 0.9	0.143
Water Stability (%)	83 ± 0.3^{a}	85 ± 0.3^{a}	86 ± 0.3^{a}	70 ± 6.4^{b}	**0.011**
Sinking Velocity (cm/s)	6.2 ± 0.24^{a}	4.8 ± 0.27^{b}	4.6 ± 0.25^{b}	7.0 ± 0.22^{a}	**<0.001**
Fat leakage (%)	8.5 ± 0.26^{b}	8.3 ± 0.36^{b}	11.7 ± 0.49^{a}	8.0 ± 0.23^{b}	**<0.001**

*S.E.M: standard error of the mean. [abcd]Significant difference among diets.

6 Discussion

Among the results the following were most important and deserve to be discussed:

- The diet with SBM induced the expected SBMIE in DI of the salmon.
- Supplementation of the SPC diet with saponin induced DI alterations typical for SBMIE.
- Gene expression of DI tissue in fish with SBMIE indicated alteration in immune functions, increased cell proliferation, damages, apoptosis and dysfunctions of the tissue such as disrupted barrier functions and fluid permeability.
- The antinutrients isoflavonoid and phytosterol did not cause inflammation when supplemented alone in SPC diet nor did they worsened the severity of the SBMIE when supplemented together with saponins in SPC diet.
- The observed differences in physical quality of the diets were influenced by the ingredients and extrusion parameters. However, the overall variations between the diets were small and thus may contribute little to the nutritional value of the pellets.

6.1 Comparison between SBM and SPC diet – Induction of SBMIE

The distal intestine of fish fed SBM diet showed the typical histological signs of SBMIE: 1) reducing supranuclear vacuolation, 2) shorting of mucosal fold height and 3) increased width of lamina propria and submucosa with infiltrated cells. This is in agreement with the findings from previous studies (Baeverfjord & Krogdahl, 1996; Bakke-McKellep et al., 2000; Chikwati et al., 2013a; Chikwati et al., 2013b; Krogdahl et al., 2003; Sahlmann et al., 2013; Van den Ingh

et al., 1991). Significantly decreased weight of DI tissue in fish fed SBM diet is also indicating enteritis, as reported previously (Bakke-McKellep et al., 2007b; Krogdahl et al., 2015b). The alcohol-soluble and heat-stable antinutrient saponin in SBM plays an important role in the development of SBMIE (Chikwati et al., 2012; Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015b). In the commercial defatted soybean meal, the content of saponins is around 5.1-7.0 g/kg, whereas there the level of saponin in alcohol extracted SPC is low (Knudsen et al., 2006). In the current study, the intestine of salmon fed SPC as negative control group was normal with no signs of enteritis. The results demonstrate the absence of saponin in SPC and is in line with the results from the studies mentioned above (Olli & Krogdahl, 1994; Van den Ingh et al., 1991).

The shortening of mucosal fold height in an inflamed distal intestine is related to the increased loss of epithelium cells and apoptosis during the development of SBM-induced enteritis, which also contribute to the decreased weight of the tissue (Bakke-McKellep et al., 2007b). Rapid apoptosis and cell loss at the tip of mucosal fold trigger the increase proliferation of enterocytes at the base of mucosal fold and migration rate of cells toward the apical area of mucosal fold (Chikwati et al., 2013b). In the present study upregulation of expression of proliferating cell nuclear antigen PCNA and apoptosis gene caspase CASP6 were observed, which indicate increased cell proliferation and apoptosis in the distal intestine in SBM-fed fish. Increased PCNA has also been suggested to be the marker for reparation process of damaged cells (Chikwati et al., 2013b). Cell damages and stress during SBM-induced enteropathy in salmon were also indicated by elevated immunohistochemical expression of heat shock protein 70 (HSP70) and tissue modeling gene collagenase3 (MMP13). These changes disturb the integrity of intestinal epithelium and might impair barrier function of epithelium, e.g. against pathogens and feed antigens. In addition to the epithelium, the tight-junction proteins occludin and claudins act as the main paracellular barrier between the epithelial cells (Jutfelt, 2011). Upregulation of tight junction protein genes cldn25b and cdh1 as well as down-regulation of cldn15 from this study further indicated that SBM interfered with barrier functions of distal intestine. Alteration of fluid permeability is indicated by the decrease of water channel gene aquaporin (AQP8AB) during distal intestinal inflammation in salmon, similar to the result from the previous study (Hu et al., 2016). Antioxidant gene catalase (CAT) which provides protection against oxidative stress were significantly decreased in the SBM-fed fish compared to SPC-fed fish, indicating an increase of vulnerability of epithelium to oxidative stress (Scandalios, 2005).

The width of lamina propria of distal intestine was increased in fish with SBM-induced enteritis due to the infiltration of immune cells. The earlier study demonstrated that these cells comprise mainly innate immune cells, i.e. macrophages and neutrophilic granulocytes (Bakke-McKellep et al., 2000). In a later study by Bakke-McKellep et al. (2007a), the result showed that a major part of the immune cells in the lamina propria were positive for CD3ε and negative for immunoglobulin M (IgM). Along with significantly increased expression of genes coding for CD3pp, CD4 and CD8b in the distal intestine, the authors concludes that SBMIE involves T-cell-like responses (Bakke-McKellep et al., 2007a; Marjara et al., 2012). This theory is further supported by significantly up-regulated gene expression of proinflammatory cytokine IL-17A in fish with SBM-induced enteritis (Marjara et al., 2012). Cytokine IL-17A is produced by T helper 17 cells (CD4+ cells), which induce immune signaling molecules and mediate proinflammatory responses to help the pathogen clearance process (Marshall et al., 2018). Up-regulation of IL-17A has also been observed in humans suffering from inflammatory bowel diseases (IBDs) (Fujino et al., 2003). The present study showed the same observation of increased IL-17A expression in fish fed SBM diet. In addition, another supportive evidence of the involvement of T-helper cells in SBMIE is the concomitant increase in the expression of interleukin 1 beta IL-1β in the distal intestine of fish fed SBM diet, in line with the result of the study by Marjara et al. (2012).

Inflammation caused by SBM in the distal intestine of salmon may interfere with macronutrient digestibility and decrease growth performance of the fish (Baeverfjord & Krogdahl, 1996; Krogdahl et al., 2003). During SBMIE, the epithelium in DI is filled up by immature cells to replace the dead or damaged cell at the apical area of the mucosal fold (Bakke-McKellep et al., 2007b). The immature cells lack supranuclear vacuolation. Since supranuclear vacuoles is considered to be involved in protein transport across the enterocytes, as reviewed in a previous report (Sahlmann et al., 2015), the absorption of intact protein might be reduced due to the less or absence of SNVs in epithelial enterocytes of distal intestine. Reduced brush border membrane enzyme activity is another dysfunction of the distal intestine related to the nutrient digestibility in fish with SBM-induced enteritis. In most relevant previous studies DI brush border membrane LAP capacity has been markedly reduced during the development of enteritis (Bakke-McKellep et al., 2000; Chikwati et al., 2013a). However, the results of the present study displayed similar LAP capacity in DI of salmon fed SBM and those fed fish SPC. Therefore, the higher apparent digestibility of CP, lipids and starch in SBM-fed fish than in fish fed SPC diet was unexpected. Soy protein concentrate has been suggested to be able to fully replace fish

meal without compromising the growth and nutrients digestibility of fish, and has been shown to support higher growth rate and nutrients digestibility than SBM (Olli & Krogdahl, 1994; Storebakken et al., 1998; Storebakken et al., 2000). The reason for the higher nutrient digestibility for the SBM in the present study can be an exceptionally good quality of the SBM, while the SPC, which undergoes many processing steps during which the protein quality might be changed, may be of a lower quality. The lower nutrient digestibility in the fish fed SPC diets might be the part of the reason for lack of clear differences in growth rate of the SBM group for which higher digestibility of nutrients might compensate the negative effects of inflammation. The explanation for the higher PISI of the fish fed the SBM diet may be a normal, physiological response to increased demands for digestive components. The size of the compartments of the intestine, under normal conditions will expand according to the needs for endogenous compounds, as shown e.g. in mice (Ge & Morgan, 1993).

Inclusion of SBM in the salmon feed has been reported to reduce bile salt and cholesterol level in blood plasma (Kortner et al., 2013; Romarheim et al., 2008), which might be the reason for reduced lipid digestibility in salmonids fed diets containing SBM (Krogdahl et al., 2003; Olli & Krogdahl, 1994; Romarheim et al., 2008). From the transcriptional profile, decreased expression of the gene coding for the fatty acid binding proteins (FABP2) gives the sign of lowered lipid absorption (Venold et al., 2013). In the present study, the observed level of bile salt in distal intestinal content was lower in fish fed SBM than in fish fed SPC. It might lead to lowered blood bile salt. However, the expression of FABP2 in DI, and the level of free fatty acids and triglycerides in blood plasma only showed non-significant numerical decreasing trend in SBM group compared to the SPC group. Interestingly, plasma cholesterol was higher in fish fed SBM than in fish fed SPC. This might partially be due to the observed high lipid and protein digestibility. Higher blood glucose level in SBM-fed fish might be a result of the slightly higher starch content in the feed than SPC-fed fish, but also the sucrose in the SBM may be contributing to higher blood glucose (Hou et al., 2009).

Previous studies have shown elevated level of pancreatic enzyme trypsin activity in the distal intestine content of Atlantic salmon with SBMIE (Chikwati et al., 2013a; Krogdahl et al., 2003; Lilleeng et al., 2007). The increase in faecal trypsin activity was reported to indicate that pancreatic secretion is stimulated by dietary SBM (Krogdahl et al., 2003). As reviewed by Chikwati et al. (2013a), soybean trypsin inhibitor (SBTI) in SBM may have regulatory effects on the secretion of trypsin. However, the work of Lilleeng et al (2007) indicated that the trypsin activity in the intestine may also come from the inflamed tissue in the DI. Inflammation

increases expression of genes coding for protein with trypsin like activity. Moreover, inflamed DI may have a reduced ability to reabsorb the pancreatic enzymes, resulting in the increasing activity measured in the chyme in this region (Chikwati et al., 2013a). However, the current study did not present significant differences of trypsin activity among diets although the mean values were numerically higher in Atlantic salmon with SBM diet. The reason for lack of significance might be high variances of this biomarker, and too few observations.

6.2 Effects of antinutrients

The reason why SBM fed fish showed more severe SMBIE symptoms than fish fed the SPC+sap, even though the latter supposedly contained much more saponins than the SBM diet, may be that other antinutrients in soybean meal enforce the saponin effects and increase the negative effects on the intestinal morphology and function. Similar results have been described in previous studies in which the intestine of fish displayed more severe signs of enteritis when saponin were supplemented with legume meals such as lupin kernel and pea protein concentrate (Chikwati et al., 2012; Knudsen et al., 2008; Krogdahl et al., 2015b). However, from the present results, these other antinutrient seem not to be isoflavonoids and phytosterols, at least not alone, as they did not worsen the enteritis observed when saponins were fed alone. The present results rather suggested certain beneficial effects of the antinutrient mix, as indicated by significantly reduced submucosal widening, and therefore reduced infiltration of immune cells. Regarding the results for diets with the single antinutrients, it was obvious that the isoflavonoids and phytosterols were not involved in the development of intestinal inflammation, giving no signs of SBMIE in the distal intestine of salmon.

Regarding the gene expression results, the SPC+sup and SPC+mix diets caused similar regulations of gene IL-8, AQP8AB, CLDN15 and CLDN25B in DI tissue as in DI of SBM-fed fish, indicating the the immune responses as well as the disturbances of permeability and barrier functions of distal intestine were similar, as mentioned in the discussion above. Isoflavonoids and phytosterols in the diet increased the AQP8AB expression in the fish, indicating increased water transport into the intestinal cells. The phytosterols in the diet upregulated the expression of IL-1β in fish when compared with SPC fish, which means that phytosterol might initiate an immune response in the fish. The supplementation of antinutrients saponins, isoflavonoids and phytosterols seemed to decrease the bile salt concentration in DI chyme, as reported earlier (Chikwati, 2007; Francis et al., 2002; Munro et al., 2003), as well as decrease plasma

triglycerides and in line with the well-known competition with cholesterol for absorption and consequences for production of bile salts from cholesterol (Kortner et al., 2013).

6.3 Physical quality of the feed pellets

Physical quality of pellets is influenced by the ingredients, extrusion parameters and screw configurations as reviewed by Sørensen et al. (2012). Although screw configuration is one of the factors, the effects contributed to the variation in pellet quality between the SPC and SBMX diets might be minor in the present study. Among the screw elements, reverse screw elements or left-pitched elements (Barres et al., 1990) and kneading polygon elements (Sørensen et al., 2010) have been reported to affect the ingredients transformation during extrusion. Regarding these elements, the parameters used for diets with SPC and SBMX were the same. Only some of the right-pitched elements were located differently in a same length range in the extrusion barrel. Smaller diameter and radial expansion of SBMX compared to SPC could be brought by the lower SME during extrusion process, which is again influenced by the ingredient changes. The components of the raw materials, both starch and protein are hydrated and turns into a melt in the extrusion process, generate the viscous property of the mash, and further affect the shear stress, temperature, SME, die pressure and thus expansion ratio and other technical quality of the final products (Forte & Young, 2016). When comparing the SPC and SBMX diet, the different portion of protein and starch from SPC and SBMX as well as from corn gluten meal and vital wheat gluten are likely to contribute to the different ingredient behavior in extrusion barrel and lower extrusion parameters. Die pressure and SME generally have positive relation with expansion rate when the steam or water addition is equal and expansion ratio in turn negatively influences bulk density (Forte & Young, 2016; Rokey & Huber, 1994). This is supported by the results of the present study. Diet SBMX with lower die pressure and SME with water addition 10kg/h gave lower expansion and higher bulk density.

Decreased radial expansion increased the length of pellets or the expansion in longitudinal direction in SBMX diet. Expansion in radial and longitudinal direction is related to the viscoelastic properties of ingredients (Launay & Lisch, 1983). Viscosity is the key factor for the flow rate in the barrel whereas elasticity is highly related to the swelling and recoil after the die exit (Dethlefsen, 2017). In the study by Launy & Lisch (1983), the authors reported that decreasing viscosity increases the longitudinal expansion, and decreasing elasticity decreases the radial expansion. This theory can be relevant for the present study. The viscoelasticity of

the melt for SBMX diet during process might be lower compared to SPC diet. It is likely that the viscoelasticity of the melt was also influenced by the content of wheat gluten. Wheat gluten consists of two fractions: gliadin which is responsible for viscosity and glutenin which is responsible for elasticity (Delcour & Hoseney, 2010). Even though wheat gluten was higher in SBMX, lower temperature, SME and die pressure might restricted the viscoelasticity of melt in the barrel. Lower water stability of SBMX was probably a result of less cooking of the mixture, which means less starch gelatinization with lower binding properties.

Due to high price of some of the ingredients, i.e. in particular the antinutrients, only small batches of the diets could be made, i.e. 40 kg. Among the SPC diets, the first diet produced had a lower temperature profile because the extruder was operated at room temperature. The batch size was not large enough to produce sufficient to make the extrusion temperature constant before collection of the diet. The temperature increased by processing time and therefore the higher temperature was seen in the process of second and third diets. In combination with lower temperature, die pressure and SME, the first diet with SPC has smaller diameter and lower expansion ratio in radial direction, and reversely, higher bulk density and sinking velocity compared to the diets produced as the second and third. The highest fat leakage in the SPC+sap diet might be related to high expansion rate. However, Sørensen (2012) suggested that fat leakage is related to the microstructure of the pellets, and not to the expansion stage (Sørensen, 2012). The microstrcuture of the pellets was not analysed in this study and therefore can not give suggestions regarding this.

7 Conclusion

- Dietary SBM induced enteritis in the distal intestine of Atlantic salmon whereas SPC did not.
- Inflammation caused by SBM showed the expected dysfunction of DI tissue such as barrier functions, fluid permeability and brush border membrane functions.
- Diet containing 0.5% pure saponins with SPC caused mild signs of SBMIE.
- Supplements of isoflavonid and phytosterol in the diets alone did not induce inflammation and when supplemented together with saponin they did not worsen the signs of SBMIE.

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