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Removal of three proteinaceous antinutrients from a soybean cultivar and

effects of inclusion in diets processed at two heat levels on nutritional value and gut health for Atlantic salmon (*Salmo salar* L)

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Author Contributions

The contributions of the authors were as follows: In the work presented, I participated in the planning stage of the experiment, feed manufacturing of all experimental diets, feeding trial two month, all the sampling procedures, all stages in the analyses for chymal bile salts, all stages in the analyses for mucosal enzyme activities, all stages in the histopathology examination, all stages in the nutrient uptake study and all the statistical analysis. I also participated a lot about gene expression, project planning, interdisciplinary and interorganizational collaboration in research and fish nutrition research.

Åshild Krogdahl: leadership, experimental design, data evaluation and interpretation, manuscript development., Trond M. Kortner: gene expression, manuscript writing regarding gene expression analyses., Elvis Mashingaidze Chikwati: morphological evaluation.

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List of abbreviations

ALAT	Alanine aminotransferase
ANF	Anti-nutritional factor
ASAT	Aspartate aminotransferase
BBI	Bowman-birk inhibitor
BBM	Brush border membrane
CCK-PZ	Cholecystokinin-pancreozymin
CF	Condition factor
CHOL	Cholesterol
CM	Circular muscle
CSBM	Conventional soybean meal
CP	Crude protein
Cq	Quantification cycle
CV	Coefficient of variation
DI	Distal intestine
DISI	Distal intestine somatic index
DM	Dry matter
ES	Esophagus
FA	Fatty acid
FE	Feed efficiency
FM	Fish meal
Glc	Glucose
GI	Gastrointestinal
HCl	hydrochloric acid
HIS	hepatosomatic index
ICPMS	Inductively coupled plasma mass spectroscopy
IECs	Intestinal epithelial cells
IFN-γ	Interferon gamma
IL	Interleukin
IgM	Immunoglobulin M
Kda	kilo daltone
KTI	Kunitz trypsin inhibitor
LAP	Leucine aminopeptidase
LM	Longitudinal muscle
LP	Lamina propria
MF	Mucosal fold
MI	Middle intestine
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MNE	Mean normalized levels
Na	Sodium
NTC	No template control
PI	Proximal intestine
PP	Plante protein
qPCR	Quantitative polymerase chain reaction
RAS	Recirculation aquaculture system
Ref	Reference
RCarc	Relative carcass
SBM	Soy bean meal

SBMIE	Soy bean meal-induced enteropathy
SBL	Soybean lectin
SFE	Specific friction energy
SMC	Submucosa cellularity
SNV	supranuclear vacuolization
SPC	Soy protein concentrate
SPI	Soy protein isolate
SGR	Specific growth rate
SME	Specific mechanical energy
STE	Specific thermal energy
ST	Stomach
SVE	Specific viscosity energy
TGFβ	Transforming growth factor beta
TGs	Triglycerides
TN	Triple null

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Sepehr Teimouri

Abstract

The worldwide, rapid grow of the aquaculture industry is imposing an increasing demand for ingredient feed resources for aquafeeds. Plant-derived ingredients like full fat soybean meal have been widely investigated in recent years as alternative protein and energy sources. However, presence of a variety of anti-nutritional factors in soybeans can lead to reduced growth performance and affect gut health and function. Researchers from the University of Arizona and the University of Illinois have developed a new soybean cultivar in which three proteins with anti-nutritional characteristics (Kunitz trypsin inhibitor, soybean lectin and recognized allergen (P34)) have been removed though a non-GMO breading program. The cultivar is named "*Triple Null*". The present study addressed the nutritional value of Triple Null (TN) for salmon production, and whether elimination of the antinutrients also eliminated the ability of soybean meals to induce enteritis in the distal intestine. In this study, also possible effect of variation in specific mechanical energy (SME) during extrusion might affect the quality of the experimental diets. Three basal diets processed at high and low SME, i.e. a total of six experimental diets, were fed to the fish in triplicate tanks (18 tanks in total with 55 fish per tank) for each diet for 56 days to evaluate the performance and efficiency of soy diets in aquafeeds for Atlantic salmon.

The results showed that removal of the proteinaceous components did not significantly alter dietary feed intake, growth, condition factor, relative carcass and organ weight, nor apparent digestibility of protein, most essential amino acids, body composition. Also, the chyme bile salt concentration in the distal sections of the intestine and plasma biomarkers of fish fed NT and CSBM were similar in the feeding period. The same was the case for several of the indicators of gut health conditions, i.e. faecal dry matter, distal intestinal morphology, activity of brush border LAP, there was no significant difference between TN and CSBM diet. For the gene expression profile, the TN fed fish showed higher expression of *tgfβ*, CD3γδ and *mmp13* but the effects were minor. indicating For the other investigated genes, i.e. *ifn γ, myd88, pcna, fabp2b, aqp8ab, sod1, cat, hsp70,* no significant difference was seen between TN and CSBM.

The likely reason for the lack of effect of antinutrient removal was fact that the heat treatment involved in typical extrusion processing used for salmon feed may be sufficient to inactivate heat labile soybean inhibitors.

High SME compared to the low SME did not induced major differences in nutritional quality but increased fecal dry matter content and apparent digestibility of protein, lipid and energy by high SME, significantly, supposedly related to structure unfolding of the protein molecules by temperature. For both ALAT and ASAT, indicators of liver stress high SME caused significantly higher values than low SME, possibly caused by the heating process might have developed components which represented a challenge to the liver.

1 Introduction

The limited supply of fishmeal could hinder future growth in the aquaculture industry, and much effort has therefore been made to find alternative protein sources that could replace fishmeal in feed formulations for carnivorous fish (Carter et al., 2000). Soy is a legume cultivated widely for food and the feed industry, particularly for agricultural commodities in the world, with a steady increase in annual production. New nutrient sources like soybean may have considerable potential with to reduce reliance on fishmeal (FM) if the obstacles of the antinutrients are overcome. FM is expensive and it is expected to be limited in its availability in the future, and thus it is unable to sustain the expected increase in aquaculture production (Hardy, 2010). The use of plant proteins (PP) as alternatives to FM in aquafeeds has been thoroughly studied during the last decades but it remains a major issue in aquaculture research and fish health (Naylor et al., 2000, Hardy, 2010). Nonetheless, soybeans are known to contain specific compounds that inhibit the absorption of other nutrients (so-called anti-nutritional factors (ANFs)) or that cause allergic reactions. Due to the presence of ANFs, the use of full-fat and solvent-extracted soybean meal (SBM) is limited in species-specific fish diets such as salmonids. Inclusion of SBM in diets produces alterations in the normal functioning of the digestive tract such as inflammation and failure to regulate mucosal integrity, often called SBM-induced enteropathy (SBMIE) (Krogdahl et al., 2010). Several investigations indicate that alcohol-soluble ANFs (Bureau et al., 1998, Ingh et al., 1996, Francis et al., 2001) especially soya saponins (Knudsen et al., 2007, Kortner et al., 2012, Krogdahl et al., 2015), are potential causative factors. Recently, researchers from the University of Arizona and the University of Illinois have bred a new type of soybean in which the levels of three proteins associated their anti-nutritional and allergenic properties are remarkably reduced compared to existing types. The three proteins addressed in this study are: Kunitz trypsin inhibitor (KTI), which affects the digestibility of soybeans, soybean lectins (SBL) and P34 or Gly m Bd 30k (mentioned as P34), a major allergen for humans (Valenta et al., 2015). A new variety of soybean was developed that contained low or zero levels of all three proteins and was therefore named "*Triple Null*" (TN).

Reducing the levels of or eliminating these proteins from soybeans may increase their nutritional value and reduce the occurrence of inflammatory reactions of the distal intestine and reduced immunological function in salmonids. Three specific proteins have been identified as major contributors to the anti-nutritional and allergenic properties of soy. Benefits of the

development of a soy variety with low levels of ANFs may include the potential elimination of the need for a heat treatment, which is normally applied before using raw soybeans. This could reduce energy costs and avoid the negative effects of heat treatment on the quality of raw soybeans. The goal of the present project is to document the potential value of TN as a soybean meal input for salmon production by measuring the nutritional value of and capacity of TN to prevent the enteritis and decreased growth that results from higher inclusion rates of standard beans.

2 Background

2.1 The history of the soybean

Soybean (*Glycine max* Linnaeus) belongs to the family Leguminosae (Figure 1) and is often called the "miracle crop" or "king of beans". The soybean originated from China with more than 5,000 years of cultivation history (Wang, 1997). Soybean can be grown in a wider variety of soil and climatic conditions than any other major world crop.

Figure 1. Soybeans legumes (Adapted from allaboutfeed).

In the last 20 years, scientists have learned how to extract a wide variety of byproducts from soybeans that are proving beneficial in animal feed, human food and industrial applications (Liu, 2004). A large part of this production is used in the extraction of oil yielding a cake of high protein quality. Soybeans undergo various processing steps (Figure 2) to obtain many different soy products such as soy bean meal (SBM), soy protein concentrates (SPC), and soy protein isolates (SPI), that have been evaluated in fish feeds (Figure 2).

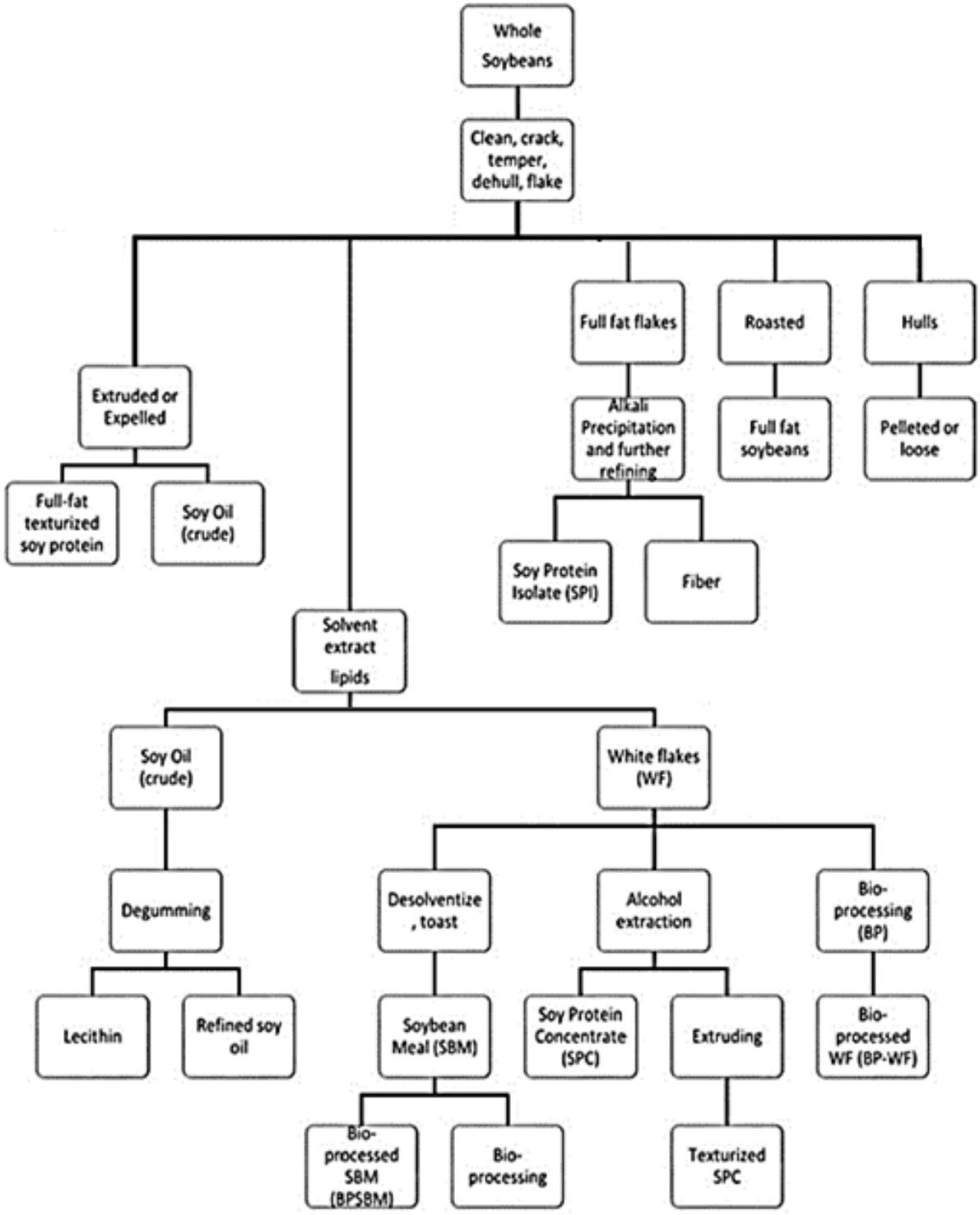


Figure 2. Processing scheme of soy products adapted from (Hammond et al., 2011).

Soy products are regarded as economical and nutritious feedstuffs with high crude protein content and a reasonably balanced amino acid profile. However, the presence of several antinutritional factors and certain nutritional characteristics leads to health issues. (NRC, 1993). The accessible price and stable supply are favorable factors for soybean to emerge as an

important source of protein for cultivated fish like Atlantic salmon. Concentrations of the 10 essential amino acids (EAA) are overall lower in SBM than in fish meal (Table 1) (Gatlin et al., 2007). However, due to the processing costs involved, these products (SPI and SPC) are not yet economical for large-scale use in aquafeeds (Table 1).

Table 1. General Concentrations of amino acids in Soybeans (Dry Matter Basis)(Orf, 1988)

Component	Unit	Range	Typical
Protein	%	30-50	40
Amino acid composition	g/100 g seed		
Non-essential			
Alanine		1.49-1.87	1.69
Arginine		2.45-3.49	2.9
Aspartic acid		3.87-4.98	4.48
Glutamic acid		6.10-8.72	7.26
Glycine		1.88-2.02	1.69
Cysteine		0.56-0.66	0.6
Proline		1.88-2.61	2.02
Serine		1.81-2.32	2.07
Essential			
Histidine		0.89-1.08	1.04
Isoleucine		1.46-2.12	1.76
Leucine		2.71-3.20	3.03
Lysine		2.35-2.86	2.58
Methionine		0.49-0.66	0.54
Phenyalanine		1.70-2.08	1.95
Threonine		1.33-1.79	1.58
Tryptophan		0.47-0.54	0.49
Tyrosine		1.12-1.62	1.43
Valine		1.52-2.24	1.83

2.2 Important antinutrients in Soybean

“The terms “antinutritional factor” (ANF) and “antinutrients” refer to endogenous substances in foods and feedstuffs that cause negative effects on health and nutrient balance when ingested by animals or humans” (NRC, 2011). Possible harmful effects might include disturbance of digestive processes and inhibition of growth, decreased feed efficiency, reduced palatability, pancreatic hypertrophy, intestinal dysfunction, altered gut microbiota, hypoglycaemia, liver dysfunction, goiterogenesis and immune suppression or modulation (NRC, 2011). This is mostly related to the existence of ANFs which affects the nutritional value, utilization and digestibility of soybean protein. In this regard, soybean must be subjected to treatment before

consumption by human beings or animals, to eliminate or reduce this anti-nutritional content. Some ANFs are easy to eliminate by processing, and others are more challenging to eliminate.

Some processing steps, such as heat treatment, may inactivate the inhibitors (proteinase, amylase, *etc*) (Table 2) but the thermal treatment process can also destroy other essential nutrients, and heating raises the cost and energy demands of soybean production. Removal of ANFs by heat-treatment has been attempted with varying success. For all ANFs, fermentation or enzyme treatments directly focusing on inactivation of a specific ANF may reduce content or activity in the feedstuff.

Selective breeding or conventional breeding and stacking of traits derived from non-biotech sources such as mutation to enhance seed composition, can also be another way to reduce ANFs effects.

Furthermore, such new varieties could reduce the number of intolerances or allergic reactions in humans (Shewry et al., 2001). However, genetic modification may also result in unintended alterations in the contents of ANFs (Cellini et al., 2004).

Table 2. Lists the major ANFs present in a variety of feedstuffs and treatments that may reduce biological activity either by elimination or by inactivation (Krogdahl et al., 2010).

Antinutrient	**Sources**	**Type of treatment**
Proteinase inhibitors	Legumes	Heat, methionine supplementation
Amylase inhibitors	Peas	Heat
Lipase inhibitor	Beans	Heat
Lectins	All plants seeds	Supplementation with specific carbohydrates
Phytic acid	All plants	Mineral supplementation
Fibre	All plants	Dehulling
Tannins	Rape seed, beans	Dehulling, restriction of heat treatment
Saponins	Legumes	Alcohol extraction
Sterols	Legumes	Alcohol/non-polar extraction, cholesterol supplementation
Oestrogens	Beans	Alcohol/non-polar extraction
Gossypol	Cotton seed	Non-polar extraction, iron supplementation
Oligosaccharides	Legumes	Alcohol/aqueous extraction
Quinolozidine alkaloids	Lupins	Aqueous extraction
Goitrogens	Rape seed	Iodine supplementation

Fish nutritionists should keep in mind that concentration thresholds of ANFs at which the harmful and beneficial effects occur has not been evaluated carefully, and in most cases there may be slow cumulative adverse effects (Cabrera-Orozco et al., 2013).

2.2.1 Proteinase inhibitors general characteristics

Protease inhibitors are proteins that are widely distributed within the plant and have the ability to inhibit the proteolytic activity of digestive enzymes such as serine-proteases (trypsin and chymotrypsin) which are characteristic of the gastrointestinal tract of animals. Protease inhibitors can inhibit endogenous proteases and enzymes of bacteria, fungi and insects (Liener, 2012). Protease inhibitors have been classified into several families based on homology in the sequence of amino acids in the inhibitory sites. The molecular structure of the inhibitor affects both the force and the specificity of the inhibitor. The two main families of protease inhibitors found in soybean are the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor (Kunitz, 1945, Bruneton, 1995). The proteinase inhibitors seem to stimulate pancreatic enzyme secretion. However, after longer term feeding, the pancreas may no longer manage to compensate for the presence of active inhibitors by increasing secretion (Krogdahl et al., 2003).

2.2.1.1 Kunitz trypsin inhibitor (KTI)

The first protease inhibitor to be isolated and characterized was the Kunitz inhibitor. It has a molecular weight between 18 to 24 Kilodaltons (kDa) and contains between 170 and 200 amino acid residues (Cabrera-Orozco et al., 2013) (Figure 3). This inhibitor has one head, meaning one molecule of inhibitor inactivates one molecule of trypsin. It is a competitive inhibitor, which means that it binds to the active sites of trypsin in the same way the substrate of the enzyme does, resulting in the hydrolysis of peptide bonds between amino acids of the reactive site of the inhibitor (Figure 3).

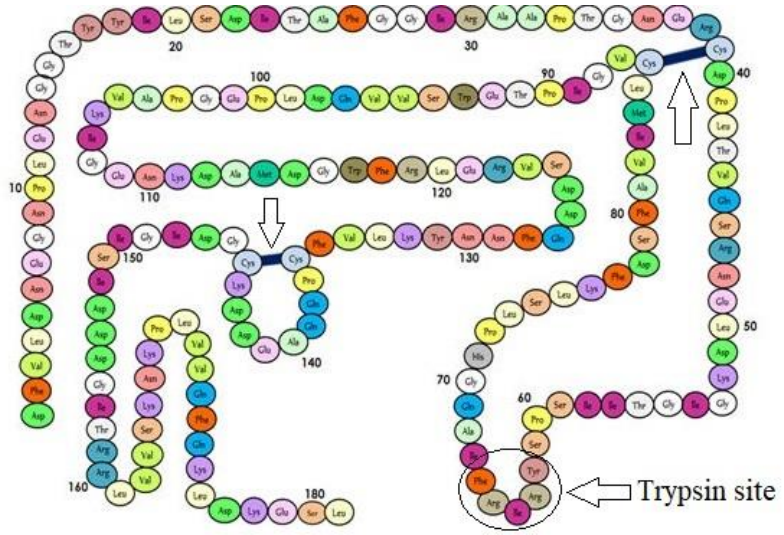


Figure 3. Primary structure of the Kunitz inhibitor from soybean Disulfide bonds are shown in two arrows (Meester et al., 1998).

2.2.1.2 Bowman-Birk inhibitor

This inhibitor has low molecular weight polypeptides (7 to 9 kDa) containing 60 to 85 amino acid residues (Figure 4)(Cabrera-Orozco et al., 2013). Bowman-Birk inhibitors (BBIs) have seven disulfide bonds which make them relatively stable to heat treatment. These inhibitors have two heads (two separate sites of inhibition) and are competitive inhibitors. They can simultaneously and independently inhibit two enzymes, thus, there are trypsin/trypsin are trypsin/chymotrypsin inhibitors (Berhow et al., 2000, Cabrera-Orozco et al., 2013).

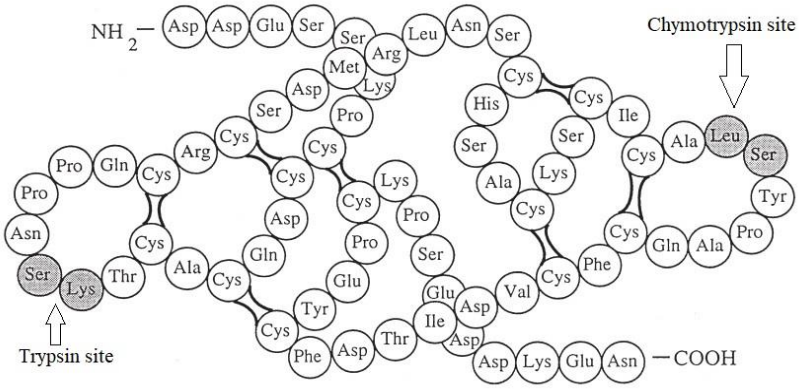


Figure 4. Primary structure of Bowman-Birk type inhibitor from soybean and the seven disulphide bonds. In the drawing, the chymotrypsin inhibitory site appears on the right side (Leu-Ser amino acids; darkened circles) and the trypsin inhibitory site appears on the left side (Lys-Ser, amino acids; darkened circles).(Odani et al., 1973).

2.2.2 Biological effects of proteinase inhibitors in fish

Proteinase inhibitors and their effects in fish have been studied comprehensively. Based on these studies, an understanding of their action has been developed entirely (Krogdahl et al., 1994). In the intestine, inhibitors first form a rather stable complex with trypsin, thus reducing trypsin activity. This in turn stimulates secretion of cholecystokinin-pancreozymin (CCK-PZ) from the gut wall. This hormone stimulates the secretion of trypsin from pancreatic tissue and stimulates the gall bladder to empty its content into the intestine. In studies with salmonids, proteinase inhibitors have been found to reduce apparent digestibility not only of protein but also of lipid (Krogdahl et al., 1994, Olli et al., 1994). The effects on digestibility correspond to a decrease in trypsin activity and presumably chymotrypsin, which is also inhibited by soybean proteinase inhibitors (Olli et al., 1994). The proteinase inhibitors stimulate pancreatic enzyme secretion causing the enzyme level of the intestinal content (trypsin protein) to increase. However, the activity in the intestinal content is not increased. The enzyme activity seems unaffected when fed diets with the lower inhibitor levels and short-term feeding, but higher levels decrease the activity. After longer-term feeding it seems the pancreas can no longer manage to compensate for decreased enzyme activity by increasing secretion. Thus, enzyme production does not appear to keep up with the increased demand. A study from 1994 (Krogdahl et al.) indicates cumulative digestibilities of protein and cysteine in intestinal segments along the gastrointestinal (GI) tract of rainbow trout are a function of dietary inhibitor level. The results support the findings of the study (Lea et al., 1989) indicating when supply of cysteine-rich pancreatic enzymes into the GI tract is increased, the level of cysteine of the digesta increases sharply, giving a negative cumulative apparent digestibility in the pyloric region of the intestine.

2.3 Lectins general characteristics

Lectins (previously known as agglutinins, hemagglutinins or phytohemagglutinins) are a group of soluble, heterogeneous (glyco) proteins that “possess at least one non-catalytic domain which binds reversibly to a specific mono- or oligosaccharide” (Peumans et al., 1995). Lectins are generally found in highest concentrations in seeds and other storage organs. Plant lectin proteins contain at least one carbohydrate-binding domain. Based on this, four major types of lectins are distinguished, namely 1. merolectins, 2. hololectins 3. chimerolectins and 4. superlectins. Lectins are one of the most important physiologically active ingredients and potent

exogeneous biological signals in the diet. Although the amounts of lectins in foodstuffs can vary considerably, they can dramatically affect the entire digestive tract, body metabolism, and health. Lectins are stable proteins that do not degrade easily. For examples, some lectins are resistant to stomach acid and digestive enzymes. Unfermented soy products contain high levels of lectins. Soy bean agglutinin (SBA) or soy bean lectin (SBL) is a highly specific carbohydrate-binding protein, with the highest affinity for N-acetyl-D-galactosamine through hydrogen bonds and Van Der Waals interactions and with one or more binding sites per subunit. Soybeans seeds contain between 300 and 600 mg/100 g of lectins (Gu et al., 2010, Lis et al., 1998), which is approximately 0.2 – 1% of the soy protein (Anta et al., 2010, Rizzi et al., 2003).

2.3.1 Biological effects of lectin in fish

Binding of soybean lectin (agglutinin; SBA) to carbohydrate of glycoconjugates, specifically to N-acetyl-D-galactosamine, on the intestinal brush border membrane of Atlantic salmon and rainbow trout has been demonstrated (Buttle et al., 2001, Krogdahl et al., 2003, Hendriks et al., 1990). In the distal intestine of Atlantic salmon and rainbow trout higher maximum binding and lower dissociation constant were observed relative to the more proximal areas. A few papers in the scientific literature have reported results of in vivo studies with fish fed purified plant lectins (Buttle et al., 2001, Iwashita et al., 2008). SBL alone in the diet did not cause any histological alteration in the Atlantic salmon intestine. However, from the existing knowledge, it appears that lectins play a minor role. Current knowledge regarding the effects of lectin in fish still needs more research and further investigation regarding interaction with other ANFs is necessary.

2.4 Saponins general structure and chemical characteristics

Saponins are a big group of glycosides which are widely distributed in green plants, including more than 100 families of plants such as soy, pea and lupin. (Anderson et al., 1995). The name saponin comes from the Latin word *sapo* which means soap, due to generating foam in aqueous solutions (Anderson and Wolf, 1995). Saponin are amphiphilic glycosides, where the polar essential constituents are sugars (pentoses, hexoses or uronic acids) that are covalently linked to a nonpolar group, which consists of an aglycone, called sapogenin, which can be either steroidal or triterpenoid (Cabrera-Orozco et al., 2013). This combination of polar and nonpolar

components in their molecular structure explains their surfactant property in aqueous solutions (Cabrera-Orozco et al., 2013). Above-mentioned, the saponins are secondary metabolites that can be classified into two groups based on the nature of the aglycone skeleton. The first group consists of steroidal saponins, which are present almost exclusively in monocotyledons angiosperms (Cabrera-Orozco et al., 2013). The second group is composed of triterpenoid saponins, which occur mainly in dicototyledonous flowering plants. Steroidal saponins comprise a steroidal aglycone, a spirostane skeleton of 27 carbons (C_{27}), which generally comprises a six-ring structure (Bruneton, 1995) . Three main types of steroid aglycones are derivatives of spirostan, furostan, and nautigenin (Figure. 5). The most well-known triterpene aglycones are derivatives of oleanan (Figure. 5) (Beleia et al., 1993).

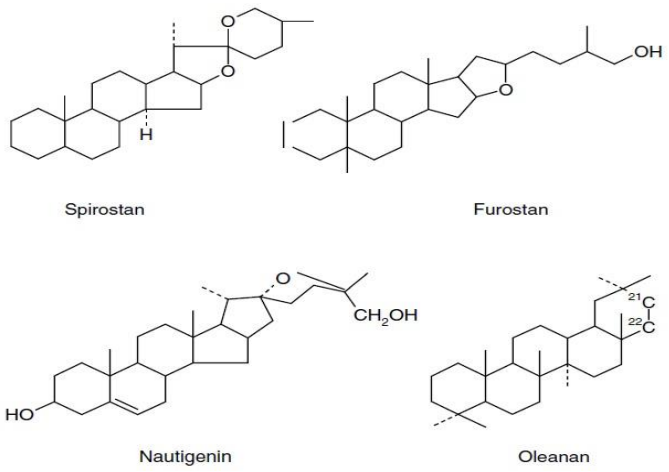


Figure 5. Structures of steroid and triterpene aglycones (Lásztity et al., 1998).

The triterpenoid saponins have an aglycone with a backbone of 30 carbons (C_{30}), which form a pentacyclic structure (Figure 6).

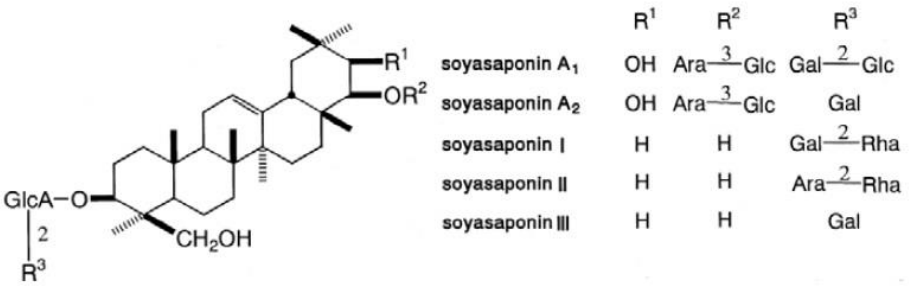


Figure 6. Chemical structures of soybean saponins showing different side chains attached to a triterpenoid backbone (Oda et al., 2000).

It has been identified that soy contains saponins with triterpenoid-type aglycones. This kind of aglycones is divided into five major groups; soysapogenol A, B, C, D and E (Figure 7), and their glycosides are correspondingly called saponins of group A, group B, etc. (Haralampidis et al., 2002). From this classification, four aglycones (soysapogenol A, B, C and E) (Kitagawa et al., 1982) were isolated after hydrolysis of soy saponins. Specifically five saponins were identified with two distinct types of aglycones: soysapogenin I (the main component), soysapogenins II and III, which contain soysapogenol B, and soysaponins A1, A2 and A3, which contain soysapogenol A (Kitagawa et al., 1982). In soybeans, the saponins containing soyasapogenol C and E have not been found. The type of sugars attached to the aglycones found in soybeans have been identified as rhamnose, galactose, glucose, arabinose, xylose and glucuronic acid (Kitagawa et al., 1982).

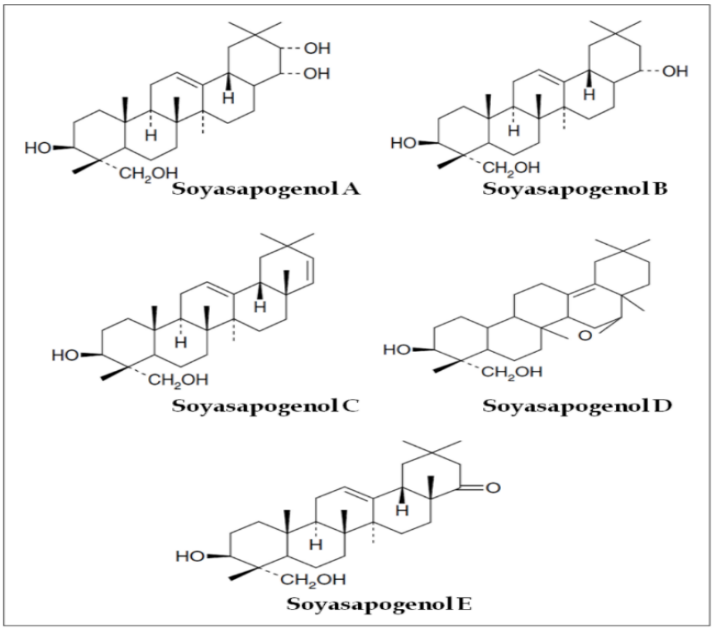


Figure 7. Structures of five types of sapogenins identified in soybeans (Lin et al., 2004)

2.4.1 Biological effects of saponin in fish

Saponins are reported to have diverse biological effects including antifungal and antiviral activity, immune stimulation, anticancer effects, antioxidant properties, inhibition of protein digestion and vitamin absorption, and glucocorticoid-like effects as reviewed by Francis et al (2002). Saponins are heat-stabile, alcohol-soluble compounds. The level of saponin in soybean is generally higher than in other common feedstuffs. Soybeans generally contain saponins in the range of 1-5 g kg^1 (Anderson and Wolf, 1995). The amphiphilic property provides saponins

the ability to bind and form nonabsorbable complexes with cholesterol (Glauert et al., 1962, Krogdahl et al., 2015). In animals, high concentrations of saponins are also capable of breaking the membrane of other cells such as those of the intestinal mucosa, which modifies the cell membrane permeability, and thus affects the absorption of nutrients and the active transport (Gee et al., 1993). On the other hand, saponins have the ability to bind to membrane cholesterol of intestinal epithelial cells and thus make holes and changes membrane permeability. This alteration in the membrane facilitates the uptake of molecules such as antigens and potential toxins which normally cannot absorb by the enterocytes (Johnson et al., 1986) (Figure 8).This ability to affect the cell membrane depends on the structural characteristics of the saponins such as the structure of the aglycone, the number of sugars in the side chains, and the side chains length (Oda et al., 2000). In Figure 8, the interaction of saponins with cell membranes is schematically shown.

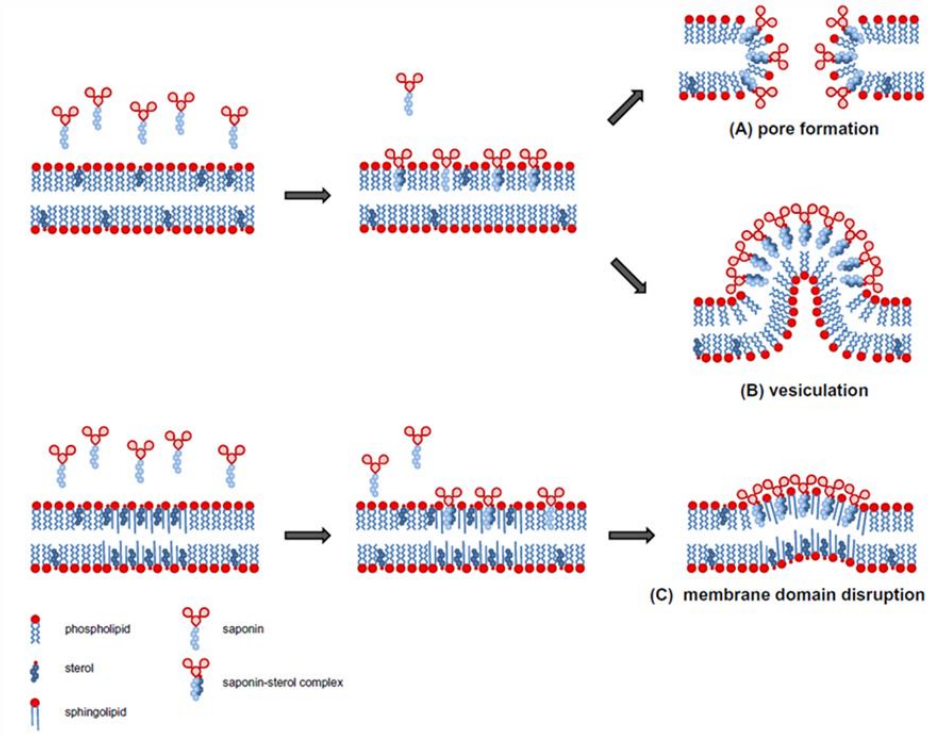


Figure 8. Schematic models of the molecular mechanisms of saponin activities towards membranes (Augustin et al., 2011) Saponins integrate with their hydrophobic part (sapogenin) into the membrane. Within the membrane they form complexes with sterols. Afterwards, they accumulate into plaques by interaction of their extra-membranous orientated saccharide residues. Sterical interference of these saccharide moieties causes membrane curve structure leading to form (A) pore in the membrane (Augustin et al., 2011). (B) Vesiculation is the result of hemi tubular protuberances in sterol extraction. As another position, after membrane integration saponins can migrate towards sphingolipid/sterol enriched membrane domains (C). Accumulation of saponins in membrane domains has been a proposed reason of deconstructive membrane curvature in a dose-dependent way (Lin et al., 2010).

Saponins have also been suggested to interfere with digestion of lipids and proteins, (Francis et al., 2002) and have a hypo-cholesterolemic effect in several animal species (Potter et al., 1993). The study of Krogdahl et al. (2015) reveal that 2−4 g/kg soya saponins in diets from a 95% purified source elicited signs of inflammation in the DI of Atlantic salmon without the presence of other legume components. Therefore, the latest study substantiates previous suggestions that the induction of inflammation by soya saponins is potentiated by other ANFs, antigens, or other components in SBM and other legumes such as peas and lupins (Krogdahl et al., 2015).

2.5 Soybean allergenic protein P34 (Gly m Bd 30K) general characteristics

Feed or food allergens are defined as substances that react with Immunoglobulin E (IgE) antibodies and induce allergic sensitization/reactions, usually via mast cell degranulation and histamine release (Verhoeckx et al., 2015). Soybean seeds contain approximately 37% protein, of which allergenic proteins (Gly m 1 to Gly m 30) have so far been registered by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (www.allergen.org). Among soybean allergens, papain superfamily member P34 (also named P34) (Kalinski et al., 1992) or Gly m Bd 30k (Ogawa et al., 2000) a thiol-protease, might be a major allergen that may affect more than 50% of soy allergic subjects (Ogawa et al., 2000).

2.5.1 Biological effects allergenic proteins (Gly m 1 to Gly m 30) in fish

A few protein components of some legume seeds and cereals elicit antigenic effects in animals and these compounds are capable of inducing intestinal mucosal lesions, abnormalities in the villi, specific and non-specific immune responses, and abnormal movement of digesta through the gut (Lalles et al., 1996). Although it has not been demonstrated that fish are able to react allergically with a type I hypersensitivity reaction, except for Perciformes (tilapia, sea bass and sea bream) (Mulero et al., 2007). Mast cells of salmonids do not contain histamine (Dezfuli et al., 2000, Mulero et al., 2007) and salmon do not react to intravascular injection of histamine. Also, teleosts do not appear to have an analogous structure to monomeric Immunoglobulin E (IgE) , only tetrameric Immunoglobulin E (IgM) and possibly monomeric Immunoglobulin E (IgD) (Mulero et al., 2007, Rombout et al., 2014).

The antigenic compounds present in feed may trigger a variety of non-specific and specific immune responses in the fish intestine (Baeverfjord et al., 1996, Bakke-McKellep et al., 2007) and this might lead to a reduction in growth. The presence of allergens or antigens that fish may react to in plant-derived feed ingredients, howbeit, remains a matter of controversy. The anticipation of allergenicity of soybean and products is limited because of a very limited number of high-quality studies performed on soy allergies in fish. Whether soy allergen may be reduced or unaffected by feed processing is still not clear yet. Apart from highly refined soybean oil and other soybean products in which the level of soybean proteins are reduced below clinically relevant levels, one-step processing may not fully abolish soy allergenicity (Verhoeckx et al., 2015).

2.6 Developing a Low Anti-Nutritional Bioactivity Soybean Seed; the Creation of Triple Null

A decade-long effort by University of Arizona scientists Monica Schmidt and Eliot Herman and University of Illinois scientist Theodore Hymowitz has yielded a new soybean with significantly reduced levels of three key proteins (Schmidt et al., 2015). The researchers (Herman, Schmidt and Hymowitz) screened 16,000 varieties of soybeans before finding one that contained almost none of the three key proteins, (Kunitz trypsin inhibitor, soybean agglutinin and P34) that are responsible for the soybean's anti-nutritional effects in aquaculture and humans. After nearly a decade of crossbreeding each variety to the soybean reference genome called Williams 82, the team has produced a soybean that lacks most of the P34 and trypsin inhibitor protein, and completely lacks soybean agglutinin. They have given a name the new variety "Triple Null." Triple Null also has applications for agriculture and livestock as vegetable protein for animal feed. A growing use of soybean is in aquaculture, which produces more than 50 percent of consumed seafood, with this number expected to rise to 75 percent by 2030 (Schmidt et al., 2015). Proteomic analysis of *Triple Null* shows that the line lacks this trio of bioactive proteins while retaining the full complement of other proteome constituents without any other collateral bioactive protein alterations (Schmidt et al., 2015). Creating this antinutritional/allergen null stack in a standard background enables its use in soybean products where there has been reluctance to use genetically modified beans in either feed or food applications.

2.7 Soybean meal-induced enteritis as a model in salmon

The international aquaculture industry makes great efforts to reduce the reliance on marine resources such as fishmeal which is regarded as the "gold standard" protein source in fish feed and great progress has been made over the past decade by increasing use of plant protein sources in diets for fish, such as solvent extracted soybean meal (SBM) and, soy protein concentrate (SPC) (Gatlin et al., 2007, Drew et al., 2007). However, the inclusion of plant-protein ingredients in diets expose fish to ANFs, leading to increased risk of nutrition-related disorders where salmonids have shown to be especially sensitive (Krogdahl et al., 2010, Francis et al., 2001). The dietary inclusion of SBM at moderate or higher levels in feed for salmonids has shown to cause inflammation in the distal intestine (DI), usually referred to as SBM induced enteritis (SBMIE) (Van den Ingh et al., 1991, Baeverfjord and Krogdahl, 1996, Krogdahl et al., 2010, Krogdahl et al., 2003). SBMIE leads to morphological changes in the distal intestine of salmonids (Baeverfjord and Krogdahl, 1996, Bakke-McKellep et al., 2007, Urán et al., 2008, Lilleeng et al., 2007, Lilleeng et al., 2009, Skugor et al., 2011, Marjara et al., 2012, Chikwati et al., 2013b, Sahlmann et al., 2013, Venold et al., 2013), such as thickening of the lamina propria (LP) due to infiltration of macrophages, neutrophils, eosinophilic granular cells, loss of the normal supranuclear vacuolization (SNV) of the absorptive cells in the intestinal epithelium, increased number of goblet cells, reduced cell height and increased cytoplasmic basophilia, widening and reduction in height of the intestinal folds (Fig. 9). The SBMIE model has also shown to be useful to evaluate alternative feed ingredients with beneficial effects on fish intestinal homeostasis (Romarheim et al., 2010, Romarheim et al., 2013).

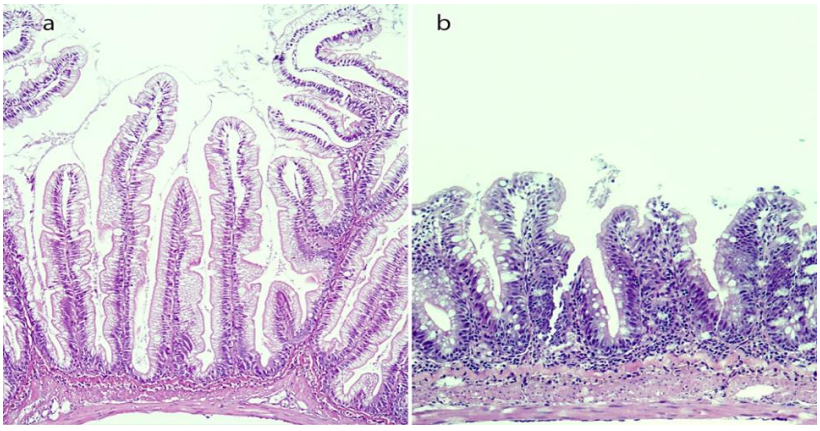


Figure 9. Representative images of comparison of distal intestine of Atlantic salmon during the enteritis, a) healthy DI mucosa normal epithelium with tall, finger-like mucosal folds (MF); SNV are normally aligned.; LP is a thin and delicate core of cells (fish fed diet Ref). b) completely disturbed epithelium, showing infiltration of inflammatory cells especially EG into the LP; SNV are no longer present; mucosal folds MF have a stubby appearance (fish fed diet TN).

2.7.1 Effects of SBMIE on epithelial cell barrier

The epithelium is the cell layer that marks the border between the outside (lumen) and inside (tissue) of the animal (Figure 10 A). The epithelium typically consists of a monolayer of epithelial cells linked at their apical ends by attachment proteins. Most cells of the intestinal epithelium are enterocytes.

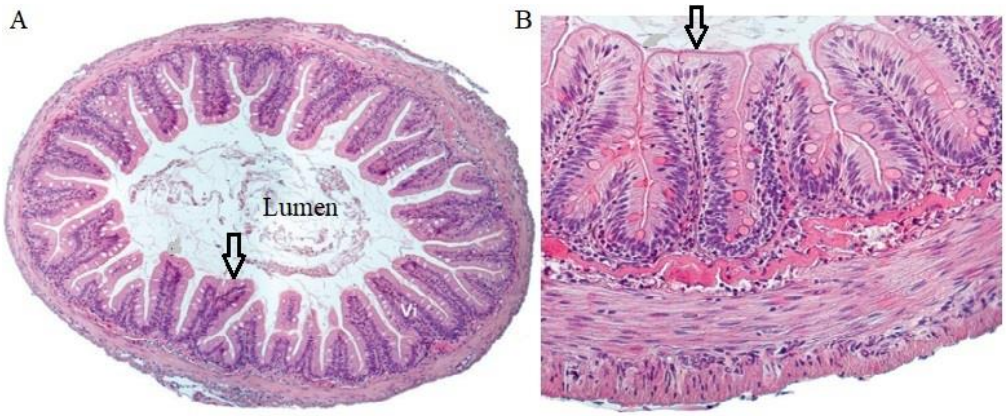


Figure 10. (A-B). Cross section of the intestine in Atlantic salmon and brush border membrane are shown in arrows (Jutfelt, 2011).

Enterocytes are columnar cells with an elongated nucleus (Figure 11 A and B). The finger-like extensions of the membrane called microvilli increased luminal surface area, which make up the brush border membrane (BBM; Figure 10B and 11). Enterocytes of salmon differ somewhat in appearance between intestinal regions. Enterocytes of the DI are characterized by large supranuclear vacuoles (SNVs) in the apical region of the cell, while these large vacuoles are absent or much smaller in enterocytes of the proximal intestine (PI) and middle intestine (MI).

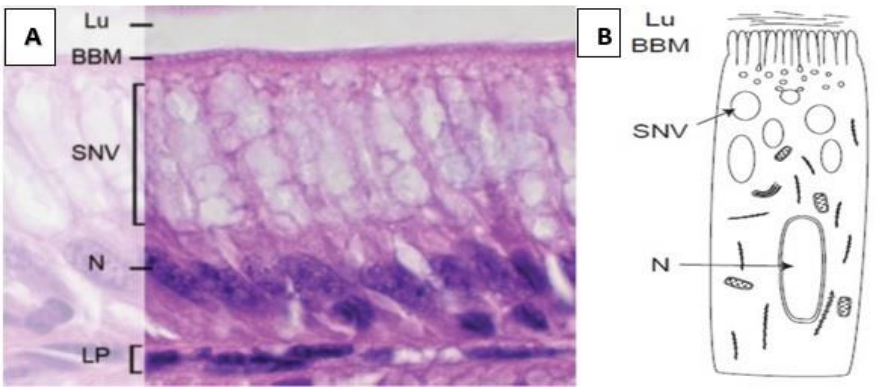


Figure 11. Structure of the distal intestinal epithelium. A: Histological section of the distal intestinal epithelium (photo taken by Michael Penn), B: Drawing of a single enterocyte. Lu: lumen; BBM: brush border membrane; SNV: supranuclear vacuoules; N: nucleus; LP: Lamina propria (Sahlmann, 2013).

Other cells of the intestinal epithelium include mucus-secreting goblet cells, endocrine cells, and intraepithelial lymphocytes. The basal membrane of the enterocytes forms a border to the lamina propria (Figure. 12). The lamina propria and submucosa are made up of connective tissue that contains blood vessels, nerves and various resident immune cells. The structure of the wall of the GI tract varies along the tract but has in common a surface facing the lumen of mucus-producing (goblet) cells between enterocytes. The latter holds digestive and transport apparatus located in microvilli facing the lumen and is responsible for the uptake of nutrients (Figure. 12).

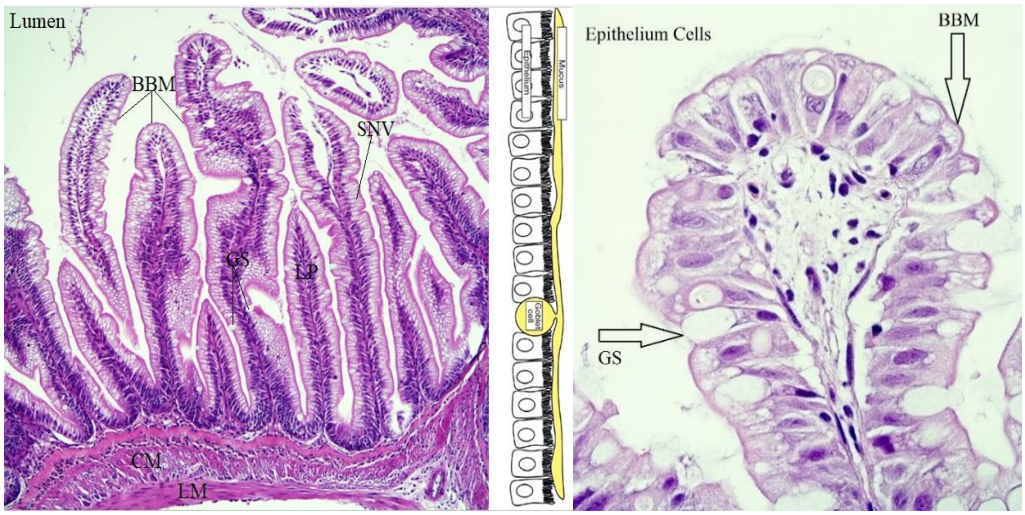


Figure 12. Representative view of epithelium cells, brush border membrane (BBM), supranuclear vacuoules (SNV) and goblet cell (GS) lining the mucosal folds, circular muscle (CM), longitudinal muscle (LM).

The morphological changes associated with SBMIE are characterized by important changes in several compartments (Figure 13) (Van den Ingh et al., 1991, Baeverfjord and Krogdahl, 1996, Urán et al., 2009, Romarheim et al., 2013). In general, SBMIE is characterized by decreased height and complexity of the distal intestinal mucosal folds, decreased size and/or amounts of SNVs, reduced cell height and increased cytoplasmic basophilia, widened lamina propria and submucosa with increased leukocyte infiltration, as well as increased amounts of intraepithelial leukocytes and diffuse immunoglobulin M (IgM) (Van den Ingh et al., 1991, Baeverfjord and Krogdahl, 1996, Bakke-McKellep et al., 2007). Morphological changes are visible after 2-5 days of feeding SBM (Van den Ingh et al., 1991, Baeverfjord and Krogdahl, 1996). After 7 days of feeding SBM, all of the individuals show signs of inflammation and after 21 days the typical characteristics are exacerbated. The inflammation in the DI is usually accompanied by impaired epithelial barrier function, increased cellular permeability (Nordrum et al., 2000, Knudsen et al., 2008, Mosberian-Tanha et al., 2018) and decreased macromolecular uptake

(Urán et al., 2008). Furthermore, loss of digestive function is indicated by alterations of digestive enzyme activities in the distal intestine. The activity of BBM enzymes are strongly reduced while pancreatic enzyme activities are increased (Bakke-McKellep et al., 2000, Krogdahl et al., 2010). Most of these functional studies describe the responses during chronic stages of SBMIE (Sahlmann et al., 2013).

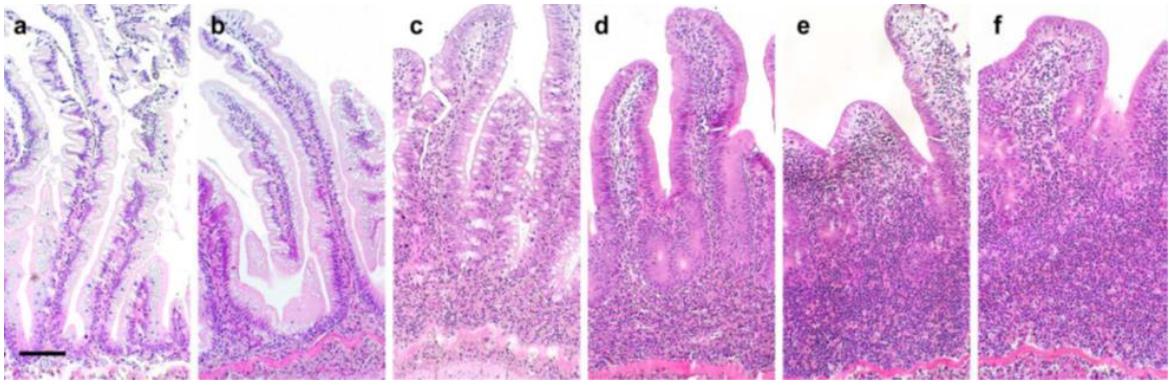


Figure 13. The morphological changes in the distal intestine of Atlantic salmon during development of enteritis. Pictures a and b show normal microscopic appearance of the distal intestine of Atlantic salmon fed on a fishmeal-based diet. Pictures c to f show early stages develop to the late stages with infiltration by inflammatory cells and the consequent widening of the submucosa and lamina propria, disappearance of supranuclear vacuolization of the epithelial cells and shortening and fusion of the mucosal folds. The scale bar in the image represents a distance of 50 μm for all images (Chikwati et al., 2013a).

2.7.2 Inflammatory responses in distal intestine during SBMIE

Atlantic salmon fed diets containing more than 5-10% full fat or defatted (extracted) soybean meal (SBM) develop inflammation in the distal part of the intestine. SBM contain ANFs, including lectins, protease inhibitors,, saponins and more (Krogdahl et al., 2015). ANFs have been demonstrated to damage the intrinsic intestinal barrier with disrupted tight junctions and sloughing of enterocytes and increasing the permeability (Krogdahl et al., 2010). This impairment of the physical barrier has been demonstrated to favor antigen entrance and lead to induction of the innate immune response (figure14).

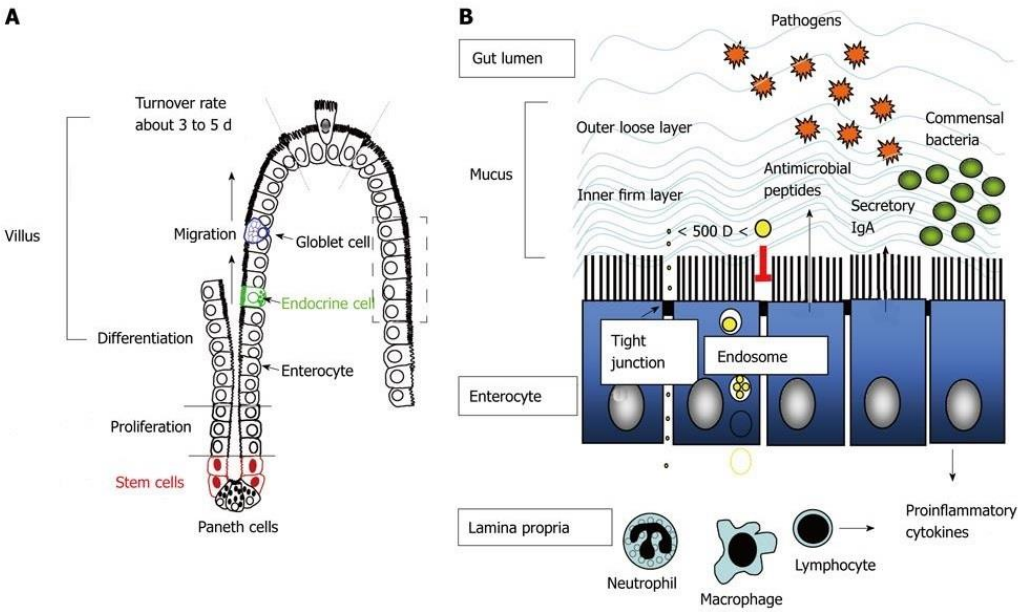


Figure 14. Mammalian intestinal enterocytes axis and formation of intestinal barriers. A: Proliferation and differentiation into columnar epithelial cells (enterocytes) with high expression of brush border enzymes and transporters, and meantime migrate upward. B: Enteric pathogens are restricted in the gut lumen by physical barriers (epithelium and mucus), chemical barriers with antimicrobial peptides, and immune barriers like secretory immunoglobulin A (IgA). A immune cells in the lamina propria such as phagocytes (macrophages and neutrophils) and lymphocytes are responsible for inflammatory responses (Yu et al., 2012).

A damaged intrinsic barrier have also been suggested to be one of the major factors behind the intestinal inflammation characterizing (Xavier et al., 2007). Intestinal inflammation involves a series of continuously expressed pro-inflammatory as well as anti-inflammatory cytokines and other immune related factors (Maloy et al., 2005). When the innate immune response is activated, pro-inflammatory cytokines like interleukin-1β (IL-1β) and interferons (IFNs) first organize an acute inflammatory response (Rombout et al., 2014). Further, IFNγ have an important role in the complex integration of the innate and adaptive processes of the immune system (Mulder et al., 2007). The immune response further includes activation of transcription factors such as nuclear factor kappa B (NF-κB) (Rombout et al., 2014). Some of the pro-inflammatory cytokines also stimulates cellular recruitment to attract more immune cells to the affected region, e.g. the chemokine IL-10 (Valenta et al., 2015, Bakke-McKellep et al., 2007). In mammals it is well known that IL-1β stimulate production of adhesion molecules that allow migration of neutrophils to the target tissue (DeForge et al., 1992). Cytokines like transforming growth factor-β (TGFβ) and IL-10 are thought to counteract the pro-inflammatory cytokines (Niklasson et al., 2011). Together with IFNγ, they regulate specific (acquired) immunity through T cell and B cell activation (Lilleeng et al., 2009, Bakke-McKellep et al.,

2007). Different subsets of T cells evoke different cytokine response patterns depending on the antigen and its source e.g. IL-10 and TGFβ are coupled to the helper T cell 2 (T_h2) and regulatory T cell (T_{reg}) responses and IFNγ to the T_h1 response (Komatsu et al., 2009, Miyara et al., 2007) (figure 15).

Long term inflammation can lead to a chronic inflammatory response characterized by both tissue degeneration and remodeling (Qin, 2012). Matrix metalloproteinases (MMPs) and other types of serine proteases that are involved in tissue remodeling have shown to induce signaling leading to intestinal inflammation. (Sahlmann et al., 2013, Cenac et al., 2002). More focused immunological analyses of intestinal transcriptome profiling during the development of enteritis have been reported previously (Kortner et al., 2012, Sahlmann et al., 2013).

During the study of the development of SBMIE in Atlantic salmon, a significant modulation in the expression levels of several immune relevant, pro-inflammatory or regulatory genes were found (Kortner et al., 2012). The identification of useful biomarkers is complicated however by comparing results of different transcriptomic studies some candidate gene genes represented in this study (table 8) cab be informative pool of potential molecular markers of the immune response in the salmon intestine.

Modulation in the expression levels of some immune relevant, pro-inflammatory genes in distal intestine included:

The up-regulation of IL-17 expression during the inflammation, with the implication that T_H17 cells are involved in the SBM-induced enteropathy, is further supported by the increase in CD4a and CD8b expression observed in previous studies in SBM-fed salmon (Bakke-McKellep et al., 2007). The up-regulation of the TNF signaling pathway and NF-kB-mediated response, a critical trigger for the release of pro-inflammatory cytokines and also the activation of a number of intracellular pathways eventually leading to apoptosis and cell survival (De Santis et al., 2015).

The main function of IFNγ is probably acting as a regulatory cytokine in both innate and adaptive immunity. The production of IFNγ in response to chronic inflammatory and autoimmune diseases is considered important factor in induction of autoimmunity of celiac disease (Monteleone et al., 2001).

Myeloid differentiation primary response 88 (*myd88*) is a protein used by most toll-like receptors (TLR) to activate nuclear factor-kappa B (NFκB) (Marjara et al., 2012) ,which can

lead to dendritic cells acquiring a pro-inflammatory phenotype and inducing production of inflammatory cytokines. NF-kB also plays an important role in the development and maintenance of an inflammatory response within cells (Brasier, 2006).

Matrix metalloproteinase (MMPs) contribute to inflammatory processes, and they do so by regulating physical barriers, modulating inflammatory mediators such as cytokines and chemokines, and establishing chemokine gradients in inflamed tissues that regulate the movement of leukocytes at sites of infection or injury. Matrix metalloproteinase 13 (*mmp13*) is involved in extracellular matrix (ECM) degradation in disease processes (Ottaviani et al., 2010).

Proliferating cell nuclear antigen (*pcna*) is expressed in the nuclei of proliferating cells and is a marker for identifying actively proliferating cells in the intestine of Atlantic salmon (Sanden et al., 2009). The cell renewal process functionally resembles the mammalian organization of crypts and villi. In the absence of distinct villi and crypts in salmon, mucosal fold bases of intestinal epithelial take on the burden of intestinal epithelial cell (IEC) proliferation. This phenomenon is especially demonstrated as a response to the inflammation caused by the SBM diet, when proliferation appears to occur along the entire length of the mucosal fold. The SBM-induced enteropathy has previously been suggested to result in enhanced IEC turnover and in apoptosis, leading to the IEC barrier being largely made up of immature cells (Krogdahl et al., 2003, Bakke-McKellep et al., 2007).

Fatty acid binding proteins (*fabp*) belong to the protein superfamily which can convey fatty acids and other hydrophobic components within the cytoplasm (Venold et al., 2013). Lipid digestibility is often reduced in salmonids fed diets containing full fat or defatted soybean meal (SBM) (Krogdahl et al., 2003). In salmon, *fabp2* is present in most tissues, except in white muscle and spleen, with the highest levels in intestinal tissues (Venold et al., 2013).

Aquaporin 8 is a versatile transmembrane channel expressed in multiple tissues of different animals where it has been suggested to be involved in the maintenance of intracellular osmotic equilibrium, transport of ammonia and small organic solutes or mitochondrial expansion during oxidative phosphorylation (Saparov et al., 2007).

Oxidative stress is caused by the imbalance between the generation of reactive oxygen species (ROS) and the ability of the biological system to neutralize and eliminate them. ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (• OH), etc. (Evans et al., 2001). In addition, environmental factors such as increase or decrease in water temperature,

elevated metal ions, and chemical pollutants can also produce ROS. Endogenous antioxidants like Cu Zn-superoxide dismutase (*sod1*) and catalase (*cat*) are easily oxidized and ensure the protection against oxidative stress in cell (Hermes-Lima, 2004).

Many studies have shown that HSPs are up-regulated in response to a wide range of stressors, as they have a role in repair and degradation of misfolded or denatured proteins. Heat shock proteins (*hsp*) have been proposed as an indicator of stressed states in fish (Iwama et al., 2004). Various heat shock proteins are up-regulated in response to a wide variety of stressors. Increased levels of *hsp70* have been observed in salmon fed soy as a replacement for fishmeal (Bakke-McKellep et al., 2007, Sagstad et al., 2008)

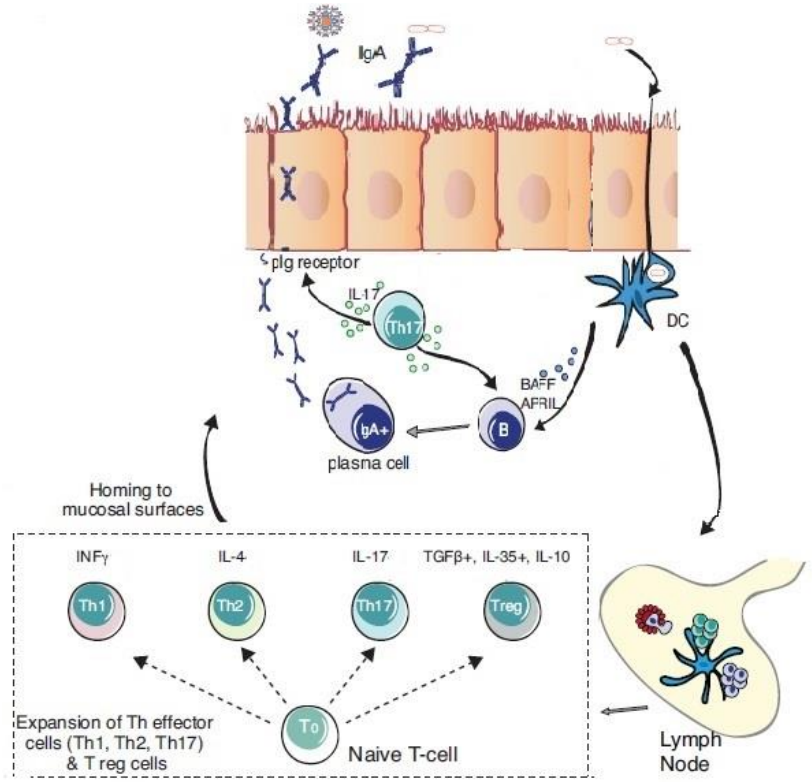


Figure 15. Mammalian mucosal immunity and a complex network of innate and adaptive immune components Continuous pathogen surveillance is mediated by antigen processing dendritic cells (DCs). Mucosal DCs are particularly important at initiating adaptive immune responses by migrating to the draining lymph node and mediating the expansion of antigen-specific naive T-cells into T helper subsets, involving an upregulation lineage-defining cytokine (INFγ, IL-4, IL-17, TGFβ, IL-35, and IL-10). Expanded T-cell subsets will come back to mucosal surfaces to perform their effector functions. T_h17 cells and IL-17 expression can upregulate polymeric Ig (pIg) receptor expression and enhancing IgA secretion. In addition, DCs and epithelial cells can promote T-cell Independent (TI) IgA class switching. Increased IgA production promotes immunity at mucosal surfaces (Lawson et al., 2011).

2.8 Life cycle of Atlantic salmon

The Atlantic salmon (*Salmo salar* Linnaeus) is phylogenetically placed in the family Salmonidae in the teleost division. Bony fish evolved from the ancestral vertebrate lineage approximately 400 million years ago (Falk et al., 2013). Atlantic salmon lives a 'double life' it starts its life in rivers, before transforming its physiology and behavior and migrating to sea to grow and accumulate resources for reproduction (Gillard et al., 2018). The life cycle of Atlantic salmon can be divided into six stages: egg, alevin, fry, parr, smolt and post-smolt salmon (Figure. 16) (Allan et al., 1977). Salmonids do not undergo a metamorphosis from a larval to juvenile stage, in contrast to many marine fish species. Some aspects of early life stages of Atlantic salmon have been intensively studied due to the commercial importance of this species. (Cowey et al., 1985, Gorodilov, 1996).

2.8.1 Definition of developmental stages of Atlantic salmon

Alevin is the period from hatching until first-feeding, also called yolk sac fry or free-swimming embryo. The yolk sac is visible externally. Fry designates the period from first feeding until the appearance of dark stripes on the lateral line (parr marks). In this stage, the yolk sac is absorbed and no longer visible outside the body. The period from the appearance of parr marks until adaptation to seawater (smoltification) is called parr. Smolt is the period of transition to seawater. Parr marks are no longer visible in this period. The period after transition to seawater is post-smolt (Stefansson et al., 2008).

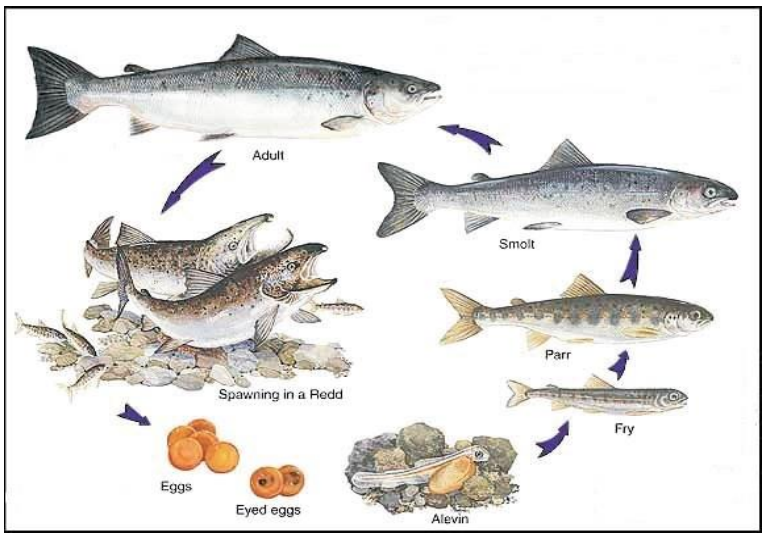


Figure 16. life stages development of Atlantic salmon (Björnsson et al., 2012).

2.9 Structure and function of the gastrointestinal tract of Atlantic salmon

The teleost gastrointestinal system differs topographically from that of mammals (Zapata et al., 2000). In mammals, the small and large intestines can easily be distinguished from each other, and the small intestine may further be subdivided into the duodenum, jejunum, and ileum. In contrast, the teleost tract exhibits profoundly varying topography in intestinal length, degree of looping, number of pyloric caeca, and presence or absence of a stomach (Kapoor et al., 1976). This makes difficult to agree upon a universal nomenclature for teleost intestinal anatomy. A general division into stomach, pyloric caeca, mid intestine and distal intestine has been introduced. However, the precise borders between divided regions are hard to distinguish (Harder, 1975). Starting from the mouth, it can be subdivided into esophagus (ES), stomach (ST), proximal intestine (PI) with adjoining pyloric caeca, mid intestine (MI) and distal intestine (DI) (Figure. 17). The ES of teleost fish has been described as a straight, thick walled tube with longitudinal folds, which can increase in diameter when large amounts of food are ingested (Wilson et al., 2010). The mucosal epithelium is stratified and includes numerous mucus secreting cells (Wilson and Castro, 2010). In most fishes, a clear border or sphincter at the transition of ES to ST is not visible (Wilson and Castro, 2010). The stomach of teleost fish is enveloped in smooth muscle and can be divided into cardiac, fundic, and pyloric region (Zambonino-Infante et al., 2008, Lazo et al., 2011). Some authors do not discriminate between cardiac and fundus of the stomach but describe the whole anterior part as cardiac region (Harder, 1975). Carnivore species show the shortest GI tract, typically less than the body length, whereas in herbivores, such as tilapia, the GI tract may be more than 20 times the body length (Ringø et al., 2014).

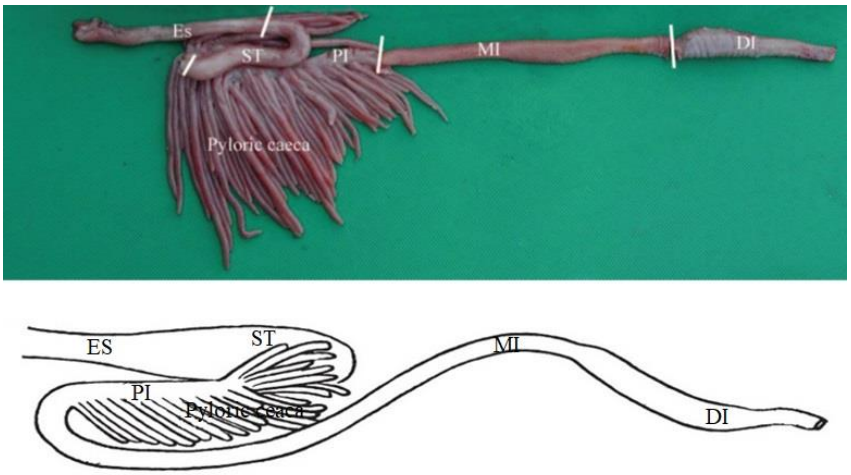


Figure 17. Gastrointestinal tract of Atlantic salmon showing esophagus (ES), stomach (ST), pyloric caeca, proximal intestine (PI) mid intestine (MI) and distal intestine (DI) (Krogdahl et al., 2003).

The tubular segment of the digestive tract (stomach, pyloric caeca, intestine) has four layers: mucosa, submucosa, muscularis, and adventia. Mucosa (RED) consists of: epithelium, lamina propria (LP) and muscularis mucosa. Surface epithelial membrane on a basal lamina, supported by connective tissue (lamina propria) and a thin muscle layer. The mucosa lining of the GI tract is an interface between the external and internal environments, in conjunction with the associated organs (e.g., pancreas, liver and gall bladder). Also the mucosa allows the functions of digestion, osmoregulation, immunity and endocrine regulation of GI tract, as well as the elimination of environmental and toxic contaminants (Mumford et al., 2007). Submucosa (GREEN) consists of coarse areolar connective tissue and elastic fibers, containing blood vessels and nerves. It allows mobility of the mucosa. Muscularis (YELLOW) contains smooth muscles circularly oriented in the inner layer and longitudinally oriented in the outer layer. The function of muscularis is moving and mixing food with digestive enzymes through peristaltic movement. The outer layer of the GI tract adventitia (BLUE) is a relatively dense layer of connective tissue, and when covered by the peritoneum it's called a "serosa". The serosa contains blood vessels, nerves, and lymphatics. In some fish, the compartments may hardly be distinguishable macroscopically, while in other species the sections are divided clearly and may be separated by valves or sphincters. The presence of valves and sphincters between the sub compartments of the intestine may greatly influence the residence time of the chyme in the GI (Mumford et al., 2007) (Figure 18).

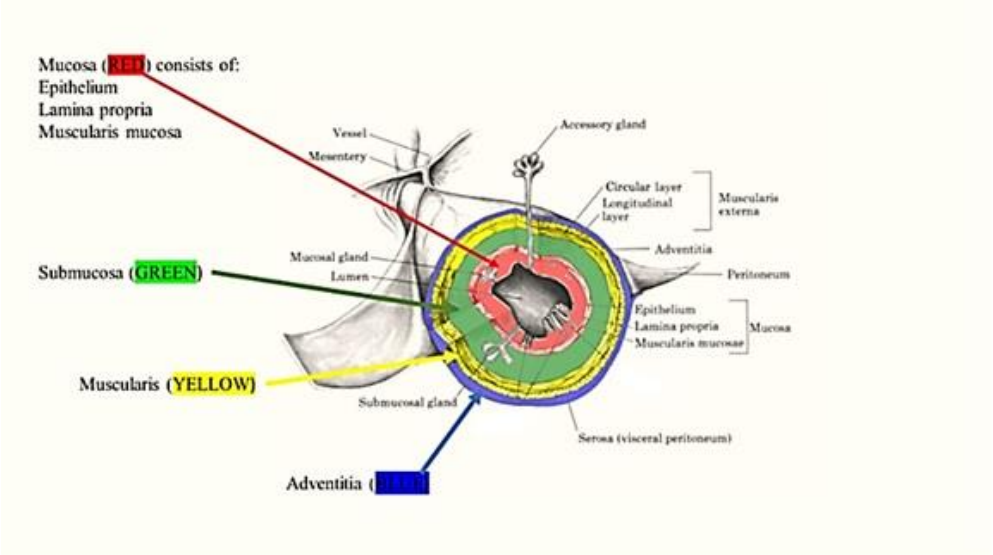


Figure 18. A general cross section of the intestine in Atlantic salmon (Mumford et al., 2007).

The proximal intestine of Atlantic salmon has fingerlike, blind appendages called pyloric caeca, which greatly increase the surface area of the proximal intestine (Figure. 15). Pyloric caeca and PI are surrounded by mesenteric adipose tissue, diffused by endocrine and exocrine pancreatic tissue. Morphological separation between the PI and MI is not clear in Atlantic salmon GI (Løkka et al., 2013). The transition from MI to DI (Figure. 15) is marked by an increase in diameter, darker color of the mucosa, and the presence of complex folds.

2.9.1 Digestive function

A common basic function of the GIT in fish is hydrolysis of macronutrients and release of their small components. Digestive enzymes are secreted from stomach and pancreas and break down macronutrients into smaller molecules. Secretion of brush border enzymes from the apical surface of enterocytes assists for further degradation and absorption (Mosberian-Tanha et al., 2018). In the stomach, secretion of hydrochloric acid (HCl) leads to protein denaturation and activation of pepsinogen, a proteolytic enzyme which hydrolyses peptide polymers. HCl ruptures plant cell walls and releases cell contents for enzymatic processes (Lobel, 1981). HCl and pepsinogen are secreted by oxynticopeptic cells in fish gastric glands. Oxyntic (parietal) cells produce HCl and chief cells are responsible for pepsinogen (Smit, 1968). Endocrine cells control secretion of gastric juices and goblet cells produce mucins with a protective role against HCl, which is the role of gastric mucosa in the digestion process (Mosberian-Tanha et al., 2018). The (chyme mixed gastric secretions) enters the intestine by peristaltic contractions for further digestion. In the intestine, digestion of the chyme is continued, and absorption of nutrients takes place gradually. Gallbladder secretions and pancreatic juice enter the intestine through the common duct containing bicarbonate and digestive enzymes. Pancreatic enzymes consist of chymotrypsinogen, trypsinogen, carboxypeptidase, proelastase, aminopeptidase, procollagenase, pancreatic lipase, α-amylase and phospholipase (Halver et al., 2002). Bile salts, which are produced in the liver, are secreted by gallbladder and assist digestion and absorption of lipids through emulsification of lipid droplets. In the lumen, brush borders enterokinase activates pancreatic trypsinogen. Active trypsin activates chymotrypsinogen and proelastase (Hidalgo et al., 1999). Enterocytes are a category of intestinal epithelial cells (IECs) with a crucial role in digestion and absorption of food. Enterocytes express Na+/K+-ATPase, with an essential role in ion regulation. On the apical surface of these cells, finger-like microvilli have the key role of increasing intestinal surface area. The digestive function of enterocytes is

performed by different digestive enzymes located in the brush border membrane (Harpaz et al., 1999). These enzymes contain aminopeptidase, carboxypeptidase, mono- and triglyceride lipase, wax ester hydrolase, and amylase. The products of these enzymes are small peptides, free amino acids, 2- monoglycerides, free fatty acids, glycerol, fatty alcohols and monosaccharides. Through pinocytosis, peptides and proteins can be absorbed directly from the lumen and transported across the brush border membrane (Storebakken et al., 1998). Endocrine cells in the intestinal region regulate the digestion process by releasing hormones such as cholecystokinin and secretin. This function is necessary to control contraction of the gallbladder and secretion of pancreatic juice (including digestive enzymes and bicarbonate) (Hidalgo et al., 1999). In fish, the digestive and absorptive function of the epithelium is reduced gradually along the intestine and replaced by the mucus secretory function of the goblet cells.

2.10 Diet Formulation and preparation method for salmonids

Diet formulation is not easy, and many factors need to be considered when formulating feed for use in aquaculture. It is a complicated process in which the appropriate feed ingredients are selected and blended to produce a diet with the required quantities of essential nutrients. To fulfil all the nutrient requirements of a cultured organism, selecting various ingredients in the correct amounts, a compounded ration which is nutritionally balanced, pelletable, palatable, easy to store and use is needed. Undoubtedly, supplying adequate nutrition for especial species require precise formulation containing essential nutrients. For salmon, the optimum protein level in feeds depends on dietary energy content and the ratio of essential to non-essential (or indispensable to dispensable) amino acids (Bjerkås et al., 2006).

2.10.1 Role of extrusion technology on aquafeed specially in Atlantic salmon

Over the course of the past 30 years, extrusion processing has become the primary technique used for fish feed production, mainly because of the high physical and nutritional quality of the resulting feed (Grosell et al., 2010). Optimization of feed technology is aimed at improving technical quality, increasing the availability of nutrients, and reducing the content of anti-nutrients in the feed. Most fish feed production in Norway today is based on extrusion technology (Samuelsen, 2015). The extrusion system consists of a barrel housing with one or two rotating screws (single or twin-screw extruder). The extrusion process can be denominate

as a reactor or activator, since it eases chemical reactions. A wide set of parameters such as temperature, moisture, retention time, pressure, shear forces, and mixing reactants / ingredients can be adjusted during extrusion (Figure 19).

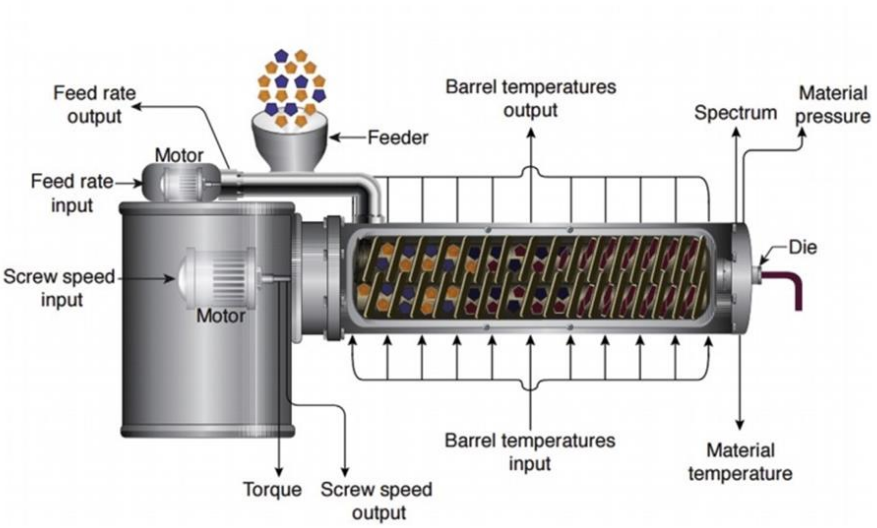


Figure 19. Schematic drawing of extrusion process (Patil et al., 2016).

In conventional extrusion, conditioning is the first step after milling, mixing, and placing the dry mash in the storage bin. For achieving the desired consistency of material in the process and make them bind together, moisture can be added. In the conditioner, the moisture is usually added both in gas and liquid phases. Gelatinization of the starch takes place in the conditioning at the range 80 - 95°C temperature (Sørensen et al., 2011a). Water is added located near the inlet of the conditioner, therefore the temperature reaches a plateau level quite quickly. The moisture content can be in the range 20 - 30% (Sørensen et al., 2011a). The retention time in the conditioner can differ within the range of 30-120 s (Singh et al., 2007). After leaving the conditioner, the material enters the extruder. Due to the high moisture content and temperature, the mash can be adhesive, therefore a force-feeding system into the extruder barrel may be present, as shown in (Figure. 20).

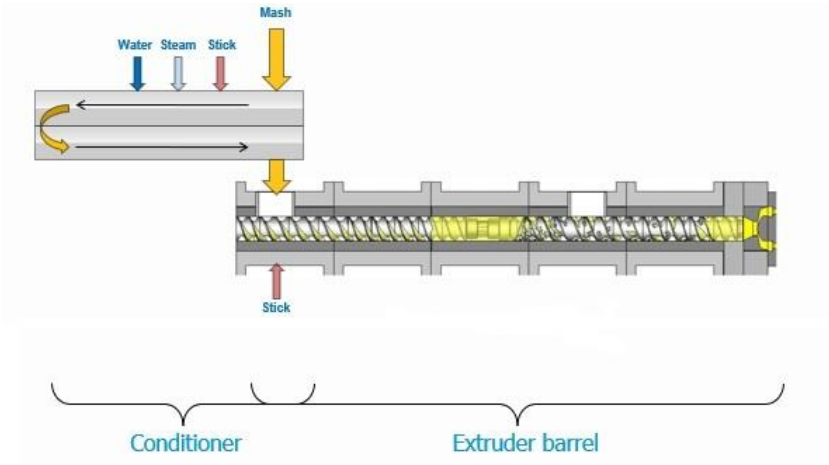


Figure 20. Conditioner and extruder compartments (Kraugerud, 2008).

The extruder barrel is generally divided into sections, and the extruder used in the current study had five sections, as shown in figure 21. The number of sections indicates how severely the extruder will treat the material, where a higher number results in more treatment. (Sørensen et al., 2011a).

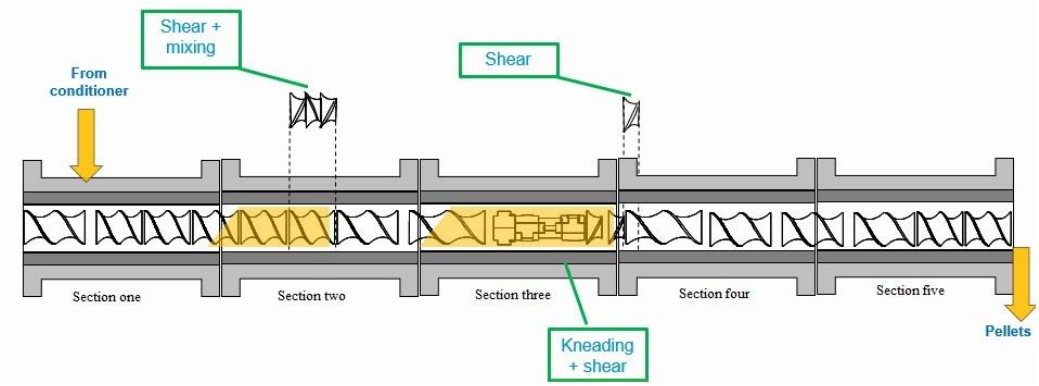


Figure 21. Different extrusion sections can be adjusted with screw elements (Kraugerud, 2008).

A common measurements of extruder length is the length (L) to diameter (D) ratio of screws, or L/D, where regular cooking has ratios of 12 – 24. The diets described in this thesis were produced on extruder with L/D of 20 (Figure 22) (Kraugerud, 2008).

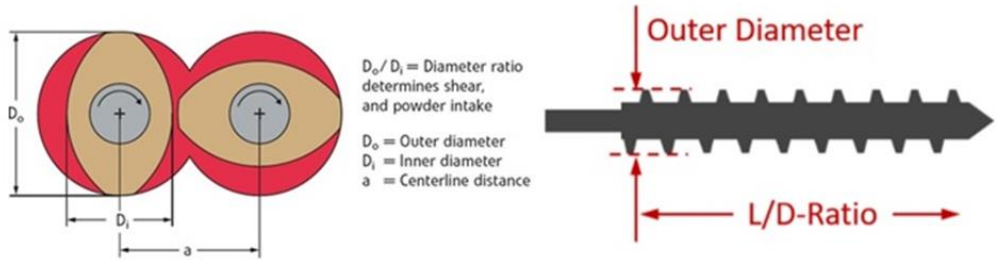


Figure 22. Cross-section of the screws in co-rotating twin-screw extruder. The red area is a hollow area in the barrel (Xtrutech company).

The hollow area in the barrel of an extruder is where the screws rotate, eventually shaping the material through a die by kneading and pushing the ingredients forward. For twin-screw extruders the hollow area in the extruder has the shape of an eight, (figure 22), leaving a small volume free for the material mixing and kneading. The pilot plant extruder utilized in this thesis was a plant extruder with a capacity of 200 – 1,400 kg h^{-1} (Buhler AG co-rotating twin -screw BCTG 62/20 D). The tasks of screws is mainly to convey the material through the barrel, which in general takes 20-40 s (Kraugerud, 2008), and kneading the material to make a homogenous melt of the meal (figure 21). Altering the formulation and processing conditions changes the bulk density of the feed so that it floats on top of, sinks slowly in, or sinks quickly in water. Bonds are formed within the gelatinized starch which results in a durable, water-stable pellet. The twin-screw configuration design has good flexibility in manufacturing, resulting in pellets that will absorb a higher amount of lipid compared to pellets produced with single screw extruders. A main advantage of the cooking-extrusion process is that it raises the digestibility of carbohydrates in the feed due to the exposure of the feed mixture to high temperature and pressures. Cooked carbohydrates play the role of an efficient binder in extruded and expanded pellets. Extruded pellets are more water-stable than steam pellets due to the fact that gelatinized starch resists disintegration in water and better suited for use with slow-feeding species and in recirculating aquaculture systems (RAS) (Sørensen et al., 2011a). Spraying oil onto the surface of pellets is better suited to extruded pellets than to steam pellets, making this another advantages of extruded pellets for many aquaculture situations (Sørensen et al., 2011b).

By heating-cooling the jackets, adding steam-water to the material, or through energy dissipation by kneading/ shear forces, the temperature can be adjusted easily. A temperature change is also a change in energy of the system. The total energy change of the material (ΔQ) in the extruder can be written as

$$\Delta Q = SME + STE \quad (1)$$

Specific mechanical energy (SME) [Wh kg^{-1}] in the extruder can be divided into specific friction energy (SFE) and specific viscosity energy (SVE). Specific thermal energy (STE) [Wh kg^{-1}] is comprised of convective heat, either through steam-water in the jacket and in the barrel (Q_h), natural convection (Q_e) from the barrel to the environment, or from steam addition directly into the material (Q_s). Q_e will usually be negative value since it represents a loss of energy (figure 23).

$$SME = SFE + SVE \quad (2)$$

$$STE = Q_h + Q_e + Q_s \quad (3)$$

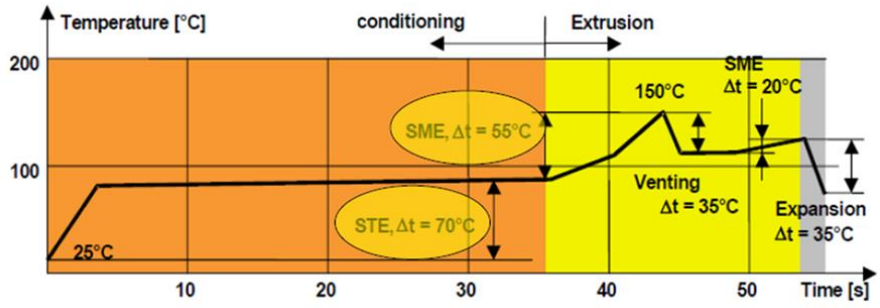


Figure 23. Different levels of specific mechanical energy (SME), to evaluate how the diets responded to differences in treatment severity (Godavarti et al., 1997). The temperature increase in the pre-conditioner is basically caused by steam and moisture addition (STE). The temperature increase in the extruder is generated from mechanical dissipation (SME). Venting remove steam (and thus temperature) causing a drop in the temperature before the last expansion. Expansion of the pellet as the feed exits the die, because moisture flash off and thus, remove heat.

SFE relies on the friction between the barrel wall, the screw, and the material. The friction is closely related to the viscosity of the material. There is a still discussion whether SFE or SVE is most significant (Godavarti and Karwe, 1997). A simple way to express SME is to let the friction and viscosity terms be included in the torque (T_q) [Wh] of the extruder, therefore it measures the energy consumption required to rotate the screw of extruders and incorporates the kneading resistance in the material.

$$SME = {}_m{}^{-1} * T_q * n * 2\pi 60^{-1} \quad (4)$$

where n represents the screw speed of extruder per 60 seconds. Therefore, SME and STE are the energy conveyed to the material as (ΔQ). The energy transferred to the material can be

divided into heat (Q_T) and enthalpy changes of the molecules (Q_r) such as denaturation of proteins and gelatinization of starch.

$$\Delta Q = Q_T + Q_r \quad (5)$$

However, there are plenty of energy components that can be considered such as torque and screw speed. That means that increasing the screw speed is a simple way to increase the SME. Albeit, increasing the screw speed also decreases the residence time (Godavarti and Karwe, 1997). Energy dissipation can occurs where the material is meeting resistance, like in the kneading area and before entering the die (Godavarti and Karwe, 1997) (Figure.24).

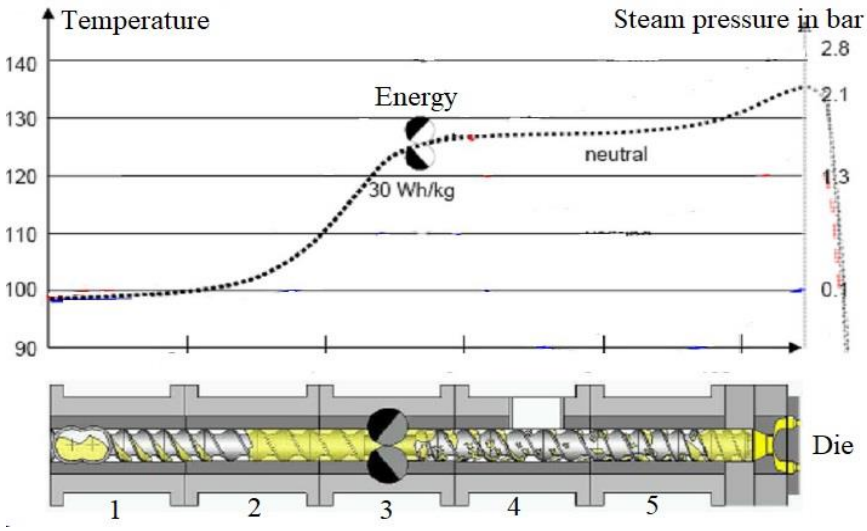


Figure 24. Energy dissipation in extrusion process (Kraugerud, 2008).

The kneading area is located in 3rd section for creating extra pressure. The temperature can decrease before leveling out in the 5th section. (Figure 24). To achieve the desired kneading of material in the barrel, the screw configuration can play a vital role by different elements. The screw configuration is major factor controlling the product (Figure 25).

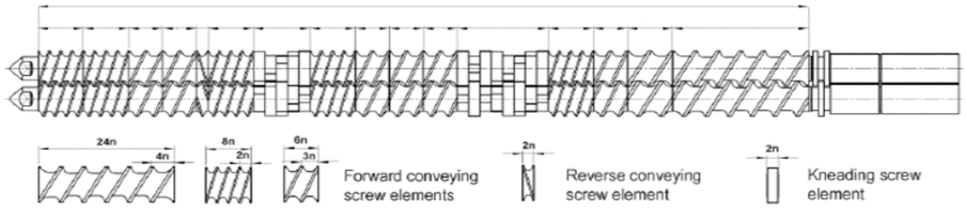


Figure 25. Example of a twin-screw configuration. The elements can be exchanged to be adapted to the type of mash and the pretreatment conditions, such as retention time and shearing force (Takizawa et al., 2011).

2.10.2 Effect of extrusion on the nutritional quality of feed

For several reasons, heat treatment during feed processing can increase the nutritional value of protein ingredients. Frist of all denaturing exposes sites for enzyme to attack and thus make a protein more digestible. Secondly heat labile proteinaceous anti-nutritive factors such as trypsin inhibitors and lectins are destroyed, that moreover enhance the nutritive value. More heat stable anti-nutritive factors such as saponin, alkaloids, phytic acid, tannins and non-starch polysaccharides (NSP) are rarely affected by heat treatment (Storebakken, 2000) and thus limit the level of inclusion of vegetable protein rich ingredients. Severe heating, particularly in combination with low moisture causes destruction in the primary structure in the protein or introduce new bonds between amino acids, which make them hardly digestible (Storebakken, 2000) (table 3).

Table 3. Effect of heat treatment on denaturation and degradation of protein (Phillips et al., 1989)

Temperature,°C	Effect of heating
≤55	Increases hydration and loss of some crystalline structure
70-80	Disulphide splitting of bonds and loss of the tertiary structure
80-90	loss of secondary structure
90-100	Formation of intermolecular disulphides bonds
100-150	Lysine and serine loss, isopeptide formation
150-200	Peptidisation and more isopeptide formation
200-250	Pyrolysis of all amino acid residues

3 Aims and strategies

The main goal of the present study was to document the potential value of Triple Null as a soybean meal input for salmon production by measuring the nutritional value of and capacity of Triple Null to mitigate the enteritis and reduced growth that results from higher inclusion rates of standard beans. The second goal was to evaluate how changes of specific mechanical energy (SME) during extrusion affect the efficiency and quality of experimental diets. Therefore, we hypothesized that high and low SME would significantly affect the performance and efficiency of soy diets in aquafeeds for Atlantic salmon.

4 Material and Methods

4.1 Fish and facilities

The experiment was conducted in compliance with laws regulating the experimentation with live animals in Norway as overseen by the Norwegian Animal Research Authority (FDU). The feeding trial was performed at the Center for Fish Research (a modern center for small-scale fish experiments), in specially adapted facilities recirculation aquaculture systems (RAS) technology for feed research at Norwegian University of Life Sciences (NMBU), Campus Ås, Norway (figure 26). In contrast to large, full-scale experiments, such as those which are run in commercial production, small scale facilities offer the opportunity to conduct experiments with a great degree of precision. Using collecting devices for uneaten feed, feed intake can be calculated, which can be related to growth of the fish with high precision. The feed used in this experiment was managed by the Department of Animal and Aquaculture Sciences (IHA) and was produced at the center for fôrteknologi, FôrTek at NMBU.

Figure 26. Representative picture of fish lab (Dyrerom F110, RAS 1 system).

A total of 1045 Atlantic salmon (Salmo salar L.) pre-smolt of the Aqua Gen (Sunndalsøra) breed with weight of 45 ± 10 g (mean ± SD) chosen from a large tank of fish that had been kept on a commercial diet were weighed and randomly allocated into 18 cylindrical fiberglass tanks (1× 1×0.6 m3, water depth 40–50 cm) with 55 fish per tank and supplied with flow-through freshwater (6–7 L min−1). The tanks had conically shaped bottoms to ensure efficient recovery of uneaten feed (Helland et al., 1996). Water temperature was 13-14 °C, oxygen content and salinity of the outlet water were monitored daily to ensure saturation above 85%. A 24-hours lighting regime was employed during the experimental period. For the establishment of a

precise experiment, and for observation of morphological, physiological and behavioral changes (smoltification) of fish, one tank was added and fed with commercial feed.

4.2 Diets and feeding

Six experimental diets were formulated (Table 4). A commercially available alcohol-water washed soy protein concentrate (SPC) was used as a negative control (Ref), a positive control diet of conventional soybean meal (CSBM), and soybean Triple Null (TN) as an experimental diet. Thus, 6 diets were evaluated, with 3 sources of soy protein, each processed at 2 SME levels during the extrusion process. All diets contained 0.1 g yttrium oxide (Y_2O_3) kg^{-1} as an indigestible marker for apparent digestibility determinations (Austreng et al., 2000). Vitamins and minerals were added to all diets to fulfill the National Research Council (NRC) recommendations (NRC, 1993). All indispensable amino acids and other nutrients protein level was above minimum requirements in all diets.

During acclimatization, the fish received standard, fishmeal-based, commercial diets (BioMar AS, Myre, Norway). The six diets were fed to triplicate (3 tanks for each diet, 55 fish each tank) groups of fish over a period of 56 days. The fish were weighed individually when allocating the fish to the experimental units to assure similar biomass in all tanks. The total biomass in each tank was determined at the middle of feeding (28 days) and after 56 days of feeding. Feed was provided continuously using automatic feeders (figure 27). Growth rate was expected to be 1% per day of body weight during the feeding period (Austreng et al., 1987), and the fish were fed 10% more than the expected need, considering a feed conversion of 1.0. Body weights and lengths of five fish from each tank were taken at the beginning and halfway through the feeding period to ensure that all the fish were growing at an acceptable rate. Fish were fed to satiation three times a day (8:00, 2700 seconds interval 12:00, 2700 seconds interval and 18:00, 14400 seconds interval). The amount of feed was recorded every day and excess feed was removed and recorded. Feed intake was estimated based on records of the amount of feed supplied and feed waste. Daily feed intake in each tank was quantified as described by Helland et al. (1996), by collection of uneaten feed from the outlet water of the tanks using wire mesh screens. The latter was collected from each tank and the weight estimated by counting the number of pellets and multiplying it by the average pellet's dry weight (Helland et al., 1996). Fish were weighed

at the end of the experiment, and weight gain, specific growth rate (SGR) and feed efficiency (FE) were determined.

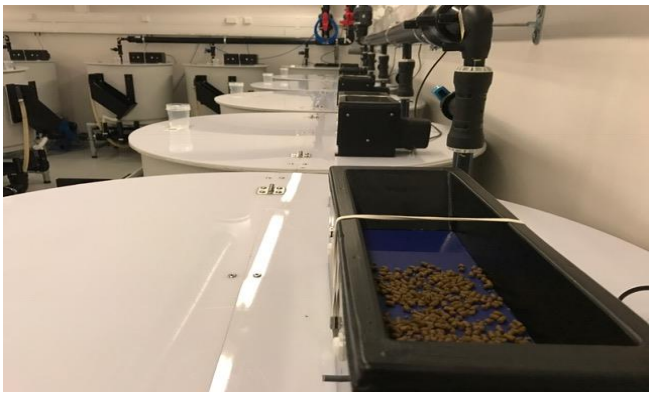


Figure 27. Automatic feeders used in this experiment

Table 4. Composition of the experimental diet.

	Diet name					
	Low SME			High SME		
	CSBM	TN	Ref	CSBM	TN	Ref
Formulation g kg^{-1}						
Fish meal[a], LT	235	230	236	235	230	236
Soyprotein concentrate[b]	0	0	190	0	0	190
Full fat soy[c], conventional	250	0	0	250	0	0
Full fat soy, TN[d]	0	270	0	0	270	0
Vital wheat gluten[e]	70	70	70	70	70	70
Wheat[f]	150	150	150	150	150	150
Fish oil[g]	186	170	240	186	170	240
Soybean oil[h]	50	50	50	50	50	50
Choline chloride[i]	0.3	0.3	0.3	0.3	0.3	0.3
Mono calcium phosphate[j]	18.2	18.2	18.2	18.2	18.2	18.2
Limestone[k]	4	4	4	4	4	4
L-Lysine[l]	7	7	9	7	7	9
DL-Methionine[m]	13	13	15	13	13	15
L-Tryptophan[n]	0.6	0.6	0.6	0.6	0.6	0.6
L- Arginine[o]	5	5	5	5	5	5
L- Threonine[p]	1.8	1.8	1.8	1.8	1.8	1.8
L-Valine[q]	3	4	4	3	4	4
Stay C 35%[r]	1	1	1	1	1	1
Yttrium oxide[s]	0.1	0.1	0.1	0.1	0.1	0.1
Premix[t]	5	5	5	5	5	5
Proximate composition						
Dry matter g/kg	971	969	972	962	965	957
Ash g/kg	79	79	79	79	78.92	76.60
Kjeldahl N g/kg	60.64	60.21	62.70	61.56	65.38	67.03
Starch %	10.58	10.84	11.55	11.13	12.12	11.52
Crude fat g/kg	267.31	258.98	267.05	264.73	250.04	267.60
Energy MJ/kg	24.03	23.97	23.86	23.75	23.49	23.74

[a]NorsECO-LT, Norsildmel, Fyllingsdalen, Norway.
[b]Lyckeby Culinar AB, Fjolkinge, Sweden.
[c]Denofa, Fredrikstad, Norway.
[d]Full fat soy, TN
[e]Gluvital 21000, Cargill, Barby, Germany.
[f]Feed grade wheat, Felleskjøpet, Kambo, Norway.
[g]NorSalmOil, Norsildmel, Fyllingsdalen, Norway
[h]Raw Soybean oil, Denofa, Fredrikstad, Norway.
[i]Choline Chloride-70%, INDUKERN, S.A., Spain.
[J]Monocalcium Phosphate Monohydrate-Feed Grade, Yara Animal Nutrition.
[k]Franzefoss Miljøkalk AS, Rud, Norway.
[l]Lysine monoHCl, 99% feed grade, Cheil Jedang, Indonesia.

4.3 Feed manufacturing

The diets were processed at the Center for Feed Technology (FôrTek) at NMBU, Ås, Norway. The CSBM and TN were ground in a Münch hammer mill (HM 21.115, Wuppertal, Germany) equipped with a 0.6-mm screen, and mixed in a Pegasus Menger (400 l, Sevenum, The Netherlands) twin shaft paddle mixer, and conditioned for 90 s in a Bühler (Uzwil, Switzerland) double conditioner (BCTC 10) to 86–90 °C before extrusion in a Bühler twin screw extruder (BCTC 62). The die hole diameter was 3 mm. The feed was dried in a Bühler fluidized bed dryer (OTW 50 05TSR2) to approximately 920 g kg^{-1} dry matter before it was oil coated in a Fortek (Ås, Norway) 6-l mini-coater. During production of the "SME level" the extruder was adjusted to produce diets at two different levels of SME (high and low), see Table 5. Samples of feed ingredients were taken for chemical analyses and feed samples from each treatment were taken during feed manufacturing for chemical analyses. The diets were extruded to give pellets 3 mm in length (figure 29).

Figure 28. Production line of experimental diets and testing bulk density of pellets.

Table 5. Processing parameters during production of the low and high SME diets.

	Diet group					
	Low SME			**High SME**		
	CSBM	**TN**	**Ref**	**CSBM**	**TN**	**Ref**
SME (Wh/kg)	520	500	386	850	852	660
Torque (Nm)	170	154	245	256	183	202
Torque (Relativ, %)	43	36	57	60	42	46
Drive power (kW)	4.9	4.8	4.4	6.2	6	7.3
Screw speed (rpm)*	315	315	175	255	310	350
Extr. water kg/h	10	10	11.2	7	7	11
Die temperature	97	101	99	108	109	93

4.4 Sampling

Feed samples were taken from all the experimental feeds and analyzed for chemical composition. All sampling of the fish was carried out on randomly selected individuals from each tank. The tanks were also chosen in random order. At the start and termination of the feeding trial, five fish from each tank were killed, weighed and body length measured. The carcasses were frozen and kept at -20°C. Three fish at the midpoint of the feeding period (day 28) and twelve fish at the termination of the feeding period were randomly sampled from each tank. The fish were anesthetized with (Finquel ® vet. 100%) metacain (tricainmesylat), weighed individually, and blood was sampled for biochemical analyses. Blood was drawn from the caudal vein with 3ml heparinized vacutainers and put on ice until centrifugation at 1500 ×g for 5 minutes (figure 30 A). The GIT was sectioned into stomach (ST), pyloric intestine (PI, from the most proximal to the most distal pyloric cecum), mid intestine (MI, from the last pyloric cecum to the distal intestine), and distal intestine (DI, from the increase in intestinal diameter, visible folds and darker mucosa to the anus). The viscera fat and connective tissue were removed, and the sections were cut open and rinsed, weighed, and frozen in liquid nitrogen and stored at −80 °C. The liver was also removed and weighed individually. Six of these fish were also sampled for histological examination of the intestinal tract. Approximately 5mm×5mmtissue samples from the DI were taken (figure 29 B). The samples were fixed in 10% neutral buffered formalin (4% formaldehyde, pH 7.4) for 24 h, then transferred to 70% ethanol and stored at 4 °C until processing. The remaining fish in the tanks were stripped for feces as described by Austreng (1978) (Austreng et al., 1987). Feces were immediately frozen at −20 °C after stripping, freeze-dried and ground to a powdery consistency before analyses.

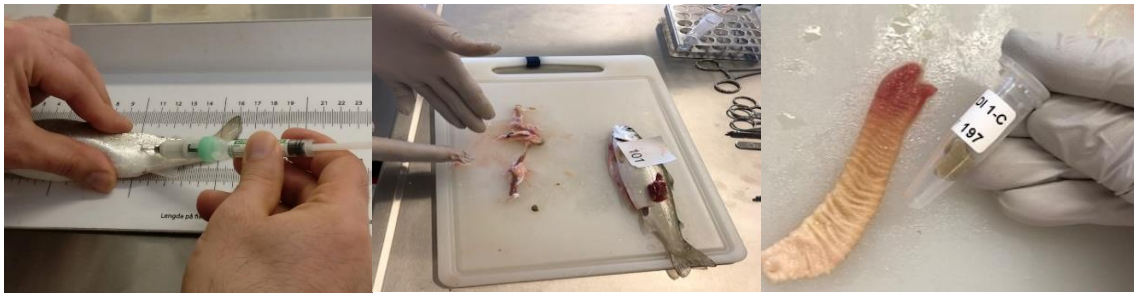


Figure 29A-C. Blood sampling, GIT sampling.

4.5 Chemical analyses

The dry matter contents of all feed and faeces samples were determined following freeze-drying and all analyses were conducted on freeze-dried material. Chemical analyses were carried out as follows: ash was analysed gravimetrically following combustion at 550 °C. Crude protein (N*6.25) was determined by the Kjeldahl method. Crude lipid was determined using pre-extraction and hydrolysis of the sample in 4 M HCl at 100 °C. Yttrium oxide concentration in feed and feces was determined by inductively coupled plasma mass spectroscopy (ICPMS). Gross energy was estimated by bomb calorimetery. Amino acid (except tryptophan) analysis of all samples was performed with a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, U.K.) after hydrolysis, according to EC Commission Directive 98/64/EC.

4.6 Brush Border Membrane Enzyme Activity

The activity of the brush border membrane enzyme leucine aminopeptidase (LAP) was measured in PI, MI and DI tissue homogenates diluted in ice cold Tris-mannitol buffer (1:20, w/v) containing 4-(2-aminoethyl)- benzenesulfonyl fluoride hydrochloride (Pefabloc SC, Pentapharm Ltd., Basel, Switzerland) as a serine proteinase inhibitor. The activity was determined colorimetrically using the substrate L-leucine β- naphthylamide hydrochloride as described by Krogdahl et al. 2003 (Krogdahl et al., 2003). LAP specific activity was calculated following tissue protein concentration analysis using the BioRad protein assay (BioRad Laboratories, Munich, Germany).

4.7 Plasma Cholesterol, total bile acids, and Other Metabolites

Analyses of plasma levels of cholesterol, total bile acids (conjugated and nonconjugated bile acids), glucose, triglycerides, free fatty acids, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) and sodium were performed by the Central Laboratory of the NMBU School of Veterinary Medicine (Oslo, Norway) according to standard protocols (Advia 1800, Siemens Healthcare Diagnostics, Erlangen, Germany).

4.8 Intestinal morphology evaluation

A histological assessment of distal intestinal DI sections of Atlantic salmon was conducted two times (intermediate sampling and final sampling). For intermediate 54 and final sampling 108 DI tissue sections were routinely dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin according to standard histological techniques. Sections of approximately 5–8 μm thickness were cut and stained with haematoxylin and eosin (H&E). The DI was sectioned longitudinally (i.e. perpendicular to the macroscopically visible circular folds). The processing of tissues was performed at the Section for Pathology of the Veterinary School (Oslo, Norway). Blind histological examination was performed using a light microscope (Carl Zeiss microscopy AX10, Germany). Tissue morphology was evaluated using a semi-quantitative scoring system. Preselected tissue parameters (mucosal fold height, lamina propria width, lamina propria cellularity (hyperplasia and/or infiltration), submucosa width, submucosa cellularity, enterocyte vacuolization, and enterocyte vacuole size disparity) were scored using a continuous scale (Table 6). The degree of histomorphological change (i.e., deviation from normal) was assessed and assigned to one of five categories: normal, slight, moderate, marked and severe. For each of the morphological characteristics, the degree of change of the DI morphological features were graded using a scoring system with a scale of 1-10 where 1 to <3 represented normal; 3 to <5, mild changes; 5 to <7, moderate changes; >7 to <9, marked changes, and 9-10, severe changes. Scoring was done using a visual analogue scale to generate the scores as continuous variables that allowed conducting one-way analysis of variance (ANOVA) statistical analyses on the data.

Table 6. Description of semi-quantitative scoring system using different parameters to assess the degree of enteritis developed by Atlantic salmon.

Score	Parameter	Score	Parameter
	Mucosal folds (MF)		**Submucosa width (SMW)**
1	Normal	1	Normal
3	Mild	3	Mild
5	Moderate	5	Moderate
7	Marked	7	Marked
10	Severe	10	Severe
	Submucosa cellularity (SMC)		**Lamina Propria width (LPW)**
1	Normal	1	Normal
3	Mild	3	Mild
5	Moderate	5	Moderate
7	Marked	7	Marked
10	Severe	10	Severe
	Lamina Propria cellularity (LPC)		**Supranuclear Vacuoles (SNV)**
1	Normal	1	Normal
3	Mild	3	Mild
5	Moderate	5	Moderate
7	Marked	7	Marked
10	Severe	10	Severe

4.9 Quantitative Real Time PCR (qPCR)

Quantitative PCR (qPCR) assays of distal intestine samples were performed according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009) standards on 3 fish from each of 3 tank replicates of the 6 diet groups (n=9 animals per diet, total number of samples=54, see Table 7).

Table 7. Sample and diet group overview

	SME	
Diet group	**High**	**Low**
CSBM	n=9	n=9
TN	n=9	n=9
Ref	n=9	n=9

Samples were stored in RNAlater® at -20°C before processing. Total RNA was extracted from DI tissue samples (~30 mg) in randomized order using a custom made Reliaprep simply RNA HT protocol (Promega) and a Biomek 4000 laboratory automation workstation (Beckman Coulter). 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 an Epoch microplate spectrophotometer (BioTek Instruments). All samples had RNA integrity numbers (RIN) >8.6, with a mean RIN value of 9.7. Total RNA was stored at -80 °C until use. First strand cDNA was synthesized from 1.0 µg total RNA from all samples using Superscript IV VILO Mastermix (Thermo Fisher Scientific) in 20µL 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 13 target genes with key roles in intestinal immune, metabolic and stress/antioxidant function were profiled. See Table 8 for details. Expression of individual gene targets was analyzed using the Light Cycler 96 (Roche Diagnostics). Each 10µL DNA amplification reaction contained 2µL PCR-grade water, 2µL of 1:10 diluted cDNA template (corresponding to 8ng total RNA), 5µL of Light cycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5µL (final concentration 500nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC) and an inter-plate calibrator. Pipetting was performed using a Biomek 4000 laboratory automation workstation (Beckman Coulter). 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, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase II *(rnapolII)* and hypoxanthine-guanine phosphoribosyltransferase 1 (*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. The geometric mean of *gapdh*, *rnapolii* and *hprt1* was used as the internal normalization factor (Kortner et al., 2011). Mean normalized levels (MNE) of target genes were calculated from raw quantification cycle (Cq) values (Muller et al., 2002).

Table 8. Target gene names, category and function.

Gene name	Acronym	Category	Function
Interleukin 1β	*Il1β*	Immune	Pro-inflammatory cytokine
Interleukin 17a	*Il17a*	immune	Pro-inflammatory cytokine, T cell marker
Interferon γ	*ifn γ*	Immune	Pro-inflammatory cytokine
Transforming growth factor β	*tgfβ*	Immune	Anti-inflammatory cytokine
Myeloid differentiation primary response gene 88	*myd88*	Immune	NF-κB activation
Cluster of differentiation 3γδ	*cd3 γδ*	Immune	T cell marker
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
glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	Reference gene	Catalytic activity
RNA polymerase II	*rnapolII*	Reference gene	Polymerization reaction
hypoxanthine-guanine phosphoribosyltransferase 1	*hprt1*	Reference gene	Catalytic activity

For primers and assay details see (Krogdahl et al., 2015)

4.10 Calculations

Feed efficiency = weight gain (g)/feed intake (g). The specific growth rate (SGR) was calculated using the tank means for initial body weight (IBW) and final body weight (FBW) and calculated as follows: SGR = [(ln FBW − ln IBW)/number of days] × 100. Condition factor of the specimens were determined by using the formula: Condition factor (K)= (body weight/fork length^3) ×100. Hepatosomatic Index (HSI) is defined as the ratio of liver weight to

body weight. HSI = liver weight x 100/Body weight. Organosomatic indices (OSI) of the DI were calculated as the percentages of the weight of the intestinal sections in relation to the fish body weight, i.e. Organosomatic index = (organ weight/body weight) × 100. Nutrient retention efficiency = 100× nutrient gain/nutrient intake. Apparent digestibilities were calculated as: Apparent digestibility coefficient (%)=100 – (100 × (nutrient in faeces/yttrium oxide in faeces) × (yttrium oxide in feed/nutrient in feed)).

4.11 Statistical analyses

Data was analyzed using two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) for post hoc comparison. This method was used to determine whether there are any significant differences between the basal diets and SME levels. The level of significance for all analyses was set at $P < 0.05$. When there was a significant interaction, a one-way ANOVA was performed to help interpretation of the interaction.

5 Results

The results of our study are presented below with the following organization according to the goals: Firstly, the differences between the results of the fish fed TN and CSBM are presented, thereafter effects of SME, and finally important differences between the two soybean meals (TN and CSBM diets) and the soy protein concentrate (Ref Diet) on the other.

The experiment was carried out without any problems, the fish accepted all diets well, and no mortalities were recorded. The model chosen for the evaluation of the differences between the TN and the CSBM gave the expected results, i.e. induced symptoms of enteritis in the fish fed the CSBM. The experiment therefore turned out to be suitable for the goal of the study.

5.1 Specific growth rate and feed efficiency

Figure 30 A-D illustrates the results regarding SGR and FE for the six diets at 28 days and 56 days of feeding. The mid-term observations for SGR showed a significant diet effect which depended on the SME level as shown by the significant interaction. TN and CSBM did not show significant difference independent of SME, whereas at low SME the Ref showed significantly higher SGR than the two soybean meals. At high SME, no significant diet effect was observed However, at the end of the experiment, there were no significant effects on SGR either of diets or SME level.

Differences in FE of the fish fed TN and CSBM during the feeding trail were not observed.

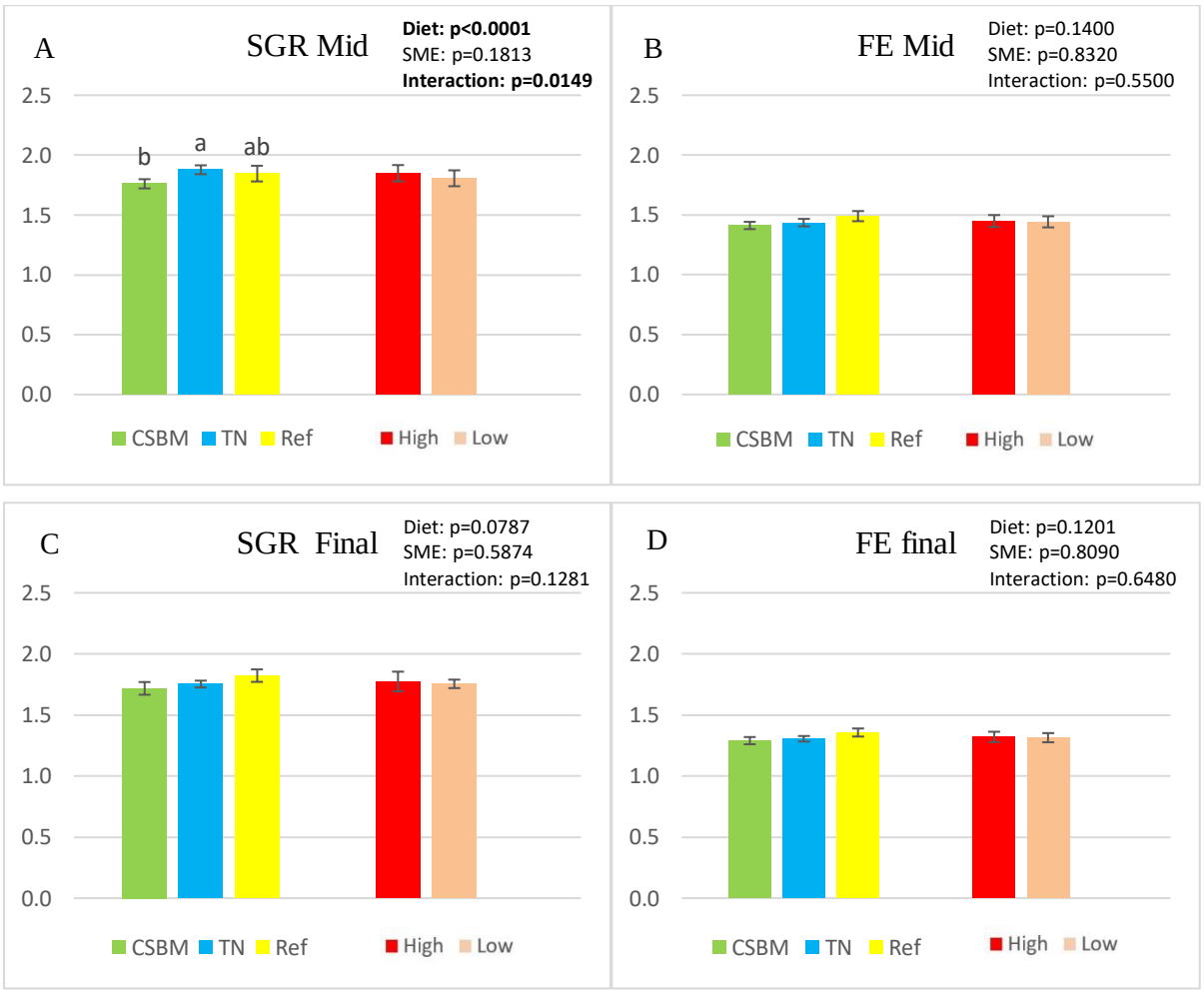


Figure 30.A-D Overall effect of diets and SME, including results of two-way ANOVA, for SGR and FE recorded after four weeks (A-B) of feeding and at the end of the feeding period (C-D). Error bars show standard error of the mean (SEM). Significant differences between diets are denoted with letters.

5.2 Condition factor, relative carcass weight, hepatosomatic index and distal intestine somatic index

Figure 31 A demonestrstes the results regarding CF and shows that fish fed TN and CSBM diet did not differ significantly for this variable. However, variation in level of SME caused significant effects on CF. Fish fed high SME diets showed lower CF than fish fed the low SME diets. The interaction between the two main factors was not significant. Moreover, there was no significant difference in CF between fish fed the Ref and the two soybean meals.

For relative carcass weight (RCarc), the statistical evaluation demonstrated that fish fed TN and CSBM diets did not show significant differences. Moreover, variation in level of SME did not cause significant effects and the interaction between the two was not significant (figure 25 B).

Significant diet effect on hepatosomatic index (HIS) was observed, whereas variation in level of SME did not cause significant effects and the interaction between the two was insignificant. Fish fed the TN diet had higher HIS value than fish fed CSBM diet (figure 25 C). Compared to the TN and CSBM diets the Ref Diet showed significantly lower values than both.

Regarding distal intestine somatic index (DISI), fish fed TN and CSBM did not show significant difference. The variation in level of SME did not cause significant effects and the interaction between the two was also insignificant (figure 25 D). DISI attained its highest value for Ref diet in comparison with the two full fat soybeans (TN and CSBM), and the difference was significant.

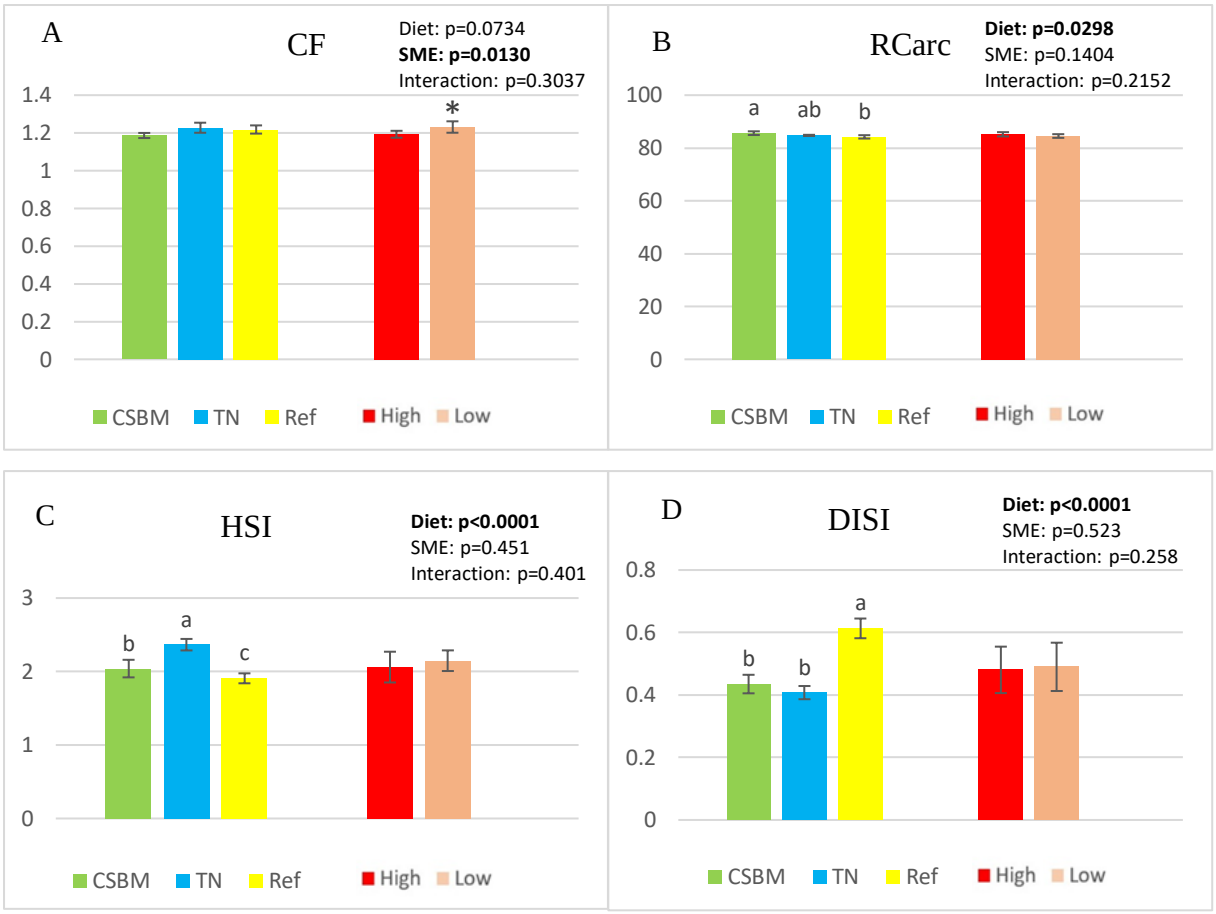


Figure 31A-D. Effects of diets and SME level are shown for CF, RCarc, HSI and DISI, P-values for the two-way ANOVA are given. Bars are standard error. Significant differences between diets are denoted with letters. Asterisk indicates significant differences between high and low SME.

5.3 Apparent digestibility

Apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets are presented in Table 9. For CP digestibility a significant interaction was observed between the two main factors. At low SEM the TN and CSBM diets did not differ significantly (87.1 and 85.6%), but the Ref diet showed a significantly higher value (90.3). At high SME no significant difference was seen between the three diets. Fish fed high SME diets had significantly higher AD of protein than those fed low SME diet.

Regarding amino acid digestibility a very similar pattern of result as for crude protein was observed for all. However, the level ranged from 97 for Met to 77 for Cys. No important differences were seen in amino acid digestibilities between TN and CSBM, neither at low or high SME, but high SME increased amino acid digestibility. At low SME the Ref diet showed

higher values than the TN and CSBM diets, whereas at high SME no such difference was apparent.

Lipid and energy digestibility of the TN and CSBM diet showed significant differences. Also variation in level of SME caused significant effects on lipid and energy digestibility. The interaction between the basal diets and SME was insignificant.

Apparent digestibility of phosphorus did not change among basal diets and either SME level or interaction did not cause significant differences.

Table 9. Apparent digestibility (AD, %) of macronutrients and energy (mean ± s.e.m) of the salmon fed diets with TN in comparison with CSBM and Ref

Diet	**CSBM**	**TN**	**Ref**			**P-value (Diet)**	**P-value (SME)**	**P-value (Interaction)**
SME				**Low**	**High**			
Crude protein	88^{b}	88^{b}	90^{a}	87^{x}	90^{y}	0.0002	<0.0001	0.0004
Crude lipid	94^{a}	93^{b}	88^{c}	91^{x}	92^{y}	<0.0001	0.0007	0.21
Energy	82^{a}	81^{b}	80^{c}	79^{x}	82^{y}	0.0007	<0.0001	0.44
Essential amino acids								
Arg	93^{b}	93^{b}	95^{a}	92^{x}	92^{x}	<0.0001	<0.0001	<0.0001
His	89^{b}	88^{b}	92^{a}	88^{x}	91^{y}	<0.0001	<0.0001	<0.0001
Ile	90^{b}	90^{b}	93^{a}	90^{x}	92^{y}	<0.0001	<0.0001	<0.0001
Leu	93^{a}	91^{b}	93^{a}	90^{x}	93^{y}	<0.0001	<0.0001	<0.0001
Lys	92^{b}	93^{b}	95^{a}	93^{x}	94^{y}	<0.0001	<0.0001	<0.0001
Met	96^{b}	96^{b}	97^{a}	96^{x}	97^{y}	<0.0001	<0.0001	<0.0001
Phe	90^{b}	90^{b}	93^{a}	90^{x}	92^{y}	<0.0001	<0.0001	<0.0001
Thr	88^{b}	87^{b}	91^{a}	87^{x}	90^{y}	<0.0001	<0.0001	<0.0001
Val	91^{b}	91^{b}	94^{a}	91^{x}	93^{y}	<0.0001	<0.0001	<0.0001
Cys	77^{c}	78^{b}	83^{a}	77^{x}	82^{y}	<0.0001	<0.0001	<0.0001
Total AA	90^{b}	91^{b}	92^{a}	90^{x}	92^{y}	<0.0001	<0.0001	<0.0001
Minerals								
P	48	50	48	49	48	0.33	0.75	0.24

$^{a, b, c}$ Significant differences between basal diets and ^{x,y} SME levels.

5.4 Retention of nutrients

Table 10 presented the proximate composition of whole fish body (%) and retention of crude protein. No significant differences were found in whole body composition between fish fed TN and CSBM independent of SME levels. Fish fed the Ref diet showed lower dry matter content than fish fed the TN and CSBM diets. Retention of ingested CP of fish fed TN diet was significantly lower than fish fed CSBM diet, while no significant effect was seen for the SME level. The interaction between the basal diets and SME level was insignificant.

Table 10. Whole-body composition1 and nutrient retentions (mean ± s.e.m) of the salmon fed diets with TN soybean in comparison with CSBM and Ref

Diet	**CSBM**	**TN**	**Ref**			**P-value (Diet)**	**P-value (SME)**	**P-value (Interaction)**
SME				**Low**	**High**			
Body composition, (kg wet weight)$^{-1}$								
Dry matter, g	321^{a}	321^{a}	305^{b}	315	317	0.0092	0.61	0.1300
Crude protein, ¦	167	167	165	168	165	0.1200	0.06	0.0093
Crude lipid, g	111	122	109	115	120	0.15	0.34	0.16
Ash, g	22	22	22	22	22	0.33	0.65	0.42
***Retention, % of intake* 0-56 day**								
Crude protein	61^{b}	57^{c}	64^{a}	60	61	<0.0001	<0.0001	0.26

$^{a, b, c}$ Significant differences between basal diets and ^{x,y} SME levels.

5.5 Bile salt concentration levels in intestinal content

Results of analysis of bile salts concentration in the intestinal content along the intestine are presented in figure 32A-B.

Overall, the results revealed that by passing the chyme from PI1 to DI1, levels of total bile acid concentration decreased significantly. However, between DI1 and DI2 a slight elevation was observed. The chyme bile salt concentration in all the segments of the PI and MI showed significantly lower values for TN than CSBM diets. In the distal segments there were not significant differences between TN and CSBM diets. Fish feed the Ref diet had higher levels than both CSBM and TN in most of the sections, figure 32-A.

As the SME level did not cause significant differences and the interaction between the basal diets and SME level was insignificant, the figure presents means for the two SME.

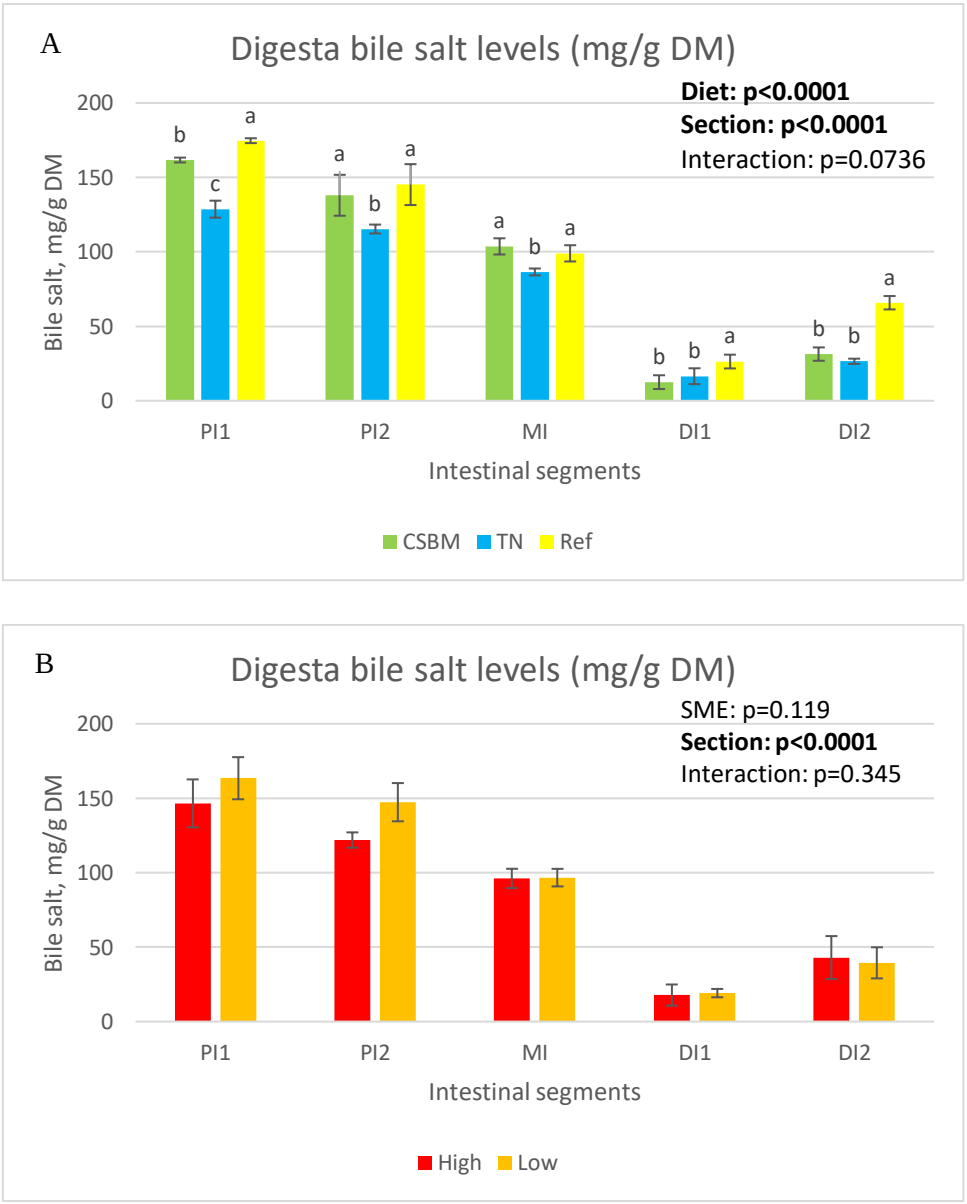


Figure 32A-B. Bile salt concentration levels in intestinal segments (PI to DI), expressed as mg/g DM. Values are means with standard errors represented by vertical bars. Different letters denote diet groups that are significantly different.

5.6 Brush border membrane leucine aminopeptidase activity in the distal intestinal tissue

Activities of LAP in distal intestinal tissue presented as total capacity (U/kg body weight LAPkg,) and specific activity (U/mg of protein, LAPprot) are shown in figure 33 A-B. The statistical analyses revealed that TN and CSBM diets did not differ significantly regarding effects on LAPkg and the result was independent of SME. However, for LAPprot, a significant interaction between the main factors was apparent as the results were dependent on SME level. Increased SME elevated the activities for fish fed the Ref diets, whereas for TN and CSBM a lower result was observed. Fish fed the Ref diet showed significantly higher values than those fed the two full fat soybeans for both LAP activities.

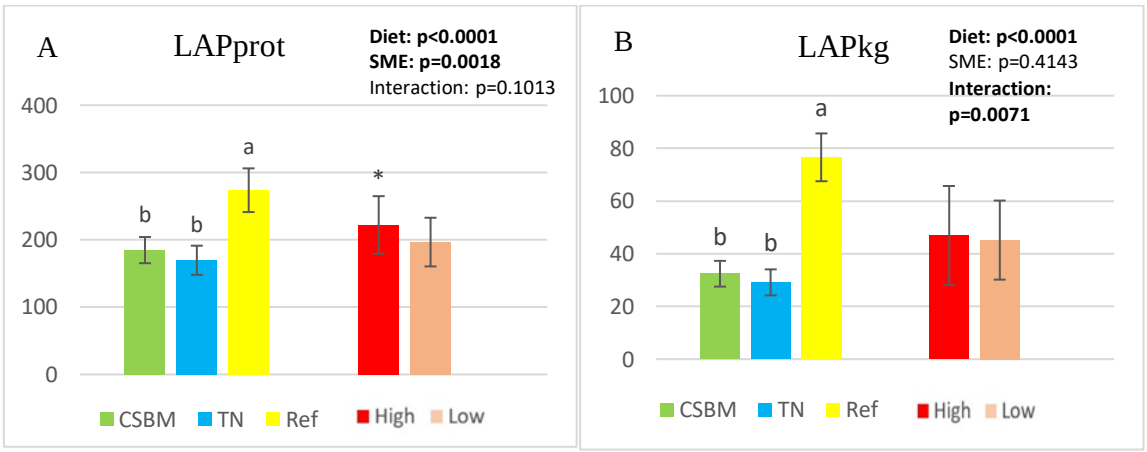


Figure 33A-C. Leucine aminopeptidase (LAP) activity in the distal intestinal tissue, expressed as per kg body weight. Values are means with standard errors represented by vertical bars. Different letters denote diet groups that are significantly different. Asterisk indicates significance differences between high and low SME.

5.7 Plasma cholesterol and other variables

The statistical analysis for plasma cholesterol (CHOL) demonstrated that there was not significant differences between fish fed TN and CSBM diets, the level of SME did not show significant effects, and the interaction between the SME and the diets was also insignificant (Figure 34 A-B).

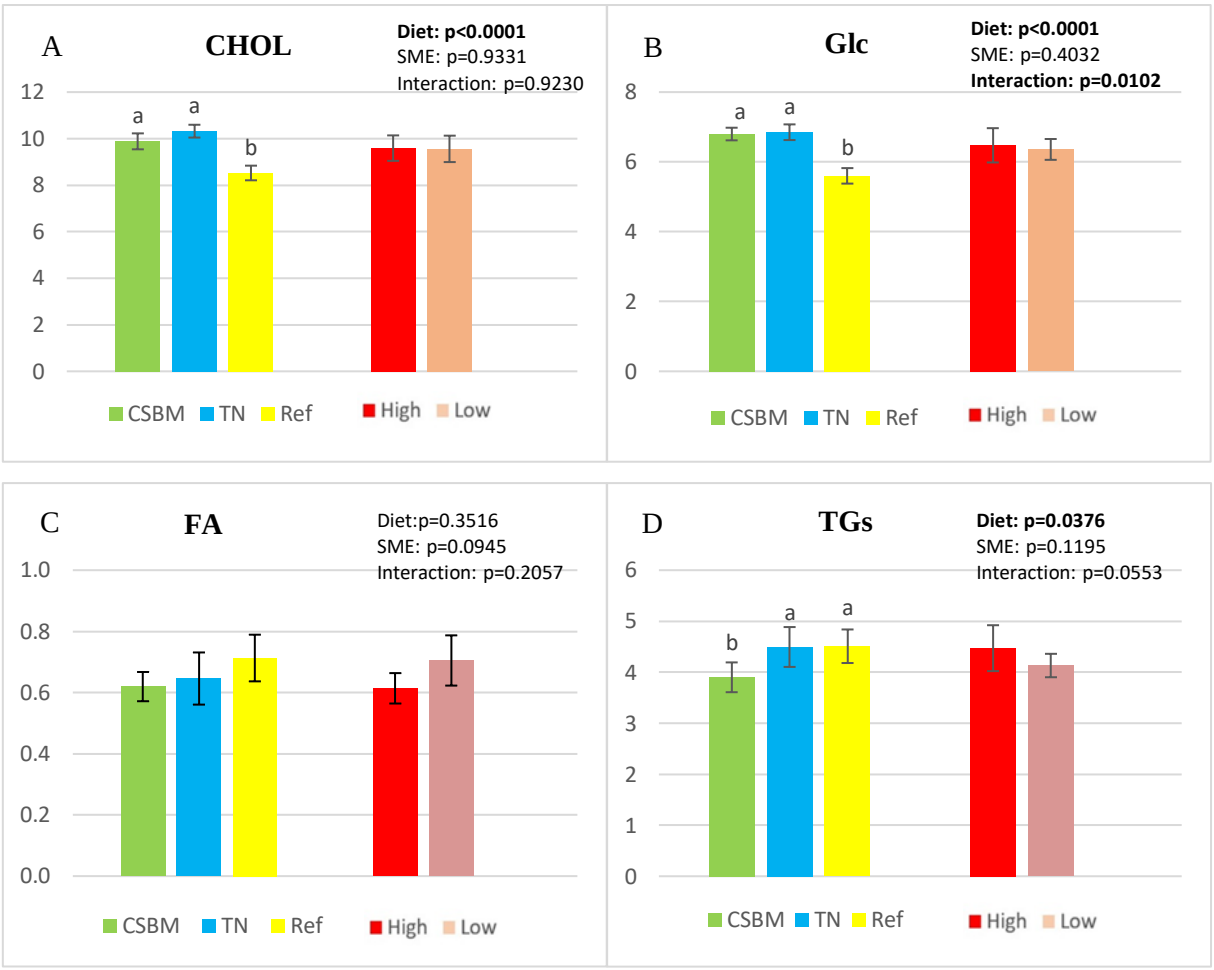
For glucose (Glc), significant interaction between the main factors was apparent, i.e the results for the basal diets depended on the SME level. The significant interaction was due to a difference in rank of the basal diets between low and high SME, and a larger difference between the basal diets at low than high SME. The results for TN and CSBM did not differ significantly,

neither at low nor high SME. The two SME levels did not differ significantly. The results for the Ref diet were significantly lower than for the TN and CSBM at both SME levels.

For fatty acid (FA), there was no significant differences between basal diets and SME levels, and the interaction between the two was also insignificant (Figure 34-C). Triglycerides (TGs) level showed a significant difference between TN and CSBM. Fish fed TN diet compared to SCBM had high value. TGs level of fish fed TN and fish fed Ref diet were at same level. The level of SME did not cause significant effects, and the interaction between the two was also insignificant for triglycerides (Figure 34-D).

Comparison of levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) did not show any significance differences among basal diets. The variation of SME level caused significant difference with the high value for high SME. The interaction between the basal diets and the SME level was insignificant (Figure 34-E-F).

For sodium (Na) significant interaction between the main factors was apparent. The significant interaction was due to the different rank for the basal diets at low and high SME and smaller difference between the basal diet at low than high SME. At low SME the TN and CSBM did not show significant difference, whereas at high SME the TN showed significantly lower value than CSBM. Between the two SME levels there was no clear difference. The Ref diet did not differ significantly from the other basal diets, either at low of high SME (Figure 34- G).


A
CHOL
Diet: p<0.0001
SME: p=0.9331
Interaction: p=0.9230
12
10
8
6
4
2
0
a
a
b
CSBM TN Ref High Low
B
Glc
Diet: p<0.0001
SME: p=0.4032
Interaction: p=0.0102
8
6
4
2
0
a
a
b
CSBM TN Ref High Low
C
FA
Diet:p=0.3516
SME: p=0.0945
Interaction: p=0.2057
1.0
0.8
0.6
0.4
0.2
0.0
CSBM TN Ref High Low
D
TGs
Diet: p=0.0376
SME: p=0.1195
Interaction: p=0.0553
6
5
4
3
2
1
0
b
a
a
CSBM TN Ref High Low

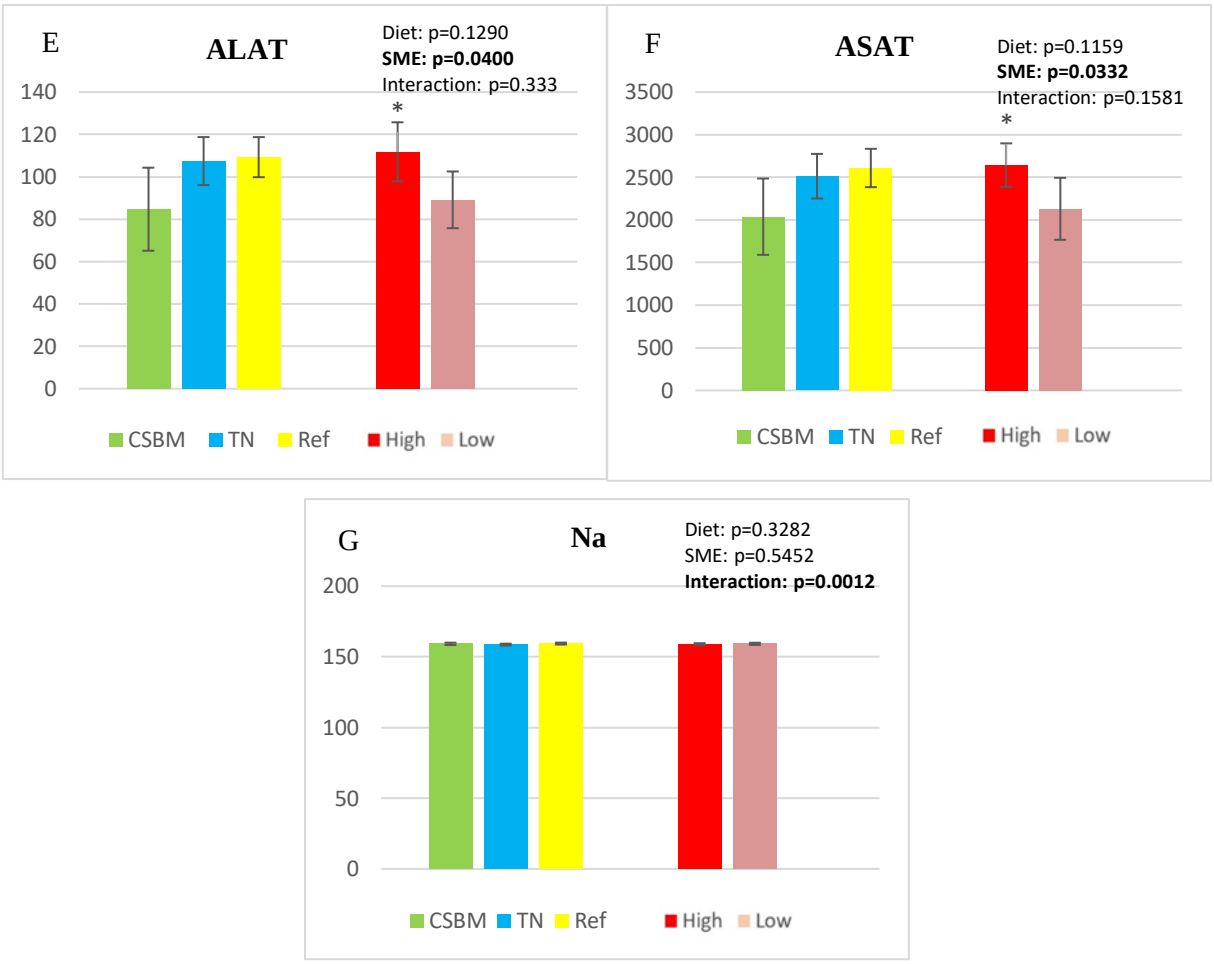


Figure 34A-G. Plasma CHOL, Glc, FA, TGs, ALAT, ASAT and Na levels in diets and two SME level. Values are means with standard errors represented by vertical bars. Different letters denote diet groups that are significantly different. Asterisk indicates significant differences between high and low SME.

5.8 Morphology of the distal intestine intermediate sampling

Figure 35 presents the impact profiles of the basal diet groups on selected DI morphological features. Histological changes in mucosal fold height, width, and cellularity of both the submucosa and lamina propria were all observed with the severity of the changes ranging from mild to marked.

A mild reduction in mucosal fold height was observed in 8 sections out of 18 from fish fed the TN diet compared to 4 out 17 of DI sections from fish fed the CSBM diet. Reduction in enterocyte supranuclear vacuolization was observed in all fish fed on diets with TN and CSBM and in none of the fish fed Ref diet. Mild widening and infiltration by inflammatory cells in the submucosa was observed in 16 out of 18 of the fish fed Diet TN and in 11 of the 17 fish fed on CSBM diet. One of the fish fed the CSBM diet showed moderate widening and increased

cellularity in the submucosa. The same pattern was observed for the widening and infiltration of the lamina propria but with fewer fish showing changes (Figure 35).

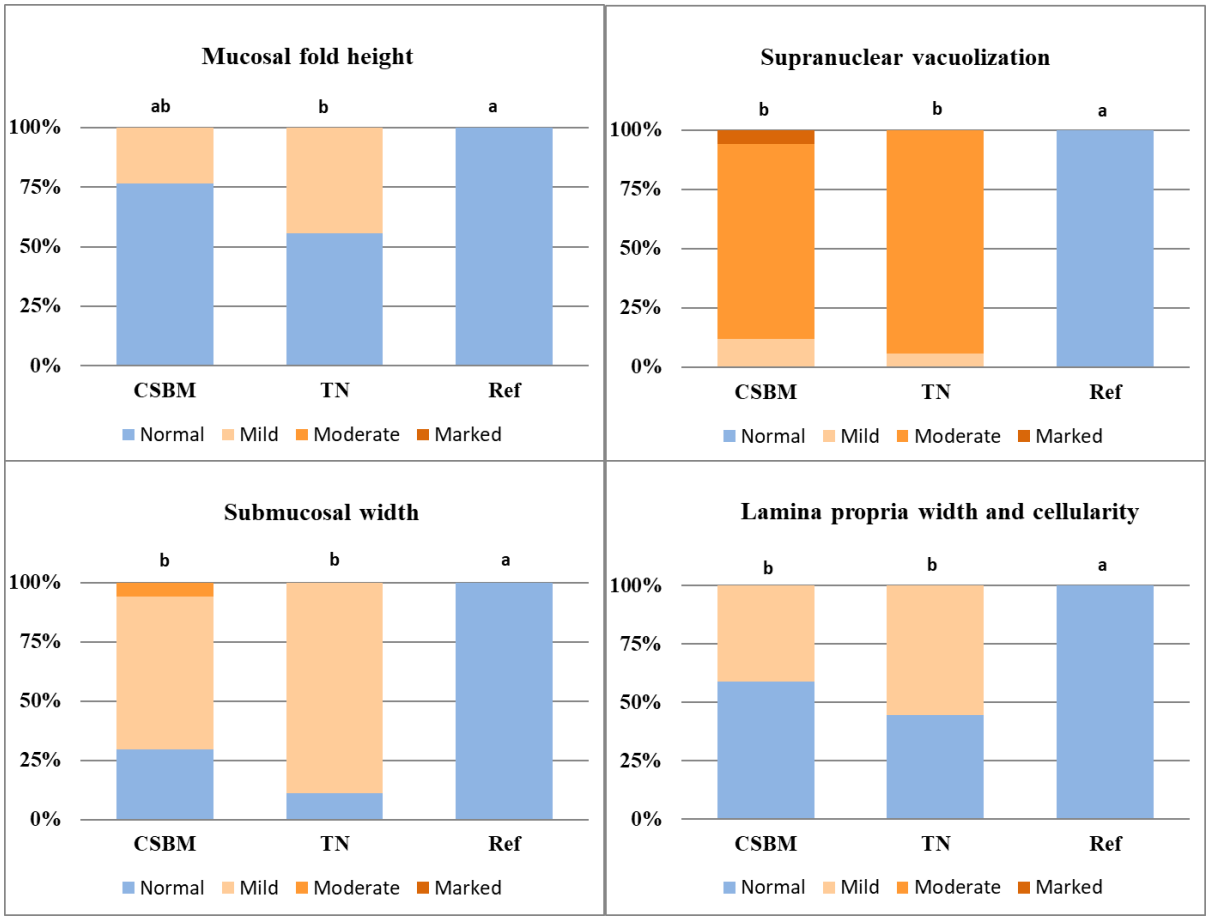


Figure 35. Proportions of sampled individuals that were scored as "normal", "mild", "moderate", "marked", or "severe" for selected morphological features of the distal intestine during the histological evaluation: The x-axis represents the diet groups CSBM, TN and Ref. Different letters denote diet groups that are significantly different.

5.9 Morphology of the distal intestine at final sampling

Figure 36 illustrates the distribution of DI morphological changes induced by the different trial diets. Diets TN and CSBM induced similar DI morphological changes regardless of the heat treatment. Both diet groups induced mostly mild inflammatory changes in more than 60% of the evaluated fish, with the exception of Diet TN with low SME that induced changes in 50% of the sections. One fish from the Diet TN in the high SME level showed moderate submucosal inflammatory changes. DI sections from fish fed on Diet Ref were predominantly normal and healthy in appearance.

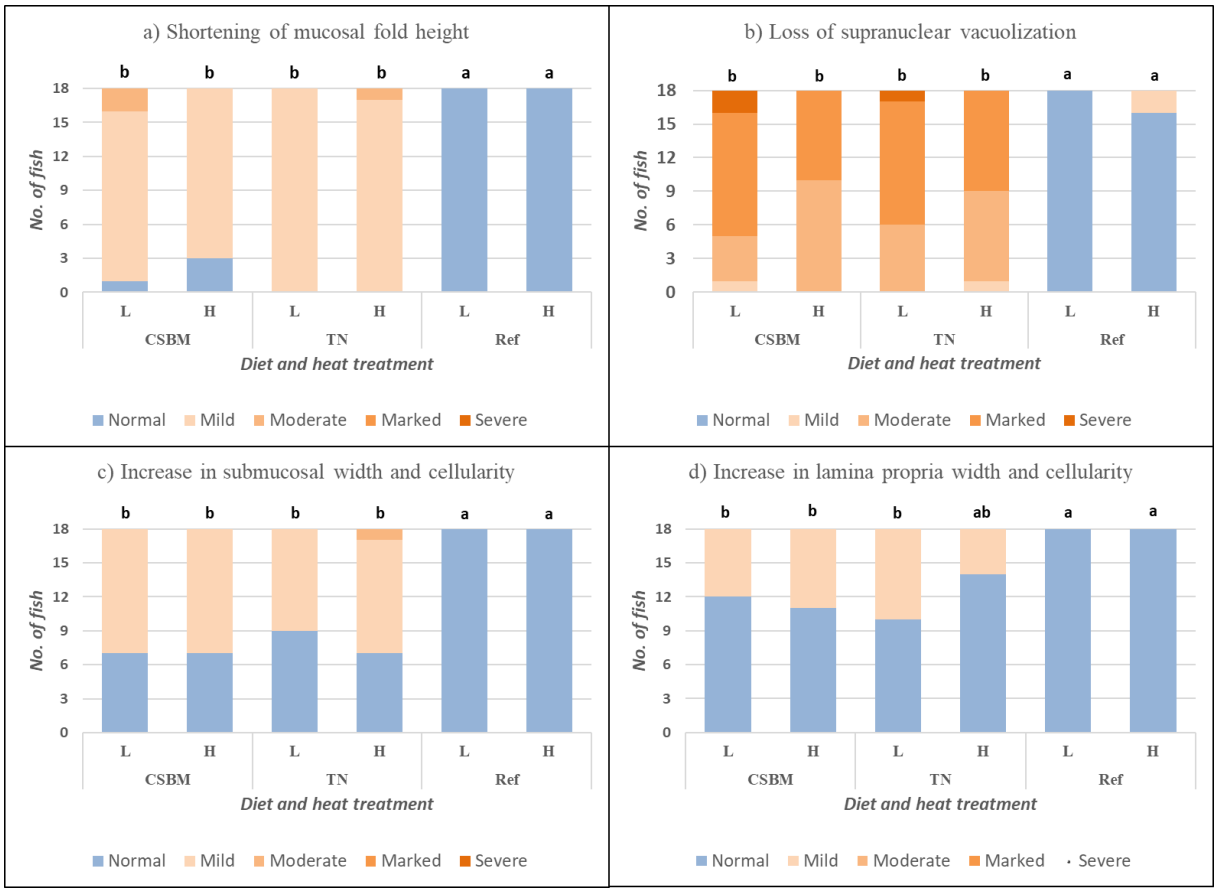


Figure 36. Proportions of sampled individuals that were scored as "normal", "mild", "moderate", "marked", or "severe" for selected morphological features of the distal intestine during the histological evaluation, the x-axis represents the diet groups CSBM, TN and Ref. Chart columns not sharing similar letters on top are statistically distinct according to methods and criteria in the 'Statistics' section above

5.10 Gene expression

Real-time quantitative PCR analyses were limited to the DI tissue, where possible effects of feed on gut immune function would be expected. The relative mRNA expression analyses of Interleukin1β (*Il1β*), Interleukin 17a (*Il17a*), Interferon γ (*ifnγ*), Transforming growth factor β (*tgfβ*), Myeloid differentiation primary response gene 88 (*myd88*), Cluster of differentiation 3γδ (*CD3 γδ*), Matrix metallopeptidase 13 (*mmp13*), Proliferating cell nuclear antigen (*pcna*), Fatty acid binding protein 2b (*fabp 2b*), Aquaporin 8ab (*aqp8ab*), Heat shock protein 70 (*hsp70*), catalase (*cat*), and CuZn-superoxide dismutase (*sod1*) in DI are shown in figure 37 A–M, respectively.

Among the immune related gene transcripts, Figure 37 A-F, significant interaction between the main factors was apparent for *Il1β*. The significant interaction was due to the different rank for

the basal diets at low and high SME and smaller difference between the basal diet at low than high SME. The results for TN and CSBM did not differ significantly, neither at low nor high SME. Between the two SME levels there was clear difference, low SME had lower value for CSBM whereas for TN was vice versa and also for Ref diet low SME had higher value. The results for the Ref diet were significantly lower than for the TN and CSBM diet at high SME level (Figure 37- A).

For *Il17a* significant interaction between the main factors was apparent. The significant interaction was due to the different rank for the basal diets at low and high SME and larger difference between the basal diet at low than high SME. The results for TN and CSBM differed significantly at low SME, but not the high SME. Between the two SME levels there was no clear difference. The result for the Ref diet was significantly lower than TN diet at low SME level but not the high SME (Figure 37- B).

For *ifnγ* significant interaction between the main factors was apparent. The significant interaction was due to the different rank for the basal diets at low and high SME and smaller difference between the basal diet at low than high SME. The results for TN and CSBM did not differ significantly, neither at low nor high SME. Between the two SME levels there was no clear difference. The results for the Ref diet were significantly higher than for the TN and CSBM at high SME level but not the low SME (Figure 37- C).

There was a significant up-regulation in *tgfβ* gene for TN fed fish compared to the CSBM. The level of SME did not show significant differences in mRNA expression level of these genes. The interaction between the basal diets and the SME level was insignificant (Figure 37- D).

Myd88 mRNA expression levels did not show significant differences between fish fed TN and CSBM diets. The level of SME did not cause significant effects. The interaction between the basal diets and the SME level was insignificant (Figure 37- E).

The difference in expression level of *CD3 γδ* for fish fed the TN and CSBM diets was significant. For fish fed TN diet had more value than fish fed CSBM diet. Neither the SME level nor interaction between basal diets and the SME level caused significant differences (Figure 37- F).

The mRNA expression level of *mmp13* was up-regulated significantly in fish fed TN diet versus the CSBM diet. Neither the SME level nor interaction between basal diets and the SME level caused significant differences, (Figure. 37-G).

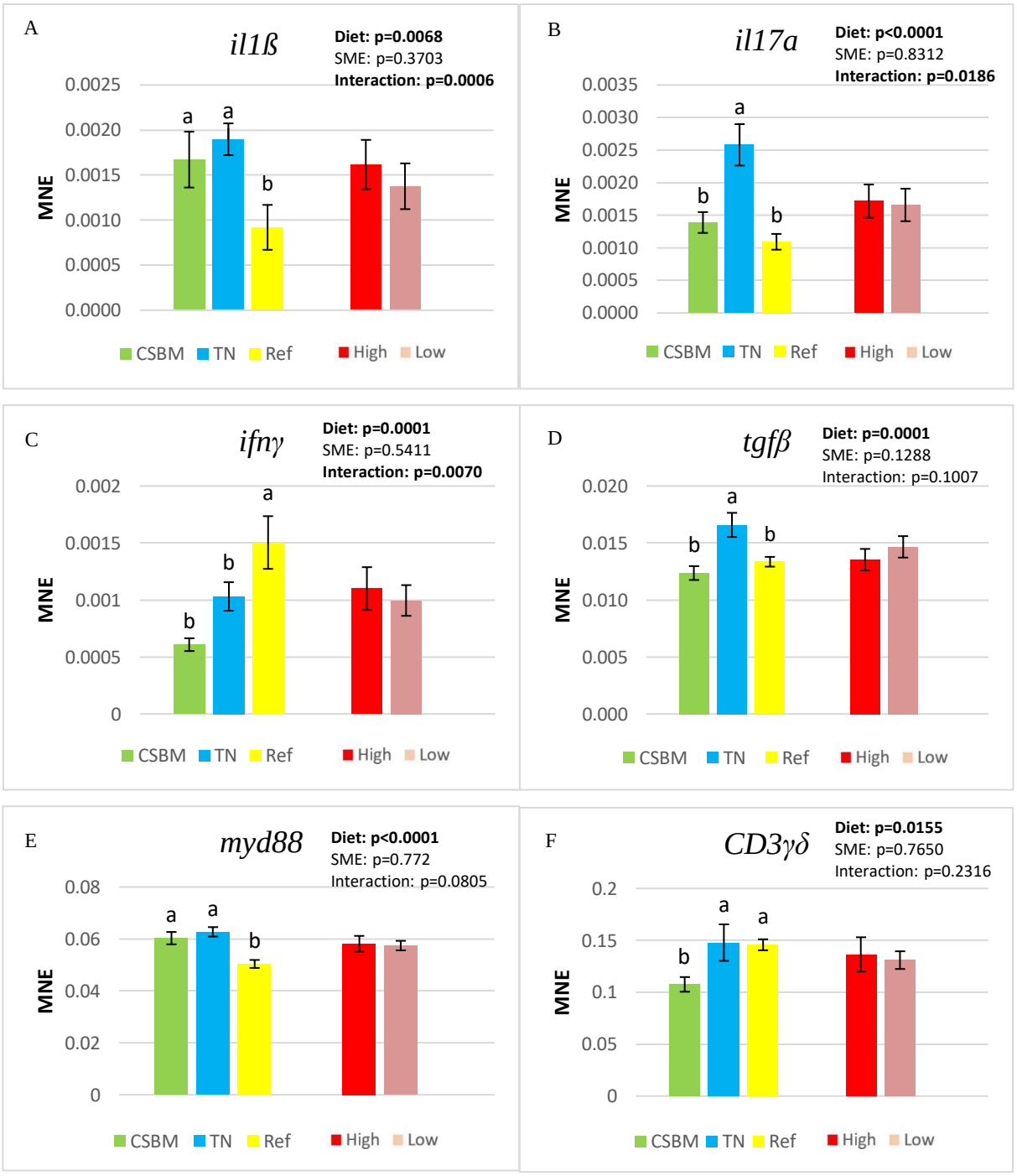
The mRNA expression of *pcna* did not show significant differences between fish fed TN and CSBM diets. Neither the SME level nor interaction between basal diets and the SME level caused significant differences for *pcna* expression, (Figure. 37-H).

The mRNA expression of *fabp2* gene did not show significant effect on fish fed TN and CSBM diets. The SME level did not cause significant effects, and the interaction between the basal diets and the SME level was also insignificant (Figure 37- I).

The mRNA expression of *aqp8ab* gene did not show significant differences between fish fed TN and CSBM diets. The SME level did not cause significant effects, and the interaction between the basal diets and the SME level was also insignificant (Figure,37-J).

For *sod1* significant interaction between the main factors was apparent. The significant interaction was due to a difference in rank of the basal diets between low and high SME, and a larger difference between the basal diets at low than high SME. The results for TN and CSBM did not differ significantly, neither at low nor high SME. The two SME levels did not differ significantly. The results for the Ref diet did not differ significantly compared to the TN and CSBM diets at both SME levels (Figure. 31-K).

The mRNA expression of *cat* and *hsp70* did not show significant effect on fish fed TN and CSBM diets, level of SME did not cause significant effects, and the interaction between the two was also insignificant (Figure. 37-L-M).


A
il1ß
Diet: p=0.0068
SME: p=0.3703
Interaction: p=0.0006
MNE
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
a
a
b
CSBM TN Ref High Low
B
il17a
Diet: p<0.0001
SME: p=0.8312
Interaction: p=0.0186
MNE
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
b
a
b
CSBM TN Ref High Low
C
ifnγ
Diet: p=0.0001
SME: p=0.5411
Interaction: p=0.0070
MNE
0.002
0.0015
0.001
0.0005
0
b
b
a
CSBM TN Ref High Low
D
tgfß
Diet: p=0.0001
SME: p=0.1288
Interaction: p=0.1007
MNE
0.020
0.015
0.010
0.005
0.000
b
a
b
CSBM TN Ref High Low
E
myd88
Diet: p<0.0001
SME: p=0.772
Interaction: p=0.0805
MNE
0.08
0.06
0.04
0.02
0
a
a
b
CSBM TN Ref High Low
F
CD3γδ
Diet: p=0.0155
SME: p=0.7650
Interaction: p=0.2316
MNE
0.2
0.15
0.1
0.05
0
b
a
a
CSBM TN Ref High Low

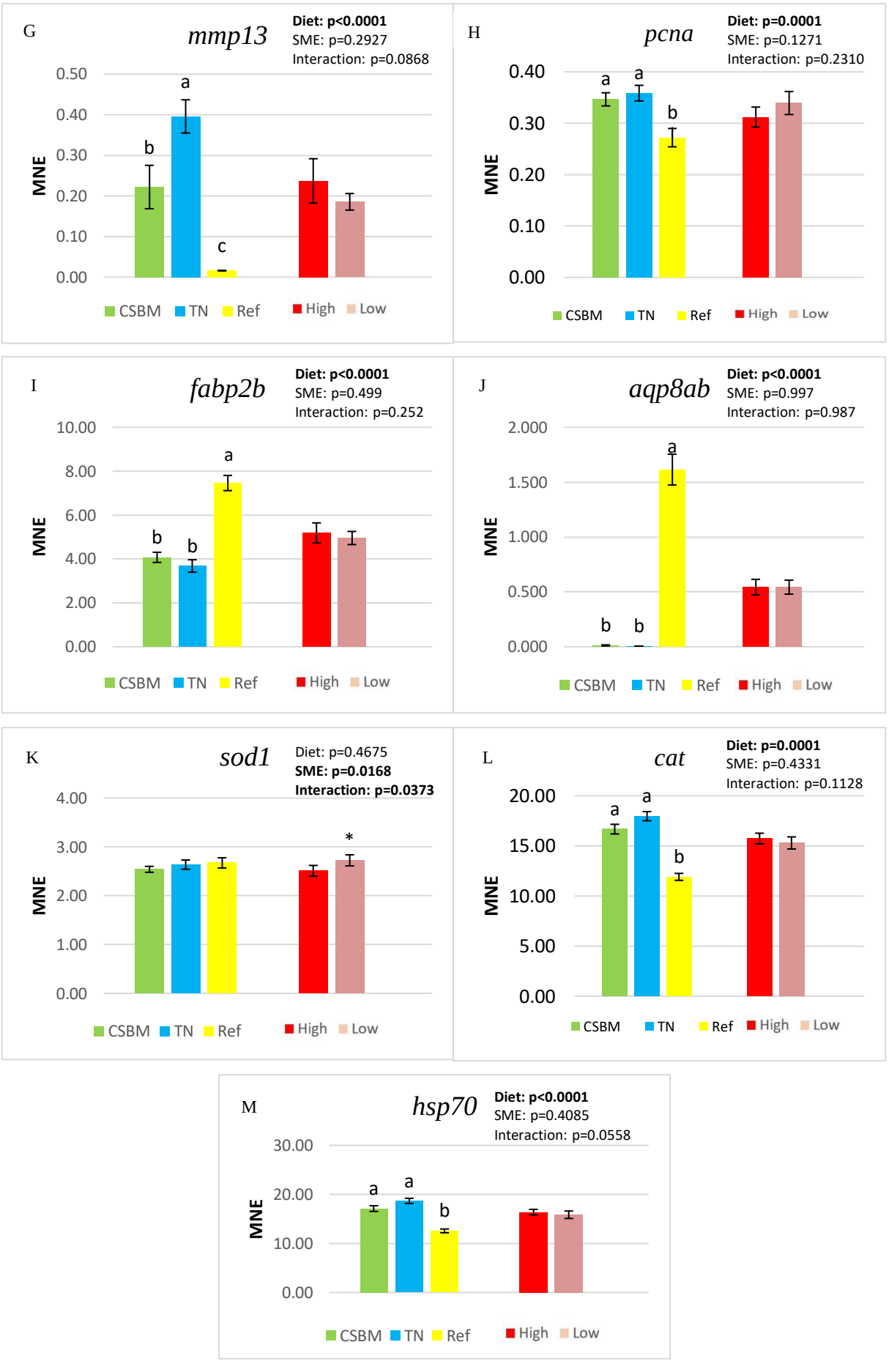

G
mmp13
Diet: p<0.0001
SME: p=0.2927
Interaction: p=0.0868
MNE
0.50
0.40
0.30
0.20
0.10
0.00
a
b
c
CSBM TN Ref High Low
H
pcna
Diet: p=0.0001
SME: p=0.1271
Interaction: p=0.2310
MNE
0.40
0.30
0.20
0.10
0.00
a a b
CSBM TN Ref High Low
I
fabp2b
Diet: p<0.0001
SME: p=0.499
Interaction: p=0.252
MNE
10.00
8.00
6.00
4.00
2.00
0.00
b b a
CSBM TN Ref High Low
J
aqp8ab
Diet: p<0.0001
SME: p=0.997
Interaction: p=0.987
MNE
2.000
1.500
1.000
0.500
0.000
b b a
CSBM TN Ref High Low
K
sod1
Diet: p=0.4675
SME: p=0.0168
Interaction: p=0.0373
MNE
4.00
3.00
2.00
1.00
0.00
*
CSBM TN Ref High Low
L
cat
Diet: p=0.0001
SME: p=0.4331
Interaction: p=0.1128
MNE
20.00
15.00
10.00
5.00
0.00
a a b
CSBM TN Ref High Low
M
hsp70
Diet: p<0.0001
SME: p=0.4085
Interaction: p=0.0558
MNE
30.00
20.00
10.00
0.00
a a b
CSBM TN Ref High Low

Figure 37A-M. Distal intestine expression of selected genes. Data are mean normalized expression(MNE) levels of n=9 fish per diet. P-values for the two-way ANOVA are given. Bars are standard error. Significant differences between diets are denoted with letters. Asterisk indicates significant differences between high and low SME.

6 Discussion of key results

The main aim of our study was to evaluate possible beneficial effects on nutritional value and gut health of removal of the proteinaceous antinutrients from a conventional soybean meal. An additional aim was to find possible modulating effects of specific mechanical energy (SME) or heat treatment during processing of the diets.

6.1 Effects of removal of the antinutrients:

Briefly summarized the results showed the following regarding comparison between TN and CSBM: removal of the proteinaceous components did not significantly alter feed intake, growth, condition factor, relative carcass and organ weight, apparent digestibility of protein most essential amino acids, nor body composition. For apparent digestibility of lipid and energy, a significantly lower value was observed for TN compared to CSBM. Also, protein retention was lower for TN than CSBM. Moreover, there was a significantly lower chyme bile salt concentration in the proximal sections of the intestine of fish fed TN compared to CSBM. No difference was seen for plasma biomarkers, except for plasma triglycerides, which was higher for TN. No significant difference was observed for ALAT and ASAT. Regarding the observed gut health conditions, the picture was the same no significant difference, regarding several of the indicators of gut inflammation, i.e. distal intestinal morphology, activity of brush border LAP. Among the gene expression results, a significantly higher value was observed in fish fed TN for *il17* and the effect was more pronounced in fish fed the low SME diets. The TN fed fish also showed higher expression of *tgfβ*, CD3γδ and *mmp13*, independent of SME, but the effects were minor. For the other investigated genes, i.e. *ifn γ, myd88, pcna, fabp2b, aqp8ab, sod1, cat, hsp70*, no significant difference was seen between TN and CSBM. All in all, the results are showing typical signs of SBMIE for both TN and CSBM with no clear difference between the two.

The lack of effect of removal of the antinutritional proteins on many of the biomarkers observed in the present study, i.e. lack of difference between TN and CSBM, can be explained by the fact that the heat treatment involved in typical extrusion processing used for fish feed may be

sufficient to inactivate most of the heat labile ANFs in the CSBM i.e. the trypsin inhibitors, lectins and allergens, all highly active in unheated soybean meal (Romarheim et al., 2005). The results of the present study indicate that extrusion processing has a potential of being the sole heat treatment to achieve acceptable feed intake and protein digestibility when unheated soybean meal accounts for a considerable part of the dietary protein.

The biomarkers which showed significant difference between TN and CSBM were few and the differences in general minor. The observation that digestibility of lipid and energy was low for TN compared to CSBM may be related to the low bile salt concentration in digesta observed for the TN fed fish. Chyme bile salts are necessary for efficient absorption of lipids and a low concentration may reduce rate of lipid digestion and absorption. The lower bile salt concentration may also explain the lower digestibility of crude protein, as bile salts are known to stabilize proteolytic enzymes and inactivate their autolysis in the digestive tract as reviewed by Lilleeng et al., (2007).

Several studies have shown that dietary SBM can lowers the lipid digestibility in salmonids (Krogdahl et al., 2003, Krogdahl et al., 1994). Bile salts have a central role in the digestion and uptake of lipids, and dietary SBM may reduce the bile acid level (Romarheim et al., 2008). In the present experiment, bile salt levels also differed between the fish fed the TN and CSBM diets, with lower values in the TN fed fish, which can be explained by plant feed ingredients, with their contents of fibers, phytosterols, phytoestrogens, and saponins all affecting cholesterol and bile salt absorption (Krogdahl et al., 2015, Krogdahl et al., 2010). In fish, bile acid absorption and metabolism have been suggested to be a cause of the frequently observed reductions in lipid digestibility of plant ingredients, in particular standard SBM (Romarheim et al., 2008).

Since proximal and mid regions of the intestine together have been reported to be responsible for more than 90% of the total apparent absorptive capacity for lipids and amino acids (Krogdahl et al., 1994), the reduction of bile acid concentration can have substantial implications for the intestinal digestion and ultimate absorption of these nutrients. The mechanisms behind the differences in chyme bile salt concentration is not clear but may be due to the involvement of antinutritional factors such as saponins (Krogdahl et al., 2015).

Lower protein retention for fish fed TN diet compared to fish fed CSBM diet can be explain by lower crude lipid digestibility and bile salt concentration was fish fed TN diet. There is less energy available for fish. And also, lower bile salt concentration for fish fed TN diet indicates

that the sterol metabolism (CHOL) is changed and that may be burden for the fish, so the protein growth is less.

The up-regulation of the expression level of IL-17 A in fish fed TN diet in comparison with fish fed CSBM is in line with the observed increase in mRNA expression of TGFβ suggests that the SBMIE in fish fed TN diet were more severe rather than less in fish fed CSBM diet.

6.2 Effects of level of heat treatment:

Briefly summarized the results showed the following regarding comparison between high and low SME: significant effects of heat treatment were observed for apparent digestibility of protein, lipid and energy, which was higher for the high SME than the low SME. Significant effects were also seen for CF, i.e. a lower value for the high SME, and for specific activity of distal intestinal LAP (U/mg protein), i.e. a higher value for high SME. Regarding plasma biomarkers, high SME caused lower glucose values, but only in fish fed the TN diet. For both ALAT and ASAT, high SME caused significantly higher values than low SME. The gene expression analyses showed significantly lower sod1 for high SME, but only for fish fed the SPC diet. For the other observed gene expressions, no significant SME effect was found.

Referring to the earlier discussion, finding optimal SME level in a production line is complicated. Any changes during the process affect the SME level. For example, a sharp rise in torque, die pressure and die temperature may have an effect on SME level. The improved digestibility by high SME can be explained by increased protein solubility due to denaturation and structure unfolding of the protein molecules (Sørensen, 2003). Such structural changes leave the protein more accessible to digestive protease (Sørensen, 2003) (see table 3). This is in line with the observed increase in the content of total AA digestibility with increasing SME (see table 9). The literature, however, reveal gap in the knowledge of how and to which extent the nutritional value of fish feed is affected by various extrusion conditions. Few studies have been published aiming at studying effects of extrusion processing on high-low salmonid feed.

Moreover, the higher nutrient digestibilities of protein, lipid, and energy could occur through physical disruption of cell walls and cleavage of non-starch polysaccharides (NSP) into smaller fragments due to the effect of extrusion, thereby substantially reduce their ANF effects (Meng et al., 2005). While temperature is one important process parameter, the amount of shear forces developed during extrusion should also be considered. It has been shown that development of

a certain amount of shear forces can increase the in vitro accessibility of SBM proteins to hydrolytic enzymes (Morken, 2011). These factors vary in practical significance, and there are a number of uncertainties with respect to the effects of SME level on fish health or performance (Phillips and Finley, 1989, Morken, 2011).

Variation in CF is most often related to a variation in abdominal lipid accumulation, which may indicate a corresponding variation in partitioning of dietary nutrients into muscle and lipid growth. In this experiment, the lower CF observed for the high SME may be explained by the higher protein and amino acid digestibility which makes more substrate available for protein growth. Protein growth requires energy, and less energy is available for lipid accumulation in the abdomen (Herbinger et al., 1991).

In this study, elevated ASAT and ALAT in fish fed high SME diets indicate that the heating process might have developed components which represented a challenge to the liver and possibly also other organs. In general, these enzymes are seen as a reflection of liver health. It reflects liver cell turn-over, where an increase in ALAT/ASAT values reflects increased cell turn-over, which is the case in inflammation (Singh et al., 2012). It is difficult to draw conclusion on whether such high SME have negatively affected the biomarkers of liver health.

A higher mRNA expression *sod1* in fish fed low SME may be explain by an influence of the amount of free superoxide radicals. Temperature and moisture both have been shown to cause extensive changes in the number of free radicals. A balance between free radicals and antioxidants is necessary for proper physiological function. If free radicals overwhelm the body's ability to regulate them, a condition known as oxidative stress ensues. Free radicals thus adversely alter lipids, proteins, and DNA and trigger a number of diseases (Lobo et al., 2010).

6.3 Comparison between Ref diet (SPC) and the two soybean meal diets (SBMs)

Briefly summarized the results showed the following regarding comparison between SBMs and the SPC: feed intake, growth, condition factor, RCarc did not differ significantly. Fish fed SBMs differed clearly from fish fed the SPC regarding body dry matter, lipid and energy digestibility, as well as liver index, which all were higher in fish fed the SPC. The SBMs showed lower values for DISI, CP, CL, CE, protein retention, and protein and amino acid digestibility. There was a significantly lower chyme bile salt concentration in all intestine sections of the intestine of fish fed SBMs compared to fish fed SPC. No difference was seen for plasma

biomarkers, except for CHOL and Glc, which was higher for SBMs diets compared to SPC diet. No significant difference was observed for ALAT and ASAT. Regarding the observed gut health conditions, there was a significant difference between SBMs diets and SPC diet. Distal intestinal morphology from fish fed on SBMs showed clear signs of soybean meal-induced enteritis in DI compared to healthy appearance in all SPC fed fish sections. Fish fed the SBMs diets showed significantly lower values than those fed the SPC for both LAP activities.

The gene expression analyses showed significant difference between the SBMs and SPC fed fish. A significantly higher value was observed for mRNA expression level in fish fed SBMs for *il1β, il17, myd88, mmp13, pcna, cat* and *hsp17* compared to fish fed SPC. The SBMs fed fish also showed lower expression of *ifnγ, fabp2b* and *aqp8ab* and *mmp13*. For the other investigated genes, i.e., *tgfβ* and *CD3γδ* no significant difference was seen between SBMs and SPC.

The major differences between nutrient composition of SBM and SPC is related to the several parameters like: crude protein level of SPC (65-67%) is higher than SBM. The high protein level of SPC makes it suitable for nutrient dense aquafeeds for marine fish and for fry and fingerling feeds of both freshwater and marine fish. SPC has protein and energy digestibility much higher than soybean meal (protein digestibility of 96-97% has been measured in trout) (Friedman et al., 2001). SPC has much lower levels of ANF's than soybean meal such as (trypsin Inhibitor, saponins, lectin, oligosaccharides). The soy antigens glycinin and ß-conglycinin are denatured during the manufacturing of SPC. Indigestible and harmful carbohydrates (oligosaccharides, sucrose, raffinose and stachyose) are extracted during SPC processing.

Lower protein digestibility, as observed for SBMs compared to SPC and at the same time higher lipid digestibility result in a lower protein to energy ratio in the supply of nutrients to the fish body which would be expected to cause high lipid accumulation. This may explain the higer dry matter content and the numerically higher lipid content of the SBMs fed fish (Nordgarden et al., 2003).

Regarding the observed gut health conditions, fish fed the SBMs diet showed lower relative weight of the distal intestine. Decreased weight of the DI, as seen for fish fed SBMs diet, is also typically observed in concert with intestinal inflammation, apparently due to loss of intestinal mucosa (Nordrum et al., 2000).Distal intestinal morphology showed clear signs of enteritis such as the disappearance of the SNV in the enterocytes, a strong decrease of microvilli height, a

swelling of the lamina propria and sub epithelial mucosa, an increase in the number of goblet cells, a strong invasion of the eosinophilic granulocytes, a loss of the mucosal fold architecture and finally a total tissue disruption, whereas all fish fed SPC showed healthy appearance.

The decrease in the activity of LAP enzymes in the DI wall indicates a severe reduction in digestive capacity of this region during SBMs exposure, most likely due to the development of the SBM-induced inflammation with concomitant dysfunction of the tissue. SBM causes shortening of microvilli of the brush border membrane in Atlantic salmon (Van den Ingh et al., 1991). Concomitantly, the activity of digestive enzymes in the epithelial cells' brush border membrane (e.g. LAP) of the salmon distal intestine are significantly and dose-dependently reduced by including soybean meal in the diet (Krogdahl et al., 1994, Krogdahl et al., 2003). Reduction in LAP activity may be one of several factors underlying the negative effect of dietary SBM inclusion on protein digestibility observed in many studies (Bakke-McKellep et al., 2007, Krogdahl et al., 2003).

In the present study, histology of the distal intestine revealed progression of typical characteristics of SBMIE on the feeding period, which was also reflected in the transcriptomic changes that can be linked to inflammatory changes (Sahlmann et al., 2013). Diets SBMs induced mostly mild soybean meal-induced enteritis in the evaluated DI sections regardless of the variation in the SME level. Fish fed SPC diet had normal healthy DI mucosa. This is in line with previous studies reporting in close relationship in SBMIE and saponins have been implicated as the causal agent (Krogdahl et al., 2015). Therefore, the current study results substantiate previous suggestions that the induction of inflammation by soya saponins is potentiated by other ANFs, antigens, or other components in SBM (Krogdahl et al., 2015). All the following biomarkers are just indication of the difference in inflammation.

The increase in the expression levels of IL-17a receptor as well as the cytokines IL-1β and TGFβ in the present study in fish fed SBMs diet compared to fish fed SPC, supports the involvement of putative T_H17 T cell by the increase in CD4a and CD8b expression observed in previous studies in SBM-fed salmon (Bakke-McKellep et al., 2007).

The results of mRNA expression levels of *ifnγ* in this study did not correlated well with the development of the histopathological signs of inflammation. Similar studies done in salmon also showed upregulated expression of *infγ* in distal intestine after SBM treatment (Martin et al., 2017).

Up-regulation of *myd88* in fish fed SBMs compared to fish fed SPC diet in the current study, which supports the activation of lamina propria dendritic cells in the induction of intestinal inflammatory responses such as pathogenic or commensal bacteria or viruses.

In this study, *mmp13* up-regulation in fish fed SBMs compared to fish fed SPC is in line with the several lines of evidence show that MMPs can either promote or repress inflammation by the direct proteolytic processing of inflammatory cytokines and chemokines (McQuibban et al., 2001). These results suggest that the functional loss of the epithelial barrier and barrier extra cellular matrix was affected by degradation and remodeling from SBM feeding which may result from the inflammation.

In the current study, the observed increased level of *pcna* expression for fish fed SBMs diets compared to fish fed SPC diet, which supports that fish with SBM-induced enteritis suffer from an excessive loss of epithelial cells in the distal intestine and the increased proliferation indicated by the intestinal cellular localization of proliferating cell nuclear antigen (PCNA) expression in Atlantic salmon cannot compensate for the rapid loss of IECs in the inflamed state.

In the current work, the lowest expression level of *fabp2* was found in the fish fed SBMs diets compared to fish fed SPC diet. The results are also in agreement with previous studies in that the rapid reduction in *fabp2* gene expression and *fabp2* protein level during the development of SBM-induced DI inflammation is consistent with the tissue malfunction that occurs. Reduced *fabp2* levels could explain the decreased lipid ADs typically observed in fish fed SBM.

Our results regarding low *aqp8* expression in fish fed SBMs compared to fish fed SPC diet is in line with the down-regulation of *aqp8* was associated with decreases in the absorption of water, suggesting a possible role of aquaporins in the pathogenesis of diarrhea accompanying intestinal inflammatory disease (Krogdahl et al., 2015).

No significant difference in *sod1* mRNA levels was seen between the SBMs diets and SPC diet in this experiment which may supports the conclusion that environmental factors such as increase or decrease in water temperature and oxygen level of water were same for all of fish.

The increased expression of *cat* gene was found in the fish fed SBMs diets compared to fish fed SPC diet in this study may indicate that the SBMIE induced stress in fish fed TN and CSBM.

In the current work, the highest expression level of *hsp70* was found in the fish fed SBMs diets compared to fish fed SPC diet, which supports the conclusion that the SBMIE involved stress in fish fed SBMs. Increased levels of *hsp70* have been observed in salmon fed soy as a replacement for fishmeal (Bakke-McKellep et al., 2007, Sagstad et al., 2008).

7 Main conclusions

Removal of the proteinaceous antinutrients from a conventional soybean meal did not improve growth performance, feed utilization, or gut and overall health in Atlantic salmon.

The effect of the high SME compares to the low SME did not induced major differences but significant increase in fecal dry matter content and apparent digestibility of protein, lipid and energy by high SME was apparent. Regarding plasma biomarkers, high SME caused lower glucose values. For both ALAT and ASAT, high SME caused significantly higher values than low SME. The gene expression analyses showed significantly lower *sod1* for high SME.

Comparison between SBMs and the SPC did not differ significantly for feed intake, growth, condition factor, RCarc. Fish fed SBMs differed clearly from fish fed the SPC regarding body dry matter, lipid and energy digestibility, as well as liver index, which all were higher in fish fed the SPC. The SBMs showed lower values for DISI, CP, CL, CE, protein retention, and protein and amino acid digestibility. There was a significantly lower chyme bile salt concentration in all intestine sections of the intestine of fish fed SBMs compared to fish fed SPC. No difference was seen for plasma biomarkers, except for CHOL and Glc, which was higher for SBMs diets compared to SPC diet. No significant difference was observed for ALAT and ASAT. Regarding the observed gut health conditions, there was a significant difference between SBMs diets and SPC diet. Distal intestinal morphology from fish fed on SBMs showed clear signs of soybean meal-induced enteritis in DI compared to healthy appearance in all SPC fed fish sections. Fish fed the SBMs diets showed significantly lower values than those fed the SPC for both LAP activities.

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9 Appendix

Basis for interpretation of the interaction between the effects of basal diets and the SME level is presented in Table 11. The interaction was significant in LAP (Leucine aminopeptidase), glucose, sodium, Interleukin1β (Il1β), Interleukin 17a (Il17a), Interferon γ (ifnγ) and superoxide dismutase (sod1).

Table 11. Interaction of basal diets and SME on LAP, plasma variable and gene expression analysis of Atlantic salmon fed the experimental diets.

Means of One-Way ANOVA									
BasalD	**SME**	**LAP Tissue**	**LAPkg**	**Glucose**	**Sodium**	***Il1β***	***Il17a***	***ifnγ***	***Sod 1***
CSBM	Low	7.66^{c}	35.08^{c}	6.91^{a}	158.0^{b}	0.0013^{bc}	0.0011^{bc}	0.0006^{b}	2.53^{ab}
TN	Low	7.28^{c}	28.53^{c}	7.14^{a}	159.1^{ab}	0.0020^{ab}	0.0030^{a}	0.0011^{ab}	2.73^{ab}
Ref	Low	11.51^{b}	71.12^{b}	5.38^{b}	159.8^{ab}	0.0011^{abc}	0.0009^{c}	0.0011^{b}	2.86^{a}
CSBM	High	7.23^{c}	29.74^{c}	6.69^{a}	160.4^{a}	0.0024^{a}	0.0015^{bc}	0.0006^{b}	2.56^{ab}
TN	High	6.88^{c}	30.39^{c}	6.59^{a}	158.2^{b}	0.0018^{abc}	0.0022^{ab}	0.0010^{b}	2.62^{ab}
Ref	High	13.62^{a}	82.90^{a}	5.82^{b}	159.0^{ab}	0.0005^{c}	0.0011^{bc}	0.0017^{a}	2.50^{b}

Abbreviations: LAP (Leucine aminopeptidase), Interleukin1β (*Il1β*), Interleukin 17a (*Il17a*), Interferon γ (*ifnγ*), superoxide dismutase (*sod1*). Mean values with different online letters a,b and c within a column are significantly different ($P < 0.05$).

Table 12. Interaction of basal diets and SME on CP, ADAA of Atlantic salmon fed the experimental diets..

Means of One-WayANOVA								
BasalD	**SME**	**adCP**	**adCys**	**adMet**	**adAsp**	**adThr**	**adSer**	**adGlu**
CSBM	Low	85.6^{b}	73.2^{b}	95.4^{b}	78.2^{b}	85.0^{b}	84.8^{b}	92.5^{d}
CSBM	High	89.9^{a}	81.9^{a}	96.9^{a}	84.0^{a}	89.8^{a}	89.8^{a}	95.5^{ab}
TN	Low	87.1^{b}	75.0^{b}	95.8^{b}	79.9^{b}	85.8^{b}	85.9^{b}	93.5^{cd}
TN	High	90.1^{a}	81.9^{a}	97.0^{a}	84.5^{a}	89.5^{a}	89.8^{a}	95.6^{ab}
Ref	Low	90.3^{a}	84.5^{a}	97.2^{a}	84.4^{a}	91.3^{a}	91.6^{a}	95.7^{a}
Ref	High	90.3^{a}	83.3^{a}	97.1^{a}	83.5^{a}	91.3^{a}	91.3^{a}	94.3^{bc}

Table 13.Interaction of basal diets and SME on ADAA and dTotAA of Atlantic salmon fed the experimental diets.

Means of One-Way ANOVA

BasalD	SME	adAla	adVal	adILE	adLeu	adTyr	adPhe	adHis	adLys	adArg	dTotAA
CSBM	Low	87.9[b]	88.4[c]	87.3[b]	88.6[b]	90.6[b]	87.6[b]	85.7[b]	91.3[b]	90.8[b]	88.4[b]
CSBM	High	92.2[a]	92.8[a]	92.6[a]	93.3[a]	94.8[a]	92.5[a]	90.7[a]	94.3[a]	94.8[a]	92.4[a]
TN	Low	89.0[b]	90.2[b]	88.9[b]	89.9[b]	91.8[a]	89.1[b]	86.9[b]	92.3[b]	92.2[b]	89.6[b]
TN	High	92.1[a]	93.1[a]	92.7[a]	93.3[a]	95.3[a]	92.6[a]	90.6[a]	94.5[a]	95.0[a]	92.5[a]
Ref	Low	92.6[a]	94.2[a]	93.8[a]	93.9[a]	94.8[a]	93.3[a]	92.4[a]	95.6[a]	95.4[a]	93.2[a]
Ref	High	92.5[a]	93.9[a]	92.8[a]	93.0[a]	94.2[a]	93.4[a]	91.5[a]	95.6[a]	95.5[a]	92.6[a]

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