Norwegian College of Fisheries Science
Faculty of Bioscience, Fisheries and Economics

Replacement of fishmeal with alternative proteins in diets for Atlantic salmon (Salmo salar L.):

A study on the microbiota, morphology and function of the intestine

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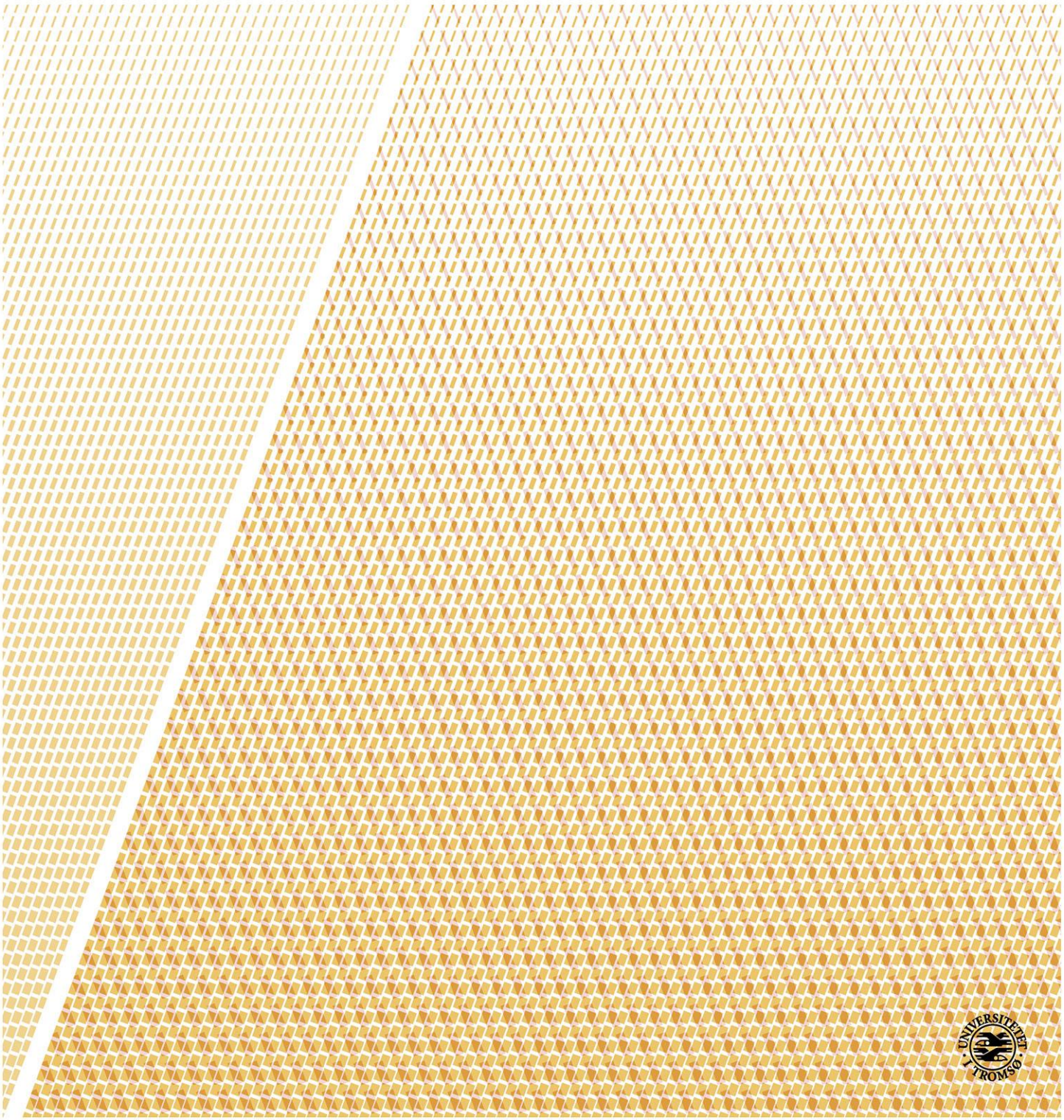
Mali Bjerkhaug Hartviksen

A dissertation for the degree of Philosophiae Doctor – March 2015



Replacement of fishmeal with alternative proteins in diets for Atlantic salmon (Salmo salar L.): A study on the microbiota and morphology of the intestine

Dissertation for the degree of *Doctor Philosophiae*

By

Mali Bjerkhaug Hartviksen

Norwegian College of Fisheries Science, Faculty of Bioscience, Fisheries and Economics

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Mali Bjerkhaug Hartviksen

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Paper I

M. Hartviksen, J.L.G. Vecino, E. Ringø, A.-M. Bakke, S. Wadsworth, Å. Krogdahl, K. Ruohonen, A. Kettunen (2014) Alternative dietary protein sources for Atlantic salmon (*Salmo salar* L.) effect on intestinal microbiota, intestinal and liver histology and growth. *Aquaculture Nutrition*, **20**, 381-398

Paper II

Mali Hartviksen, Anne Marie Bakke, José G. Vecino, Einar Ringø, Åshild Krogdahl (2014) Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (*Salmo salar* L.): Digestive and metabolic investigations. *Fish Physiology and Biochemistry*, **40**, 1621-1637

Paper III

Hartviksen M, Vecino JLG, Kettunen A, Myklebust R, Ruohonen K, Wadsworth S, Ringø E Probiotic and pathogen ex vivo exposure of Atlantic salmon (*Salmo salar* L.) intestine from fish fed four different protein sources. *Journal of Aquaculture Research and Development, submitted.*

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Abbreviations

Selected abbreviations used in the synopsis

Nutritional abbreviations

ANF	Anti-nutritional factor
CFI	Cumulative feed intake
ESF	Extracted sunflower meal
FCR	Feed conversion rate
FeM	Feather meal
FM	Fishmeal
MOS	Manan-oligosaccharides
PBY	Poultry by-product meal
PPC	Pea protein concentrate
SGR	Specific growth rate
SPC	Soy protein concentrate

Morphological and physiological abbreviations

Af	Actin filaments
BBM	Brush border membrane
C	Cytosol
DI	Distal intestine
ECP	Extracellular products
GIT	Gastrointestinal tract
H&E	Haematoxylin and Eosin
HCl	Hydrochloric acid
IROMP	Iron regulated outer membrane protein
LAB	Lactic acid bacteria
LAP	Leucine amino peptidase
Li	Liver
Ma	Macula adherence
MI	Mid intestine
Mv	Microvilli
PI	Pyloric intestine
Tw	Terminal web

Methodologically relevant abbreviations

CFU	Colony forming units
CI	Confidence interval
Ct	Threshold cycle
DGGE	Degenerative gradient gel electrophoresis
GCMS	Gas chromatographic mass spectrometry
LM	Light microscopy
PCR	Polymerase chain reaction
qPCR	quantitative PCR
TEM	Transmission electron microscopy
TVC	Total viable colonies

Summary (English)

Hartviksen, Mali (2014). Replacement of fishmeal with alternative proteins in diets for Atlantic salmon (Salmo salar L.): A study on the microbiota, morphology and function of the intestine

Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, UiT The Arctic University of Norway

To be able to take advantage of the foreseen rapid increase in demand for seafood in a situation with limited production of marine raw materials for use in feed, the aquaculture has explored new alternative feed ingredients. There are however many feed ingredients in commercial use today for which there is very little knowledge available on their effect on gut microbiota, digestive physiology or potential effect on disease susceptibility. This present thesis aims therefore to increase the knowledge on some of these raw materials: pea protein concentrate (PPC), soy protein concentrate (SPC), extracted sunflower (ESF), hydrolysed feather meal (FeM) and poultry by-product (PBY).

The first study of this thesis explored the effect of 200 g/kg inclusion of the feed ingredients on the performance, gut microbiota (paper 1) and digestive physiology (paper 2) of the Atlantic salmon. The fish grew well and showed improved growth with the inclusion of pea and poultry. It was very interesting to observe that in all fish, regardless of dietary group, *Lactobacillaceae* dominated the allochthonous (transient) bacteria, whilst *Corynebacteriaceae* dominated the autochthonous (adhered) gut microbiota indicating that 1) carefully chosen raw materials can be chosen to keep the microbial profile close to that of fishmeal, and 2) qPCR is a useful tool for investigating the intestinal microbiota of fish. Furthermore, there was a clear correlation of use of plant proteins and production of short chain fatty acids in the fish gut, indicating increased carbohydrate utilization by the gut microbiota and a potential source of energy for the fish. From examinations into the digestive physiology it became apparent that feed ingredients which resulted in growth equal to or better than the control also showed few deviations from that observed in fish fed fishmeal. Feather meal however is an exception, showing comparable growth, but also increased leucine aminopeptidase activities, increased distal intestine weight and persistent high nitrogen indicating an ability to compensate for the low digestibility. The intestine was examined for morphological effects which might indicate inflammatory changes or possible breach of the mucosal barrier, but this was not observed in any of the fish examined in the present thesis.

The intestine has an important barrier function against pathogen bacteria and the second trial used *ex vivo* methodology to investigate the effects of the feed ingredients on the relationship between a pathogen, a probiotic and the intestinal morphology (paper 3). *A. salmonicida* ssp. *salmonicida* is a familiar pathogen of salmonids and known to use the intestine as a portal of entry. *Carnobacteria*

divergens, an indigenous lactic acid bacterium in salmon gut, is known to provide the host with some protection against this pathogen. There is however limited information on the effect of diet on the susceptibility of the fish to the pathogen or the potential protective effect of the probiotic. The present thesis shows that the ultrastructure of the intestine was not modulated by the use of alternative feed ingredients and it was interesting to also observe that the raw materials did not modulate adherence of *C. divergens* or *A. salmonicida*. Results however indicate that although *A. salmonicida* is more efficient at adhering to the mucosal lining than *C. divergens*, the probiotic is more efficient at displacing the pathogen if allowed to adhere to the mucosal lining first. The result of the present thesis therefore suggests that using the feed ingredients tested in the present trial may not affect the beneficial effects of *C. divergens* against invasive intestinal pathogens.

To relieve the dependency on marine protein the aquaculture industry has made good progress in replacing the fishmeal with alternative raw material. As shown by the present thesis, careful evaluation and use of alternative ingredients in diets for Atlantic salmon diets may result in high inclusion replacements, however caution should be used when adding new raw materials to the raw material basket to avoid compromising fish health, welfare and growth.

Sammendrag (Norwegian)

For å kunne benytte seg av den spådde økningen i etterspørsel for sjømat, kombinert med en begrenset produksjon av marine råvarer tilgjengelig for fôrproduksjon, har akvakulturindustrien forsket på nye alternative fôr ingredienser. Flere alternative ingredienser er allerede i kommersielt bruk i dag som det er veldig lite informasjon tilgjengelig på tarmmikrobiota, fordøyelsesfysiologi og den potensielle effekten på sykdomsutvikling. Denne avhandlingen vil derfor belyse effekten av noen kommersielt brukte råvarer som erteprotein konsentrat (PPC), soyaprotein konsentrat (SPC), ekstrahert solsikke (ESF), hydrolysert fjørmel (FeM) og bi-produkter av fjørfe produksjon (PBY).

Den første studien undersøkte effekten av 200 g/kg inklusjon av de alternative fôr ingrediensene på vekst og tarmflora (paper 1) samt fordøyelsesfysiologien (paper 2) hos Atlanterhavlaksen. Fisken viste forbedret vekst ved inklusjon av erter og fjørfe. Det var interessant å observere at i all fisk, uansett diettgruppe, ble den alloktone (transient) tarmmikrobiotaen dominert av *Lactobacillaceae* og den autoktone (festede) tarmmikrobiotaen dominert av *Corynebacteriaceae* noe som indikerer at 1) nøye valgte råvarer kan holde den mikrobielle profilen lik den for fiskemel, og at 2) qPCR er ett nyttig verktøy for undersøkelser av tarmmikrobiotaen i fisk. Det ble også observert en klar korrelasjon mellom bruken av plantemel og produksjonen av kortkjedet fettsyrer i tarmen, som indikerer en økt utnyttelse av karbohydratene og en potensiell kilde til energi til fisken. Fra undersøkelsene av fordøyelsesfysiologien kom det frem at fisk som hadde vist god vekst lik eller bedre enn fiskemel også viste få avvik fra det observert i fiskefôret basert på fiskemel. Fjørmel er ett unntak, hvor veksten var på nivå med fiskemel, men også økt leucine aminopeptidase aktivitet, økt vekt av bak tarm og ett vedvarende høyt nivå av nitrogen som indikerer en evne til å kompensere for den lave fordøyeligheten av råvaren. Tarmen ble også undersøkt for morfologiske endringen som kan indikere inflammatoriske endringer eller mulig brudd av mukosa barriere, men dette ble ikke observert i noen av de undersøkte fiskene.

Tarmen har en viktig barrierefunksjon mot patogene bakterier og det andre forsøket tok i bruk en ex vivo metode for å undersøke effekten av alternative fôringredienser (PPC, ESF og FeM) på forholdet mellom en patogen bakterie, en probiotisk bakterie og tarmmorfologien (paper 3). *A. salmonicida* ssp. *salmonicida* er en vanlig patogen bakterie hos salmonider og er tidligere vist å bruke tarmen som inngangsportal. *Carnobacteria divergens*, en endogen bakterie i tarmen hos laks, er tidligere vist å gi en viss beskyttelse mot den patogene bakterien. Denne avhandlingen viser at tarmens ultra-struktur ble ikke modulert ved bruk av alternative fôringredienser, og det var interessant å observere at heller ikke *C. divergens* og *A. salmonicida'* festeevne ble endret ved bruken av alternative råvarer. Resultatene indikerer at selv om *A. salmonicida* var mer effektiv til å feste seg til mukosa enn *C. divergens* var probiotika bakterien mer effektiv til å hindre at den patogene bakterien festet seg hvis den fikk mulighet til å feste seg først. Denne

avhandlingen forslår derfor at bruken av de undersøkte råvarene ikke vil påvirke den fordelaktige effekten av C. divergens mot invaderende tarmpatogener.

For å letne avhengigheten av marine råvarer har akvakulturnæringen gjort god progresjon i å finne nye råvarer og som vist av denne avhandlingen kan nøye valg av råvarer resultere i høy inklusjon av alternative fôringredienser i fôret til Atlanterhavlaksen men også at varsomhet må brukes ved innføring av nye råvarer for å unngå kompromiss av fiskens helse, velferd og vekst.

Scientific background

The research presented in this thesis aims to gain knowledge on the impact of a variety of alternative feed ingredients for which there is an increasing use for in fish feed. In focus were effects on the community population and metabolism of intestinal microbiota, its ability to compete with pathogens, gut physiology and health as well as fish performance and feed utilization. The introductory chapters presents the scientific 'state of the art' regarding availability of alternative protein sources, relevant aspects of intestinal structure and function, diet-microbiota relationships as well as effects of probiotics and interaction with pathogens. Furthermore the reader will find a discussion of key methods used in the present work. Discussions on the main results of the fish trials is aimed at giving the reader an understanding of the impacts of the alternative feed ingredients and also provide increased knowledge for the feed industry in the potential and optimized use in diets for Atlantic salmon.

Aquaculture

Aquaculture has long been important in the effort of feeding the increasing global population. As the global population is expected to reach 9 billion people in 2050 it is become increasingly clear that the intense production of seafood will be paramount in covering the demand for protein. The definition of aquaculture is the cultivation of marine and freshwater species for human or animal consumption. In most production associated with marine organisms, the flesh is funnelled into the market for human consumption whilst the by-products and offal such as viscera, bones and other inedible parts are processed for use in animal feed. Many species of marine and freshwater fish, crustaceans, molluscs and algae are cultivated in a variety of environments from ponds and rivers to open oceans (FAO, 2012). Whereas carp, tilapia and catfish dominate the freshwater fish species, Atlantic salmon (*Salmo salar* L.) is the most intensely cultivated marine species and Norway and Chile are the core producers of salmonids in the world (Tacon & Metian, 2008). Although aquaculture is ancient, decreased wild fisheries (Naylor *et al*., 2010) and increased global population concomitant with increased purchasing power in densely populated counties, have stimulated a rapid growth in the last three decades exceeding 142 million tons produced marine aquaculture products in 2008 (FAO, 2010). Globally the Asian market accounts for 89% of the aquaculture consumption, whereas the Americas and Europe account for 4.6% and 4.5% respectively (Bostock *et al*., 2010). Production of seafood through aquaculture has grown by 8-9% annually since the early 1980's whilst in comparison capture fisheries levelled out at 90

million tons. Sustainability of the growth in aquaculture has been discussed both in terms of environmental aspects such as coastal damage, pollution (Cho & Bureau, 1997; Naylor *et al*., 2000), reduced natural fish stocks (Welcomme *et al*., 2010) and the use of available raw materials in diets (Hardy, 2010).

Atlantic salmon (*Salmo salar* L.)

Atlantic salmon is an anadromous species which means it spends the first part of its lifecycle in freshwater (Fig. 1). As a hatchling the larvae are relatively large compared to other fish larvae, and the large yolk sac adhered to the belly at hatching allows the larvae to postpone first feeding. This allows the larvae to be able to accept and digest formulated feeds at start feeding which is a benefit in the intensive cultivation of the species, and negates the potential challenges of using live feed at first feeding. First feeding feeds are usually formulated using high levels good quality marine protein which may add a greater constraint on the use for grower fish when supply is low.

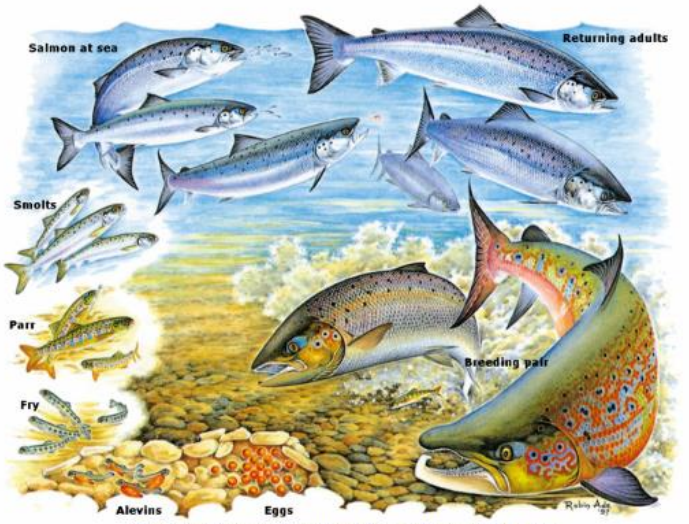

Figure 1. Illustration of the life cycle of the anadromous Atlantic salmon (*Salmo salar* L.) (North Atlantic salmon conservation organization, no date).

Fishmeal

Fish meal (FM) and fish oil (FO) are major dietary components in feeds for many cultivated species and historically feeds for Atlantic salmon were formulated to contain high levels of both. The nutrient profile of FM is well balanced to the nutritional needs of the naturally carnivorous salmon. High quality FM supplies all the necessary amino acids, especially methionine, lysine and tryptophan which can be

deficient in various plant proteins (Watanabe, 2002). Moreover, FM is highly digestible and palatable to fish ensuring a high feed intake and nutrient utilization. Fish meal however has become a limited commodity since the wild stock landings levelled out in the early 1980's and also as a result of the consumers purchasing abilities and demand for seafood has increased. As FM becomes increasingly limited, prices will also increase and successful replacement of the FM with alternative proteins will assist in stabilizing feed production costs as well as sustain future growth in the sector.

Increased need for alternatives to fishmeal and potential raw materials

Alternative raw materials have been a focus for the aquaculture feed sector for the last decades. Growing fish using alternative raw materials has however raised many questions regarding sustainability of the sector growth and product quality, especially the consequence of reduced omega-3 fatty acids in the fillets. A lot of research has allowed the industry to grow continuously using protein from non-marine sources (Gatlin *et al*., 2007; Glencross *et al*., 2007; Hardy *et al*., 2010).

Promising sources of alternative proteins include plants (e.g. legumes, oilseeds, cereals) some of which are already incorporated into commercial formulations for technical or nutritional purposes and by-products of meat and poultry production, e.g. poultry and feather meal, porcine meal, blood meal (Glencross *et al*., 2007). Other protein sources from various species of insect and krill, (e.g. *Euphasia superba* and *Meganyctiphanes norvegica*) (Storebakken, 1988) are also tested concomitantly. Although studies are carried out frequently to investigate partial replacement of FM with alternative raw materials, only a limited number of studies have reported replacing all the FM in diets for salmonids (Espe & Njaa, 1991; Gomes *et al*., 1995; Adelizi *et al*., 1998; Espe *et al*., 2006). The challenge lies in finding a source of protein that the fish can digest and utilize, will supply most of the necessary nutrients and that do not contain harmful substances. Sustainability, availability, price, nutrient profile, nutrient availability through high digestibility and, in the case of plant materials, the presence of anti-nutrients (ANF's) are all important criteria in identifying alternative raw materials.

Soybean (SBM), wheat, corn, barley, cottonseed, canola and peas have been investigated as potential replacements for fishmeal (Gatlin *et al*., 2007). SBM meal is considered especially promising based on its favourable amino acid profile, competitive price, high protein content and sustainable production (Carter & Hauler, 2000) however use of the lesser refined SBM has been limited due to the presence of ANF's (Baeverfjord & Krogdahl, 1996; Knudsen *et al*., 2008). ANF's such as saponin, chlorogenic factors, trypsin inhibitors and protease as well as high levels of oligosaccharides amongst others may be a limiting factor for higher inclusion levels of plant materials in diets for Atlantic salmon. Although it has been suggested that salmon can tolerate low levels of saponin (Knudsen *et* al., 2008) a good alternative to SBM is the more refined product from alcohol extracted SBM, namely soy protein

concentrate (SPC). The process of using alcohol to extract carbohydrates from dehulled and defatted SBM also removes ANF's such as saponin (van der Ingh *et al*., 1996) leaving a product high in protein (>65%) and low in ANF's. Pea is another promising alternative protein sources and is often de-hulled and air classified to remove fibre and carbohydrates to produce pea protein concentrate (PPC) which is useful in diet for fish (Drew *et al*., 2007). Use of the raw material in high inclusion levels are however limited by the presence of saponin (Penn *et al*., 2010) and relatively high price compared to other commercially available alternative protein sources. Digestibility and the nutrient profile of the raw material suggest that rendered animal proteins are prime candidates for fishmeal replacement (Bureau *et al*., 1999). Although the ban on the use of processed animal protein (PAP) in aqua feeds was lifted for non-ruminants (poultry and porcine) in 2013 (Regulation 56/2013) consumer reluctance in using such feed is predicted to limit commercial use for some time still.

One of the concerns for the industry in replacing the marine protein with alternative protein sources in formulated feeds is the effects it may have on fish growth which is an important indicator of feed utilization and fish welfare. During a disease outbreak or suboptimal environmental conditions fish will often stop eating and hence stunt growth for a limited or prolonged period of time. For a commercial site low or no growth over any period of time is costly. Growth is therefore an important indicator to a researcher investigating the impact of alternative feed ingredients. In the present study growth and feed intake are used as indicators both for feed utilization and welfare, but also on the quality of the fish feeding trial.

Intestinal morphology and function

The gastrointestinal tract (GIT) of fish in early development is basically a tube running from the mouth to the anus, which soon differentiates to accommodate the needs for food processing and nutrient absorption. In most fish species, the tube differentiates into a stomach, a foregut with a various number of blind appendages known as pyloric caeca (PC) opening into pyloric intestine (PC), mid intestine (MI), distal intestine (DI) and rectum. Each of these compartments is specialized for different roles in the digestive processes. Presence of sphincters between each compartment influences retention time as well as the inner environment of the compartment. Salmon have a multitude of PC placed directly after the stomach and pyloric sphincter, which allows for an increase in digestive area and capacity (Buddington *et al*., 1986) and is also a prime site for absorption of nutrients (Nordrum *et al*., 2000). Earlier investigations into the relative lengths of the intestine between herbivorous and carnivorous fish revealed that intestine in herbivorous fish tend to be longer and

thinner whilst carnivorous fish tend to allocate the absorptive tissue to PC and/or a thick intestinal mucosa (Buddington, 1987).

The layers of the intestinal wall

The intestinal wall can be divided into four main layers – mucosa, submucosa, muscularis and serosa (Kryvi & Totland, 1997). The characteristics of these layers are visible at relatively low magnification and as such easily evaluated using light microscopy. The mucosa is the layer that is in direct contact with the intestinal lumen, and consists of enterocytes in a single layer, which makes up the epithelium or epithelial layer (Fig. 2, no. 6), and with an underlying lamina propria (Fig. 2, no. 2). The mucosa is oriented in folds, which extend into the lumen to increase the surface area. The layer underlying the mucosa is the submucosa (Fig. 2, no. 7). This layer supports the mucosa, and consists mostly of loose connective tissue, but which also has a prominent layer of compact connective tissue called stratum compactum (Fig. 2, no. 8). On both sides of the stratum compactum there are eosinophilic granular cells (Fig. 2, no. 9), which contain lysozymes and proteases, enzymes proposed to be active in the defence against pathogenic microorganisms.

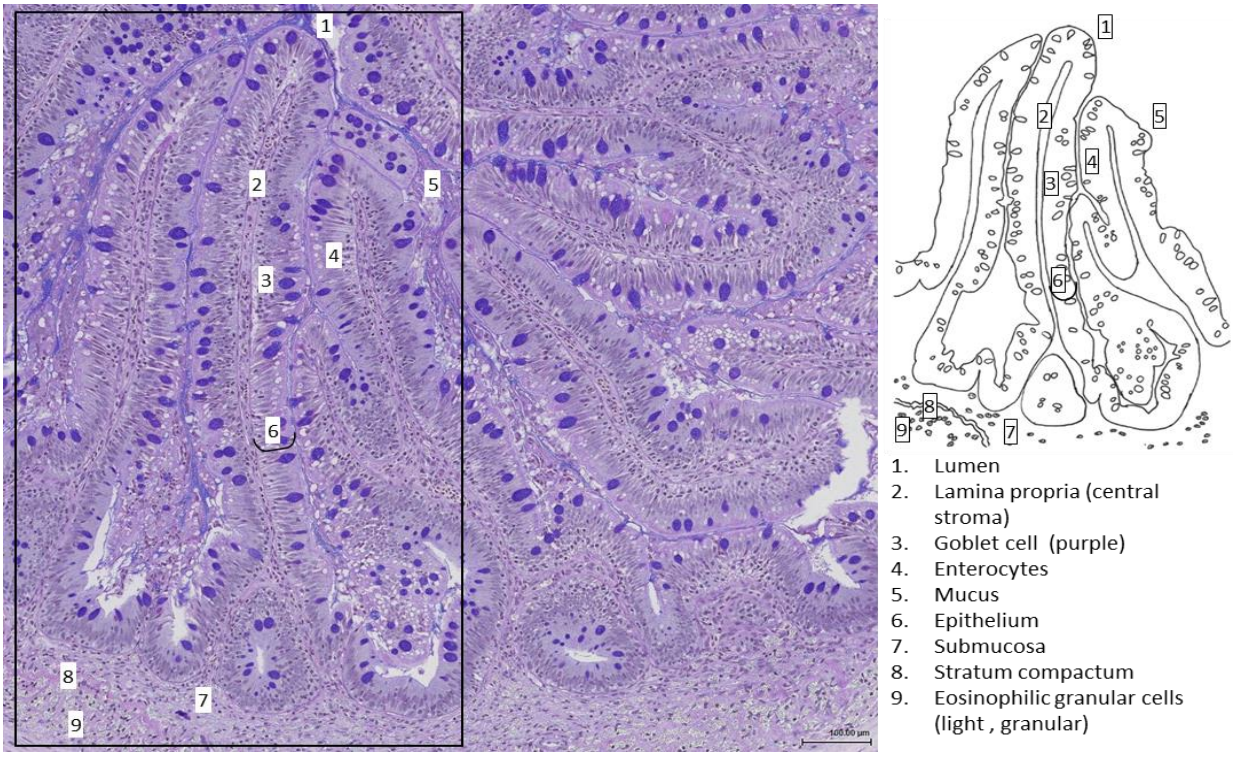

Figure 2. Intestinal epithelium of the Atlantic salmon distal intestine stained using Alcian blue (AB) and Periodic acid Schiff (PAS). 10x magnification. (Hartviksen, unpublished results).

The stratum compactum is considered the border between the submucosa and the muscularis which is a layer of circular and longitudinal muscles running the length of the intestine. Between the muscular layers are ganglia cells from the enteric nervous system. On the outside of the intestine is a single layer of cuboidal cells surrounded by connective tissue that forms a layer known as the serosa. Extending from the apical membrane of the enterocytes are numerous extensions called microvilli (Fig 3) which is collectively called the brush-border membrane (BBM). Because of the small size and tight packaging of these protrusions, visualization using light microscopy (LM) shows only a fuzzy lining of the mucosa, hence the name. The function of the BBM is to increase the area of digestion by membrane-bound enzymes as well as absorption of the finely digested nutrients. Goblet cells are located embedded in between the enterocytes. The cells secrete their content, mucus, from between the enterocytes, emptying it into the intestinal lumen.

Epithelial morphology

Transmission electron microscopy (TEM) is a visualization of the ultrastructure of tissues, cells and even organelles and can be used to evaluate structural and cellular changes at higher magnification than LM (Olsen *et al*., 2000, 2001, 2002). Histological changes involving the microvilli, tight junctions, mitochondria, nucleus, lymphocytes and the presence of alternative structures such as rodlet cells (Ringø *et al*., 2007; Salinas *et al*., 2008; Salma *et al*., 2011; Løvmo Martinsen *et al*., 2011; Harper *et al*., 2011) as well as the presence of bacteria (Ringø *et al*., 2003) are often observed using electron microscopy. Criteria such as damaged microvilli, presence of rodlet cells and intra-epithelial lymphocytes, disintegrated tight junctions and loss of epithelial integrity, edema and lipid droplets can be used to evaluate the structural status of the mucosal epithelium (Ringø *et al.*, 2004; Salinas *et al.*, 2008; Salma *et al.*, 2011).

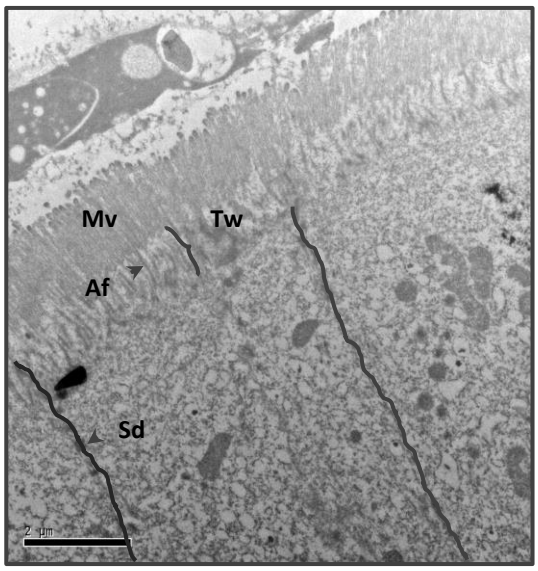

Figure 3. Transmission electron microscope micrograph (TEM) showing mid intestine of Atlantic salmon fed FM and exposed to saline. The TEM shows normal undamaged enterocytes with clear terminal web (Tw) and microvilli (Mv) that is straight and unruffled. Cell borders shows intact spot desmosomes (Sd). Apically in the cell the microvilli are anchored to the cell by active fillaments (Af). Original magnification x8000. (Hartviksen, unpublished results).

Inflammation and bacterial disruption of the intestinal tissue

The barrier function of the intestine has evolved to prevent invasion of opportunistic pathogens and other microorganism as well as maintain tissue homeostasis. Should the barrier function fail, the response of the intestine is an inflammatory response (Krogdahl *et al*., 2000) which is aimed at eliminating the invading agent and or repairing damage. There are two stages of an inflammatory response – acute and chronic. If the causative agent or insult is not removed and the acute inflammation persists, chronic inflammation develops. Inflammatory responses can be initiated by infection, tissue injury, or presence of antigenic compounds.

In salmon the presence of saponin in plant raw materials such as SBM (van der Ingh *et al*., 1996) have shown to induce a type of intestinal inflammation described as "non-infectious sub-acute enteritis" in the distal intestine and the effects seen in the intestine are visible at light microscopy level (Baeverfjord & Krogdahl, 1996; Úran *et* al., 2008, 2009; Penn *et al*., 2010). The change is observed as decreased microvilli and mucosal fold height, widened lamina propria and submucosa with an increased infiltration of immune cells, such as T-lymphocytes, macrophages, neutrophils and eosinophilic granular cells into the central stroma, sometimes increased appearance of goblet cells, and decreased presence and/or size of absorptive supranuclear vacuoles in the apical part of the enterocytes (van den Ingh *et al*.,

1991, 1996; Baeverfjord & Krogdahl, 1996; Bakke-McKellep *et al*., 2000, 2007; Penn *et al*., 2010). Knudsen *et al* (2008) suggested that the mode with which saponin induced enteritis of the intestine was connected to shifts in the intestinal microbiota and that the inflammation is a secondary effect of the membrane disrupting abilities of saponin. Increased intestinal permeability caused by the saponin would expose the mucosa to foreign antigens present in the gut lumen and induce migration of eosinophilic granular cells, widening of the lamina propria and shortening of the mucosal folds. This may be correlated to the high levels of microbes in the DI which utilizes the indigestible carbohydrates. Inulin has also shown to cause damage to the intestinal morphology of Arctic charr (Olsen *et al*., 2001) which was suggested to affect the bacterial population in the DI of fish fed inulin at 15% (Ringø *et al*., 2001).

Another function of the mucosal interphase is as a potential portal of entry for pathogen bacteria. Although fish has developed numerous protective features to prevent adherence and invasion by opportunistic pathogens, the bacteria has developed an arsenal of strategies to by-pass these and gain entrance to the hosts' enterocytes, blood stream and ultimately inner organs. *A. salmonicida* has several bacterial factors which is essential for the virulence of the bacteria. These include exotoxins and endotoxins, extracellular products (ECP's) released into the medium by the pathogen bacteria (see review by Ellis, 1991). The effects of the toxins may include apoptosis of the enterocytes, a mechanism the bacteria may use to gain entrance to the blood circulation system of the host. Translocation is another mechanism by which pathogens cross the mucosal barrier and has been observed using *in vitro* methods such as the Ussing chamber (Jutfelt *et al*., 2006). Due to the increased use of alternative feed ingredients in commercial aqua diets, dietary effects on adherence of pathogen bacteria has been studied. Following feeding with soybean and linseed oil, Lødemel *et al*. (2001) reported decreased mortality in fish fed soybean oil (20% mortality) compared to the control fish fed marine oil (48% mortality) after cohabitant challenge with *A. salmonicida* indicating increased adherence and/or translocation of the pathogen. Furthermore, Ringø *et al*. (2002a) showed that the autochthonous (adhered) bacterial profile of the DI was different between the soybean oil and linseed oil groups, as well as before and after cohabitant challenge with *A. salmonicida*, and also that the indigenous bacteria showed improved growth inhibition towards *A. salmonicida*, *Vibrio salmonicida* and *Vibrio anguillarum*. Dietary induced inflammation has also been suggested to be a contributory factor influencing mortality from pathogen bacteria as increased mortality has previously been reported in fish expressing enteritis-like signs of the DI following feeding with soybean products and cohabitant challenge with *A. salmonicida* (Krogdahl *et al*., 2000). These previous reports indicate the importance of minoring the effects of alternative feed ingredients in the morphology, and hence, the barrier function of the GIT in Atlantic salmon.

Digestive function and transepithelial transport of nutrients in the intestine

The GIT of any vertebrate is a true multifunctional organ. The digestive tract has an essential role in digestion, secretion, osmoregulation, antigen sampling, immune reaction and is an important barrier against invasion by pathogenic and opportunistic microorganism. The main function however is the assimilation of nutrients essential for bodily functions, health and welfare by the enzymatic and mechanical breakdown of feed components into smaller molecules absorbable by the intestinal transport mechanisms. Ingested feed is digested in three stages – pre-digestion in the stomach, a course pancreatic digestion in the intestinal lumen and a fine digestion by enzymes located in the BBM.

Pre-digestion of ingested food and feed start in the stomach as distention of the stomach triggers secretion of the non-specific digestive agent hydrochloric acid (HCl) as well as the protein specific pepsinogen, which is activated by the HCl to pepsin. As fish cannot chew, mastication of the food is non-existent and this sets high demands for this initial pre-digestion. Once the food is hydrated, it passes through the pyloric sphincter and enters the upper GIT. Distention of the intestine and the presence of feed components such as peptides and amino acids initiate release of the digestive hormone cholecystokinin (CCK). This hormone is released into the bloodstream from the basolateral side of specialized CCK producing cells in the mucosa. Secreted bile fluids, pancreatic digestive juices, mucus and hormones aid in the digestion and passage of the chyme and are all components vital to the processing of ingested food and feed (Bakke *et al*., 2011). Protection of the organism from the acidic components secreted from the stomach as well as opportunistic bacteria is provided by the bile secreted from the gall bladder (Shephard, 1994) as well as the mucus which lines the luminal side of the mucosa. Despite pre-digestion in the stomach and pancreatic digestion in the lumen, feed components may still be too large for absorption and a fine digestion at the BBM may be necessary for absorption to take place.

Digestive enzymes

Enzymatic breakdown of the food occurs in the digestive tract into which digestive enzymes are secreted from the pancreas and bound to the intestinal epithelium (Souza *et al*., 2007; Bakke *et al*., 2011). Enzymes such as trypsin, chymotrypsin, lipase and carboxypeptidase are produced in the pancreas and secreted into the upper intestine as a response to the presence of nutrients in the lumen. Peptidases, disaccharides and other digestive enzymes, such as leucine amino peptidase (LAP), maltase and alkaline phosphatases on the other are located in the luminal (microvillus) membrane of the epithelial cells in the intestinal wall. LAP activity (chyme associated LAP; C-LAP) may also be detected in the gastrointestinal contents and may therefore be an indication of sloughing of the intestinal

epithelium. In addition, enzymes such as cellulase and chitinase, as well as others, may also be provided by the indigenous intestinal microbiota (Ray *et al*., 2012). Furthermore, supplementation of digestive enzymes exogenously by natural prey may contribute to the digestion in fish (Kuz'mina, 2008) although this is not relevant for farmed fish fed formulated feed.

Absorption

Absorption, the uptake of solubilized nutrients as result of the enzymatic digestion, occurs at the BBM located apically on the intestinal enterocytes. Nutrients are transported across the membrane by specialized transporters into the cells which then releases them basolaterally into the bloodstream. This transport may be a transfer of the nutrients as they are, or include an intracellular digestion step.
Uptake of amino acids and sugars are in herbivores regulated phenotypically in response to the dietary content of carbohydrates or protein, whilst trout did not seem to express these same responsive abilities (Buddington, 1987). This may be a reflection of the fact that herbivorous fish may also feed on flesh when available, however a carnivorous fish will not feed as much on plant materials. This apparent inability to respond to changing nutritional profile and the relatively short intestinal lengths compared to herbivorous fish may represent a limitation in the carnivorous Atlantic salmon to respond phenotypically to the changing nutritional profiles of diets containing alternative raw materials, especially plant materials containing higher carbohydrate levels.
As described above, the digestive tract has an essential role in digestion and assimilation of nutrients. Previous studies have shown that inflammation of the GIT decreases enzyme activity (Krogdahl *et al.*, 2000) and thus intact integrity of the intestinal morphology is important to sustain optimal feed utilization. As such the focus of the present thesis in the area of intestinal morphology and function is to evaluate the morphological integrity for signs of inflammation as well as investigate enzyme activities in the tissue and luminal content for indications that the use of pea and soy protein concentrate, sunflower, feather meal or poultry by-product has affected digestive function and feed utilization. Utilization of the feeds can be monitored by the content of nitrogen, carbon and sulphur in the intestinal content as well as plasma content of metabolites.

Intestinal microbiota in fish

Colonization and "normal" microbiota

Similar to mammals following birth, fish larvae are colonized once it hatches from the egg by bacteria present in water and adhered to the egg (Hansen & Olafsen, 1999). Although the presence of an indigenous microbiota in fish larvae has been historically questioned it is now commonly accepted that

the GIT of fish larvae is colonized once it hatches from the egg even before first feeding (Hansen & Olafsen, 1999; Ringø & Birkbeck, 1999). In fish it was long assumed that the gut microbiota was less abundant and diverse than what was reported for endothermic mammals (Trust & Sparrow, 1974; Neish, 2009). Although fish microbiota is comparatively less diverse, increased knowledge in the area of fish microbiology has revealed a higher complexity than previously assumed. As a result of co-evolution, fish, as in other animals, have developed a symbiotic relationship with its intestinal microbiota which has the intent to provide benefits for both the host and the microbes. The mucosal surface represents the main site of interaction between the host and the bacteria (Cahill, 1990; Denev *et al*., 2009; Péréz *et al.* 2010) and provides the bacteria with substrates and binding sites. Furthermore, the gut microbiota may also assist in digestion of non-digestible nutrients such as certain carbohydrates (Ray *et al*., 2012), plays an important role in the protection of the host against pathogen bacteria (Ringø, 2008; Ringø *et al*., 2008, 2010), stimulates the immune system (Kanther & Rawls, 2010) and has shown to be essential for the maturation and differentiation of the intestinal epithelium (Bates *et al*., 2006). Furthermore, meta-analysis of the intestinal microbiota revealed that the gut microbiota more closely resembled that of terrestrial animals than the environment, an indication that the intestinal microbiota of fish is subject to more than just environmental influence (Sullam *et al*., 2012). In fact the intestinal microbiota in fish has been reported to be influenced by diet (Heikkinen *et al*., 2006; Bakke-McKellep *et al*., 2007; Ringø *et al*., 2012; Desai *et al*., 2012; Askarian *et al*., 2013), including non-nutritional compounds (Ringø, 1993a, b), season (Hovda *et al*., 2012), water salinity (Ringø & Strøm, 1994), development (Sugita *et al*., 1988), antibiotics (Austin & Al-Zahrani, 1988; Bakke-McKellep *et al*., 2007), presence of pathogen bacteria (Ringø *et al*., 2002a) and the difference between dominant and subordinate fish (Ringø *et al*., 1997).

The microbial density of the GIT is higher than that of the surrounding environment, indicating that the intestine consists of numerous niches well suited for colonization by bacteria (Denev *et al*., 2009). Colonization of the GIT in fish is influenced by both host and microbial factors. A bacterium that enters the host system through eating or drinking will sooner or later reach the GIT, where some will have the ability to withstand the acid, bile and enzyme rich environment to adhere to the mucosal lining and become part of the autochthonous (adherent) bacterial. Van der Marel *et al*. (2013) suggested that sugars in the mucus are meant as attractants for bacteria to enhance adherence to the mucus lining. Bacteria without the ability to adhere to the mucosal lining will be flushed through the digestive system along with the digesta, after having been excluded from the GIT by the hosts' own beneficial microbiota or anti-microbial compounds. This forms the basis of one of the most common classifications of intestinal bacteria – resident bacteria classified as autochthonous and transient bacteria classified as allochthonous (Savage, 1977; Ringø & Birkbeck, 1999; Ringø *et al*., 2003; Kim *et al*., 2007). Hence studies which

report only bacterial species and genera isolated from intestinal contents do not provide an adequate representation of the intestinal microbiota (Savage, 1977) indicating the need to investigate the autochthonous and allochthonous communities separately. Following a study investigating the allochthonous and autochthonous bacterial microbiota of Arctic charr (*Salvelinus alpinus* L.) the authors reported a difference in the abundance and diversity between the different communities and suggested that some bacteria colonize the GIT poorly (Ringø *et al*., 2006c). Furthermore some studies report different bacterial profiles (Ringø *et al*., 1995) and densities (Ringø *et al*., 2006c; Bakke-McKellep *et al*., 2007) in the proximal and distal autochthonous microbiota suggesting that the different compartments should be studied separately.

There are several decisive factors for how the intestinal microbiota in fish is composed. Factors such as environment (location, temperature), diet, health status and colonization influence the composition from hatching and continuously during the life time of the fish. In fish GIT, the most predominant species and genera isolated have earlier been classified as either aerobes or facultative anaerobes (Ringø *et al*., 1995) which is in contrast to endotherms which is predominantly dominated by obligate anaerobes (Zoetendal *et al*., 2004). Huber and co-authors (2004) however raised the issue of an underestimation of the number of anaerobic bacteria due to the difficulty in culturing such bacteria. As the laboratory tools for identifying bacteria in fish intestine has improved, so has our understanding of the complexity of the intestinal microbiota of fish. Indigenous intestinal microbiota of Atlantic salmon and other salmonids has been investigated using both cultivation (Strøm, 1988; Ringø & Strøm, 1994; Ringø *et al*., 1995) and molecular based methods (Hovda *et al*., 2007, 2012). An early study on free-living salmonids in freshwater lakes revealed that the predominant culturable microbiota were Gram negative bacteria of the genera *Enterobacter*, *Aeromonas* and *Acinetobacter* (Trust & Sparrow, 1974). It was discovered that two or three genera predominated in the various freshwater salmonids regardless of species, fish weight or sex of the fish. Whilst all of the above genera were detected in all of the GIT compartments, species of *Pseudomonas, Bacillus, Achromobacter, Alcaligenes* and *Micrococcus* were detected mainly in the anterior intestine. The study also showed that there were no significant differences in intestinal microbiota of different species of fish on the same location, or between fish from geographically different location. In contrast, a study using degenerative gradient gel electrophoresis (DGGE) to investigate intestinal microbiota in farmed Atlantic salmon in sea cages revealed that the dominant groups to be *Lactobacillus, Lactococcus, Photobacterium. phosphoreum*, Bacillus and an unidentified bacterium (Hovda *et al*., 2007).

The difference in intestinal bacteria between marine and freshwater fish is supported by many studies that have characterized the intestinal microbiota in fish in either environment. Culturable bacteria of the genera *Aeromonas*, representative of the *Enterobacteriaceae* as well as *Flavobacterium* and

Pseudomonas were reported to dominate the indigenous microbiota of the freshwater salmonids (Yoshimizu & Kimura, 1976), with a high percentage *Lactobacillus* spp. reported in Arctic charr (Ringø, 1993a, b, c) and Atlantic salmon (Strøm, 1988). Although *Vibrio* is a commonly reported genus in fish, some studies report intestinal microbial community without the presence of *Vibrio* (Ringø *et al*., 2006a, 2008).

Probiotics and the host-bacteria relationship

Some intestinal bacteria are potentially more beneficial than others. A host has a close relationship with its resident microbiota and the intestinal microbiota in fish and endothermic animal play an important role in the protective and metabolic functions of the fish (Denev *et al*., 2009). Bacteria which are beneficial to the host are commonly termed probiotic (pro-for, biotic-life). By definition a probiotic is a viable living organism that when added to the intestinal environment of a host provides health benefits to the host including improved digestion, protection against potential pathogens and alleviation of damage caused by invading pathogen microbes in endothermic animals (Salminen *et al*., 1999; Salma *et al*., 2011). Probiotics are a major focus for the aquaculture industry to replace antibiotics following the restrictions on its use due to fear of development of anti-biotic resistant strains (Sapkota *et al*., 2008). Selection of probiotic candidates are evaluated in lieu of a long list of both essential and favourable selection criteria before being regarded as a potential candidate (Merrifield *et al*., 2010; Lauzon *et al*., 2014). The beneficial roles of the commensal microbiota includes production of anti-microbial substances, competition with pathogen bacteria for binding sites (Harper *et al*., 2011; Løvmo Martinsen *et al*., 2011) and nutrients (Ringø *et al*., 2010; Perez *et al*., 2010), production of digestive enzymes which aid digestion (Ray *et al*., 2012), breakdown of indigestible carbohydrates to produce easily absorbable short chain fatty acids (Sakata *et al*., 1978) and strengthening the immune system by being in constant immune-regulation with the host (Gómez & Balcázar, 2008). Through their ability to withstand low pH in the environment and release either organic acid or other anti-microbial compounds, probiotics are able to inhibit the growth of or expel potential pathogens from the GIT of fish (Ringø, 2008) hence acting as an important protective factor in sustaining health of the host. There are different views on what characteristic is most important in a probiotic. Saarela *et al*. (2000) suggested that colonization within the intestinal mucus later is a pre-requisite for the functional success of a probiotic, whilst two other independent studies suggest that strain specificity is more important (Lee *et al*., 2003; Gueimonde *et al*., 2006).

Lactic acid bacteria

Lactic acid producing bacteria (LAB) are a natural part of the indigenous microbiota in fish (Ringø & Gatesoupe, 1998; Ringø *et al*., 2000, 2004) and some have shown to be promising probiotic candidates (Irianto & Austin, 2002). The bacteria are characterized as Gram-positive, usually non-motile bacteria which produce lactic acid as a sole or major product of the fermentation of carbohydrates (Ringø & Gatesoupe, 1998). Although some LAB bacteria are known fish pathogens, most are harmless, and even beneficial to the host (Gatesoupe, 2008). These groups of bacteria have been reported extensively in endothermic animals and are commonly used in production of preserved foods such as cheese, sauerkraut, meat, yogurt and silage (Gibbs, 1987; McKay & Baldwin, 1990). The bacteria are not characterized by classification in one genera, and LAB representatives have been discovered in *Lactobacillaceae*, *Carnobacteriaceae*, *Streptococcaceae*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Enterococcus* and *Vagococcus* (Ringø & Gatesoupe, 1998; Irianto & Austin, 2002). LAB and other probiotic strains have been isolated from several species of fish (Ringø and Gatesoupe 1998; Ringø, 2004; Ringø *et al*., 2005; Balcazar 2007; Michel *et al*., 2007; Liu *et al*.. 2008; Merrifield *et al*., 2014). Some LAB are fastidious in their nutritional and growth requirements and are therefore often not reported in cultivation studies. A good example is a study on the gut microbiota in Atlantic salmon using traditional cultivation and degenerate gradient gel electrophoresis (DGGE) where LAB was reported only from the DGGE analysis (Hovda *et al*, 2007). Benefits of LAB against pathogen bacteria include growth inhibition (Balcázar *et al*., 2006), production of anti-microbial peptides (Ringø *et al*., 2002b), reduction in the mucosal adherence abilities (Merrifield *et al*., 2014), exclusion and displacement from intestinal bindings sites (Harper *et al*., 2011) and competition for nutrients.

Carnobacterium divergens

Carnobacterium is a natural part of the intestinal microbiota of salmonids (Strøm, 1988; Ringø & Gatesoupe, 1998; Jöborn *et al*., 1997, 1999; Ringø & Olsen, 1999; Ringø *et al*., 2006c, 2008; Cantas *et al*., 2011; Merrifield *et al*., 2014) and a ubiquitous LAB. This group is seemingly less fastidious than other LAB and *C. maltaromaticum*, *C. divergens*, *C. inhibens*, and other various *Carnobacterium* ssp, are often reported from cultivation studies (Merrifield *et al*., 2014). Despite suspected pathogenic species in the midst such as *C. piscicola* (formerly *Lactobacillus piscicola*; Austin & Austin, 1993) and *C. maltaromaticum* (Loch *et al*., 2008) the genus is also regarded as a beneficial intestinal bacteria. *C. divergens* has shown to alleviate damage caused by pathogenic bacteria to the mucosal lining (Ringø *et al*., 2007) inhibit growth of pathogen bacteria (Jöborn *et* al,. 1997; Ringø, 2008). For these reasons *C. divergent* was chosen as a model bacterium for probiotic bacteria in the *Atlantic salmon* intestine.

In fish, as the marine proteins in feeds are replaced with alternatives, the modulations which occur as a result of this change may be regarded as a destabilization of the current systems, whether it is the bacterial profile, the digestive physiology or the susceptibility to invasion by opportunistic microorganisms through disruption of the intestinal barrier. The beneficial abilities of probiotic bacteria may alleviate or even mitigate the negative influences of the alternative raw materials and should therefore be considered carefully as a symbiotic in-feed functional component along with alternative feed ingredients.

Aeromonas salmonicida ssp. salmonicida

Aeromonas salmonicida ssp. *salmonicida* is a well-known pathogen for salmonids and is the causative agent for furunculosis (Austin & Austin, 1993). The name is derived from the characteristic furuncles clearly seen in the muscle of fish in chronic stages of the disease. Furunculosis is a potentially very costly disease as acute outbreaks often result in fish dying in 2-3 days (Austin & Austin, 1993). However stringent vaccination of all fish that are transferred to sea have contributed to decreasing the frequency of outbreaks of the disease (Midtlyng *et al*., 1996a, 1996b; Smith & Hiney, 2000). *A. salmonicida* has three potential portals of entry into the host: 1) skin, 2) gills and/or 3) intestine. Data has previously been reported to suggest that viable *A. salmonicida* bacteria are able to translocate across the intestinal epithelium of rainbow trout (*Oncorhynchus mykiss*) (Jutfelt *et al*., 2006). Furthermore earlier reports suggest that the mode of action for translocation is one that causes damage to the host's epithelium (Ringø *et al*., 2004, 2007).

The intestine is an important barrier against micro-organisms, however the functionality of the barrier is dependent on good intestinal health and morphology. As mentioned above, use of raw materials of non-marine origin may modulate the intestinal morphology and possible compromise the barrier function (Krogdahl *et al*., 2000). As such it is important to monitor the effect of the alternative raw materials on the mucosal morphology and also adherence of probiotic and pathogen bacteria. In order to generate more knowledge about the effects of the chosen alternative raw materials the mucosal morphology will be monitored using TEM.

Dietary effects on intestinal microbiota

In lieu of the fact that FM is a limited commodity and considering that intestinal microbiota is known to be affected by dietary modulations, it is important to gain better knowledge on the effects on the intestinal microbiota of using alternative raw materials. A variety of methods have been applied, although perhaps the most commonly used method is cultivation. Soybean meal (SBM) has been tested by several researchers due to its availability and competitive price (Heikkinen *et al*., 2006 ; Ringø *et al*.,

2006b ; Refstie *et al*., 2006 ; Bakke-McKellep *et al*., 2007; Merrifield *et al*., 2009, 2011; Cai *et al*., 2012). Pea products have been evaluated for its usefulness in diet for Atlantic salmon (Øverland *et al*., 2009; Penn *et al*., 2010), rainbow trout (Thiessen *et al*., 2003) and juvenile European seabass (*Dicentrarchus labrax*) (Gouveia & Davies, 1998). Although the results suggest that pea is suitable for use in aqua diets, none have investigated the effect of the raw material on the intestinal microbiota. Similar statement can be made for sunflower meal (Olvera-Novoa *et al*., 2002; Sànchez Lozano *et al*., 2007; Nogales Mérida *et al*., 2010), poultry meal and feather meal indicating that there is a lack of information available on the effect of commercially available alternative protein sources on the intestinal microbiota of farmed fish.

Some raw materials, usually of plant origin, contain carbohydrates that are too complex to be digested by the salmon digestive system. These nutrients tend to be transported to the distal part of the GIT where intestinal microbiota utlize the nutrients for fermentation or putrefaction. Short chain fatty acids (SCFA's) are end-products of the anaerobic microbial breakdown of carbohydrates and proteins that are not digested by the host, a process known as fermentation. In fish, fermentation takes place mainly in the hindgut (Mountfort *et al.*, 2002) and the nutrients can be indigenous in the raw materials used in the feed (e.g. structural carbohydrates in plant materials, starch used as binders etc) or added in the feed as a indigestible compounds known as prebiotics. Although fish have the enzymatic capability to digest some carbohydrates, more complex carbohydrates are resistant to the activities of the host enzymes limiting the fish's ability to digest them (Hemre *et al*., 1995; Krogdahl *et al*., 2003, 2004). In these cases the nutrients are digested by indigenous bacteria in the hindgut to produce various metabolites such as SCFA. SCFA's are known to modulate the physical environment of the intestine by amongst others decreasing pH.

Ethics

In fish trials as in all animal studies, there is a question of ethics. Fish trials tend to be large including a large number of fish to obtain a good representative data set. For the most part standard fish trials investigating alternative raw materials are not considered ethically wrong. For those studies the European regulation for the protection of vertebrate animals used for experimental purposes of 18 March (ETS no 170) compels the researcher to following the three R's – replace, refine and reduce. Studies which involve subjecting animals to disease or pain are especially subject to follow the three R's. Furthermore challenging fish in vivo sets high demands on the number of fish involved in each experimental group to ensure that a proper number of fish are infected.

One of the means to follow these 'rules' is use of in vitro and ex vivo methodology. By using for example the intestinal sac method (See Materials and Methods) it is possible to reduce the number of

fish needed. Unfortunately for some studies it is impossible to replace the animal with adequate replacements (eg cell lines) as the tissue response cannot be duplicated without the real animal involved. However, by sacrificing the fish, and excising the tissue in question, in this case the intestine, and then exposing the tissue to pathogen bacteria post mortem, it is possible to run the test without necessarily exposing the fish and causing an outbreak. These methods are however for short term evaluation as the tissue has a limited time it is still viable after excising and also as all connections to the immune system and vascular system are severed.

Summary

In summation, use of fishmeal in diets for Atlantic salmon has been steadily decreasing as a response to declining natural fish stocks and increased failure of fisheries to supply the needed fishmeal. To alleviate the dependency on marine protein, the aquaculture industry has explored using alternative raw materials in diets for Atlantic salmon. However, replacing fishmeal with meals of plant and animal origin has proved challenging, and there is a lack of information available on the effects of commercially available protein sources on the intestinal microbiota, ultrastructure and digestive function.

As a carnivore, Atlantic salmon may limited capability to adapt to dietary variations and may restrict its ability to utilize the diet. Potential changes in gut microbiota or ultrastructure due to diet composition can also affect both the physiology and health of the host, particularly when challenged by bacterial pathogens such as *A. salmonicida* that uses the intestine as a route of infection. Probiotics play an important role in the protection of the host and *C. divergens* is an indigenous bacterium previously isolated from Atlantic salmon intestine, and this bacterium has been suggested to provide some protection against intestinal damage caused by pathogenic bacteria, f. ex. *A. salmonicida*.

The overall objective of the present thesis was therefore to increase the knowledge and scientific understanding on the use of some alternative commercially available protein sources - extracted sunflower, concentrates of pea and soy, and by-products from poultry product (feather and poultry) in diets for Atlantic salmon. Of special interest are the effects on intestinal microbiota, morphology, and digestive physiology.

Project Aims

The overall objective of the thesis has been to increase the knowledge and understanding on the impact of a variety of alternative feed ingredients used in feeds for Atlantic salmon. Special focus has been on intestinal microbiota, intestinal morphology and physiology and possible effect on disease susceptibility.

The dependency on marine protein has been alleviated with the use of alternative feed ingredients. Use of alternative raw materials however has shown to potentially affect fish health and welfare through impacts on the intestine. A feeding trial was therefore carried out in order to monitor the effect of some commercially available alternative raw materials on the intestinal microbiota, digestive function and morphology. The first aim of the present thesis was thus to:

Investigate the bacterial profile of the allochthonous bacterial microbiota and the allochthonous and autochthonous bacterial microbiota of the distal intestine in Atlantic salmon fed either a plant based diet (sunflower, pea protein concentrate, soy protein concentrate) or a diet based on an animal by-product (poultry by-product, feather meal) at 200 g/kg inclusion.

Examine the effects of the alternative feed ingredients on the prevalence of diet induced enteritis of the distal intestine as well as steatosis (increased vacuolization) of the liver.

Examine the effects of using alternative raw materials on performance parameters such as growth, specific growth rates, feed intake and feed conversion rate as an indication of the acceptance of the feed as well as the utilization.

Investigate the fish's ability to adapt to the challenges of digesting new raw materials by examining the effect on some intestinal digestive enzymes both from the pancreas (trypsin, lipase) and the brush border membrane (leucine amino peptidase). Furthermore the utilization of the feeds are examined as indicated by plasma levels of triglycerides, free fatty acids, cholesterol, sodium, phosphorus, calcium and magnesium and various minerals (inorganic phosphate, calcium, magnesium and sodium).

Pathogen bacteria are problem for both wild and farmed fish, and disease outbreaks may be very costly for the farmer. The intestinal mucosa has an important barrier function to prevent invasion by pathogen bacteria. It is therefore important investigate if the use of alternative raw materials compromise this barrier function. Furthermore indigenous probiotic bacteria may alleviate or mitigate the effects of pathogen bacteria and it is useful to know if this is affected by the use of alternative feed ingredients. The second purpose of the thesis was thus to:

Use the *ex vivo* intestinal sac technique to study the effects of pea protein concentrate, extracted sunflower and hydrolysed feather meal on the adherence of indigenous *A. salmonicida* and *C. divergens* to the distal intestine of Atlantic salmon using qPCR.

Assess the use of *C. divergens* as a probiotic to protect against *A. salmonicida* by displacement and exclusion after subsequent exposure of intestinal tissue through use of ex vivo methodology

Use the *ex vivo* intestinal sac method to investigate if diets based on alternative feed ingredients may modulate the pathogenic effect of *A. salmonicida* or benign effects of *C. divergens* on the intestinal morphology of the mid intestine using transmission electron microscopy.

Examine the potential alleviating and/or mitigating effect of exposure of the distal intestine morphology to probiotic (*C. divergens*) prior to and after (*A. salmonicida*) in combination using transmission electron microscopy.

Methods and Methodological considerations

Feeding trial 1

The object of the first feeding trial was to evaluate the effect of plant and animal products on growth and intestinal morphology, digestive function and microbiota as well as utilization of feed in Atlantic salmon.

Feed formulation

For the first feeding experiment one control diet and five experimental diets were produced at EWOS Innovations' production facilities in Dirdal, Norway. The control diet contained 450 g/kg^{-1} Group 1 Scandinavian FM from Egersund whilst each of the experimental diets contained 250 g/kg^{-1} FM and an additional 200 g/kg^{-1} of the alternative protein sources. The alternative sources were extracted sunflower (ESF), soy protein concentrate (SPC), pea protein concentrate (PPC), poultry by-product (PBY) and hydrolysed feather meal (FeM). These raw materials were chosen based on the fact that they are commercially available to use in aquafeeds, are currently used in various inclusion levels in commercial diets and for which there is very little information available on the effect on intestinal microbiota and digestive physiology. The feeds were formulated with the aim to keep the protein to energy ratio constant and to supply the dietary need for protein but was not formulated to be isocaloric or isonitrogenous. By replacing 200 g/kg^{-1} of the FM with the alternative raw materials instead of any of the basal feed ingredients it is ensured that any results is a direct effect of the raw material replacement. Balancing the diets may result in masking of the true effect of the raw material and was for that reason not carried out. It should be mentioned however that the inclusion level of ESF and FeM used in the present study was higher than the highest recommended commercial inclusion level (2-4% and 4-5% respectively) suggesting that the results of the present study may not be comparable to commercial standards.

Trial facilities and fish husbandry

The fish feeding experiment was undertaken at EWOS Innovations' facilities in Dirdal Norway in 24 sea-water tanks with a total water volume of $0.5m^3$ and an ambient temperature of 10^oC throughout the 12 week feeding period. Fish were tagged using a passive integrated transponder (PIT) for identification. Prior to feeding the experimental diets the fish were allocated to the tanks and allowed 8 weeks to complete smoltification and acclimatize to their environment. Each tank was stocked with 41 unvaccinated Atlantic salmon (SalmoBreed) with an initial mean weight of 305g. Unvaccinated fish

were used to avoid the severe adherence of the viscera, a characteristic often related to the oil adjuvant component of vaccines and as such complicates the sampling of the GIT.

Experiment 2

The objective of the second fish trial was to evaluate the adherence of *C. divergent* and *A. salmonicida* to intestinal mucosa and effect on intestinal morphology in fish fed alternative raw materials.

Feed formulation

The feed formulation used for the second fish trial was the same as that for the first trial although the feeds were reproduced, which may result in batch variability. Also for this trial feeds were not isonitrogenously or isocalorically balanced. Based on the results of the first fish trial four raw materials were chosen to test in the second trial – PPC, ESF and FeM in addition to FM as control.

Trial facilities and fish husbandry

The first part of the study was undertaken at EWOS Innovations' facilities in Lønningdal, Norway in a trial lab containing eight sea-water tanks. The fish were fish were tagged using a passive integrated transponder (PIT) for identification and acclimatized for a period of four weeks on a commercial EWOS diet fed twice a day to satiation. Following acclimatization the fish were fed four experimental diets for a period of seven weeks prior to transfer to Institute of Marine Research, Bergen for the *ex vivo* bacterial exposure. The challenge was carried out two weeks after transfer to the challenge facility in Bergen.

Material sampling

Overview of the samples taken in feeding trial 1 and 2 is shown in Tables 1 and 2 respectively. Collection and handling of the various samples will be addressed in the following sections.

Gastrointestinal tissue for histology and enzyme activities

The main organ in focus in the present thesis is the GIT which is sectioned into oesophagus, stomach, pyloric intestine (the anterior most part of the intestine associated with PC; PI), mid intestine (MI) and distal intestine (DI) (Fig. 4). The reason the GIT was divided into compartments is that each compartment has a distinct role in the digestion of ingested food and it allows for an evaluation of the gradient from the anterior to the posterior GIT. Furthermore due to the large size of the pyloric and distal intestine these were for the enzyme activity assays divided in an anterior (PI1 and DI1) and a posterior (PI2 and DI2) section.

Table 1. Samples taken and analyses carried out in experiment 1

Species/ Strain	Mean size at trial start	Type of sample	Analysis	Paper no
Atlantic salmon *Salmo salar* L. SalmoBreed 2007-3 (Unvaccinated)	305 ± 69 g	Intestine and liver histology	H&E staining and LM evaluation	I
		Stripped digesta from distal intestine	qPCR for bacteriology of allochthonous bacteria	I
		Plasma	Metabolites	II
		Intestinal contents from PI1, PI2, MI, DI1 and DI2	Excreted mineral and bile salts, pancreatic enzymes	II
		GIT tissue from PI1, PI2, MI, DI1 and DI2	BBM enzyme activity	II

Table 2. Samples taken and analyses carried out in experiment 2

Species/ Strain	Mean size at trial start	Type of sample	Analysis	Paper no
Atlantic salmon *Salmo salar* L. SalmoBreed Lygrepollen (Unvaccinated)	328 ± 68 g	Mid intestine	McDowell fixed and staining for TEM	III
		Stomacher samples from distal intestine	qPCR for bacteriology of autochthonous bacteria	III

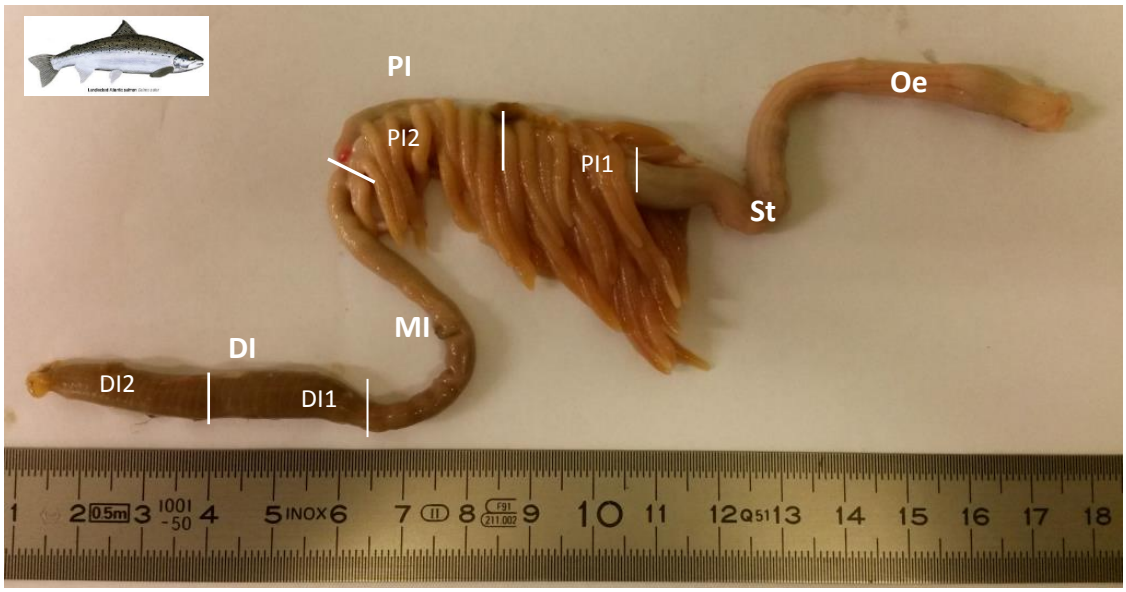

Figure 4. The complete gastrointestinal tract (GIT) of Atlantic salmon divided into several distinct compartments: Oesophagus (Oe), stomach (St), pyloric intestine (PI), mid intestine (MI) and distal intestine (DI). PI and DI are large compartments, as as gradient variations can be observed from the proximal to the distal end of these compartments they were divided in two (PI1 and DI1 form the proximal end; PI2 and DI2 from the distal end of the compartment).

Sampling for intestinal microbiota

Intestinal bacteria were sampled separately from the mid and distal intestine and also autochthonous and allochthonous community. Allochthonous bacteria were samples by stripping anaesthetized fish in pools of 10 individuals and freezing the samples in liquid nitrogen. Autochthonous bacteria were sampled by first excising the entire GIT, then flushing the intestine with physiological saline to avoid allochthonous bacteria contaminating the sample. Saline was used instead of freshwater to sustain ion balance in the intestinal tissue. The mid and distal intestine was then placed in separate plastic bags fitting the Stomacher® machine, weighted and 3 ml physiological saline added to each sample. Each intestinal compartment was then processed separately in a Stomacher® which dislodged autochthonous bacteria, releasing them into the saline solution. The solution was then frozen in liquid nitrogen to preserve the bacterial cells.

Analytical methods

This section will address the main methodology for evaluating the feed ingredients effect on intestinal morphology, microbiota and digestive function. In addition an ex vivo method to evaluate the adherence to and effects of a probiotic and a pathogen strain of bacteria on the intestinal morphology is described.

Histology using light and electron microscopy

Histology is a valuable tool in evaluating morphological structure of the organ and can be applied to any organ of choice. Inflammation due to dietary factors is frequently reported in the DI of Atlantic salmon (eg. saponin; van der Ingh *et al*., 1996; Knudsen *et al*., 2008) and therefore this study also focuses on DI to evaluate the effect of the alternative raw materials on enteric morphology. The tissue was fixated in 4% buffered formaldehyde to prevent autolysis or putrefaction, a fixative that was chosen over others due to its good penetrating abilities, fair price and good availability. One of the objectives in evaluating the morphology was to assess the migration of enteric eosinophilic granular cells, and therefore the sections were stained using Heamatoxylin & Eosin (H&E) which stains nuclei blue and counterstains eosinophilic components different shades of red, orange and pink. The sections were evaluated using light microscopy at 40x and 200x magnifications. Changes in the morphology were scored based on six parameters previously developed for the purpose of evaluating occurrence and severity of enteritis in Atlantic salmon fed soybean meal (Baeverfjord & Krogdahl, 1996; Urán *et al*., 2008). The characteristics that were scored were for mucosal fold height (MF) and lamina propria width (LP), submucosal height (SM) and the occurrence of eosinophilic granulocytes (EGC) in both submucosa and lamina propria, presence of goblet cells (GC) and status of supranuclear absorptive vacuoles in the apical end of the enterocytes.

Liver samples were fixed in 4% buffered formalin and stained using H&E. Liver was evaluated using light microscopy for degree of vacuolization indicating increased lipid storage or steatosis of the liver. The vacuolization were scored as a percentage of the total area within each hepatic cell as well as a percentage of total hepatocytes (Martinez-Rubio *et al*., 2013).

Transmission electron microscopy (TEM) is designed to evaluate the ultra-structural morphology of the cells and organelles of a tissue and is used to magnify characteristics from 1K to 500K times. Magnification at this magnitude is achieved by either coating samples with gold and heavy metals (such as in the case of scanning electron microscopy; SEM) or staining tissue sections with uranyl (such as in the case of transmission electron microscopy; TEM) to allow electrons to be reflected as they are fired at the sample. It is the reflection of the electrons that creates the image of the sample. TEM was used in the second trial to evaluate the effect of a pathogen and probiotic bacteria as well as alternative protein sources on the ultra-structural morphology of the intestinal mucosa.

Enzyme activity assays

Activity of certain enzymes may give an indication of the digestibility of nutrients such as protein, carbohydrates, and lipids. The basic principle of the enzyme activity assays is to use extracted enzymes to degrade enzyme specific enzymes then measure the product using spectrophotometry (Fig. 5). The reaction is allowed a specific amount of time (30 seconds) and is terminated by enzyme inhibitors.

The enzymes were extracted from either chyme or intestinal tissue that was frozen immediately following sampling to avoid change in enzyme. Pancreatic enzymes were isolated from intestinal content whereas BBM associated enzymes were isolated from tissue. Enzymes bound to the BBM were extracted by mechanically processing the samples using an Ultra Turrax® homogenizer prior to sonication to disrupt cell membranes and release of the cell contents. Enzyme extraction was carried out at 0°C to avoid changes in the enzyme concentration. Once the enzyme was extracted it was allowed to react with the substrate for 30 seconds before an inhibitor was added to the reaction to halt it. Table 3 shows an overview over the substrates used to evaluate enzyme activity. The amount of substrate bound to by the enzyme is quantified by spectrophotometry, which is then calculated to the amount of enzyme present. These methods were chosen as they are previously published methods.

Table 3. Enzymes activities analyzed for in the present study, source from which the enzyme was extracted and substrate used to facilitate enzyme activity. 1 enzyme unit (U) = 1 μmol min^{-1} LAP: Leucine aminopeptidase; C-LAP: Chyme associated LAP; PI: Proximal intestine; MI: Mid intestine; DI: Distal intestine.

Enzyme	**GIT compartment**	**Source**	**Substrate**	**Unit (**ΔOD)
Trypsin	PI, MI and DI	Chyme	Benzoyl-arginine-*p*-nitroanilide	U per mg DM
Lipase	PI, MI and DI	Chyme	*p*-nitrophenyl myristate	U per mg DM
C-LAP	PI, MI and DI	Chyme	L-leucyl-β-naphthylaminde	U per minute (U/min) U /min/mg
LAP	PI and DI	Tissue	L-leucyl-β-naphthylaminde	Unspecific activity (mmol/h/kg) Specific activity (μmol/h/mg)

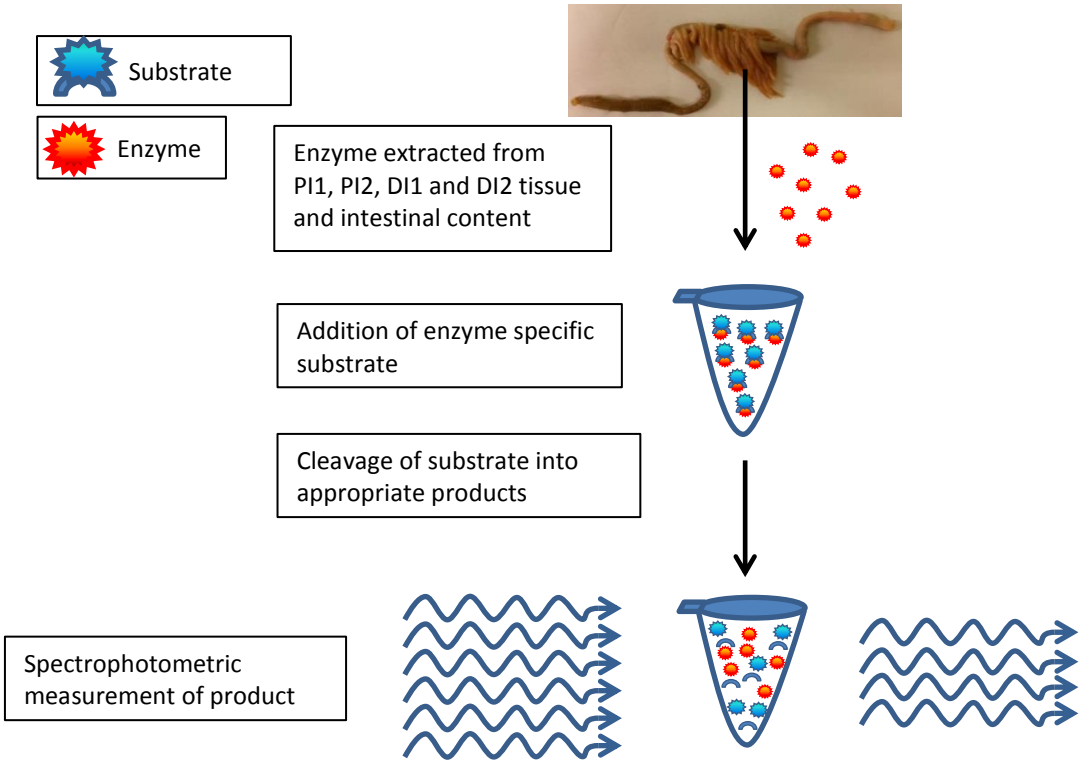

Figure 5. Simplified diagram of the general method for analysis of enzyme activities. Pancreatic and brush-border associated enzymes were extracted from intestinal content and intestinal tissue respectively. At the start of the reaction, the enzyme is added to a known concentration of substrate specific to each enzyme (see Table 7). The reaction is terminated with an enzyme inhibitor, and enzyme activity measures spectrophotometrically as a result of cleaved product.

Real-time quantitative PCR(qPCR)

Quantitative real-time PCR (q-rtPCR; qPCR) is a real-time measurement of the amplification of genomic material in the form of a fluorescent signal proportional to the amount of product made in the exponential phase of the reaction. Templates can include genomic or complementary DNA or single strand RNA. In many respects qPCR is similar to conventional end-point PCR (PCR). Both methods require primers (sequence specific or degenerate), nucleotides (dNTP's), enzyme, salt (MgCl) and template. The difference between the two methods is the fluorescent dye (SYBR green or probe) added to the mix in the qPCR analysis, which allows for a real-time measurement for fluorescence indicating replication. In conventional PCR the product is often measured qualitatively on gel electrophoresis however as the product is a measurement of the end-point product it is not possible to assess if the

amplification has run optimally for all cycles and as such compromised the amplification. Common uses of RT-qPCR include viral load, gene expression and in studying the microbial ecology of the intestine (Spanggaard *et al*., 2000; Holben *et al*., 2002; Ringø *et al*., 2000, 2001, 2006b; Verner-Jeffreys *et al*., 2003; Pond *et al*., 2006).

The cycling processes of qPCR are the same as for PCR (Fig.6). Following an initial heat activation of the enzyme, the DNA strands are separated in a denaturation step. Once the strands are separated, the primers hybridize to the complementary DNA strand in the annealing step and a new complimentary DNA strand is synthesised as the DNA polymerase attaches dNTP's that are complementary to the template in a 5' to 3' direction in the elongation step. As the new strand is synthesised and the two strands anneal, the SYBR green dye molecules hybridize to the minor grooves of the new double strand DNA (Fig. 7) and emits a fluorescent signal which is registered by sensors in the qPCR machine. The number of cycles in which denaturation, annealing and elongation are repeated is dependent on the amount of starting template.

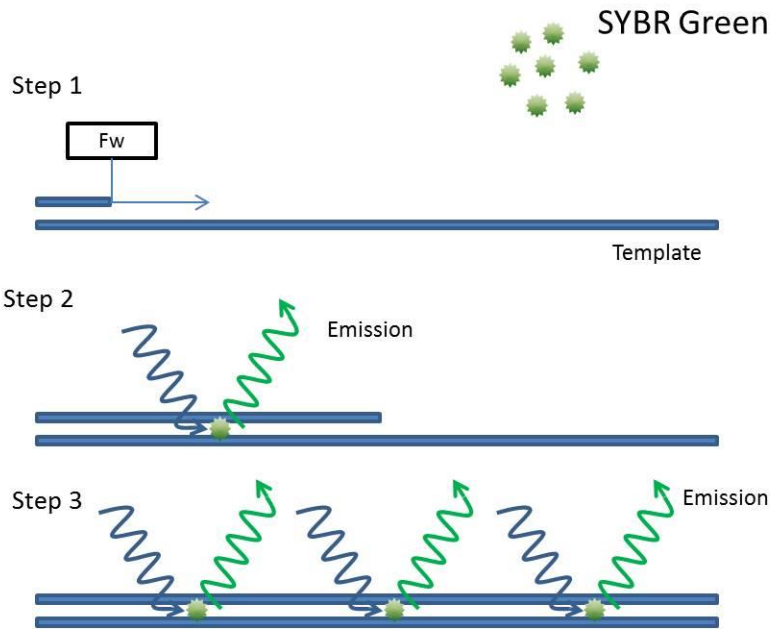

Figure 6. Amplification of DNA by quantitative PCR. Step 1: Primer, forward in this case (Fw), attaches at a specific transcript, and transcription of the DNA strand starts. Step 2: As the new strands start to elongate, SYBR Green, an intercalating fluorescent agent attaches to the minor grooves of the DNA and omits a fluorescent signal. Step 3: As the fluorescent product reaches a certain threshold level the fluorescent signal is detected.

In the present study, SYBR green was chosen as fluorescent dye. SYBR green is an intercalating nucleic acid stain and was used due to the unspecific nature of the primers. This means that the dye will bind un-specifically to all DNA and may provide a less accurate result (eg more chance of primer dimers being formed). Probes can only be used when the target is sequence specific.

Figure 7. Mode of action for the use of SYBR green to quantify amplification. As the DNA strands are separated by denaturation, primers are hybridized to the DNA strand and a new strand is synthesized by a DNA polymerase.

The target for the analysis of intestinal microbiota was in this study the small ribosomal unit (16S rRNA). The 16S rRNA is highly conserved within the bacterial genome but within this area there are nine hypervariable regions which are species-specific and can be used for identification of the bacteria (van der Peer *et al*., 1996; Chakravorty *et al*., 2007). In bacteria however the gene is often present in more than one copy therefore enumeration of bacteria based on number of 16S copies requires pure culture samples. As the objective of the present study is to characterize the microbial community of the Atlantic salmon, a more unspecific approach was taken.

The primers used in the present study have previously been developed with the purpose of characterising dominant groups of the intestinal microbiota of the Atlantic salmon. Coverage of the primers of the different families reported as part of the gut microbiota is shown in table 4. The primers were based on a sequencing study performed on the entire Atlantic salmon intestinal bacteria genome, and based on this study, dominant families with a certain similarity in their genome were pooled and a degenerate primer designed to capture the intended families. The primers were checked *in silico* against a public database by running a probe check using the Ribosomal Database Project (http://rdp.cme.msu.edu/).

Table 4. Coverage of the assay primers of the 10 degenerate primers pairs use in the preset study (paper 1).

Assay Name	***Phylum***	***Class***	***Order***	***Coverage***
Corynebacteriaceae	Actinobacteria	*Actinobacteria*	*Actinomycetales*	Family *Corynebacteriaceae*
Bacilli-like	Firmicutes	*Bacilli*	*Lactobacillaceae*	Families *Bacillaceae, Planococcaceae, Staphylococcaceae, Carnobacteriaceae Enterococcaceae*
Lactobacillaceae	Firmicutes	*Bacilli*	*Lactobacillaceae*	Families *Lactobacillaceae Leuconostocaceae*
Streptococcaceae	Firmicutes	*Bacilli*	*Lactobacillaceae*	Family *Streptococcaceae*
Peptostreptococcaceae	Firmicutes	*Clostridia*	*Clostridiales*	Family *Peptostreptococcaceae*
Mycoplasmataceae	Tenericutes	*Mollicutes*	*Mycoplasmatales*	Family *Mycoplasmataceae*
β – Proteobacteria	*β – Proteobacteria*	*β – Proteobacteria*		
Pseudomonadaceae/ Xanthomonadaceae	*γ - Proteobacteria*	*γ - Proteobacteria*	*Pseudomonadales/ Xanthomonadales*	Families *Pseudomonadaceae* and *Xanthomonadaceae*
Enterobacteriaceae	*γ - Proteobacteria*	*γ - Proteobacteria*	*Enterobacteriales*	Family *Enterobacteriaceae*
Vibrionaceae	*γ - Proteobacteria*	*γ - Proteobacteria*	*Vibrionales*	Family *Vibrionaceae*

Location of the primers in the source species' genome is shown in Table 5.

Table 5 Species source and primer location of the 11 specific assays used in the present study.

Assay name	Source species	Forward primer	Reverse primer	Annealing T
Corynebacteriaceae	*Corynebacterium effenciensis*	576-594	818-834	60
B- proteobacteria	*Burkholderia glumae*	560-576	872-887	60
Pseudomonadaceae/ Xanthomonadaceae	*Stenotrophomonas maltophilia*	534-552	821-840	60
Enterobacteriaceae	*Escherichia coli*	818-838	877-894	60
Mycoplasmataceae	*Mycoplasma microti*	666-685	757-774	58
Vibrionaceae	*Photobacterium phosphoreum*	562-578	854-874	60
Bacilli-like	*Kurthia zophii*	616-631	876-890	58
Lactobacillaceae	*Lactobacillus crispatus*	347-365	676-692	60
Streptococcaceae	*Streptococcaceae coccus bovis*	523-542	729-746	60
Peptostreptococcaceae	*Peptostreptococcaceae anaeribus*	608-629	723-739	60

Initially, the first knowledge of the intestinal microbiota in fish has come from cultivation studies where medium was used to grow bacteria from serially diluted samples from intestinal contents or tissue from GIT. This has led to knowledge and understanding of the aerobe and facultative anerobe bacteria in GIT of various fish species (Cahill, 1990; Ringø *et al*., 1995; Spanggaard *et al*., 2000). Although cultivation is important in that the method provides information on the requirements of the bacteria (nutrients, temperature, pH optimum etc), which is essential for the classification of new species (Suau *et al*., 1999; Hovda *et al*., 2007), identification and phylogenetic characterization has leaped ahead with the use of cultivation-independent genetic molecular methods. There are however strengths and weaknesses with both methods one should be aware of. The advantages and disadvantages of culture methods (Table 5) and molecular methods (Table 6) have been summarized by Furrie (2006) and some biases can be revealed also in this study.

When applying molecular based methods, one of the advantages is in the use of a genomic template, that the method not necessarily relies on viable organisms. By freezing samples in liquid nitrogen all cellular activities are either stopped or slowed, preserving the genetic material and one can avoid bias from taking the microbes from its natural environment. It also excludes the bias of losing unculturable bacteria. When sampling for autochthonous bacteria however, this bias may be relevant. Sampling for autochthonous bacteria require that the intestine is excised, rinsed and then processed in a Stomacher® before the bacterial sample is flash frozen. This means that the bacteria are exposed to the 'outside' environment for up to several minutes before it is frozen which is likely to affect at least obligate

anaerobe bacteria. Although freezing samples preserve the genetic material, it also represents a potential source of loss of genetic material due to lysis of the cells.

Table 6. Advantages and disadvantages of using culturing methods to analyze intestinal microbiota as shown by Furrie (2006)

Advantages	Disadvantages
Relative inexpensive	Slow, time consuming and labor intensive
Widely available	samples require immediate processing
Allows quantification of bacterial populations	Restricted to culturable organisms
Can provide good indication of ecosystem complexity, if carried out by skilled and experiences microbiologists	Extensive expertise and specialized equipment need to isolate strict anaerobes
Physiological studies are possible	Selection of growth media can greatly affect results. Not all viable bacteria can be recovered
Biochemical studies are possible	Once isolated, bacteria then require identification using a number of techniques

Table 7 Advantages and disadvantages of using molecular based techniques to study intestinal microbiota as summarized by Furrie (2006)

Advantages	Disadvantages
High throughput and relatively short learning time for most techniques	Difficult to standardize extraction of genetic material from each species equally. Severe bias possible in mixed populations
Anaerobic handling and expertise not required	Can be very expensive
Samples can be frozen for later analysis	Selection of primers and probes can introduce severe bias in detection
DNA can be transported easily between laboratories	Impossible to model ecosystem
Unculturable species are detectable	Some methods are very insensitive
In theory, only one molecule of target DNA is needed for quantification	Many methods are not quantitative so confirmatory analysis is needed

Cultivating bacteria using tradition medium growth is important to gain knowledge on the physiological and biochemical properties of bacteria. A previous study has indicated however that intestinal microbiota has a high portion of unculturable bacteria. In a study by Huber *et al*. (2004) it was estimated that only 11-50% of the intestinal bacteria in fish were cultivatable, and in one case only 2% of the total

bacteria were grown. These studies indicate that the level of cultivatable bacteria in fish intestine may be variable and that the total number of bacteria may be grossly underestimated using cultivation methods alone. As the objective of the study was to characterize the enteric microbial community, the method of choice was qPCR.

Molecular methods are however not without bias, present study included. In using molecular based methods to analyse for enteric microbiota it is common practice to pool samples to avoid inter-individual variation (Hovda et al., 2007; Navarrete et al., 2013) which is considered a constraint in microbial studies in fish (Spanggaard et al., 2000). By pooling samples, knowledge on the individual complexity is lost. Furthermore, as the samples are frozen, there is an increased risk of loss of genetic material from Gram negative bacteria. Another potential source of bias is the qPCR, where a lot of pipetting may be a source of human error, as well as suboptimal qPCR analysis due to annealing temperature, salt concentration or primer design.

Ex vivo methodology

The intestinal sac method is what the name implies – using the intestine as a sac to expose the mucosal lining to organisms which may affect the morphology, permeability or absorption abilities of the intestinal tissue. *Ex vivo* (Latin, meaning 'outside the living') is in this study the removal of the intestine from the fish and using this tissue only to investigate the effect of bacteria suspended in a solution. Study of the pathogenicity of invasion of pathogen bacteria often implies subjecting the host animal to painful disease outbreak and researchers has therefore developed *ex vivo* methodology to avoid subjecting the fish to the pain.

The intestinal sac method has been described in several studies thus far (Ringø *et al*., 2004; 2010; Harper *et al*., 2011; Kristiansen *et al*., 2011; Løvmo Martinsen *et al*., 2011; Salma *et al*., 2011). The principle behind the intestinal sac method is to isolate the intestine and expose it to only one treatment in order to relate the changes to that treatment alone. When using fish as a whole to investigate intestinal response the potential effectors may be multifactorial. Furthermore, when administrating bacteria to an aqueous organism it is difficult to quantify how much bacteria reaches the intestine. By excising the intestine and using it as a sack, or pouch, to incubate bacterial solutions it is possible to correlate the response of the intestine to the specific bacteria.

Prior to doing the challenge bacterial suspensions were made of *Carnobacterium divergens* and *Aeromonas salmonicida* ssp. *salmonicida.* The strain of *C. divergens* used in the present study (Lab01) was originally isolated from the DI tract from Atlantic salmon fed a commercial diet (Strøm, 1988). The bacterium has been identified on the basis of 16S rDNA sequence analysis and amplified fragment length polymorphism (AFLP^{TM}) fingerprinting (Ringø *et al*., 2001). The pathogen used in the study was

Aeromonas salmonicida ssp. salmonicida strain VI-88/09/03175 from the culture collection at the Central Veterinary Laboratory in Oslo, Norway. The bacterium is a known pathogen to salmonids (Samuelsen *et al*., 1998) and has previously been shown to use the GIT as a portal of entry (Jutfelt *et al*., 2006). Both bacteria were cultured in tryptic soy broth added 5% glucose for 48 hours at 12°C. Exposure dose was measured by plate counts of viable colony forming units (CFU) and the exposure dose for *C. divergens* was 3.2×10^7 CFU ml^{-1} and 8.6×10^6 CFU ml^{-1} for *A. salmonicida*.

Briefly, the entire intestine, from behind the last pyloric ceaca to the anus was removed aseptically from fish fed ESF, PPC, FeM or FM based diets. The intestine is then rinsed by flushing the intestine with sterile physiological saline, a step that was carried out to remove remaining allochthonous bacteria from the intestine. The distal end of the tube is then sealed, creating a sack of the intestine which then can be filled with a bacterial solution. Once the intestinal sac is sealed it is incubated for a period of 60 minutes. By limiting the incubation time to 60 minutes, the morphological changes in the tissue as result of autolysis will be minimal and may therefore be caused by the bacteria. In studies where the interaction between two bacteria species are used, such as the present trial, the intestine is first incubated with one bacteria, opened, rinsed and then incubated with the second bacteria. The intestine is incubated with each bacteria for a period of 30 minutes so as to not exceed the 60 minute limitation. During the incubation the sealed intestine is immerged in saline in a Falcon tube, and the tube submerged in water with the same temperature of the holding water of the fish.

The purpose of exposing the intestinal mucus to various bacteria is to investigate the adherence of bacteria to the intestinal lining. This proposes a problem of harvesting the bacteria for analysis. One option is to open the intestinal longitudinally and scraping the mucus. This type of sample however contains a substantial amount of mucins which may disrupt the extraction and analysis of the bacteria. To avoid this, a Seaward Stomacher® was used to detach adhered bacteria in the present study. The Stomacher® machine works in that inside there are two paddles that beat the intestinal sample and thus dislocates the bacteria, which are then released into a saline solution.

A comparison of three different *ex vivo* methods is shown in Table 7. The difference between this method and other *ex vivo* methods such as everted sleeve or the using chamber, and a *limitation* of the intestinal sac method, it that the other two methods also includes oxygenating the tissue, and as such the tissue can be incubated for a longer period of time.

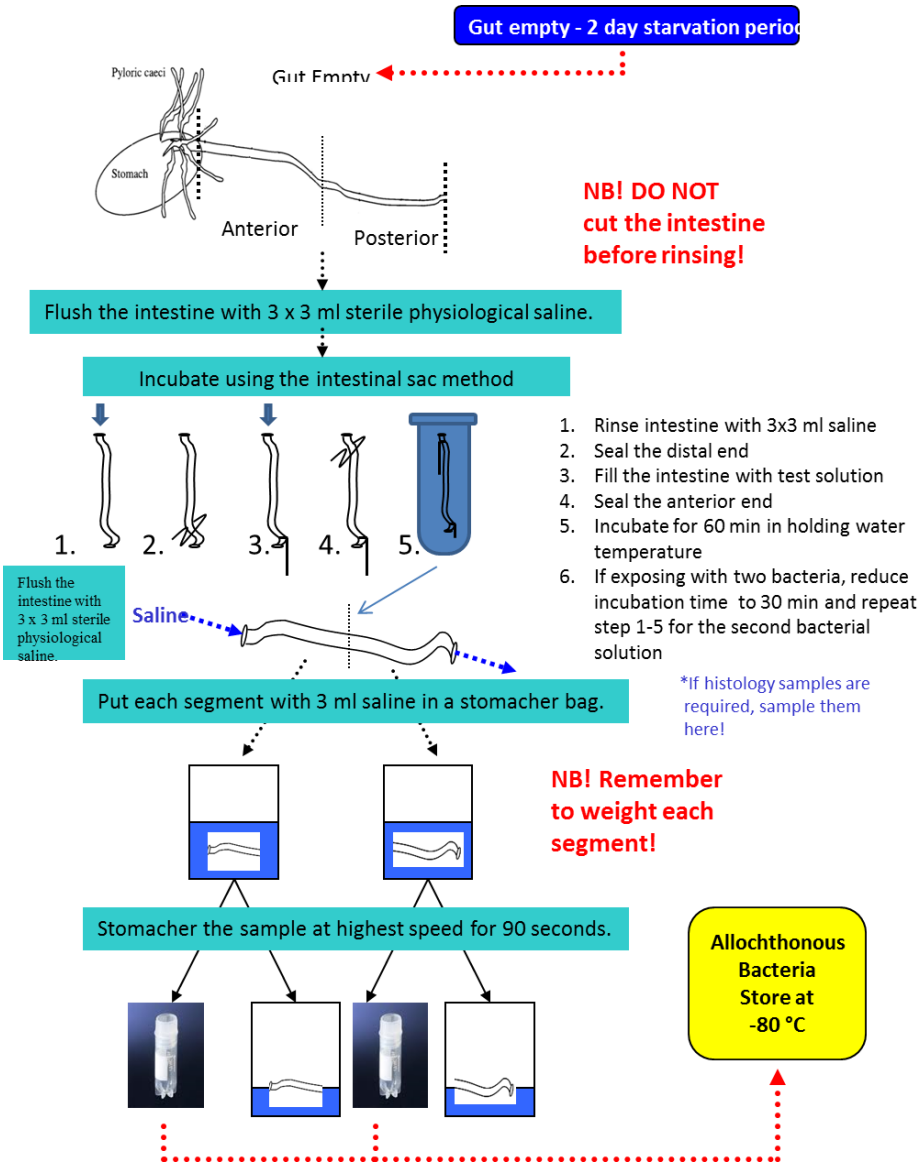

Figure 8. Schematic workflow of the intestinal sac method. The intestine is excised aseptically from the ventral cavity of the fish then rinsed using sterile physiological saline to remove allochthonous bacteria. The intestine is then sealed in the distal end, and filled with bacterial solution. The anterior end is immediately sealed and the whole 'sac' immerged in physiological saline and incubated in the same temperature as the fish holding temperature. Incubation time for exposure with a single bacterial solution is 60 minutes. Incubation time for using two different bacterial solutions is 30 minutes with each solution. When exposing the intestine to two solutions the sac is opened and rinsed in between treatments. Following incubation, the intestine is rinsed and the mid and distal cavities processed separately in a Seaward Stomacher machine which detached autochthonous bacteria. Bacterial solutions were stores at -80 degrees until analysis.

The intestinal sac method is a relatively cheap method that requires very little methodological knowledge and has a high throughput. As it does not require any specialized equipment it can in many cases easily be carried out in the facilities that hold the fish. There is however potential for more data output from the other methods. The presence of electrodes in the Ussing chamber allows measurements of transepithelial resistance and with reservoirs of Ringers solution on either side of the tissue allows measurements of absorption and bacterial translocation. As the focus of the present thesis was to investigate bacterial adherence, the intestinal sac method was chosen as the method of choice.

Table 8 Advantages and disadvantages of three *ex vivo* methods for studying bacterial adherence and translocation, morphology and absorption of the intestine –Intestinal sac, Usssing chamber and everted sleeve.

	Intestinal sac	Ussing chamber	Everted sleeve
Labour	High throughput	Labor intensive	Labor intensive
Equipment	Relatively inexpensive, no special equipment necessary	Needs a relatively expensive Ussing chamber and accessories	Relatively inexpensive, some specialized equipment needed
Complexity	Simple, widely available	Needs qualified and experiences personnel	Needs qualified and experienced personnel
Data output	Bacterial adherence and intestinal morphology	Transepithelial resistance, absorption, bacterial translocation and morphology	Absorption and morphology
Tissue oxygenation	Not oxygenated	Oxygenated	Oxygenated

There are several reasons for choosing an *ex vivo* method. By excising the tissue, in this case the intestine, rinsing it and exposing it to a bacterial or compound solution one is assured that the effect seen in the tissue is caused by the incubation solution, and also that each intestine is exposed to the same amount/number bacteria per ml. Also the method allows for a reduction of the number of experimental animals and negates the need to subject the animal to the stress and pain of being exposed to pathogen bacteria.

Statistics

The present study includes several different types of data sets which require that different statistical methods are applied for correct analysis of the data. The complexity of the data was decisive for which method was applied. An overview of the methods used in the present trial is shown in Table 9.

Table 9. Use of statistical methods in the present study.

Data	Software	Analysis	Paper no
Microbial community composition	R language[1]	General linear model using diet as predictor	I
Growth	R language[1]	Multilevel modelling on repeated measurements for each tank and using feed and days as predictors	I
Intestinal scores	R language[1]	Ordinate logistic regression	I
Liver vacuole scores	R language[1]	Model for multinomial response using Markov Chain Monte Carlo package	I
Organ weight	R language[1]	General linear modelling using diet as predictor	I
Short chain fatty acids	R language[1]	General linear modelling using diet as predictor	I
Enzyme activity	R language[1]	General linear model using diet as predictor	II
Bile salt concentration of chyme	R language[1]	General linear model using diet as predictor	II
Dry matter and mineral content of chyme	JMP[2]	ANOVA and Tukeys	II
Plasma metabolites	R language[1]	General linear model using diet as predictor	II
Adherence of *C. divergens* and *A. salmonicia*	R language[1]	General linear models. Effect of diet, treatment and interaction of these was analysed using likelihood tests on nested models	III
Electron microscopic evaluation	R language[1]	Spearman rank correlation, Multilevel binomial regression model	III

[1] R language, 2012

[2] Zar, 2010

Summary of Results

Feeding trial 1

The results are addressed in the following papers:

"Alternative dietary protein sources for Atlantic salmon (Salmo salar L.) effect on intestinal microbiota, intestinal and liver histology and growth."

"Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (Salmo salar L.): Digestive and metabolic investigations"

Performance

Growth (g and % compared to FM control group), feed conversion rate (FCR) and specific growth rate (SGR) is shown in Table 10. Growth and cumulative feed intake decreased significantly with the use of ESF whilst cumulative feed intake increased in fish fed PPC. FCR was not modulated significantly with use of alternative raw materials. The good growth in the present trial indicates that the feeding trial was of good quality.

Table 10. Wight gain (g and %) compared to FM control group, cumulative feed intake (CFI), feed conversion rate (FCR) and specific growth rate (SGR) of fish a FM based control diet and experimental diets containing 200 g/kg alternative protein sources

	FM	ESF	PPC	SPC	FeM	PBY
Weight gain (g)	257[a]	193[b]	284[a]	227[a]	234[a]	268[a]
Weight gain (%)		-24,9	10,5	-11,7	-8,9	4.3
CFI (g/fish)	193[a]	171[b]	217[c]	183[a]	182[a]	196[a]
FCR[¤]	0,75	0,89	0,76	0,81	0,78	0,73
SGR[¥]	6,93	6,58	7.06	6,78	6.81	6,98

[¤]FCR = Weight of feed consumed/live weight gain

[¥]SGR =100(ln final wt – ln start weight/days of experiment)

*Significant change (p=0.05) compared to FM control group

Histology

Histologically there were no signs of structural changes or damage to the intestinal morphology in any of the feeding groups and enteritis score in all individuals were low. In general submucosa (SM) and lamina propria (LP) were narrow, and consisted of loose connective tissue and some scarce erythrocytes easily recognizable by their cell nuclei. Some scarce eosinophilic granular cells (EGC's) were observed in the submucosa, but were not observed in the lamina propria. The effects of the alternative feed ingredients on the mucosal folds (MF) were negligible, and were in general tall and slim although some folding and

branching were observed due to the orientation of the tissue during cutting. Goblet cells (GC) varied somewhat between individuals but in general number of cells were low in the basal parts of the mucosal folds and increased in density towards the upper part of the fold The upper 'tip' of the fold was usually the part of the mucosal most dense in goblet cells. Supranuclear vacuoles (SNV's) are highly variable and may seemingly vary independent on for example diet. In the present study the scores for this parameter was low indicating basal size SNV's with very little reduction in size.

Liver was evaluated using light microscopy for degree of vacuolization indicating increased lipid storage or steatosis of the liver. In the present study the examined livers showed no signs of increased vacuolization in the hepatocytes and the percentages of affected hepatocytes were then also naturally low.

Intestinal microbiota

Total bacteria

The results revealed that the total allochthonous bacteria were significantly increased with the inclusion of extracted sunflower (ESF), feather meal (Fe) and poultry by-product (PBY) and insignificantly with the use of pea protein concentrate (PPC) and soy protein concentrate (SPC) compared to the FM control. Total autochthonous bacteria in the proximal intestine remained unmodified following feeding with the alternative feed ingredients, whilst in the distal intestine total autochthonous bacteria significantly increased following use of PBY and PPC.

Response of the allochthonous bacteria to alternative protein

qPCR analysis of the bacterial community revealed that *Corynebacteriaceae* dominated the allochthonous bacteria of the distal intestine and *Lactobacillaceae* dominated the autochthonous microbiota of both proximal and distal intestine in all diet groups, however the levels were significantly affected by which raw material had been used. In the allochthonal bacterial community use of ESF significantly increased the level of *Corynebacteriaceae* and *Lactobacillaceae* whilst concomitantly decreasing *β-proteobacteria, Bacilli, Peptostreptococcaceae* and *Streptococcaceae*. Use of FeM in the test diet resulted in significantly increased *Corynebacteriaceae, Lactobacillaceae, Streptococcaceae* and *Vibrionaceae*. Addition of PBY to the experimental diets gave a significant increase in *Corynebacteriaceae* and a simultaneous decrease in *β-proteobacteria, Bacilli, Peptostreptococcaceae* and *Streptococcaceae*. Use of PPC resulted in a significant increase in *Vibrionaceae* whilst the addition of SPC the diets did not modulate any of the investigated allochthonal bacterial groups.

Response of the autochthonous bacteria to alternative protein

In the proximal intestine *Lactobacillaceae* dominated the autochthonous bacterial community in all groups but was only significantly increased with the use of SPC. *Corynebacteriaceae* increased significantly with

use of PBY, and FeM resulted in an increased level of *Pseudomonadaceae/Xanthomonadaceae*. *Vibrionaceae* decreased in all of the experimental feeding groups albeit not significantly using SPC. *Enterobacteriaceae* and *Streptococcaceae* both increased significantly with the addition of PPC and SPC, whilst use of PBY also increased *Streptococcaceae*. *Bacilli* increased following addition of SPC to the diets whilst *B-proteobacteria*, *Mycoplasmataceae* and *Peptostreptococcaceae* remained unmodified after feeding with the alternative raw materials.

In the distal autochthonal community all the significant changes observed were increases of the affected bacterial groups. Addition of ESF to the experimental diets resulted in an increase in *Streptococcaceae,* whilst use of SPC modulated the level of *Streptococcaceae* and *Bacilli*. Use of PBY in the diet increased the level of *Pseudomonadaceae/Xanthomonadaceae* and *Peptostreptococcaceae,* whilst showing a concomitant increase in *B-proteobacteria, Vibrionaceae, Lactobacillaceae, Streptococcaceae* and *Peptostreptococcaceae* following use of PPC. FeM did not modulate any of the bacterial groups on the distal intestine compared to the FM control group.

Short chain fatty acid production

Gas chromatographic mass spectrometry (GCMS) analysis of SCFA revealed a promising correlation between the addition of plant protein to the diets (PPC, SPC or ESF) and the increased production of acetic acid indicating a potential of the intestinal microbiota to utilize the carbohydrates in the plant materials. PPC resulted in the highest measured level of acetic acid with a concentration of 1.26 nM, whilst addition of ESF and SPC resulted in 1.10 nM and 1.06 nM respectively. In comparison fish fed the control FM diet had 0.80 nM, FeM had 0.91nM and PBY had 0.80 nM in the intestinal content. Other volatile fatty acids were also analysed for (butyric acid, isobutyric acid, isovalericacid, lactic acid, 2-metylbutyric acid, propionic acid and valeric acid) however there were not detected over the detection limit of 0.05 nM.

Organosomatic index

The organosomatic index of stomach (St) was not affected by use of alternative raw materials and liver showed significant decrease in weight only with use of FeM compared to the FM fed control fish. The organosomatic index of pyloric (PI)-, mi (MI),- and distal intestine (DI) significantly increased with the use of FeM and SPC compared to that observed in FM fed fish, whilst the weight of DI was increased in fish fed PBY.

Dry matter content of chyme

Dry matter (DM) content of chyme is an indication of the prevalence of diarrhoea and as such is an indication of the digestibility of the feed. Evaluation of the DM content measured in the chyme from the various compartments showed no significant effects of alternative feed ingredients in the proximal regions compared to FM, ie. the stomach (St) and pyloric intestine (PI1 and PI2). In the mid intestine (MI), DM was significantly increased compared to the FM control following feeding with ESF and SPC, whilst in the distal intestinal region (DI1 and DI2) SPC significantly decreased DM in DI1 and DI2, FeM decreased the DM in DI2. PBY on the other hand increased the DM content of both distal regions. Replacement of marine protein with PPC did not significantly affect DM compared to FM in any of the measured compartments.

Element content of chyme

The elemental concentrations compared to the DM content of the diet, generally increased in the St, and for some elements were also higher in the PI and other intestinal regions. This was particularly evident for sulphur (S) which showed a three-seven fold increase in the PI and MI regions and a two-fold increase in the DI regions. Compared to the FM control group, PPC was the only diet to significantly increase S in the regions PI1, PI2, MI and DI1 and numerically increase the element in DI2. Nitrogen (N) was also significantly altered by the use of alternative raw materials, and both ESF and FeM resulted in significant increases in all regions. PBY on the other hand decreased the N content in PI1, PI2, MI and DI2.

Pancreatic and brush-border membrane enzyme activities

Various effects were observed of the alternative raw materials on the intestinal enzyme activities. For the pancreatic enzymes trypsin and lipase, trypsin was significantly decreased in DI1 and DI2 by ESF and in MI by SPC. Lipase was concomitantly increased in DI1 by PPC, MI, DI1 and DI2 by FeM and DI1 and DI2 by PBY.

Leucine aminopeptidase (LAP) is a brush-border membrane (BBM) associated enzyme, however in the present study the enzyme was also measured in the chyme as an indication of sloughing of the mucosal layer. By adding ESF to the diet, activity of chyme associated LAP (C-LAP) decreased in all compartments. PPC also resulted in decreased activity in DI1, SPC in PI1 and PBY in DI2. FeM on the other hand increased the activity significantly in MI and DI1. Also for the BBM-LAP use of SPC and FeM resulted in a significant increase in the total enzymatic activity in PI, and in DI of fish fed FeM. The combined activity of PI+DI increased significantly only in FeM fed fish. The specific activity of the BBM-LAP on the other hand increased in the PI of SPC fed fish, and concomitantly decreased in the DI of

the same fish. FeM fed fish showed a two-fold increase in DI, but no significant alteration in the activity in PI.

Plasma minerals and metabolites

Plasma minerals and metabolites were evaluated as an indication of the utilization of the feed. From the results it is apparent that cholesterol is decreased following use of ESF, SPC and FeM, whilst feeding with PBY results in a significant increase in plasma triglycerides and free fatty acids. Glucose was only significantly modulated by FeM.

Feeding trial 2

The results are addressed in the following paper:
"Probiotic and pathogen ex vivo exposure of Atlantic salmon (Salmo salar L.) intestine from fish fed four different protein sources. "

Histology following ex vivo exposure to bacteria

Transmission electron microscopic evaluation of the mucosal structure of fish fed ESF, PPC, SPC, FeM or PBY and exposed to saline shows a normal looking mucosa with health undamaged enterocytes and microvilli. Mitochondria were generally dense and clear, a usual sign of health mitochondria. As such the alternative raw materials did not alter the intestinal morphology.
Transmission electron microscopic (TEM) evaluation of intestines exposed to *C. divergens* from fish fed FeM, PPC and ESF revealed that intestinal structure appeared normal and similar to that of the FM fed saline exposed control group. Feeding PBY however results indicate an apparent improvement compared to that of the FM fed saline exposed control group. The intestines show a general reduction in intraepithelial leukocytes (IEL's), less debris and a higher frequency of healthy mitochondria.
Intestines exposed to *A. salmonicida* showed clear signs of damage: disrupted microvilli, damaged enterocytes and cell debris present in the lumen. These modulations were observed in low frequencies in fish fed FM, PPC and ESF. In fish fed FeM however the morphological changes were observed in medium frequency indicating a worsening of the effect of the pathogen by raw material. Following feeding with ESF and exposure to *A. salmonicida* an aggregation of rodlet cells were observed which was not observed in any of the other groups.

Adherent intestinal microbiota

Results show that using ESF, PPC, SPC, FeM or PBY in the treatment diet and exposing the intestine to sterile saline endogenous levels of *C. divergens* and *A. salmonicida* were not modulated. Exposure of the

intestine to C. divergens alone or to the combination treatments (*C. diverges* + *A. salmonicida*; *A. salmonicida* + *C. divergens*) show significant increase in adherent *C. divergens*. Following exposure to *A. salmonicida* alone did not modulate the adherence of *C. divergens*. Use of alternative raw materials prior to the intestinal sac exposure did not significantly affect the adherence and there were no interaction between exposure treatments.

Exposure of the intestine to *A. salmonicida* alone and *A. salmonicida* prior to *C. divergens* significantly increased adherence of *A. salmonicida* compared to the FM fed saline exposed control group. Exposure of *C. divergens* prior to *A. salmonicida* resulted in a numerical insignificant increase of the pathogen. Diet did not affect the adherence of *A. salmonicida* and there were no interactions between diet and treatment exposures.

Discussion of main results

Reliance on FM has steadily decreased with the increased use of alternative feed ingredients in aquaculture feeds. It is common knowledge that as FM is replaced with alternative feed ingredients, various effects are observed in the fish, and sometimes optimizing the replacement to sustain appropriate growth may be challenging in that nutrient profiles, digestibility, presence of ANF's, and fatty acid profiles are different from FM.

At the start of this project there was very little information available on the effect of alternative feed ingredients such as the ones used in this present thesis, despite the fact that all of the raw materials are in use in various degrees in commercial diets today. It is commonly accepted that the composition of a diet affects the intestinal microbiota and host in different manners, and the raw materials tested in this study were no exception. As the use of alternative raw materials is constantly increasing in both volume and variety, it has become important for the feed industry to look closer into the effects observed in the host. As such the object of the study was to increase the knowledge of the effect of extracted sunflower, soy protein concentrate, pea protein concentrate, feather meal and poultry by-product on the intestinal microbiota, digestive physiology and bacterial adherence of pathogen and probiotic bacteria in farmed Atlantic salmon.

Can the fish utilize and grow on the chosen alternative raw materials?

The answer to that question is 'yes'. The paramount question for a fish farmer however, whose income is dependent on good growth and reasonably priced feed, is 'how well'. In the present study a variety of raw materials which might be considered 'good or bad' ('good' being a raw material which provide growth equal to FM and 'bad' being a raw material resulting in reduced growth compared to FM) was included in diets. The feeds were not balanced according to protein content or amino acid profile (isonitrogenous) nor energy content (isocaloric) but within the nutritional requirements of the salmon in order to induce a more pronounced effect of the different alternative feed ingredients.

In the present thesis growth was measured as an indication of feed utilization, the feeds ability to provide the fish with the required nutrients, but also as an indication of the quality of the trial. In suboptimal trial conditions variations in growth may be due to other factors than the feed (eg stress due to temperature, oxygen variation, handling stress). Throughout the trial the growth trajectory of the FM control group, as well as for the experimental groups, increased evenly giving a good indication that the trial was of good quality, meaning that the fish were not subjected to factors other than feed which could affect the growth.

The raw material which resulted in the best growth in the present trial was PPC, which grew 10.5% better than the FM control group. At least three separate studies, including the present study have shown that PPC results in comparable or even improved growth, compared to FM (Øverland *et al.*, 2009; Hansen *et al.*, 2011, paper 1) indicating that there are other non-biological reasons for the low commercial inclusion of ~0.4%. The significant increase in feed intake following feeding with PPC however, indicate that more feed is needed to obtain the improved growth, and even though FCR is not different from that observed in FM fed fish, it may not be cost effective. As it is outside the scope of the present thesis to investigate cost-effectiveness of the feed ingredients, an economic study into cost and use is merited to investigate this further. Increased feed intake also indicates favourable palatability and as such, PPC may be a useful feed ingredient to mask other unpalatable feed ingredients, eg medicines.

The low growth of fish fed SPC in the following study was however unexpected. SPC is also considered a good raw material for use in feed for Atlantic salmon, and is frequently used in levels up to 20% in commercial diets. In the present thesis however the group fed SPC grew 11.7% less than the FM control group. In contrast to the present study previous studies have shown that replacing up to 100% of the FM with soybean concentrate for rainbow trout (Kaushik *et* al., 1995; Stickney *et al*., 1996) and inclusion levels up to 56% for salmon (Olli *et al*., 1994; Bureau *et al*., 1998) did not affect growth. It is therefore uncertain why the inclusion level used in the present study had such negative effects on growth, and more in depth study into uses of high SPC may be merited.

Inclusion of ESF significantly reduced growth and resulted in 25% less growth than the FM control group. Sunflower contains very few of the a ANF's found in oilseeds (eg lectins, phytic acids, phytoestrogens) but it is known to contain low levels of sponin, chologenic acid (Francis *et al*., 2001), have a high fibre content and a deficient amino acid profile (Dong *et al.,* 2000). Furthermore, a significantly low feed intake may indicate a palatability issue. Saponin is bitter, and as such make the feed itself bitter to the taste which fits well with the reduced cumulative feed intake (total feed eaten per fish) shown by this group. Another reason may be through growth inhibition of saponin itself, which is one of the negative effects of this ANF. At 20% inclusion rate the raw material used in the present study is much higher in ESF than the commercial level of 2-4%, however Sanz *et al*. (1994) reported satisfactory growth of rainbow trout at 40% inclusion rate, and tilapia (*Sarotherodon mossambicus*) apparently grew well on 75% inclusion of sunflower (Jackson *et al*.,1982). These studies indicate that salmon may have a lower tolerance for ESF than other species which merits further study in order to increase the inclusion rate of ESF in commercial diet. Although FCR for ESF did not increase significantly according to the chosen statistical method at p=0.05, FCR of 0.9 using ESF in comparison to 0.75 using only FM is still a substantial increase in feed usage, which is important to the farmer.

Animal proteins are generally considered promising protein sources, however quality and as such digestibility has been variable. In the present study, inclusion of FeM and PBY resulted in 8.9% decreased and 4.3% increased growth respectively compared to the FM control group (paper 1) indicating differences in digestibility and utilization of the raw materials. Digestibility of PBY has through a previous digestibility study shown to be somewhat higher than FeM (76-87% *vs* 87-91%) (Bureau *et al*., 1999) which may partially explain the differences in growth between the animal product groups. However as neither group was significantly different from the FM group, the differences are most likely indistinguishable. Previous studies show that use of FeM and PBY for rainbow trout (Fowler, 1991) and chinook salmon (*Oncorhynchus tshawytscha*) (Fowler, 1990) result in growth comparable to FM based diets, although inclusion of amino acids methionine and lysine are necessary at higher inclusion levels of the raw materials (Steffens, 1994) indicating poor utilization of the amino acids at higher inclusion levels. The experimental diets used in the present study were not balanced according to amino acid profile in order to evaluate the true effect of the feed ingredient, and as such may be a factor limiting growth in fish fed FeM. However as none of the diets were deficient according to the nutritional requirements of the salmon this is unlikely. Digestibility of the FeM may have been low in the present study as indicated by the two-fold increase in BBM-LAP in the DI as well as the as the specific activity in the PI and the combined PI+DI activity (paper 2). The digestive physiological responses to the alternative feed ingredients will be discussed more thoroughly later on. Observations of the present thesis thus confirm PBY as a promising alternative feed ingredient for Atlantic salmon however care should be taken with use of FeM to ensure that growth is not limited due to poor amino acid utilization.

Gut morphological responses to alternative feed ingredients

The intestine is a dynamic organ and a good indicator of the gut and general fish's health when fed alternative raw materials. The present thesis has shown that inclusion levels of 20% of various products of pea, soy, sunflower and poultry can be included in the diets for Atlantic salmon without initiating a gut morphological response (paper 1). These current results are very promising as the industry are looking for alternative protein sources for use in commercial salmon diets and plant materials especially have been studied closely for their potential negative influences on the intestinal morphology due to the importance of the intestine in digestion, nutrient assimilation and barrier function. Previously a commonly reported negative effect of using plant materials such as SBM (Úran *et al*., 2008, 2009) and higher levels of pea (35%, Penn *et al*., 2010) is the onset of enteritis which has been attributed to the presence of ANF's in the raw material (van der Inghi *et al*., 1996). SPC however has been reported used at 22.2% (Krogdahl *et* al., 2000) and 30% (Penn *et al*., 2010) without causing detrimental effects on the intestinal morphology. The lack of response using the present plant materials is most likely due to the processing of the feed

ingredients prior to use. Various extraction (ethanol, aqeous) and classification (air) methods have been developed to remove excess fibre, carbohydrates and other ANF's. The remaining product is high in protein and low in ANF's. ANF's are however rarely completely removed which is how extracted raw materials may still induce enteritis at high levels (Penn *et al*., 2010). Thus, according to the present thesis, PPC, SPC, ESF, FeM and PBY can be added to the diets at currently investigated inclusion levels without any adverse histomorphological changes to the intestine.

Evaluation of alternative feed ingredients on digestive physiology

Feather meal

The digestibility of a feed can be considered in two manners – the ability of the fish to digest the feed based on the enzymes present in the GI tract and the effect of the raw material on the enzyme activity. FeM is considered a low digestible raw material (Hardy *et* al., 1996; Bureau *et al*., 1999), however results of the present study suggests that when fed to Atlantic salmon in excess of ordinary commercial inclusion levels (20% vs 3%) the fish is able to modulate its digestive physiology to compensate for the sub-optimal diet digestibility (paper 2). Following feeding with FeM the fish expressed a two-fold increase in the specific BBM-LAP activity in the DI concomitantly with an increase in the total BBM-LAP activity (paper 2) and an organosomatic increase in all intestinal compartments (paper 1) suggesting a stimulation of gut mucosal growth. Furthermore, observations of the intestinal morphology reveal that the feed ingredient did not cause adverse structural changes. The total enzymatic capacity is a function of the activity of the enzyme and the weight of the fish and as such can be explained at least partially with the increased intestinal weight. Increased specific enzyme activity however suggests a true increase in digestive capacity and as such a compensatory mechanism of the digestive physiology of the fish in response to the low digestibility. This may contribute to explain why growth in fish fed 20% FeM was not significantly different from that of FM fed fish. A possible feedback mechanism may aim to increase the scavenging ability of the DI as suggested by the increased D weight, increased BBM-LAP activity in DI and the persistent high nitrogen content of the chyme. Increased C-LAP activity indicate increased sloughing of the MI1 and DI of fish fed FeM which may be correlated with the increased intestinal mass, however increased intestinal mass of various compartments were also observed in fish fed ESF, SPC and PBY, without the concomitant increase in C-LAP activity. As the increased intestinal mass in fish fed FeM were substantially higher than in fish fed the other alternative feed ingredients, this may explain the discrepancy.

Extracted sunflower

Extracted sunflower, in contrast to FeM, did not seem to initiate the same compensatory reaction as was seen in fish fed FeM. Sunflower is a raw material which is usually used in low levels in commercial diets with inclusion level of up to 2-4% and as such the present inclusion of 20% in the diet is not realistic from a commercial point of view. Growth of Atlantic salmon fed ESF was lower than that observed for fish fed FM (paper 1), which is in contrast to what has been observed previously for salmon and other species. One study previously reported using 27% sunflower meal for Atlantic salmon without adverse effects on growth (Gill *et al*., 2006) and the herbivore tilapia (*Sarotherodon mossambicus*) showed improved growth when fed 75% inclusion of sunflower indicating an increased capability to digest and utilize the feed ingredient. Sunflower contains saponin and chlorogenic acid (Akande *et al*., 2010) a substance that is known to inhibit digestive enzymes including trypsin, chymotrypsin, amylase and lipase. It is possible that the almost 10-fold increase in experimental inclusion level compared to the commercial level has increased the level of ANF's in the feed to a level at which digestion is inhibited, observable by the decreased in trypsin activity of the DI in fish fed ESF (paper 2). Furthermore an increased level of nitrogen in all compartments of the GIT and increased DM content in DI of ESF fed fish suggest that the digestion of the feed ingredient is inhibited possibly by the high inclusion rate of the raw material. Consistently high nitrogen levels may also be a reflection of increased pepsin or other proteins secretion in the stomach as indicated by the 30% increase in nitrogen, which is far higher than observed in fish fed PPC, SPC, FeM or PBY. Digestibility of sunflower has previously been reported as high for major carps (*Labeo rohita)* when fed sunflower at a level of 36.7% of the diet (Salim *et al*., 2004) and Atlantic salmon smolt when fed sunflower at 27% dry weight inclusion level (Gill *et al*., 2006). These studies suggest that there is a potential for higher inclusion of sunflower in feeds for Atlantic salmon, however more information is needed as to the potential effects of ANF on the enzyme activities, and thus support the current inclusion of 2-4%.

Soy protein concentrate

Soy protein concentrate is one of the most used alternative feed ingredients in commercial formulations today, and can be used in levels as high as 30%. This is in part due to good nutritional profile and digestibility which results in good growth but also in part due to competitive prices and steady supply. In the present study addition of SPC at 20% showed a reduction of more than 11%. It is uncertain what raw material affects may have caused this. In accordance with previously reported studies (Olli *et al*., 1994; Krogdahl *et al*., 2000; Penn *et al.*, 2011), use of SPC did not cause adverse structural changes in the histomorphology of the distal intestine (paper 1). Furthermore, evaluation of the digestive physiology

revealed that although chyme carbon and hydrogen, DM and trypsin and BBM-LAP activities were significantly altered following addition of SPC to the diets, these differences were relatively small (paper 2). It is interesting to note however that use of SPC showed a more pronounced proximal-to-distal variation of BBM-LAP activity than observed in FM fed fish which is in contrast to what Penn *et al.* (2011) observed which was a narrowing of the proximal-to-distal variation. Furthermore, these results of the present study correlated with an increased organosomatic index if PI and MI (paper 1), indicating increased digestive and absorptive processes in the proximal part of the GIT, resulting in less substrate in the DI chyme, and a down-regulation in BBM-LAP activity. Bacterial metabolism may also have been affected by the reduction of substrate reaching the DI as evident by the somewhat lower production of acetic acid compared to the PPC fed fish (paper 1). Although increased digestive processes may explain the decreased trypsin and BBM-LAP activity in the present trial, down-regulation of activities is also a typical sign of dietary induced enteritis (Krogdahl *et al*., 2003) and as such may in the present study indicate subclinical effects of the SPC on the DI. This is also supported by the low DM content of the DI, a clinical sign generally accompanied by SBM-inflammation and which indicate a tendency for diarrhea (Beaverfjord & Krogdahl, 1996; Krogdahl *et al*., 2003) as well a higher carbon and hydrogen concentrations (paper 2). However, with the reported increase in organosomatic index of all intestinal compartments (paper 1) no apparent significant decrease in LAP activity was observed. As such, the results of the present thesis further indicate that SPC is an acceptable feed ingredient for Atlantic salmon at 20% inclusion rate, as opposed to full fat or hexane-extracted SBM (Baeverfjord & Krogdahl, 1996; Bakke-McKellep *et al.*, 2000, 2007; Chikwati *et al*., 2013)

Pea protein concentrate

Pea protein concentrate showed very little impact on the digestive physiological parameters investigated in the present study. At 20% inclusion rate the raw material resulted in improved growth and increased feed intake compared to FM as well as the other experimental feed ingredients but did not affect FCR (paper 1). At least three separate studies, including the present study have shown that PPC results in growth at least as well as that provided by fishmeal (Øverland *et al.*, 2009; Hansen *et al.*, 2011, paper 1). Evaluation of the intestinal histomorphology showed no signs of inflammation (paper 1) or any other accompanying clinical signs of inflamed, dysfunctional tissue (Krogdahl *et al*., 2003) including trypsin activity of the DI (Krogdahl *et al.*, 2003; Lilleeng *et al.*, 2007; *Penn et al*., 2011). Higher inclusion of PPC has previously shown to induce clinical signs of inflamed tissue (35%, Penn *et al.*, 2011) indicating that inclusion levels exceeding 20% should be monitored carefully for adverse effects on the intestinal morphology. Use of PPC did however result in increased chyme levels of sulphur indicating increased secretion of sulphur containing compounds either in the form of the sulphur-rich taurocholate in the bile (Bogevik *et al*., 2009)

or the sulphur containing pancreatic secretions proteases, glutathione etc. Indications for increased pancreatic secretions were only otherwise supported by increased lipid activity in the two DI compartments, although trypsin activity and bile salt level were often numerically, albeit not significantly, higher. Despite these promising results however, use of PPC in commercial formulations remain low with an inclusion level of around 0.4% indicating that there are other non-biological limiting factors for the increased use of this feed ingredient. Increased feed intake may be a factor in that the feed will no longer be cost efficient at higher inclusion levels. Furthermore as price and availability is more variable and potentially unfavourable than for other alternative ingredients such as SPC, inclusion of PPC may still below despite favourable biological properties.

Poultry by-product

Poultry by-product resulted in very few significant changes compared to the FM control diet. Consistently low nitrogen in the intestinal compartments indicates high digestibility and the high sulphur content indicates stimulation of pancreatic and bile secretion (paper 2) which may indicate good nutrient gain and explain the slight weight gain over the FM control fish. The increased pancreatic secretion may explain the increased observation of lipase in the DI compartments, but which may also be a reflection of the high levels of lipid in the raw material. High lipid content in the raw material may also explain the increased level of plasma TG and FFA and indicates that the nutrients in the raw material are highly utilizable. In contrast to the present study, a recent paper report that when added to the diets for Atlantic salmon posts-molts, although growth was not affected, based on lower retention of protein and energy the author suggested the raw material to have low digestibility (Hatlen *et al*., 2014). Despite this though, the study concluded that PBY is suitable as feed ingredient for Atlantic salmon. As such the results of the present study indicate that PBY is a suitable alternative feed ingredient for Atlantic salmon at inclusion levels up to 20%.

Dietary effect on microbial load and community composition

Microbial load

The present findings revealed that some raw materials have the potential to cause changes in the bacterial load of the various intestinal compartments compared to fish fed the FM control diet. Following the use of alternative raw materials it is evident from the present study that the total bacteria load of the proximal intestine remained unaltered compared to the load in the FM control fed fish (paper 1). Other previous reports have also documented an apparent insensitivity in the total cultivable population levels in response to addition of cellulose or non-starch polysaccharides (Ringø *et al*., 2008) or soybean meal (Merrifield *et*

al., 2009). One possible reason for this may be that the bacteria associated with the proximal intestine are subjected to many alterations in environment resulting in an ability to resist possibly damaging changes and variation in the environment. Bacteria associated with the PI is under constant influence by factors such as bile salts, gastric acidity, digestive enzymes (Ringø *et al*., 2003) and mechanical stress through peristalsis and passing of the chyme. Autochthonous bacteria inhabiting the PI may therefore have mechanisms in place to counter these influences. One example of a protective mechanism is bile tolerance. This ability allows the bacteria to adhere and proliferate in locations otherwise unattractive to other species of bacteria. These mechanisms may also render the bacteria insensitive to dietary alterations although it is uncertain how these correlate. One hypothesis indicates that the bolus is not present in the intestine long enough for the bacteria to be influenced by the source, type or length of the carbohydrates or fibres or other nutrients present in the food. Retro peristalsis increases the retention time of the digesta in the upper part of the intestine which indicates that this is not a likely reason. Furthermore as the proximal intestine is one of the major sites of digestion and absorption in the GI tract of fish, it may be that bacteria associated with the PI rather utilize pre-digested nutritional components already digested by the host into absorbable components such as triglycerides, peptides and amino acids releasing resources within the bacteria which can be used to counter the potentially negative influences of the before mentioned chemical and physical influences of the GI tract. Because of the potentially harsh environment the PI it becomes an unlikely preferred location of adherence for most bacteria and hence harbours a lower bacterial load that that found in the distal intestine.

In contrast to the apparent insensitivity of the bacterial load in the proximal intestine, the use of alternative feed ingredients resulted in a modulation of the distal intestine bacterial load. A higher bacterial load in the distal intestine where most of the microbial activity in the GIT occurs may be a possible theory for these results. Use of ESF, FEM and PBY all resulted in an increased bacterial load of the allochthonous gut microbiota in the DI, whilst addition of PBY and PPC to the diets increased the autochthonous gut bacteria of the DI indicating these are favourable substrates for the indigenous microbiota of the Atlantic salmon intestine. One should also consider the fact that although the fish has some ability to digest the more complex carbohydrates which are present in feed with increased inclusion of plant materials, some of the food components will migrate downstream to the distal intestine and become substrate for bacteria indigenous to that part of the intestine. This may assist in explaining the apparently higher bacterial density in the distal intestine than in the proximal intestine especially for the plant based materials high in carbohydrates and low digestible ABP's.

Modulations of the autochthonous gut microbiota can be induced by changes in the nutrient profile or as result of histomorphological changes. By replacing dextrin with inulin at 15% adherent gut microbiota of the DI decreased from Log 5.68 to Log 4.55 in the Arctic charr (Ring ø *et al.*, 2006c). A previous report

from the same trial showed that inulin caused damaging effects to the enterocytes (Olsen *et al*., 2001) suggesting that alterations in the morphology affected the bacterial population. In contrast, Bakke-KcKellep *et al* (2007) showed that inulin at 75 g/kg for Atlantic salmon did not affect the autochthonous CFU/g, but did decrease the allochthonous TVC of MI and DI. As previously mentioned none of the alternative feed ingredients tested in the present thesis caused morphological changes to the intestine or reduced autochthonous bacteria in the DI (paper 1), and suggesting that bacterial load may change in response to substrate profile. The relationship between changes in histomorphology of intestine of fish however and the autochthonous bacteria load merits further study however, as previous reports have shown contradictory results. Krill (Ringø *et al*., 2006a) and SBM (Bakke-McKellep *et al*., 2007) in diets for Atlantic salmon both increased the autochthonous TVC DI, however whilst SMB caused detrimental changes to the histomorphology of the DI, krill did not indicating that morphological changes may not be a significant factor influencing the adherent bacterial load.

Use of alternative feed ingredients may not always modulate the bacterial load of the gut. In the present study, use of SPC apparently did not modulate bacterial load in PI or DI (paper 1). Following use of the less refined SBM, at least two reports show no significant modulation to the autochthonous and allochthonous bacterial load in Atlantic salmon at 50% inclusion (Merrifield *et al*., 2009; Navarrete *et al*., 2013). A previous study also showed that feeding non-starch polysaccharides or cellulose did not significantly affect autochthonous or allochthonous TVC (Ringø *et al*., 2008) indicating that dietary effects on gut microbiota may not necessarily affect the total load, and that it is therefore important to also investigate the population dynamics as result of the dietary changes.

Based on the results of the present trial, the methods used in the present study to extract genomic material and enumerate total bacterial load may provide a more accurate picture of the bacteria resident in the gut of fish. Most of the previous studies report total numbers as Log CFU or TVC and are often in the area between Log 2.99 and 6.10 TVC (Heikkinen *et* al., 2006; Ringø *et al*., 2006a,c, 2008; Bakke-McKellep *et* al., 2007; Merrifield *et al*., 2009) whilst in the present study the total bacterial load ranged from Log 6.11 to 8.14 (paper 1). Furthermore, as enumerations are based on the ability of bacteria to form colonies on a growth medium, this often excludes strict anaerobic bacteria suggesting an underestimation of the number of bacteria traditional cultivation studies. It is important however to mention that analyzing for total number of 16S rRNA copies, one does not take into consideration that there are usually several 16S copies in each bacteria, and as such the Log number generated in the present trial is not equivalent with number of bacteria.

The total number of bacteria either associated with the mucosal lining or the gut content provides some information on the effect of dietary alterations. As shown by some of the previously mentioned studies however, dietary effects may not necessarily affect the bacterial load and it is therefore also important to

investigate the composition of the bacterial population of the autochthonous and allochthonous gut microbiota.

Gut bacterial community composition

qPCR analysis of the transient bacteria in the intestinal content revealed that *Carnobacteriaceae* dominated both in the proximal and distal compartments, also regardless of which diet the fish was given. Furthermore analysis of the allochthonous bacterial community revealed that *Lactobacillaceae* was the dominating group regardless of which diet the fish had been fed (paper 1). This is to the present author's knowledge the first mention of *Corynebacteriaceae* and *Lactobacillaceae* as sustainable dominating groups in the Atlantic salmon bacterial community. Although *Corynebacteriaceae* and *Lactobacillaceae* are not previously reported as dominant groups, a recent study reported *Shewanella* to dominate the bacterial community in fish fed FM, SBM or SBM and probiotics (Navarrete *et al*., 2013) suggesting that the bacterial communities of the Atlantic salmon intestine may be more stable than previously believed. Furthermore, the same authors also reported a few years earlier that either *Pseudomonas* ssp. or *Shewanella* were the dominating group along the digestive tract in Atlantic salmon (Navarrete *et al*., 2009). The fact that they reported two different dominating groups in the same bacterial community is a tribute to the different methods applied – use of 16S rDNA PCR-TTGE resulted in dominance by *Pseudomonas* ssp. whilst aerobic culture analysis of the samples revealed dominance by *Shewanella*. As such, the results of the mentioned papers also indicate that the choice of methods may also influence the results generated. The assays (primers) used in the present study were developed with the intention to cover ~90% of the bacterial community in the Atlantic salmon intestine by using family based degenerate primers. Sequence studies of Atlantic salmon faecal studies where 11 dominant groups were identified were the basis for the design of these primers. Although the coverage of the assays provides new knowledge on the microbial community composition in the Atlantic salmon gut microbiota, information on the effects on the gut microbiota on species level is limited.

Lactobacilli ssp. is shown through previous studies to be difficult to cultivate. A study by Hovda e*t al,* (2007) reported *Lactobacillus* by using DGGE, but not by using traditional cultivation. The present thesis shows however that by applying qPCR as a quantitative method, *Lactobacilli* are dominant in the autochthonous microbiota. Furthermore, this dominance was observed regardless of diet, although in some groups (SPC in MI and PPC in DI) the level of *Lactobacilli* significantly increased compared to the FM control group (paper 1). *Lactobacilli* were also detected in the autochthonous microbiota of MI and DI indicating a ubiquitous presence in the Atlantic salmon intestine. These result indicate that biomolecular based methods may be useful, or even required, for a more accurate quantification of *Lactobacilli* than that provided by cultivation alone.

Corynebacteriaceae

Mycoplasmataceae is a group of omnipotent bacteria with very complex growth requirements and which lack a cell wall. For these reasons the bacteria are difficult to cultivate and are rarely reported in fish microbial studies, despite their presence as an environmental bacteria. In the present study, *Mycoplasmataceae* were observed in all feeding groups indicating the usefulness of molecular based methods to capture un-cultivatable bacteria. The levels in which the bacteria were observed in the present trial were several magnitudes lower than the other groups which may be a reflection of the difficulties in extracting genomic material from bacteria with no cell wall. Following a phylogenetic study in pen-raised salmon, *Mycoplasmataceae* were suggested to be a natural part of the gut microbiota in fish (Holben *et al.*, 2002) as well as a common environmental bacterium. As the fish used in the present study were tank raised in controlled conditions, in which very few environmental bacteria are present, this may be reflected in the low levels of *Mycoplasmataceae* in the tank raised fish.

The use of molecular based enumeration in combination with flash freezing of microbial samples have shown to have some benefits over cultivation as suggested by the observation of *Mycoplasmataceae* in all groups albeit in low levels, as well as *Peptostreptococcaceae*, a strict anaerobe bacteria observed in both allochthonous and autochthonous communities (paper 1). There was however few dietary correlations in the alterations of the gut microbial communities indicating the need for more in-depth investigations into the assays used in the present thesis.

Dietary effect on probiotic and pathogen adherence to intestinal mucosa

Exposing intestine from Atlantic salmon to increased levels of *C. divergens* or *A. salmonicida* resulted in increased adherence of the bacteria to the mucosal lining of Atlantic salmon DI compared to the saline exposed intestines, indicating an inherent ability to adhere to intestinal mucosa (paper 3). Both *C. divergens* (Strøm, 1988; Ringø & Gatesoupe, 1998; Bakke-McKellep et al., 2007; Lauzon & Ringø; 2011) and *A. salmonicida* (Ringø *et* al., 1997) are indigenous bacterial species to the gut of the Atlantic salmon however in vivo studies are however required in order to gain more information about the effects of the alternative feed ingredients on the long term adherence of the bacteria to the intestinal lining of fish.

Exposing the DI of Atlantic salmon to pure cultures of *C. divergens* and *A. salmonicida* revealed however that the pathogen apparently adhered with a greater efficiency than the probiotic bacteria, despite that the concentration of the incubation solution was slightly lower for the pathogen than for the probiotic - Log $8.6x10^6$ *vs* $3.2x10^7$ respectively (paper 3). This suggests that the pathogen bacteria have an advantage over the probiotic in the competition for binding sites. As a pathogen enters the GIT it is recognised as foreign and potentially harmful by the immune system. One of the observed pathomorphological effects of the presence of pathogen bacteria is an increased occurrence of goblet cells suggesting an increased

production and secretion of mucus to prevent adherence of the foreign bacteria (Lødemel *et al*., 2001). To strengthen the barrier effect of the mucus layer, the mucus is reported to contain a range of antibacterial compounds (eg, complement proteins and antibacterial peptides, lysosomes, antibodies, lectins and pentraxins) (Dalmo *et al*., 1997; Saurabh & Sahoo, 2008) which the pathogen bacteria must pass to make the host-bacteria interaction. Pathogen bacteria such as *A. salmonicida* therefore need to develop abilities to reach and adhere to the mucosal lining despite the increased mucus production and immune reaction. Once the bacteria have gained access to the mucosal interphase it may gain access to binding sites through exclusion of the adherent indigenous bacteria. Ringø et al. (2002a) suggested that *A. salmonicida* was able to exclude the indigenous bacteria through its production of exo- and endotoxins which would liberate binding sites for the pathogen. As such, pathogen bacteria need to be more opportunistic than probiotics which may explain the increased adherence of *A. salmonicida* compared to *C. divergens*.

In the present thesis, use of alternative feed ingredients did not affect the adherence of *C. divergens* or *A. salmonicida* (paper 3) indicating that the alternative raw materials cannot be used to enhance probiotic adherence or alternatively that the fish will not be more susceptible to adherence and invasion from A. salmonicida when fed PPC, ESF, or FeM. It is uncertain why the alternative raw materials affected the endogenous intestinal microbiota but not the adherence of *C. divergens* or *A. salmonicida*, or both. Time may be a factor here, and the differences between using in vivo and ex vitro methodology. Feeding alternative raw materials to fish allows the bacteria to utilize the nutrients along with the host, whereas ex vitro methodology demands the rinsing of the intestine prior to exposure to bacteria, and hence removing the nutrients otherwise present in the intestine. As such, adherence of the bacteria and modulation thereof, is most likely correlated with modulations of the mucosal morphology and the prevalence of available binding sites for the bacteria. The fact that the adherence of the bacteria did not change is in correlation with the results that none of the experimental raw materials changed the mucosal morphology (papers 1, 3). As the inclusion rates of the alternative feed ingredients were substantially higher than commercial levels these results indicate that commercial use of these raw materials may not inhibit the adherence of the probiotic or pathogen bacteria. Although there is very little information on the effect of diet on adherence of bacteria following *ex vivo* exposure, at least one other study report having investigated the effect of diet on the gut microbiota before and after cohabitant challenge with *A. salmonicida* (Lødemel *et al*., 2001; Ringø *et al*., 2002a). Based on this study the authors suggest that the reduced bacterial load of the DI following cohabitant challenge was as a result of increased mucus production by the pathogen (Ringø *et al*., 2002a) which was supported by the report of an increased prevalence of goblet cells following the cohabitant challenge (Lødemel *et al*., 2001). Following the cohabitant challenge, *A. salmonicida* was detected only in MI of fish fed marine oil prior to the challenge, indicating that use of alternative feed ingredients (albeit oils and not meals in this case) may indeed affect the adherence of the

pathogen to the mucosal lining of salmonids. Another reason why the pathogen was detected in only one group in the previous studies and in high levels in the present study is probably a reflection of the use of the difference in methodology - *in vivo* in the previous and *ex vivo* in the present study.

Evaluation of the histomorphology of the MI using TEM revealed that exposing the intestine to high levels of *C. divergens* did not cause damaging changes to the morphology and was comparable to saline exposed control group (paper 3). This is in accordance with a previous study where Atlantic salmon intestine exposed to *C. divergens* at concentrations up to $6x10^6$ showed no signs of histomorphological damage (Ringø *et al*., 2007). Some signs of improved histomorphology were observed following *C. divergens* exposure including decreased registration of IEL's. Although intestinal morphology in fish fed ESF, PPC and FeM was comparable to saline exposed intestine, decreased IEL's was not observed in these groups. It is uncertain why a reduction in the observations of IEL's following exposure to the intestine to *C. divergens*. Studies into the effect of in-feed administration of *Pediococcus acidilactici* on intestinal morphology of rainbow trout show increased migration of IEL's in response to the presence of the probiotic (Harper *et al*., 2011) indicating an enhancement of the immune system. It is possible that the short time in which the intestine was exposed to the probiotic in the present study compared to the previous study (1 hour *vs* 2 weeks) was not adequate enough to allow for migration of the IEL's. However as exposure of beluga (*Huso huso*) intestine to *Leuconostoc mesenteroides* and *Lactobacillus plantarum* increased the observation of IEL's after only 45 minutes of exposure (Salma *et al*., 2011) time may not be a decisive factor for the apparent decrease on IEL's observed in the present study.

The damaging effect of *A. salmonicida* to the MI of the Atlantic salmon was as previously described for the DI (Ringø *et al*., 2004, 2007; paper 3). Use of FeM however intensified the damage caused by *A. salmonicida* (paper 3). It is uncertain how the uses of FeM lead to the worsening of the damage by the A. salmonicida as the raw material did not show signs of morphological changes following a 12-week feeding period with the same inclusion level (paper 1). Addition of FeM to the diet did however result in increased organosomatic index for all GIT compartments and increased BBM-LAP activity possible to compensate for low digestibility or poor amino acid utilization which may compromise the enterocytes making them more susceptible to invasion by opportunistic pathogenic bacteria. A possible mechanism for this however merits further investigation to ensure that high levels of new raw materials does not render the host more susceptible to intestinal damage from the presence of opportunistic pathogen bacteria.

C. divergens and *A. salmonicida* – exclusion, displacement and effect on the intestinal mucosa

Incubation of the DI of Atlantic salmon to *C. divergens* prior to *A. salmonicida* revealed that adherence of *A. salmonicida* was lower than observed for intestines exposed to *A. salmonicida* alone (paper 3)

indicating an ability of *C. divergens* to exclude the pathogenic bacteria. As a probiont, *C. divergens* have shown to inhibit growth of *A. salmonicida* (Jöborn et al., 1997) through production of inhibitory substances which is released into the surrounding environment. When the pathogenic bacteria were allowed to adhere to the intestine prior to the probiont however, it is apparent that the probiont was only able to displace a small amount of the adhered *A. salmonicida* (paper 3). These results indicate that it is apparently more difficult for the probiont to displace pathogenic bacteria already present at the entrance of the probiont than to exclude the pathogenic bacteria when already adhered. This may be due to the pathogen's abilities as an opportunistic bacterium to breach the defense systems of both the host (eg. antimicrobial substances in the mucus, tight junctions, complement system), and the hosts indigenous bacteria (eg. production of antimicrobial substances) as well as the competition from the latter for nutrients and binding sites and achieve adherence at the mucosa-bacterial interphase. Specialized adherence proteins located in the outer membrane of the bacteria known as S-layer is an important factor for *A. salmonicida* adherence (Garduño *et al*., 2000) and may explain why exclusion of the pathogenic bacteria more difficult for the probiotic than displacement.

Appearance of the intestinal morphology following exposure to *C. divergens* prior to *A. salmonicida* showed similar appearance to that of saline exposed control samples (paper 3) indicating that the probiotic is able to prevent the adverse effects of adhering pathogenic bacteria. This correlates well with the probiotics' ability to exclude the pathogen before adherence can occur. In fish fed FeM however, TEM micrographs show signs of excess lipid vacuoles, although it is uncertain how this can be related to exposure to bacterial strains. The alleviating effects of *C. divergens* against *A. salmonicida* and *V. anguillarum* in salmon foregut has been documented previously (Ringø *et al*., 2007) although alleviation of the clinical signs were only observed when the probiotic was allowed to adhere to the mucosal lining prior to the pathogenic bacteria. This is similar to what was observed in the present study where intestines exposed to *A. salmonicida* prior to *C. divergens* showed the same appearance as to that observed in samples exposed to *A. salmonicida* alone (paper 3) although a somewhat lesser severity of the damage indicates that the probiotic bacteria may alleviate, but not mitigate, the clinical signs. Probiotic bacteria, such as *C. divergens*, have shown to enhance disease resistance and stimulate the immune system in both fish (Irianto & Austin, 2003; Ringø *et al*., 2005; Salinas *et al*., 2005) and higher vertebrates (Madsen *et al*., 2001) which explains the apparent lack of damage observed when the probiotic is adhered to the mucosal lining prior to pathogenic bacteria. As the probiotic bacteria adhere to the mucosal lining, antimicrobial compounds are secreted into the surrounding environment, and as such inhibit the growth and toxin producing abilities of *A. salmonicida*. As such, use of FM, PPC and ESF does not seem to increase the susceptibility of the fish to invasion by *A. salmonicida*, nor do they intensify the adverse effects of the pathogen once adhered to the intestinal lining. FeM however intensified the harmful effects

of the pathogen bacteria and should therefore be used with care in periods where infection of A. salmonicida is possible. *In vivo* challenge studies will clarify if the use of FeM in commercial levels will intensify to the same degree as observed in the previous study when the fish is exposed to more natural level of the bacteria.

Conclusions

Fishmeal was for a long time the only protein source used in diets for farmed fish, however constraints in fishmeal production and availability has spurred the use of alternative feed ingredients to replace the marine protein. Terrestrial protein from sources such as plants (eg legumes, oilseeds) and animal production (eg offal from poultry production) has been tested for their usefulness as fishmeal replacements. Use of alternative feed ingredients affects the gut microbiota, intestinal digestive physiology and the fish's susceptibility to invasion from opportunistic gut pathogens. Furthermore as cultivation of gut microbiota has shown to have its limitations in gut microbiota investigations, a new approach using quantitative qPCR has been applied.

- The present thesis reveals that dominance of *Corynebacteriaceae* of the allochthonous gut microbiota in distal intestine and *Lactobacillaceae* of the autochthonous gut microbiota of the mid and distal intestine was unaltered by the use of alternative feed ingredients. This is a reflection of use of qPCR and is unprecedented in previous cultivation studies.

- Pea protein concentrate showed superior growth to the fishmeal group however the subsequently increased feed intake may question the cost-efficiency of the feed ingredient. Use of this raw material resulted in very few alterations in digestive physiology, histomorphology or gut microbiota of the fish. Furthermore, use of pea protein concentrate did not increased the fish's susceptibility to disease from invasion of *A. salmonicida*. The current commercial inclusion level is ~0.4% and as the present thesis show that pea protein concentrate can be used at levels up to 20% with very alterations compared to the fishmeal group, this also suggests that there are non-biological reasons for the low commercial inclusion rate.

- Soy protein concentrate is the feed ingredient which is the mostly used in feed for Atlantic salmon. In the present thesis this alternative feed ingredient was included in the diets at 20% without potential adverse effects on growth or digestive physiology. Furthermore as production of soy protein concentrate from soybean meal removes most ANF's, no adverse effects were observed in the intestinal morphology. However the results of the present thesis showed that the raw material modulated digestive enzyme activities and should therefore be used with caution at higher levels

- Use of extracted sunflower depressed growth by almost than 25% compared to the fishmeal fed control fish and may be a reflection of low digestibility and possibly presence of chlorogenic factors and other ANF's present in the raw material. Caution should therefore be used when using this raw material in levels as high as 20% in diets for Atlantic salmon.

- Poultry by-product is considered a promising alternative feed ingredient, and in the present thesis the feed ingredient is used at 20% without adverse effects on growth, histomorphology of the intestine, intestinal digestive physiology and gut microbiota. Susceptibility to *A. salmonicida* adherence was not affected by the inclusion or poultry by-product. As such, PBY is highly useful as feed ingredient for Atlantic salmon.

- Feather meal is a cheap, readily available raw material which is in commercial diets is only used in low levels due to low digestibility and high quality variation. In the present thesis however use of the feather meal at 20% showed growth comparable to fishmeal. This may be a reflection of the fish's ability to compensate for the raw material as shown by the enhanced effects on BBM-leucine amino peptidase activity and increased organosomatic index of the GIT compartments. Although the fish performed well on feather meal in the present thesis, further investigations into the possible compensatory mechanism is merited in order to use the raw material in comparable levels in commercial diets.

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