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In vitro effects of antinutrients on gut microbiota from farmed Atlantic salmon

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Abbreviations and acronyms

ANF - Antinutritional factor

BBM - Brush border membrane

bp - Base pair

DE - Digestible energy

DGGE - Degenerative gradient gel electrophoresis

DHA - Docosahescaenoic acid

DI - Distal intestine

DNA - Deoxyribonucleic acid

DP - Digestible protein

EFAs - Essential fatty acids

E_h - RedOx (Reduction oxidation)

EPA - Eicosapentaenoic acid

ES - Oesophagus

FAO - Food and Agriculture Organization

FISH - Fluorescence in situ hybridization

FM - Fishmeal

FO - Fish oil

GALT - Gut associated lymphoid tissue

GE - Gross energy

GI - Gastrointestinal

GIALT - Gill-associated lymphoid tissue

GIT - Gastrointestinal tract

GM - Genetic modified

IECs - Intestinal epithelial cells

IFN - Interferon

ILS - Interleukins

KTI - Kunitz' trypsin inhibitor

LAB - Lactic acid bacteria

LC-PUFAs - Long chain polyunsaturated fatty acids

MALT - Mucosa-associated lymphoid tissue

MI - Mid intestine

MBL - Mannose-binding lectin

mRNA - Messenger RNA

NCC - cytotoxic cells

NGS - Next generation sequence

NSPS - Non-starch polysaccharides

PA - Phytic acid

PAMP - Pathogen-associated molecular pattern

PCR - Polymerase chain reaction

PI - Proximal intestine

PP - Peyer`s patches

PRR - Pathogen recognition receptor

PUFA - Polyunsaturated fatty acids

qPCR - Quantitative polymerase chain reaction

RNA - Ribonucleic acid

SALT - Skin-associated lymphoid tissue

SBM - Soybean meal (Standard solvent-extracted)

SBMIE - Soybean meal induced enteritis

SCFA - Short chain fatty acids

SNP - Single Nucleotide Polymorphism

SPC - Soy protein concentrate

TCR - T-cell receptors

TGGE - Temperature gradient gel electrophoresis

TLR - Toll-like receptor

TNF - Tumor-necrosis factor

T-RFLP - Terminal-Restriction Fragment Length Polymorphism

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Summary

Currently investigations on gut microbiota of animals and humans have received increased emphasis as it is thought to be a key factor in metabolism of nutrients, immune system, growth and protection against potential pathogens. Investigations on the effects of plant ingredients and fibers/NSPs on gut microbiota have been widely studied and implicated that their role shifts the level and composition of gut microbial communities. However, the presence of ANFs in plant ingredients effect on fermentation and gut microbiota is not clearly known. It was hypothesized that, inclusion of different levels of purified ANFs affect gut microbes including their proliferation, numbers, total composition and metabolic activity. As Atlantic salmon don not have functional enzymes to digest carbohydrate diets, the study of such changes is difficult to have *in vivo* study to ascertain the cause-effects of purified ANFs, because it is not possible to distinguish whether the outcome is due to the fibers/NSPS in SBM or due to the tested ANFs. Therefore, in this study, the use of *in vitro* simulation model mimicking microbial processes in salmon intestine was supposed to offer a suitable alternative to avoid such possible confounding effects as the results of these assays would be caused by the direct effects of individual and combined effects of purified lectin, saponin, isoflavonoid and phytosterol on fermentation and gut microbiota of farmed Atlantic salmon.

The changes in the bacterial levels and composition of farmed Atlantic salmon in response to the different ANFs were investigated by qPCR analysis. Parameters such as gas production, change in pH, redox potential, and levels of metabolites released were also used to estimate the effects on microbial fermentation. The total microbial counts tended to decrease linearly with increasing combination of antinutrients, particularly with saponin and isoflavonoid but their effects were not significantly different either from the control or the other levels. Saponin was relatively the most efficient ANF to decrease the total microbial levels. However, the lactic acid bacteria including lactobacillaeceae, and Bacilli were the most resistant towards the current levels of ANFs, instead they tended to increase their proportion with increasing ANF concentrations. In addition, though their level was very low, the aerobic bacteria represented a major cluster in microbial community, were resistant and would probably further increase their proportion. Of all the individual and combined antinutrients, saponin and isoflavonoid were the ones that showed relatively greater effects both in metabolic and microbial populations. Although, high level of

lectin and low level of phytosterol were very effective to inhibit the growth of Vibrionaceae bacteria that contains some pathogenic species. At mid-concentration of all ANFs, pH was numerically lower than either the non-amended control, or low or high concentration, whereas the values for the high concentration was always numerically higher than non-amended control. This may indicate that at medium level these ANFs have stimulated the gut microbiota and increase fermentation process as reflected by pH reduction. On the contrary at high level of ANFs may affected the gut microbiota and inhibited the fermentation process. Although, the concentrations of the tested ANFs in this in vitro simulation was most likely exceed many-fold the concentrations in authentic salmon GI tract fed soy bean based feed, their effects both individually and in combination affected the microbial fermentation only little, which is different from what was predicted in the hypothesis. However, as there were some variabilities in regard to the effects of these ANFs, it is very difficult to generalize their effects on gut microbiota. Finally, it was suggested that the low incubation temperature and the high proportion of the frozen samples used as inoculum for the simulation model may affected the current results. Therefore, further studies with more samples and advance identification methods such as next generation sequencing is recommended to detect the high proportion of microbes remained uncaptured by the current method and smaller variations that may occur in the gut microbiota of fish.

1. Introduction

According to the Food and Agriculture Organization (FAO), the global population is estimated to reach 9.7 billion by 2050. Thus, FAO has predicted that 70% more food must be produced globally by 2050 to secure the increased demand (FAO, 2016). Aquaculture production is expected to play a major role as an important source of protein. Aquaculture can be defined as the breeding and harvesting of aquatic organisms, both marine and freshwater species for human or animal consumption. It is the fastest growing animal food-producing sector globally, contributing to the increasing demand for seafood (FAO, 2014). Especially with the developing aquaculture production around the world, there is a large potential of further increases in fish supply an important source of animal protein for human consumption. Globally, fish represents about 16.6% of animal protein supply and 6.5% of all protein for human consumption (FAO, 2012). While, fish produced by aquaculture industry covers around half (50.3%) of all fish supplies destined for direct human food consumption (FAO, 2012). Farmed fish production is expected to continue to increase and intensify to meet the world's growing demand for protein (Naylor et al., 2000; Lech et al., 2012). The World Bank developed a scenario analysis in their report `*Fish to 2030*` (Msangi et al., 2013) predicting that aquaculture will continue to fill the supply-demand gap and that by 2030, 62% of fish for human consumption will come from this industry.

The Norwegian aquaculture has grown from its pioneering days in the 1970s to become one of the world`s leading intensive farming industry primarily based on Atlantic salmon and rainbow trout production (Taranger et al., 2015). However, despite having achieved good progress in terms of expansion and intensification, in many countries the aquaculture sector has confronted with many growing constraints including lack of feedstuffs, fish vulnerability to diseases and adverse environmental conditions (Taranger et al., 2015). In 1980s, bacterial and viral disease outbreaks have been the major challenge in Norwegian aquaculture, but now because of vaccine development and other measures, the situation for most bacterial diseases seems to be under control. Nevertheless, there are many viral infections and sea lice problems still reported in Norway and many other countries affecting many aquatic animal species, resulting in partial or sometimes total loss of production (FAO, 2012). Moreover, another main challenge is to produce adequate quantities of aquatic feed, as the fish feed industry has relied heavily on fishmeal (FM) and fish oil (FO) supplied by capture fisheries as their important source of protein and essential

fatty acids (EFAs). Thus, a growing aquaculture industry is placing an increasing pressure on global fisheries by feeding wild fish to farmed fish (Tacon and Metian, 2009; Taranger et al., 2015). Furthermore, farming of carnivorous species has been perceived as a net fish consumer rather than producer, raising concerns about the long-term sustainability of the industry (Barlow, 2000; Naylor et al., 2000; Tacon and Metian, 2009; Hardy, 2010).

Consumption of high volumes of marine based feed in the aquaculture industry, especially for carnivorous species such as the Atlantic salmon (*Salmo solar*), has been justified not only because of their high levels of dietary essential amino acids (EAAs) and EFAs but also due to their high palatability and digestibility (Lech et al., 2012). However, due to the rising cost of marine resources and limits on capture fishery production (Naylor et al., 2000), researchers are investigating other protein sources that could be used as alternatives to FM for the aquaculture industry (Knudsen et al., 2008; Torstensen et al., 2008b; Desai et al., 2012; Green et al., 2013; Hartviksen et al., 2014b). Hence aquaculture feed industry has made substantial progress in developing more plant-based diets to substitute for marine products in aquafeeds, and the inclusion level of FM has been reduced from 50-60% to the existing inclusion level of about 10-20%, and this will continue as the growth in production continues (Hardy, 2010). For some herbivorous and omnivorous farmed fish species, complete replacement of FM with plant protein ingredients has been accomplished in research studies (Hardy, 2010). Similarly, some achievements have also been reported with complete FM replacement using blend of diverse plant proteins ingredients for late juvenile stage of Atlantic salmon (Burr et al., 2012). Some of the alternative protein sources for carnivorous fish, such as soybean meal (SBM), soy protein concentrate (SPC), maize gluten, wheat gluten, pea protein, horse beans, potato protein concentrate, sunflower, canola/rapeseed, lupines, flax/linseed and cottonseed meal, have been investigated as potential replacements for FM (Naylor et al., 2000; Francis et al., 2001a; Gatlin et al., 2007; Rosamond et al., 2009; Ytrestøyl et al., 2015). SBM is potentially the most promising plant-based substitute, widely available at a relatively low price with good amino acid profile for fish production (Van den Ingh et al., 1991; Storebakken et al., 2000b; Lech et al., 2012).

Plant ingredients and their chemical constituents may have significant effects on the composition of gut microbiota (Ringø and Olsen, 1999; Ley et al., 2008). The change in the level and composition of gut microbiota in fish caused by plant feed ingredients, is currently receiving more research attention. One of the research studies that have been carried out to investigate the effect

of commercial diets on the levels and composition of microbiota in Atlantic salmon showed that, fish harbour a great diversity of gut microbiota (Gajardo et al., 2016). Regardless of their importance in terms of microbial biodiversity, the function of microbiota in the digestive processes of the Atlantic salmon is not clearly known. The gut microbiota of humans and most animals are considered important for their enzymatic functions in digestion of especially cellulose and other complex carbohydrates, but also simple and complex proteins, and lipid ingested with different foods and feeds. Like other animals, the gut microbiota of fish is suggested to play important roles in the nutrition and health of the host, promoting nutrient supply through their metabolic activities, preventing the colonization of infectious agents and maintenance of normal mucosal immunity (Ringø et al., 1995; Sugita and Ito, 2006; Ley et al., 2008; Denev et al., 2009; Nayak, 2010; Merrifield et al., 2010b).

The effect of anti-nutritional factors (ANFs), including fibres and other non-digestible carbohydrates, present in most plant ingredients are challenging due to their negative impact in digestion and absorption of nutrients, growth and ultimately fish health (Ringø et al., 1995; Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003; Knudsen et al., 2008; Mansfield et al., 2010; Geay et al., 2011; Desai et al., 2012). Some of the most important ANFs present in plant ingredients used in aquafeeds include lectins, saponins, isoflavones and phytosterols (Knudsen et al., 2008; Hartviksen et al., 2014; Krogdahl et al., 2015). Predictive studies on the levels of the individual ANFs and their interaction effects are important to consider their effect on gut and animal health. Like other monogastric animals, Atlantic salmon don`t have enzymes to digest complex carbohydrates/fibres and these may therefore influence gut microbiota, and hence gut and animal health (Bakke-McKellep et al., 2007; Ringø et al., 2016). However, it is often not possible to discriminate whether gut microbiota response to plant ingredients is caused by indigestible carbohydrates or by other ANFs present (Bakke-McKellep et al., 2007; Merrifield et al., 2010b; Gajardo et al., 2016; Gajardo et al., 2017).

Therefore, in this study, a simulation model mimicking microbial processes in salmon intestine was used to investigate the individual and combined effects of four different purified ANFs on gut microbiota of farmed Atlantic salmon. The four ANFs purified from soybean including lectin, saponin, isoflavonoid and phytosterols and their combination were used to investigate the direct effects of ANFs on gut microbiota of farmed Atlantic salmon. One of the approaches employed during this *in vitro* study was to investigate the ANFs effects in metabolic processes. This was

assessed by various parameters including measurement of gas production, pH change and the level of metabolites released such as SCFAs that are normally produced by fermentation process in the hindgut of fish (Clements et al., 1994; Clements and Raubenheimer, 2006). The evaluation of gas production in the different treatments vessels was carried out during fermentation processes while the other parameters were measured at the end of the fermentation. These parameters such as gas production, change in pH, redox potential, and levels of metabolites released were used to estimate the direct effect of ANFs on gut microbiota. Collected data have been analysed and representative results and their implications are described in this paper.

2. General structure and function of fish alimentary tract

2.1 Anatomy and digestive physiology

The Gastrointestinal Tract (GIT) of Atlantic salmon, just like in other teleost fish, is a tube that passes through the body and is anatomically, functionally and histologically differentiated into different segments: oesophagus (ES), stomach, proximal intestine (PI) with adjacent pyloric caeca, mid-intestine (MI), distal intestine (DI) and rectum (Fig. 1). The lining of the tract is a mucous membrane and represents an interface between the external and internal environments. In conjunction with the associated organs (e.g., pancreas, liver and gall bladder), it provides the functions of digestion, osmoregulation, immunity, endocrine regulation of GIT and systemic functions, as well as the elimination of environmental contaminants and toxic metabolites. Carnivorous species in general show the shortest GI tract, typically less than the body length, whereas in herbivore, such as tilapia, the GI tract may be more than 20 times the body length as reviewed by Ringø et al. (2016) and Wang et al. (2017).

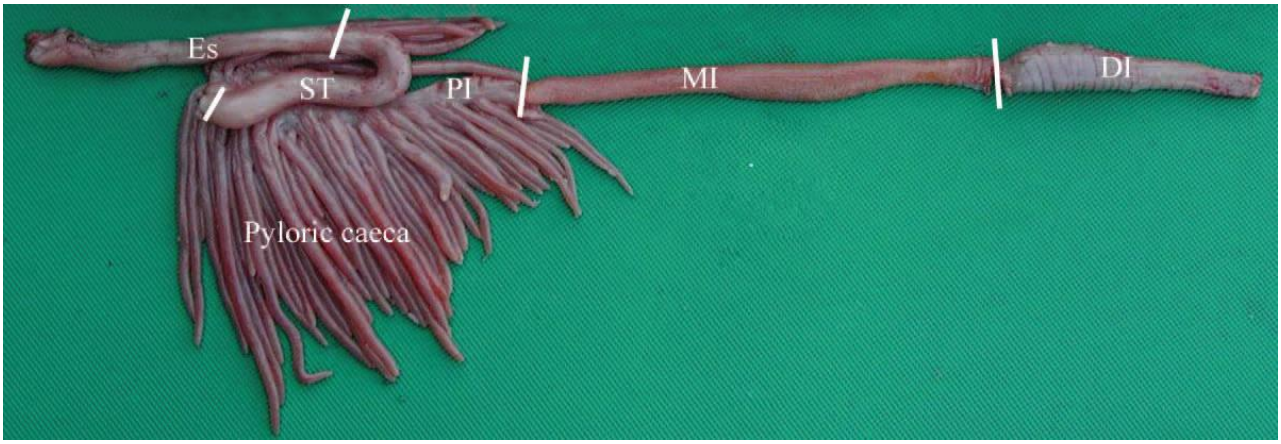

Figure 1. Illustration of the GIT of Atlantic salmon.
Esophagus (Es), stomach (ST), proximal intestine (PI) with pyloric caeca, mid (MI) and distal intestine (DI).(Original photo taken by Krogdahl Å, shown by Sahlmann (Sahlmann, 2013).

The function of the oesophagus is mainly to pass food from the mouth to the stomach. The oesophagus of salmon, like in most fish, is short and of small diameter, but with the possibilities to expand greatly. Numerous mucus-producing cells located in the lining supply mucus that aids in food passage. A common feature of carnivore fish species is great elasticity and strong musculature in the stomach wall. In some fish species, the muscles of the stomach seem to function as a grinder. Digestion is initialised in the stomach with its acidic environment and by the digestive enzyme pepsin. Pyloric caeca and PI are surrounded by mesenteric adipose tissue with

interspersed, diffusely organized endocrine and exocrine pancreatic tissue. When ingested nutrients are passed on into the PI, the release of pancreatic enzymes such as trypsin, chymotrypsin, elastase, α-amylase and lipases, as well as bile from the liver via the gallbladder, is initiated. The pH of the stomach in fish is higher compared with that in mammals, and may be of relevance for microbial survival in the stomach with higher survival during passage of the stomach in fish (Ringø et al., 2016). The lack of acidification in the foregut of stomachless fish species makes it even more likely for microbes to survive the passage to the more distal parts of GIT in these fish compared with salmon and other fish with stomach. In cod, lower pH has been reported in the DI compared with the other mid and pyloric regions and it was speculated as the result of microbial activity (Ringø et al., 2016). The mucus-producing cells covering the intestinal mucosal folds have many functions such as providing physical barrier by restricting the motility and attachment of pathogens and toxins.

In Atlantic salmon, the increased mucus and bicarbonate secretion from the pancreas and bile are important to buffer the acidic chyme coming from the stomach, which creates an optimal environment for pancreatic enzymes, while final digestion of peptides and digestible carbohydrates is completed by brush border membrane enzymes in the epithelial lining as reviewed by Nayak (2010). Nutrient absorption can occur throughout the intestinal tract via the epithelial lining, however majority of the nutrients are absorbed in the PI with the adjacent pyloric caeca, and to a lesser extent in the following regions including the distal intestine (Krogdahl et al., 1999; Bakke-McKellep et al., 2000a). Moreover, recent reviews by Ringø et al. (2016) and Wang et al. (2017) indicated that the mucus itself also contains antimicrobial peptides, lysozyme and immunoglobulins and hence acts as a connection between the physical, chemical and immunological barriers. Furthermore, some enzyme-producing microbiota from fish GI tract, and extensive range of enzymes (e.g. amylase, cellulase, lipase, proteases, chitinase and phytase) produced by GI bacteria might have a significant role in digestion (Ray et al., 2012).

2.2 Immune function in the GIT of fish

Fish have evolved with both non-specific (innate immunity) and adaptive (acquired) immune mechanisms. The innate immune system generates a fast, non-specific reaction to the pathogen infecting the host organism. It gives the first line of defense by means of epithelial barriers such as the mucus membranes and physiological barriers like stomach pH, gut microbiota and chemical

mediators secreted by the mucus (defensins, lysozyme, transferrin, complement system, etc.) (Pérez et al., 2010; Trichet, 2010; Rombout et al., 2011). Activation of the innate immunity is dependent on the recognition of structural motifs expressed only by pathogens. These motifs are known as pathogen-associated molecular patterns (PAMP) that have specificity for structures shared by different classes of pathogens such as bacterial or fungal glycoproteins and lipopolysaccharides (Trichet, 2010; Boltaña et al., 2011). These motifs are recognized by the pattern/pathogen recognition receptor (PRRs), receptors expressed on the surface of fish phagocyte (macrophages and neutrophils) that recognize PAMPs and activate an innate immune response (Rombout et al., 2011). Hence, the innate immunity is limited in specificity with the germline encoded PRRs that respond to PAMPs. Phagocytes and natural cytotoxic cells (NCC) are the main cellular elements of the innate immunity. Natural cytotoxic cells possess receptors that recognize proteins expressed at the surface of virus-infected cells (Rombout et al., 2011). Involvement of these cells and inflammatory response through the release of chemical mediators represents a second line of defense that is initiated if the pathogen has been able to pass the epithelial and physiological barriers. The actors of the inflammatory response are interferon (IFN), interleukins (ILs), chemokines and factors like tumor-necrosis factor (TNF-α) (Trichet, 2010; Rombout et al., 2011).The complement system appears to be one of the central immune responses in fish involved in the control of inflammation, opsonisation of immune complexes and microorganisms, and lysis of pathogens. The non-specific immune elements not only act as first line of defense against pathogens, but also play an instructive role in the development of acquired immune response (Boltaña et al., 2011; Ringø et al., 2016). The adaptive immune system of fish is similar to other animals divided into cell-mediated and humoral immunity. Cell-mediated immune components consist of thymus-dependent lymphocytes, or T-cells, which express T-cell receptors (TCR) on their surface and provide specificity against intracellular pathogens, while antibodies, or immunoglobulins (Ig), produced by B cells are the primary effector molecules of humoral immunity that give specificity. In contrast with mammals, the adaptive immune component of fish have been reviewed by Trichet (2010) as a less specific immune system with a shorter response, a limited immunoglobulin repertoire, a weak memory and a mucosal response (whose importance in comparison with the systemic response is not really known. Fish do not have lymph nodes; most likely, their kidney, spleen, and gut-associated lymphoid tissue (GALT) play an equivalent role to the lymph system in mammals with respect to antigen processing and presentation. Teleost fish

are the most primitive bony vertebrates that produce immunoglobulins. In contrast to mammals and birds, these species are not only devoid of immunoglobulin A (IgA) or a functional equivalent (Yong-An et al., 2010), but also lack an organized GALT, and thus, have no Peyer's patches (PP) or mesenteric lymph nodes (reviewed by Rombout et al. (2011) and Salinas et al. (2011)). In addition, until recently, teleost fish B cells were thought to express only two classes of immunoglobulins, IgM and IgD, in which IgM was thought to be the only one responding to pathogens both in systemic and mucosal compartments. However, a third teleost immunoglobulin class, IgT/IgZ, has recently been shown to behave as the prevalent immunoglobulin in gut mucosal immune responses (reviewed by Salinas et al. (2011)). Based on anatomical location, the mucosa-associated lymphoid tissue (MALT) in teleost fish is subdivided into GALT, skin-associated lymphoid tissue (SALT), and gill-associated lymphoid tissue (GIALT). However, the GALT which represents an essential part of an organism's adaptive defense system is considered to protect the host against pathogens not only by fighting the intruding bacteria but also by modulating the composition of the resident gut microbiota (Trichet, 2010).

Furthermore, the gut microbiota is believed to have significant effects on normal functioning of the immune apparatus of the GIT and resistance of the fish towards pathogens and other foreign factors constantly influencing the fish via the intestine (Sugita et al., 1996; Montalban-Arques et al., 2015). The gut microbiota of fish and their metabolites also play important roles in host digestive function, amino acid production, secretion of inhibitory compounds, gastric mucosa development, mucosal tolerance and immunity development that protect against bacterial pathogens in the intestine (Ringø and Gatesoupe, 1998; Merrifield et al., 2011).

3. Dietary requirements in fish

Feeds and feedstuffs that contain nutrients and energy are essential for fish growth, reproduction, health and high quality product. The main dietary requirements of fish, as other animals, include protein, lipid, carbohydrate, mineral and vitamins. Deficiencies of these substances can reduce growth rates or lead to diseases (Craig and Helfrich, 2002). In some cases, excesses can also cause a reduction in growth rate (NRC, 1993; Craig and Helfrich, 2002). Fish nutrition has been advanced dramatically in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health. The development of new species-specific diet formulations supports the fish farming industry as it expands to satisfy increasing demand for affordable, safe, and high-quality fish and seafood products (Craig and Helfrich, 2002; Tacon and Metian, 2009).

3.1 Energy requirements

Energy intake is a basic nutritional requirement because energy is vital for maintenance of life processes, which takes priority over growth and other functions. Energy is not a nutrient; it is a product of metabolic oxidation of carbohydrates, fats, and proteins. The chemical energy stored in feeds ingredients is measured in a bomb calorimeter by combustion and the energy liberated as heat is measured as calories (cal) or joules (J), and expressed as gross energy (GE) content (NRC, 2011). The calculated mean gross energy values for lipid, protein and carbohydrate (in kJ/g) are respectively: 39.5, 23.6 and 17.2 (Blaxter, 1989). The digestible energy (DE) content corresponds to the gross energy (GE) ingested, less the GE excreted with the faeces. Ratios of digestible protein to digestible energy (DP/DE) for maximum live weight gains for several fish species have been reported (NRC, 2011). Furthermore, the energy requirement for maximum growth is influenced by water temperature, type and size of fish, diet composition and nutrient availability (Storebakken, 2002; NRC, 2011). Since lipids are the primary non-protein energy source in salmonid and marine fish feeds, the protein-energy allowance for these feeds are sometimes reported as the ratio of protein to lipid (Lall and Dumas, 2015).

3.2 Carbohydrate requirements

Digestion and absorption of nutrients may vary among fish species due to differences in the morphology of the digestive tract, enzymatic digestion, gut pH, and other factors (Lall and Dumas,

2015). Carbohydrate is the cheapest source of energy, however, the digestibility of the carbohydrates in grains is highly variable among fish species (Bakke et al., 2010). There are mainly two types of carbohydrates in commercial salmon diets: starches and non-starch polysaccharides (NSPs). However, the digestible carbohydrates are found in plant feed ingredients such as grains or legume also contain other compounds, including NSP that are indigestible and may inhibit digestion and utilization of nutrients (Krogdahl et al., 2005). The value of carbohydrate as an energy source is variable among species (NRC, 2011). Omnivorous and herbivorous species derive a high amount of energy from grain starch. While, carnivorous fish fed with high starch diets seem to have a poor ability to take care of excess glucose (Hemre et al., 1995b). Starches are commonly used as ingredients in salmon diets mainly for gelatinisation during extrusion so that to improve availability, but salmon still have a limited ability to hydrolyse gelatinized starch (Lee, 2015). On the contrary, a review by Hemre et al. (2002) indicated that carnivorous species do show improved growth if fed with a low-starch diet compared with a diet having no starch as an ingredient. One reason can be that Atlantic salmon have limited activity of α-amylase in the intestine due to mutational defects at proximity to the active site of the enzyme that could impair substrate binding (Frøystad et al., 2006). Intestinal brush-border disaccharidases are active in salmon, and maltase has the highest activity. Most of the disaccharidase activity is found in the pyloric caeca and the proximal part of the intestine, which is also the main site of starch hydrolysis (Krogdahl et al., 1999). Insoluble NSP, such as cellulose, mainly act as fillers in the stomach and intestine do not affect uptake of nutrients (Storebakken, 2002). Soluble NSP, for instance mixed β-glucans and arabinoxylans in grains, and pectic and acidic polysaccharides in legumes, increase the viscosity of the digesta and the water content of the faeces and reduce digestibility of water and lipid-soluble components.

3.3 Protein and amino acid requirements

Protein is the most expensive macronutrient of fish feed. The protein requirements, meaning the minimum amount required as the major source of the dietary amino acids and to achieve maximum growth, have now been estimated in juvenile fish of many species (NRC, 1993, 2011). Protein diets are usually lower for herbivorous and omnivorous fish than for carnivorous fish, and are higher for fish reared in high density (like in circulation system) than low density (pond aquaculture) systems (Craig and Helfrich, 2002; NRC, 2011). Amino acid requirements also refers

mainly for the absolute requirement of 10 amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) has been demonstrated in most fish species (NRC, 1993). Based on size, the amino acids are generally required in higher amount for smaller fish. The level of the 10 essential amino acids in several protein diets that are commonly used as alternative ingredients have been compared to their level in FM are shown by under Table 1. Digestion and absorption of protein are highly different from one feed ingredient to another, or even within feed ingredients processed by the same method (Storebakken, 2002). Salmon digest protein efficiently, and more than half of the protein is hydrolysed and absorbed in the pyloric region (Krogdahl et al., 1999).

Table 1. Amino acid composition in some common plant protein ingredients compared to the FM. Source. Adapted from Sørensen et al. (2011a).

g (16 g N)$^{-1}$	Fish meal [a]	Soybean [b]	Soy protein concentrate [c]	Rapeseed [d]	Sunflower [e]	Pea [f]	Lupin [g]
Arginine	5.4	6.7	6.4	2.1	3.6	8.2	11.2
Histidine	2	2.4	2.5	1	1	2.7	1.8
Isoleucine	3.6	4	4.1	1.4	2.1	4.5	3.9
Leucine	6.3	6.7	6.6	2.6	3	7.5	7.7
Lysine	6.6	5.1	5.5	2.1	0.7	7.4	4.9
Methionine	2.5	1.1	1.2	0.7	0.8	0.9	0.5
Phenylalanine	3.5	4.6	4.5	1.4	2.2	4.9	3.8
Threonine	3.9	3.7	3.5	1.6	1.7	3.7	4.0
Tryptophan	1	1.5	1.3	0.4	0.6	0.9	0.7
Valine	4.1	4.1	4.1	1.8	2.3	4.8	3.5

[a] Low-temperature dried FM (Romarheim *et al.* 2005), [b] Hexane-extracted & toasted SBM with hulls (Romarheim *et al.* 2005), [c] ADM, Nederland, [d] Defatted rapeseed meal (Hertrampf & Piedad-Pascual, 2000), [e] Defatted and Dehulled sunflower meal (Hertrampf & Piedad-Pascual, 2000), [f] Pea protein concentrate, 350 g kg $^{-1}$ CP (Øverland *et al.* 2009), & [g] White luin (Hertrampf & Piedad-Pascual, 2000).

It is important to know the protein and the amino acid requirements for each size and species of fish reared. The proportion of DP to DE for maximum growth have been measured using practical diets and the optimal *DP/DE* ratio for growth and feed utilization in Atlantic salmon is around 23 g MJ^{-1} for fingerlings, 20 g MJ^{-1} for smolts, 19 g MJ^{-1} for fish weighing 1 to 2.5 kg, and 16–17 g MJ^{-1} for fish weighing 2.5 to 5 kg, are suggested to be optimal (Einen and Roem, 1997; Storebakken, 2002). Particularly, since the start of the salmon industry, it has been common practice to include high proportion of crude protein in diets for juvenile salmon and to reduce the

dietary protein content in grower diets, provided the protein-rich feed ingredients are of high quality (Storebakken, 2002). As fish grow larger, their protein requirements usually decrease. Protein requirements also vary with rearing environment, water temperature and water quality, as well as the genetic composition and feeding rates of the fish (Craig and Helfrich, 2002).

3.4 Lipid and essential fatty acid requirements

Dietary lipids are important sources of energy, EFAs and phospholipids, the latter as components of the cell membrane. Lipids also assist in the uptake of lipid-soluble nutrients such as fat-soluble vitamins. Salmon must have oil with a low melting-point, as saturated fats are poorly digested. The pyloric caeca and the proximal intestine are the main sites of fat digestion and absorption but some absorption also occurs in the distal portion of the intestine (Krogdahl et al., 1999). The digestibility of fish-oil (FO) from fishmeal (FM)-based extruded diets ranges from 90 to 95% in salmon (Storebakken et al., 2000a). Most animal species including most freshwater fish, can readily elongate and desaturate a dietary supply of C-18 EFAs to its higher homologues 20:5 n-3 and 22:6 n-3 and fulfil their n-3 EFA requirement (NRC, 2011). But several marine fish including Atlantic salmon must be provided as dietary supply of 20:5 n-3 and 22:6 n-3 in diet (Storebakken, 2002; NRC, 2011) because marine fish lack a functional Δ5-desaturase (NRC, 2011). The need for high fat content in the diet for Atlantic salmon reflects the body composition of the fish. The high dietary lipid level stresses the need to use high-quality oils and to know the effects of the lipid source on growth and salmon health, as well as product quality. The EFAs are required for proper functioning of many physiological processes, reproduction, health, and flesh quality of fish as well as for normal growth and development (NRC, 2011).

The EFAs include Polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series, such as alpha - linolenic acid, 18:3n-3 and linoleic acid, 18:2n-6. Generally, long chain PUFA requirements of freshwater fish and salmonids can be met by the supply the precursor fatty acids 18:3n-3 and 18:2n-6 in their diets, because they are desaturated and elongated into the longer PUFA, such as typical ‘marine’ FAs: 20:5n-3 (eicosapentaenoic acid (EPA)) and 22:6n-3 (docosahexaenoic acid (DHA)) and 20:4n-6 (arachidonic acid) by the endogenous enzyme systems. Whereas, marine fish lack or have very low activity of D 5-desaturase, thus they can only be met by supplying the EPA and DHA (NRC, 1993; Storebakken, 2002; NRC, 2011).

Although omega-3 PUFAs are abundant in FO, due to over-fishing of wild species and other marine environmental issues (Miller et al., 2008), together with the increasing global human population, other alternative sources are increasingly being considered (Gatlin et al., 2007; Hardy, 2010; Lenihan-Geels et al., 2013). Many efforts have been carried out to investigate certain plant oils as possible sustainable partial substitutes for FOs in compound fish feeds (Montero et al., 2005; Miller et al., 2008; Nichols et al., 2010; Lenihan-Geels et al., 2013). The common plant oils used for fish feed have been soybean, linseed, rapeseed, sunflower, palm oil and olive oil (Lall and Anderson, 2005; Tacon et al., 2011). Soybean and rapeseed oil are considered possible alternative lipid sources for fresh water and salmonid fish since they are rich in FAs, especially linoleic and oleic acid, but devoid of long-chain n-3 PUFA (Montero et al., 2005). However, the use of plant oils is not widely accepted, as consumers finally will receive lower levels of EPA and DHA from the farmed fish products. Therefore, for the future the most promising alternative approach is being developed from single cell organisms, mainly microalgae such as heterotrophic dinoflagellates, thraustochytrids, some species from other algal groups, and genetically modified crops (Miller et al., 2008; Lenihan-Geels et al., 2013).

3.5 Main mineral and vitamin requirements

Most essential elements required by other animals are also assumed to be indispensable for Atlantic salmon, and requirements have been reported for phosphorus, magnesium, iron, copper, manganese, zinc, selenium and iodine (Lall and Milley, 2008). Calcium and phosphorus are directly involved in the development and maintenance of the skeletal system and take part in several physiological processes. The calcium requirement of fish is met largely by absorption through gills and skin in fresh water and by drinking seawater. Though, the need for calcium is affected by the water chemistry and species differences, the concentration of dietary calcium rarely seems critical for salmonids, and a dietary requirement has not been demonstrated (NRC, 1993). The concentration of phosphorus is low in natural waters. Therefore, feed is the main source of phosphorus for fish. Thus, it is important to supplement salmon in fresh water through diets to cover the phosphorus requirement (Storebakken, 2002). The availability of phosphorus to the salmon is highly variable depending on the form in which it is fed. For example, phytic acid phosphorus in plant-feed ingredients has low availability to salmon, while some inorganic phosphorus salts are easily available (Storebakken et al., 1998). Moreover, the function of

phosphorus in carbohydrate, lipid, and amino acid metabolism, as well as in various metabolic processes involving buffers in body fluids, also well documented (NRC, 2011). Thus, the dietary supply of phosphorus is more critical than that of calcium because fish must effectively absorb, store, mobilize, and conserve phosphorus in both freshwater and seawater environments (Lall and Milley, 2008).

Vitamins are organic compounds that are different from amino acids, carbohydrates, and lipids. They are required in trace amounts from different diet sources for normal growth, reproduction, and health. Vitamins are commonly classified as water-soluble and fat- soluble vitamins. Water-soluble vitamins are found in cereal grains, fresh organ meats, citrus fruit (rich in vitamin C) and legumes. Most water-soluble vitamins are required in relatively small amounts, have primarily coenzyme functions, and are known as the vitamin B complex. Some water-soluble vitamins such as choline, inositol, and vitamin C, are required in larger quantities and have wider applications and functions other than coenzymes (NRC, 2011). The fat-soluble vitamins, A, D, E, and K, are absorbed in the intestine along with dietary fats; therefore, conditions favourable for fat absorption also enhance the absorption of lipid-soluble vitamins (NRC, 2011). Good sources of fat-soluble vitamins are FOs oils and meals, some grains and leafy green vegetables. Among the lipid-soluble vitamins, vitamin A and E have received most attention in salmon diet (Storebakken, 2002; Hamre et al., 2010). Deficiency of the antioxidant vitamins, vitamins A, C, E and b-carotene, generally reduces resistance of farm and laboratory animals to bacterial infections (Halver and Hardy, 2002; NRC, 2011).

4. Commercial fish feed formulation

The nutrient balance of feed ingredients influences feed utilization and growth of aquaculture species. Feed formulation is the process of combining feed ingredients to form a mixture that will meet the specific goals of production. Ingredients used in commercial fish diets can be classified as sources of amino acids, EFA, carbohydrates, vitamins and minerals (NRC, 1993, 2011). Although, the source of dietary ingredients may vary from region to region based on the availability, it is very important to determine the ratio of protein to energy of commercial feeds separately for each fish species (Storebakken et al., 2000b).

During feed production, numerous materials that act as binders in fish feed are incorporated to improve stability in water, increase pellet fitness, handling and shipping (Hansen and Storebakken, 2007; Sørensen et al., 2011b). Some binders are by-products of cereal grains or plants which provide energy or nutrients to the diet. For example, 20% pre-gelatinized potato starch is added to diets to increase durability and water stability of pellet. Formulations of pellets by extrusion process do not need pellet binders, as gelatinized starch provide sufficient binding capacity (NRC, 2011). Moreover, extrusion can have positive effect on digestibility of all nutrients in plant feedstuffs, attributed to a partial degradation of NSP and thus improved energy utilization (Francis et al., 2001a; Sørensen et al., 2011b). Feed formulations for salmon and trout have changed greatly since extrusion pelleting has been introduced. Extruded pellets are formed by extrusion of a moist mixture heated from 100°C to 150°C under pressure (20%-24%), followed by drying to reduce the moisture content to 10% or less (Hardy, 2010). Prior to the late 1980s, diets were produced using compressed (steam) pelleting a process that produces a hard, dense pellet. The compressed pellets cannot absorb as much added lipid as can extruded pellets, limiting total lipid to about 20%, while for extruded pellets total lipid levels up to 35-40% can be realized (Hardy, 2010). For example, the feedstuffs previously used for Atlantic salmon were mainly FM and FO. However, due to increased costs for these feedstuffs, alternative plant proteins are used as indicated in Table 2 (Ytrestøyl et al., 2014).

Under commercial diet formulation, it is not uncommon to have some other additives including attractants, carotenoids and enzymes. For instance, carotenoids such as synthetic astaxanthin and certain natural supplements from yeast or algae (phytoplankton) are useful to develop an attractive pink-red colour to the salmon flesh (Ambati et al., 2014). Such pigments are also added to salmon

feeds to improve growth, digestibility, osmoregulation, palatability and preservation of the feed (NRC, 2011). Antioxidants are used as additives to preserve fats and oils by hindering deterioration and rancidity due to oxidation. Furthermore, dietary inclusion of exogenous enzymes that improve digestibility of nutrients, such as NSPs and phytate phosphorus is gaining relevance in aquafeeds to increase digestibility and the bioavailability of nutrients in oilseed proteins (Sebastian et al., 1998; Lei et al., 2007; Lei and Porres, 2011).

4.1 Plant ingredients in Salmonid feeds

The protein sources that are used in fish feed must cover the nutritional requirement of fish for essential amino acids. The development of new aquafeeds formulations that contain plant ingredients differ dramatically from the natural diets of fish, because they lack the correct amount of all the essential amino acids. The shift from the natural marine based proteins sources to new salmonid feed formulation is met with the inclusion of various plant materials, such as land-based plant proteins, including soybean, canola and peas, with the addition of amino acids, vitamins and minerals are used, even in carnivorous fish species, such as salmonids (NRC, 1993; Gatlin et al., 2007; NRC, 2011). Oil crops like canola/rapeseed, soybean and sunflower, cereal-co-products like wheat gluten, corn gluten, barley, rice, pea meals, lupin seed, and various other plant proteins, as well as yeast, insects and algae are also among the commonly considered sources (Naylor et al., 2000). Apart from gluten meals that contain 60%, the protein content of many other plant ingredients such as soybean meal (45%) and lupin (26-30%) is lower than that of FM, which is 60-70% (Pratoomyot et al., 2010; Burr et al., 2012). Although, plant ingredients offer the global fish farming industry with possibilities of adequate supply of feed raw materials, their inclusion level can be limited due to their poor digestibility and low content in certain essential amino acids such as lysine and methionine (Storebakken et al., 1998) .

To increase the chance of maintaining the essential requirements, protein from several plants ingredients must be mixed together depending on the composition of their amino acids or it is possible to supplement with commercially available amino acids when the raw materials contain too low levels of these EAAs. In addition, further processing of many plant feedstuffs to protein concentrates have great potential for use in aquafeeds because of their high protein content and because they are almost devoid of anti-nutritional factors. SPC, which contains about 65% crude protein, becomes one of the potentials to replace FM at an increasing proportion in commercial

diets (Gatlin et al., 2007; Lech et al., 2012). Corn gluten meal is currently used in feeds for salmonid fish, with upper inclusion limits of 20-25% (Gatlin et al., 2007). Wheat gluten have higher price and its binding proprieties of its protein causes undesirable effects on pellet quality. Thus, it is incorporated in diets at lower levels than corn gluten (Storebakken et al., 2000c; Gatlin et al., 2007). Peas are rich in starch (>40%) and when considering their incorporation in to diets this high carbohydrate level must be taken in to consideration, as salmonid fish have limited ability to use dietary carbohydrate(Tacon et al., 2009; Tacon et al., 2011). However, pea protein concentrate, which is processed/dehulled form of pea is a good protein source (Lech et al., 2012). According to Tacon et al. (2009), legumes are incorporated in diets for carnivorous fish up to 15-25%, with mean values of 10-15%. Cereals have low protein content (8-12%) and are rich sources of starch (~ 60%).

Plant feedstuffs are the major dietary protein sources for omnivorous and herbivorous fish and have been second to FM in diets for carnivorous species (Tacon et al., 2009; NRC, 2011; Oliva-Teles et al., 2015). However, according to Ytrestøyl and colleagues, in 2012 three major feed companies in Norway; BioMar, Ewos and Skretting, used around 1,630,000 tonnes of ingredients to produce salmon feed in Norway (Ytrestøyl et al., 2015). Out of the total feed only 31% was of marine origin and 66% was derived from plants. The level of plant protein source from the total production was 37%, which was mainly soy protein concentrate, followed by sunflower expeller and wheat gluten, while rapeseed oil was the only plant oil used in the salmon diet in 2012 as shown under Table 2.

The limited supply of fish meal and fish oil makes this shift from marine to plant ingredients necessary to be able to produce increasing amounts of salmon. Information about the ingredients used for feed production in 2012 and 2013 was obtained from three feed producer companies (BioMar, EWOS and Skretting) who have a market share of 90% of salmon feed in Norway (Ytrestøyl et al., 2015). Furthermore, feed composition in the Norwegian intensive salmon farming has changed substantially since sustainability issues started. Until 1990, around 90% of the feed in the Norwegian salmon industry was composed of ingredients of marine origin whereas less than 30% of the diet was of marine origin in 2013 (Fig. 2).

Table 2. The most common plant ingredients used in Norwegian salmon feed production in 2012 & 2013. Source: Original data from EWOS, BioMar & Skretting and analysed and shown by Ytrestøyl et al. (2014).

Plant ingredients (tonnes)		**2012**	**2013**
Protein sources	Soy protein concentrate	346 730	364 980
	Wheat gluten	94 137	99 348
	Sunflowermeal	97 137	65 039
	Peaprotein concentrate	12 936	7917
	Fababeans	30 753	24 971
	Dehulled horse beans	4442	
	Maize	12 509	28 640
Sum plant protein sources		*598 861*	*590 896*
Oil sources	Rapeseed oil	298 991	309 497
	Other plant oils	0	0
Sum plant oil		*298 991*	*309 497*
Binders	Wheat	161 432	158 992
	Pea	16 466	22 055
	Tapioca	3396	
Sum plant ingredients		**1 079 146**	**1 081 439**

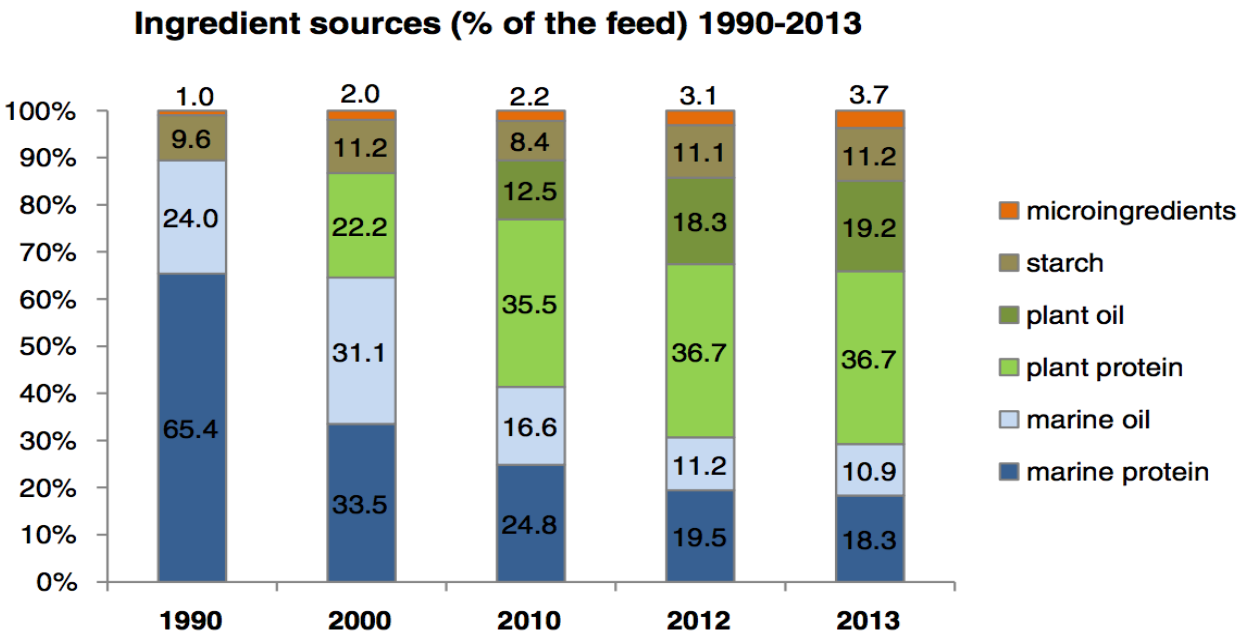

Figure 2. Development of salmon feed in Norwegian salmon farming from 1990 to 2013.
This figure illustrates that the use of marine proteins is declining from time to time, for example from 65.4% inclusion in 1990 dropped to 18.3% in 2013. Whereas, the inclusion of plant ingredients was increased from 22.25% (in 2010) to 36.7% in 2013. Due to the inhibitory substance in the plant ingredients, supplementation of essential microingrents was increased from 1% to almost 4%. Although, plants oils lack PUFA (EPA and DHA), since 2010 the inclusion of plant oils is growing as indicated from 2000 (12.5%) to 2013 (19%) as indicate in the figure. Source: Adapted from Ytrestøyl et al. (2015).

4.2 Soybean meal

The potential for alternative plant protein sources to replace limited marine ingredients in fish feeds is important for the future of the fish farming industry. For the past several years researchers have been investigating for suitable protein and oil alternatives for carnivorous fish to avoid the use of fish products in the feed (Refstie et al., 1999). Plant ingredients that contain high protein content, such as cereals and oil seeds have been tested as alternative feeds for fish meal. Soybean is the leading oilseed crop produced globally and used to produce a wide range of soybean products, such as soy flour, SBM and soy protein concentrate (SPC) that have been assessed in fish (Francis et al., 2001a; Gatlin et al., 2007; Ringø et al., 2009).

SBM has high protein content and good amino acid profiles with high palatability to most species of fish that makes it a potential alternative to replace FM (Booth et al., 2001; Francis et al., 2001a; Naylor et al., 2009; Tacon et al., 2011; Lech et al., 2012; Krol et al., 2016). SBM-based diets implicated in changes in the gut structure and function of fish leading to enteritis, the severity of which depends on the source (Urán et al., 2009a) and inclusion level of the SBM (Urán et al., 2009b). Although when heat-treated and supplemented with limiting amino acids, full-fat as well as defatted (standard; hexane-extracted) SBM-containing feeds lead to decreased growth, feed intake, energy and fat digestibility, and fecal dry matter in all salmonid species have been reviewed (Eriksen et al., 2009). Moreover, previous observations on SBM used as supplementary diet in salmon has proven that the fish gut microbial community are sensitive to dietary manipulation (Bakke-McKellep et al., 2007; Ringø et al., 2008). This is due to, the contents of various ANFs such as trypsin inhibitors, lectins, saponins, phytic acid, oligosaccharides, phytosterols and phytoestrogens, and are major impediments toward increased use of soybean products in diets for fish (Storebakken et al., 2000a; Francis et al., 2001a; Krogdahl et al., 2010; NRC, 2011; Krol et al., 2016). Thus, the effect of ANFs must be removed or inactivated by extrusion cooking and solvent extraction methods that give more refined feed ingredients in fish feeds (Storebakken et al., 2000a; Hardy, 2003; Lech et al., 2012). For example, one of the more refined product with high protein source can be obtained through application of ethanol extraction of soybean meal into SPC, which eliminates most of the soluble carbohydrates and ANFs that can negatively affect digestion of soybean in fish (Lech et al., 2012).

4.3 SWOT analysis of plant ingredients in aquafeeds

Strength

Plant ingredients are currently the most promising alternative protein and lipid sources in fish feed. Various grain legumes and cereal crops have great potential as new protein and oil sources (Gatlin et al., 2007). They are feasible alternative feedstuffs to replace FM because they possess certain characteristics, including widespread availability. Sustainable production, competitive prices, plus their ease of handling, shipping, storage and use in feed production. The use of plant ingredients has helped the aquaculture industry to grow at a lower inclusion level of FM and FO in diets (Storebakken et al., 2000a; Tacon et al., 2011). Compared with the cereal grains, the oilseeds such as rapeseed and soybean and their oil-extracted products are rich sources of protein making them well suited in salmon feed used at low environmental temperatures (Sørensen et al., 2011a). Moreover, there are a large variety of protein and lipid sources from crops including transgenic plants with a potential use in aquafeeds as substitutes for FM and FO respectively (Olsen et al., 2004; Robert, 2006).

Weakness

The use of plant feedstuffs in fish feeds has increased, but the presence of endogenous ANFs within plant feedstuffs is one of the major factors limiting their use in animal feeds including aquaculture feeds (Francis et al., 2001a). These ANFs can negatively affect the intestinal health of fish (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Knudsen et al., 2007; Knudsen et al., 2008). The inclusion of oils of plant origin that lacks the long-chain PUFA is leads to loss of acceptance by consumer of fish and fish products. This perception together with the negative impacts of ANFs present in plant origin ingredients are some of the weakness in using plant ingredients. Some of the problems particularly in Atlantic salmon is due to the high content of NSPs and negligible starch in these plant ingredients represents a major challenge due to limited carbohydrate enzymes in this species (Frøystad et al., 2006) such indigestible materials may negatively affect nutrient utilization and reduce feed efficiency in salmonids (Gatlin et al., 2007; Hansen and Storebakken, 2007). For instance, the oligosaccharide component of SBM has been linked with reduced growth performance and increased rate of SBM-induced enteritis in several salmonid fish species (Refstie et al., 1998; Krogdahl et al., 2000; Bakke-McKellep et al., 2007; Krogdahl et al., 2015; Krol et al., 2016).

In salmonids, inclusion levels above 5-10% –full-fat or defatted (hexane-extracted) soybean meal can lead to signs of inflammatory response in the distal intestine (Krogdahl et al., 2003). However, the main and significant weakness of the plant ingredients is that plant oils lack the LC-PUFAs mainly the EPA and DHA that are rich in FO. On the contrary, some plant oils such as palm oils, have high content of saturated FAs causing digestibility problem in cold water species (Torstensen et al., 2008a). Furthermore, despite the abundant supply of plant ingredients with high nutritional quality, the aquaculture sector is faced with criticism that some of these ingredients can be used directly for human consumption (Naylor and Burke, 2005; Tacon et al., 2011). The main challenges associated with replacement of FM with plant protein ingredients is: the low level of proteins, high level of carbohydrates, unfavourable amino acids profiles and mineral contents and presence of ANFs in plant ingredients (Gatlin et al., 2007; Bakke-McKellep and Refstie, 2008; Ringø et al., 2009).

Opportunities

The application of common processing techniques, such as dry and especially wet heating, extracting with water, and addition of feed supplements are crucial elements in maintaining product quality and successfully used to reduce/eliminate the concentration of antinutrients in plant feeds (Francis et al., 2001a; Barrows et al., 2007; Barrows et al., 2008). In addition, the use of protein concentrates after removal of NSPS and sometimes ANFs has resulted in the production of feed ingredients that have optimum nutritional content to be included in the feed formulation (Aslaksen et al., 2007; Gatlin et al., 2007). Furthermore, supplemental enzymes, now commonly used to improve the nutritional value of most commercial feeds, will become more functional under a variety of feed manufacturing conditions and feed system strategies for different animal species (Sebastian et al., 1998; Naylor et al., 2009; Lei and Porres, 2011).

Threats

Many alternative proteins including oilseeds (like soybean, rapeseed, sunflower, cottonseed), legumes (such as soybeans, other beans, peas, lupins) and miscellaneous processed plant protein products (including corn gluten meal and concentrates made from potatoes and leaves) have been reported as potential FM replacers (Tacon, 1994; Naylor et al., 2009). However, the use of plant based proteins in aquaculture faced major constraints identified including: lack of palatability, presence of ANFs in poorly processed plant legumes and oilseeds, limited availability and high

cost for miscellaneous processed plant proteins (Tacon, 1994). The production of krill, algae and GM based plant oils may be costly and competition with human needs may challenge their consumption by aquaculture feed suppliers (Miller et al., 2008; Olsen, 2011; Tacon et al., 2011). In addition, from consumer point of view, if the non-acceptance of GM products is continued in many parts of the world, the use of ingredients based on genetic modified plants would remain widely unaccepted as feed ingredients and can be considered as a major threat to the aquaculture industry (Sørensen et al., 2011a).

4.4 Anti-nutritional factors in plant based feed ingredients

The term "Anti-nutritional factor" (ANF) and "anti-nutrient" is defined as an endogenous substance found in foods and feedstuffs that produce negative effects on health and nutrient balance when ingested by animals or humans (NRC, 2011). Various ANFs that are found in a wide range of plant-based feed ingredients including legume seeds, oilseeds and other types of cereal grains used in aquafeeds as reviewed by Francis *et al*. (2001a) and Krogdahl *et al*. (2010). The soybean is a good example of known feedstuff containing ANFs such as phytate, trypsin inhibitors, lectins, and several other heat-stable components that have the ability to act as ANF when fed to fish (Francis et al., 2001a; Barrett, 2006; Ringø et al., 2016). Various ANFs have summarized under four groups (Francis et al., 2001a) i. Factors affecting protein utilization and digestion, such as protease inhibitors, tannins, lectins; ii. Factors affecting mineral utilization, which include phytates, gossypol pigments, oxalates, glucosinolates, iii. Antivitamins and IV. Miscellaneous substances such as mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogens and saponins. Some of the most common antinutrients that play major roles either by themselves or through their metabolic products are shown in table 3. However, the levels of all ANFs may be reduced by processing such as heat treatment and fermentation, treatment with enzymes that specifically inactivate the compound, selective breeding and genetic modification (Krogdahl et al., 2010).

Table 3. Antinutritional factors that are commonly found in alternative plant feeds sources for fish and treatment methods required to eliminate/ reduce their activities/effects.
(Adapted from Krogdahl et al (2010)).

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

4.4.1 Effects of individual anti-nutritional factors

4.4.1.1 Lectins

Lectins are also known as agglutinins or hemagglutinins because they cause agglutination by binding to the cell surface. Lectins are typically carbohydrate-binding proteins that are widely distributed in nature, including plants and crops which are commonly consumed in the diet of man and animals (Liener, 1997). They are also found in animals and microorganism. However, they are found in high concentration in most plant feedstuffs in a range of 1-5 g lectins kg^{-1} but the level in some seeds particularly in legumes and cereals such as in soybean, it is known as soybean lectins (soybean agglutinin or SBA), which the content may reach 20 g kg^{-1} on a dry matter basis

(Krogdahl et al., 2010; Krogdahl and Bakke, 2015). The most characteristic property of the lectins is their ability to bind reversibly with sugars and glycoconjugates in a highly specific fashion (Liener, 1997; Francis et al., 2001a). This specific recognition of carbohydrates is one of the lectins' special features that has been used in their classification (NRC, 2011). Hence, lectins have increased our understanding of the functions of carbohydrates in various biological systems (Van Damme, 2014).

Lectins have diverse effects on cell functions and responses such as on digestive and absorptive processes, agglutinate cells, modulate the functioning of enzymes, transport proteins, receptors, act as growth promoters and immunostimulants, and mimic or block endogenous signalling substances (Francis et al., 2001a). The main negative effects of lectins such as SBA in the GIT is their ability to bind glycoproteins on the surface of microvilli lining the small intestine, associated with a disruption of the brush border, reduced absorption of nutrients and epithelial cell viability, hyperplasia in the crypts and an increase in the weight of the tissue (Liener, 1994; Liener, 1997). Moreover, their harmful effect may be more potent when present along with other antinutrients (Francis et al., 2001a; Knudsen et al., 2008). For example, an involvement of lectin in soybean meal-induced enteritis (SBMIE) has been suggested first in Atlantic salmon (Van den Ingh et al., 1991) and later in rainbow trout (Rumsey et al., 1993; Yamamoto et al., 2007).

However, in plants they are believed to be involved in the symbiotic relationship between legumes and N-fixing bacteria, and as part of their defense mechanism against predators (Liener, 1997). In addition, lectins, either in solution or in an immobilized form, have proved extremely useful for the detection and identification of many diverse glycoconjugates (Liener, 1997). Animal lectins have a role in many biological functions including development and immunity. Matsushita et al. (2012) reviewed the role of soluble host-defense lectins in animal species. For instance, collectins are proteins that consist of a collagen-like domain and a carbohydrate-recognition domain and include mannose-binding lectin (MBL) bind to PAMPs of pathogens as a recognition molecule and elicit immune effector mechanisms including enhancement of phagocytosis and activation of the complement system. Enhancement of phagocytosis involves specific receptors for soluble host-defense lectins present on the membranes of phagocytes reviewed by many authors (Ewart et al., 2001; Francis et al., 2001a; Matsushita et al., 2012).

4. 4.1.2 Saponins

Saponins are naturally occurring structurally and functionally diverse phytochemicals mainly present in legumes have diverse biological functions (Francis et al., 2002a; Moses et al., 2014; Gu et al., 2015). They are amphipathic molecules, containing a hydrophobic steroid or triterpenoid aglycone to which one or more hydrophilic sugar chains are attached (Sparg et al., 2004; Krogdahl et al., 2010). Saponins are secondary metabolites produced in both monocotyledonous and dicotyledonous plants (Vincken et al., 2007). However, most saponin-producing plants are dicotyledons, especially legumes such as soybean, pea, and lupin that produce the triterpenoid-type saponins, while the non-sugar steroidal-type saponins are synthesized by monocotyledonous medicinal plants (Fenwick et al., 1991; Sparg et al., 2004; Moses et al., 2014). However, some lower marine animals such as sea cucumbers, starfish and some bacteria also produce these molecules (Bordbar et al., 2011). The level of triterpene saponins that are found in many of the potential alternate plant-derived feedstuffs for fish varies between 1–5 g kg^{-1} but the level in soybean is higher than in other common plant feedstuffs (Anderson and Wolf, 1995).

Saponins are natural surfactants that form a foam in an aqueous environment. Together with their surface-active activities, saponins in water are highly toxic to fish because of the detergent action of the saponins that cause damage to the respiratory epithelium of the gills (Francis et al., 2001b). However, saponins might potentially also increase the digestibility of carbohydrate-rich foods because of their detergent-like activity, which reduces viscosity and thus prevents digestive disturbances resulting from highly viscous digesta (Francis et al., 2001a). Due to their amphipathic character, saponins form micelles and can intercalate into cholesterol containing membranes, forming holes (reviewed by Krogdahl et al. (2010)). Thus, saponins added to water rapidly causes paralysis and death of fish (Murthy et al., 2010). Other effects of saponins may include increased permeability of small intestinal mucosal cells and inhibition of active nutrient transport (Francis et al., 2002a). This may be the mode of action that saponins have in inducing soybean meal-induced enteritis (SBMIE), a well-described condition in the distal intestine of salmonids, in which saponins have been associated as the causal agent (Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015).

On the other hand, saponins are known for their diverse biologically beneficial effects. Some of these beneficial effects are reviewed as cholesterol-lowering agents in several animals (Francis et al., 2002a; Couto et al., 2015b), antifungal and antiviral activity, immune stimulation, anticancer

effects, antioxidant properties, inhibition of protein digestion and vitamin absorption, and glucocorticoid (Oda et al., 2000; Francis et al., 2002a) and adjuvant activity (Oda et al., 2000). Specifically, the triterpenoids affect fundamental cellular processes in both plants and animals (Francis et al., 2002a; Moses et al., 2014). In addition, saponins can modulate both the cell mediated and humoral immune systems, thus saponins at lower doses may be used as adjuvants during vaccine production (Oda et al., 2000). Moreover, growth-promoting effects of saponins have been reported in common carp and tilapia after diets containing saponins at 150 mg/ kg and 300 mg/ kg have been supplied to these respective species (Francis et al., 2005).

4.4.1.3 Protease inhibitors

Protease inhibitors are widespread ANFs found in plant-derived diets that can be used in fish feed, particularly the legumes and oilseeds (Liener, 1994; Francis et al., 2001a; Ringø et al., 2009). The potency of enzyme inhibitors depends on their origin and the target enzyme. For example, in soybean, there are two important protease inhibitors: the Kunitz inhibitor that is relatively heat and acid sensitive, and the more stable Bowman–Birk inhibitor (Francis et al., 2001a). The molecular weight of proteinase inhibitors range between 6000 and 50 000 kDa and this affects their specificities. Most of the well-characterized plant proteinase inhibitors belong either to the Kunitz inhibitor or to the Bowman–Birk inhibitor family (Krogdahl et al., 2010). By comparison, the Kunitz inhibitors are larger (21,000 kDa) proteins with one or two disulfide bonds and has only a single reactive site, thus inhibits only one type of enzyme, e.g. trypsin or chymotrypsin but they are relatively heat and acid sensitive, thus less stable complex (Francis et al., 2001a; Ringø et al., 2009). Whereas, the Bowman-Birk inhibitors are smaller (approximately 8000 kDa) proteins characterized by the seven disulfide bridges and two reactive sites that stabilize the molecule and make it relatively stable to proteolytic breakdown, acid denaturation as well as heat reviewed by Krogdahl et al (2010). The Bowman–Birk inhibitor- inhibit two or three types of enzymes, for instance either two trypsin or chymotrypsin molecule or on one trypsin and one chymotrypsin molecule at the same time as reviewed by Ringø et al. (2009) and Krogdahl et al. (2010)).

Proteinase inhibitors including trypsin, chymotrypsin, elastase and carboxypeptidase inhibitors, are proteins that form complexes with the respective enzymes and interfere with the digestion of proteins resulting in decreased animal growth (Liener, 1994; Kim et al., 2009). The antinutrient activity of protease inhibitors is associated with growth inhibition and pancreatic hypertrophy (Kim et al., 2009). The sensitivity of animals to trypsin inhibitors in feeds is different among

species, for example, Goslings and chickens are more sensitive to trypsin inhibitors than piglets and calves (Sarwar Gilani et al., 2012).

The proteinase inhibitor in salmonids have been found to reduce the digestibility of proteins (Krogdahl et al., 1994). It has been suggested that these trypsin and chymotrypsin inhibitors are involved in the SBM-induced inflammatory response in the distal intestine of salmonids (Baeverfjord and Krogdahl, 1996). However, the activity of trypsin inhibitors in soybean products may be reduced by food processing methods such as heating. But, such processes can denature and destroy the nutritional value as well, thus heating process should be carefully regulated to minimize the loss of EAAs availability like lysine and methionine, and the reduction of protein digestibility due to excessive heat denaturation (Francis et al., 2001a; Sarwar Gilani et al., 2012). Therefore, instead of dry heat application, other methods of heating process such as moist heat treatment are options to reduce the amount of trypsin inhibitors to below the critical levels.

4.4.1.4 Isoflavones

Isoflavones are one type of phytoestrogens, found in plant foods such as berries, wine, grains and nuts, but most notably in soybeans and other legumes (Kris-Etherton et al., 2002). Isoflavones that are present in soybean are naturally occurring heterocyclic phenols that are structurally and/or functionally similar to mammalian estrogens and their active metabolites and bind to estrogen receptors (Kris-Etherton et al., 2002; Barrett, 2006). The isoflavones in soybean and soy products have three types: daidzein, genistein and glycitein in three isomers and three forms; whereas the most dominant isoflavone in soya is genistein (Wang and Murphy, 1994; Barrett, 2006). Total isoflavone content of soybeans can reach levels above 4 g kg^{-1} (Wang and Murphy, 1994), but considerable variation exists; levels are influenced by variety, location, and variation in environmental conditions (Krogdahl and Bakke, 2015). A study performed using purified isoflavone suggests that, they may negatively affect growth performance, intestinal function, liver metabolism and bone formation of salmon fry (Gu et al., 2015). On the contrary, many potential health benefits of isoflavones in soya products have been investigated in human study, including effects on cancer, vascular disease, osteoporosis, menopausal symptoms, and cognitive function (Anderson and Garner, 1997). Moreover, Collins (2014) summarized various effects of isoflavones such as antioxidative, anti-inflammatory potential and their ability in modulating of inflammatory signalling pathways.

4.4.1.5 Phytosterols

Phytosterols (also called plant sterols) are naturally occurring plant compounds that are structurally similar to cholesterol, which can therefore reduce intestinal cholesterol absorption (Woyengo et al., 2009). The most common phytosterols are β-sitosterol, campesterol and stigmasterol and the similarity of their chemical structure with cholesterol is depicted below (Ryan et al., 2007; Woyengo et al., 2009). Of these plant sterols, β-sitosterol is the most abundant phytosterol, followed by campesterol (Ryan et al., 2007).

Couto et al. (2015a) revealed that high level of phytosterols in sea bass diets induced enteritis. However, most research findings indicated the beneficial effects phytosterols have been widely indicated in different species. In mammals, the effects of phytosterols are known to lower plasma cholesterol by competitive inhibition of cholesterol uptake by the enterocytes, which lead to increasing fecal cholesterol excretion and bile acid loss (Awad and Fink, 2000). Other beneficial effects of phytosterols in humans have been shown to inhibit various forms of cancer (Woyengo et al., 2009). Similar to the cholesterol-lowering effects in mammals, low-level phytosterol supplementation in sea bass have been reported to promote similar beneficial effects (Ryan et al., 2007; Couto et al., 2015a). In addition, decreased plasma cholesterol levels has been reported in Atlantic salmon when their diets supplemented with phytosterols (Chikwati, 2007).

4.4.1.6 Phytic acid (Phytates)

Phytic acid (PA) is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol with molecular formula $C_6H_{18}O_{24}P_6$, sometimes it is abbreviated with IP6. It is the main storage form of phosphorus in plants accounting for up to 80% of the total seed phosphorus (Bohn et al., 2008). Protein concentrates and beans generally have higher phytic acid content than their unprocessed counterparts, but the level of phytic acid in ingredients that are commonly used in feeds for monogastric animals generally ranges from 1 to more than 10 g kg^{-1} reviewed Krogdahl and Bakke (2015). A comprises largely negatively charged phosphate groups, which are best known to chelate several nutritionally essential nutrients in the gastrointestinal tract of humans and animals, making them less bioavailable as reviewed by Francis (2001a) and Bohn et al. (2008)). The chelating effect of the phosphate groups, causes PA to bind readily to mineral cations, including calcium, magnesium, potassium, iron, copper and zinc rendering them unavailable to monogastric animals (Francis et al., 2001b; Bohn et al., 2008; Lei and Porres, 2011). The binding characteristics of phytic acid, combined with the inability of digestive enzymes of monogastric animals to hydrolyse

phytic acid, also decreases the activity of various enzymes, including pepsin, trypsin and amylase, and consequently it reduces the availability of protein, amino acids, starch and energy (Sebastian et al., 1998; Lei and Porres, 2011). Furthermore, PA-bound phosphorus is not available to fish`s enzymatic digestion (Francis et al., 2001b).

However, the availability of PA bound minerals can be improved by the addition of enzymes such as microbial phytase, which increase digestibility of PA and the availability of phosphorus and other crucial ions (Sebastian et al., 1998; Lei et al., 2007; Lei and Porres, 2011). Phytases are phosphohydrolytic enzymes that initiate the stepwise removal of phosphate groups from *myo*-inositol hexakis phosphate (Lei and Porres, 2011). Thus, its supplementation has become an efficient tool to improve bioavailability of phosphorus and other cations present in cereal feedstuffs and neutralize the other negative effects of phytate in animals (Francis et al., 2001a; Lei and Porres, 2011). Four different classes of phytases including histidine acid phosphatases, β-propeller phytases, cysteine phosphatases, and purple acid phosphatases are known to degrade PA and exhibit different catalytic efficiencies, structure, and mechanism of action and biochemical properties (Lei et al., 2007). For example, histidine acid phosphatases are the most widely used phytases in animal feeds (Lei and Porres, 2011). The increased availability of PA phosphorous at the same time decreases phosphorous excretion and hence reducing the phosphate load in water supplies in regions with intensive rearing of animals (Bohn et al., 2008). Although no studies have reported phytic acid effects on immune responses or disease susceptibility in fish, most minerals and amino acids have functions in the maintenance of the immune apparatus. Therefore, phytic acid causing nutrient deficiencies is suggested to affect disease defense mechanisms (Krogdahl and Bakke, 2015). However, the ability of PA to chelate minerals has been reported to have some protective effects, such as decreasing iron-mediated colon cancer risk and lowering serum cholesterol and triglycerides in experimental animals (Zhou and Erdman Jr, 1995).

4.4.2 The effects fibres and other non-digestible carbohydrate

Plant products incorporated into feeds as alternative protein sources also contain non-digestible carbohydrates and fibre. The non-digestible feed components comprise mainly fibres (non-starch polysaccharides) and some oligosaccharides. Fibres can be defined as part of cell wall structure of plants materials such as cereals and legumes having various degrees of water solubility, size, and structure (Dhingra et al., 2012). The water insoluble fibre fraction include cellulose,

galactomannan, xylan, and lignin, while the water-soluble fibres include pectin, arabinogalactan, arabinoxylans, and β-glucans. The best-known example of dietary fibre is cellulose, a straight-chain polymer consisting of glucose molecules linked together by β1 → 4 glycoside bonds, which makes the cellulose non-digestible for monogastric animals due to their lack of the enzyme cellulase (Hansen and Storebakken, 2007). High quantities of indigestible carbohydrates, fiber, and ANFs in a feedstuff can be expected to negatively affect protein and energy digestibility (Lech et al., 2012). The level of NSPs in diets for salmonids has increased due to the increased use of plant ingredients. Soybean contains a high concentration of carbohydrates, consisting predominantly of NSP and oligosaccharides (Gatlin et al., 2007), but starch is present at less than 1% (Choct, 1997). Based on dry matter analysis, SBM contains about 30% of NSPs, including 14 and 16% of soluble (mainly of pectic polymers) and insoluble (mainly cellulose) NSPs respectively (Smits and Annison, 1996).

Effects of fibres on digestive function and physiology has been studied in man and other monogastric, omnivorous homoeothermic animals. However, less is known regarding effects in salmonids and other carnivorous fish species (Ringø et al., 2009). NSPs provide selective media for the growth of different bacterial species, thus inducing changes in bacterial metabolism and virulence mechanisms (Lim et al., 2008). Many dietary fibre are widely used as thickeners, gelling agents and binders in nutritional products but also can trap pathogenic bacteria and prevent their access to gut mucosa (Trichet, 2010), because their hydroxyl groups can interact with nearby water molecules (Ringø et al., 2009). Cellulose type fibres may apparently be present in salmonid diet at levels above 15% without effects on feed intake, nutrient digestibility and growth (Hansen and Storebakken, 2007).

On the other hand, dietary fibres are believed to increase the viscosity of the chime in the digestive tract and alter flow and restrict uptake of nutrients, particularly fat and minerals, in salmonid species (Baeverfjord and Krogdahl, 1996; Refstie et al., 1998; Refstie et al., 2005). Hence, one of the main constraints in the utilisation of plant ingredients such as SBM in aquaculture diets is the presence of such indigestible carbohydrates (Gatlin et al., 2007). The NSPs, particularly the soluble NSPs are believed to be more detrimental to growth of fish than the oligosaccharides (Refstie et al., 1999) because they can trap water and form gum-like masses in the intestine which increase the viscosity of intestinal contents. Moreover, their negative effects in

fish may be either due to binding to bile acids or obstructing the action of digestive enzymes and movement of substrates in the intestine (Storebakken et al., 1998).

Oligosaccharides are another low molecular weight carbohydrate containing α- galactosidic and β-fructosidic linkages. Stachyose and raffinose are the two most predominant oligosaccharides in SBM which have been indicated as indigestible to fish and has been linked with reduced growth performance (Refstie et al., 1998) and with the occurrence of SBM induced enteritis in several salmonid fish species (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Van Den Ingh et al., 1996). The oligosaccharide levels in SBM and in SPC is respectively about 15% and 3% (Russett, 2002). The removal of the ethanol-soluble components (mostly oligosaccharides) of SBM significantly elevated the true metabolizable energy of the diet and markedly improve fish health (Gatlin et al., 2007). This may indicate that soybean oligosaccharides are indigestible and are linked with reduced nutrient uptake and growth performance as well as with the occurrence of soybean-induced enteritis (Refstie et al., 1998). Therefore it is desirable to have a soybean product where the oligosaccharide component has been altered or removed completely (Gatlin et al., 2007). Moreover, supplementation of NSP-degrading enzymes in feed is indicated mitigating the adverse effects of NSPs as reviewed by Oliva-Teles (2012).

4.4.3 Interaction effects of anti-nutritional factors

More detailed knowledge on the effects of interactions between ANFs would be particularly useful, as many of the plant-derived materials that have the potential to be used as fish feed ingredients contain more than one of the antinutrients (NRC, 2011). Plant derived antinutrients are responsible for many deleterious effects related to the absorption of nutrients and micronutrients; however, at low concentration antinutrients may exert beneficial health effects (reviewed by Bakke (2011)). However, the interaction effects between various ANFs may have substantial influences in synergizing or annulling the individual effects. Sometimes the interaction of ANFs is advantageous as they can reduce the effect of the interacting antinutrients (Francis et al., 2001a). Tannins are some of the known ANFs to interact with other antinutrients and can have various effects. For instance, the interaction effect of tannin with lectin, tannin with cyanogenic glycosides and tannin with saponin have been tested separately by different researchers. These have shown that tannins removed the inhibitory effect of lectins on amylase (Fish and Thompson, 1991), while interactions between tannins and cyanogenic glycosides (Goldstein and Spencer, 1985) or tannin and saponin (Freeland et al., 1985) showed that tannins have reduced the deleterious effects.

However, diet containing both tannins and saponin caused mice to lose their weight similar to, or greater than, those of mice fed diets containing either of the two ANFs alone (Freeland et al., 1985). Complex formation between saponins and other ANFs could however lead to the inactivation of the effects of both substances (Makkar et al., 1995; Francis et al., 2002a). In addition, simultaneous consumption of tannin and saponin (in the right proportions) may promote chemical interactions that inhibit the antinutrients` absorption from the intestinal tract (Freeland et al., 1985). It has been speculated that such beneficial effects may be due to chemical reactions between them, leading to the formation of tannin–saponin complexes, inactivating the biological activity of both (Francis et al., 2001a). Whereas, Knudsen and colleagues (Knudsen et al., 2008), demonstrated Atlantic salmon fed soya saponins in combination with lupin kernel meal displayed significant enteritis. At the same time the authors have been observed increased epithelial permeability after salmon fed hexane-extracted soybean meal as well as soya saponin concentrate independent of the protein source in the feed. The authors concluded that soya saponins, in combination with one or several unidentified components present in legumes, induce an inflammatory reaction in the distal intestine of Atlantic salmon.

Similarly *in vitro* glucose uptake study has been conducted by Bakke and colleagues (2014) to investigate the effect of ex vivo exposure of mid and distal intestinal tissue of salmon to soybean saponins, lectin and Kunitz' trypsin inhibitor (KTI), singly and in combination at different glucose concentrations. The effects of lectin and KTI were investigated under the presence or absence of bile and saponins. In the absence of bile and saponins, both absorption and transport values were shown somewhat higher in the combined effects of both lectin and KTI, than when tissue was exposed to lectin and KTI individually. When tissues exposed to lectin + bile, KTI + bile and lectin + KTI + bile exhibited increased glucose uptake at the higher glucose concentrations, the authors suggested that that was due to markedly increased tissue permeability. The addition of saponins, however, debilitated the response, which was hypothesized because of restricting bile parts. Saponins + bile, also in combination with lectin and/or KTI, as well as lectin, KTI and lectin + KTI without bile often reduced transcellular glucose uptake pathways, while maintaining low tissue permeability. Of all the effect two combinations, saponins + LEC + KTI + bile, LEC and KTI caused the most marked reductions mainly in the DI, mimicking the restriction of in vivo SBM-induced inflammatory changes to this region (Bakke et al., 2014). Further combined effects of defatted SBM, casein-based semi-purified diets supplemented with soya saponin, soya lectin,

and cholyltaurine on rainbow trout (*Oncorhynchus mykiss*) has been investigated (Iwashita et al., 2009). The occurrence of hyperplastic connective tissue in the mucosal folds of the DI was observed in fish fed a diet containing both saponin and lectin but not cholyltaurine. However, intestinal histological features in fish fed diet supplemented with cholyltaurine and lectin and/or saponin were similar to those in the control diet group. From these findings, it was suggested that the abnormal features of the DI of rainbow trout fed SBM-based diets were caused by the combination of soya saponin and soya lectin, and that supplemental cholyltaurine plays certain roles in normalizing the intestinal abnormalities caused by the saponin and lectin (Iwashita et al., 2009).

4.5 Effects of ANFs on gut microbiota

Like in other vertebrate animals, under normal conditions gut microbiota of fish may prevent pathogen colonization and play vital role in fish health (Ringø et al., 2016). However, the use of plant-derived proteins as ingredients of fish diet may affect the activities and composition of gut microbiota as revealed by Bakke-McKellep et al. (2007) and more recently by Gajardo et al. (2017). Such diet-associated changes in the gut microbiota could be linked to ANFs present in plant proteins, which may contribute to the negative effects on digestion and protein utilization, growth and health of salmonid fish (Krogdahl et al., 1994; Bakke-McKellep et al., 2007; Messina et al., 2013; Krogdahl et al., 2015). However, there is currently no in depth information about the effects of isolated ANFs on fish microbiota, thus investigation is needed to elucidate.

4.6 Soybean meal induced enteritis (SBMIE)

Due to these various soybean compounds, higher inclusion of full fat and standard (solvent extracted) SBM as feed ingredient have been implicated in hindering digestion in fish (Van den Ingh et al., 1991; Olli and Krogdahi, 1994; Bureau et al., 1998; Storebakken et al., 1998; Refstie et al., 1999; Knudsen et al., 2008; Kortner et al., 2012; Krogdahl et al., 2015), especially when included in the diets of salmon even at 5 - 10% inclusion rate has been indicated to induce gut inflammation (Krogdahl et al., 2003; Urán et al., 2009a; Krol et al., 2016). Feeding experiment by Knudsen *et al.* (2008) have shown that soya saponins has been induced inflammatory reaction in the distal intestine of Atlantic salmon only when it consumed with one or several components in legumes, which is different from what has been revealed by Krogdahl et al. (2015), who have shown that soy saponin can induce SBMIE whether the diet contained other legume components or not and

saponins have been implicated as the causal agent. These contrasting results may be due to variations in the number of samples collected and/or environmental conditions experienced in these studies. Similarly, Refstie et al. (1999) have showed the negative effect of soybean NSPS on digestion and absorption of lipid in Atlantic salmon. However, similar disorders has been reported in other fish species (Merrifield et al., 2009), affecting the digestion and absorption of process of the fish, which has been reflected by a dose-dependent negative impact of SBM on growth performance in rainbow trout, sharp snout sea bream (*Diplodus puntazzo*) and cobia (*Rachycentron canadum*) (Refstie et al., 1997); Krogdahl et al., 2003).

The main characteristics of gut inflammation characterized by shortening of the intestinal villi, rapid enterocyte turnover, and an infiltration of inflammatory cells in the lamina propria, detected by inflammatory markers such as proliferating cell nuclear antigen, immunoglobulin M, beta-actin and interleukin-1 beta (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000a; Krogdahl et al., 2003; Bakke-McKellep et al., 2007; Merrifield et al., 2009). The degree of enteritis can be graded as normal, mild to moderate and sever enteritis, where a graded morphological change to increasing dietary SBM levels has been demonstrated by many authors (Krogdahl et al., 2003; Urán et al., 2009b; Silva et al., 2015). The lowest SBM inclusion level of 5-10% resulted in moderate pathohistological changes in the distal intestine and each subsequent increase in SBM level increased the number of fish displaying severe changes (Krogdahl et al., 2003). Such grading of enteritis is based on the degree of changes in the intestinal morphology, such as: widening and shortening of the intestinal folds, loss of the supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium, widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue and infiltration of a mixed leucocyte population in the lamina propria and submucosa (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003).

Although, the effects of gut microbiota in fish health is not clearly known, it has been speculated that these pathological changes shown at the various degree may be related to alterations in gut microbiota (Ringø et al., 2006b). Furthermore, the involvement of various immune cells including lymphocytes, macrophages and polymorphonuclear granules have been suggested as part of SBMIE development (Bakke-McKellep et al., 2007). Although the exact cause for the development of SBMIE is not understood, SBMIE in the distal intestine of the Atlantic salmon

(*Salmo salar L.*) and other salmonids may be considered as a model for diet-related mucosal disorders in other animals and man (Bakke-McKellep et al., 2007; Gajardo et al., 2016).

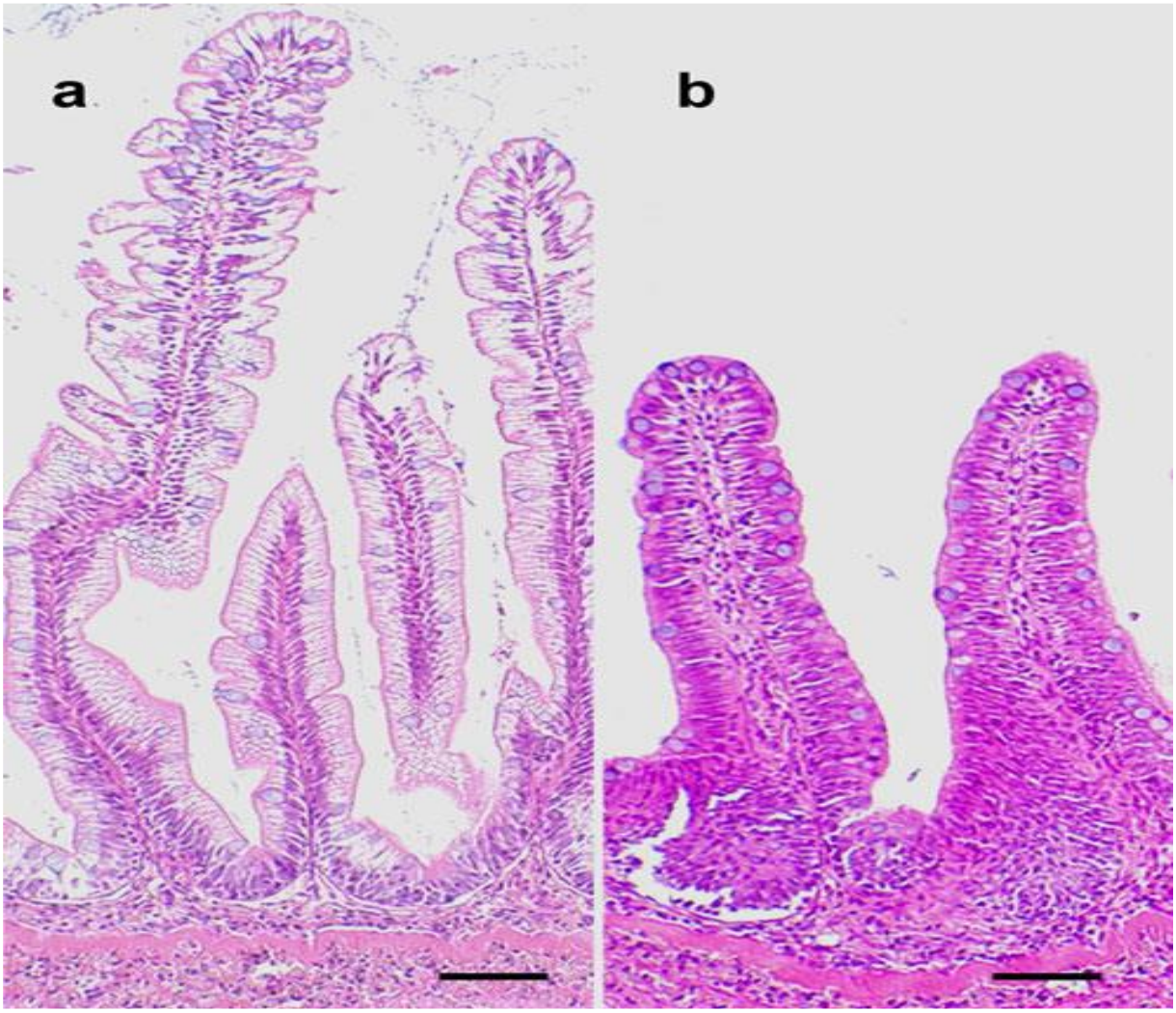

Figure 3.The morphological changes in the distal intestine (DI) of fishmeal (FM)-fed (**a**) and Soybean meal (SBM)-fed (**b**) Atlantic salmon; haematoxylin and eosin staining

The morphological changes in the distal intestine (DI) of FM-fed (a) and SBM-fed (b) Atlantic salmon; haematoxylin and eosin staining. The DI morphology of the FM-fed fish is considered as being normal, whereas the DI of the SBM-fed fish reflects an inflammatory response characterised by cellular infiltration of the lamina propria and submucosa, shortened mucosal folds, and a loss of supranuclear vacuoles in the enterocytes. Source: Adapted from (Chikwati et al., 2013).

5. Microbiota in fish intestine

Using different techniques different studies have characterized the gut microbiota of marine and freshwater fish in aquaculture. Both phenotypic and molecular methods used to identify and/or quantify microbe and their variation is in the level of resolution. The traditional culture-based characterization of the intestinal microbiota of fish have been carried out using cultivation on selective or non-selective media followed by isolation and phenotypical and biochemical characterization (Cahill, 1990; Ringø et al., 1995). However, analyzes of bacterial community by this method is complex work which is time consuming and requires various techniques and resources. In addition, bacteria identified using these methods can represent only a small fraction of the natural microbial communities (Izvekova et al., 2007). The use of molecular techniques for characterizing gut microbiota of fish is advancing from time to time and considered fundamental for research purposes. The PCR based on 16S RNA, fingerprinting and others have been most popular for long time in the characterization of microbial communities, but recently next generation sequencing (NGS) is becoming more popular in terms of its ability to capture microbes that are not identifiable by the previous methods. Based on the advancement of techniques and methods in the study of microbial community, the degree and depth of knowledge about microbes is also growing.

Several studies have reported that diverse microbial communities exist in the GIT of carnivorous, herbivorous and omnivorous fish species (Ringø et al., 1995; Ringø and Gatesoupe, 1998; Dimitroglou et al., 2009; Merrifield et al., 2009; Nayak, 2010). A recent study on gut microbiota of Atlantic salmon showed that the microbial richness and diversity in the digesta was significantly higher than the mucosa; where two bacterial phyla, Proteobacteria and Firmicutes were the most abundant in the digesta while the Proteobacteria was highly dominating in the mucosa-associated microbiota (Gajardo et al., 2016). Some members of the autochthonous group of bacteria, which are members of the mucosa community, are supposed to play various beneficial effects such as in the case of lactic acid producing bacteria (LAB), of genera *Streptococcus, Enterococcus, Lactobacillus, Aerococcus, Carnobacterium, Leuconostoc, Lactococcus, Pediococcus* and *Carnobacterium* (Ringø et al., 1995; den Besten et al., 2013).

5.1 The diversity of gut microbiota in Atlantic salmon and other fish species

The gut microbiota of marine and freshwater fish species has been reviewed by Cahill (1990) and Ringo et al. (1995) and the most common bacterial genera reported in salmonid gut include *Acinetobacter, Enterobacteriaceae, Aeromonas, Flavobacterium, Pseudomonas* as well as *Lactobacillus* (Ringø et al., 1995). While at higher taxonomical level Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria and Bacteriodes are the most commonly reported phyla in the salmonid gut (as reviewed by Nayak, 2010). However, most of the available information on these reports has been collected by methods which would isolate only aerobic or facultative anaerobic bacteria (Cahill, 1990). On the other hand, Trust et al. (1979) demonstrated that strictly anaerobic bacteria was also present in the intestines of fresh water species and marine species (rainbow trout). The later studied reported the presence in the gut of species of *Actinomyces, Bacteroides, Clostridium, Eubacteria, Fusobacterium, Pepgostreptococcu*s (from fresh water species) and *Bacteroides melaninogenicus, Clostridium* and *Fusobacterium* from rainbow trout, indicating that fish have a resident non-pathogenic anaerobic microbiota. Furthermore, Romero and co-workers (2014) reviewed the most common anaerobic bacteria from both marine and fresh water species as shown below (Fig. 4).

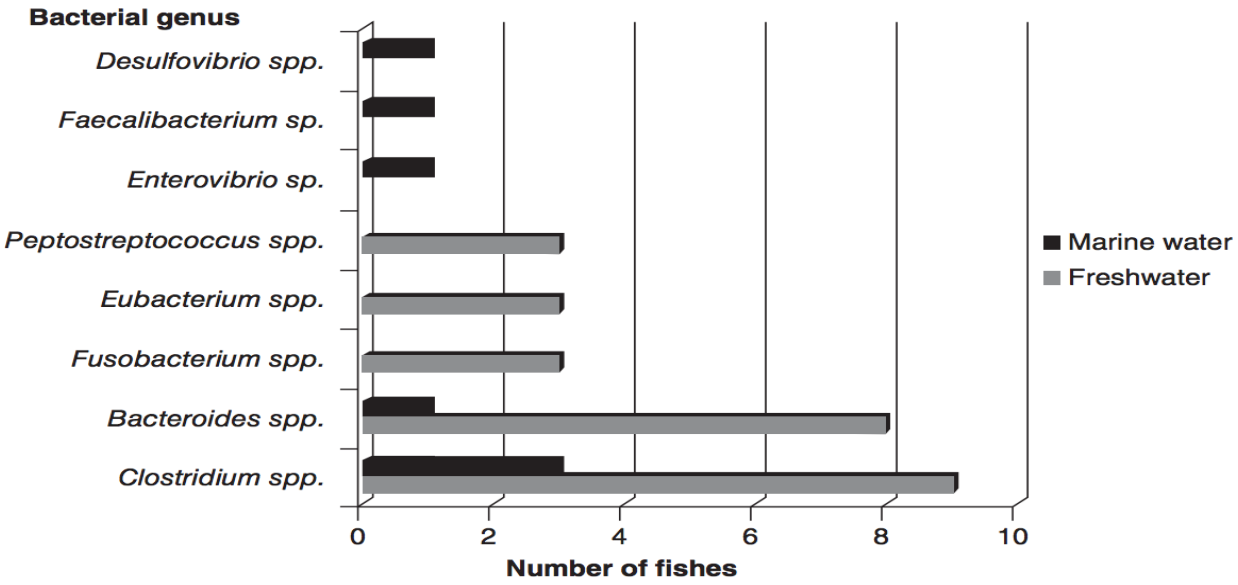

Figure 4. Anaerobic bacteria species of the major genera reported in the GIT of marine and freshwater fish. This figure compares the degree of similarity in the composition of anaerobic bacteria species isolated in both marine and freshwater species. Variation is clearly visible as the degree of similarity between the two-species indicated zero in many bacterial species (black shaded = anaerobic bacterial spp. in marine fish and grey shaded shows anaerobic bacterial spp in freshwater fish) ((Source: Data from Izvekova et al. (2007), shown by Romero et al.(2014)).

In addition, gut microbiota on both marine and freshwater fish species has been studied at genus levels (Austin, 2006), and species of the genera *Acinetobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Serratia, Aeromonas, Alcaligenes, Eikenella, Bacteroides, Citrobacter freundii, Hafnia alvei, Cytophaga/Flexibacter, Bacillus, Listeria, Propionibacterium, Staphylococcus, Moraxella,* and *Pseudomonas* were reported in freshwater fish. While, the bacterial species of genera *Aeromonas, Alcaligenes, Alteromonas, Carnobacterium, Flavobacterium, Micrococcus, Photobacterium, Pseudomonas, Staphylococcus* and *Vibrio* were found in the gut of marine fish (Austin, 2006). Moreover, Romero et al. (2014) have reviewed bacteria of both fish species based on several criteria such as structural and metabolism to get graphical image of the most commonly reported microbes in marine and freshwater fish. Thus, a range of aerobic microorganisms have been evaluated and grouped into Gram-negatives (A) and Gram-positives (B) as they have been observed in the GIT of marine or freshwater fish (Fig. 5).

The characterization of gut microbiota of marine and freshwater fish has been explored by various methods (Cahill, 1990; Ringø et al., 1995; Austin, 2006; Zhou et al., 2016; Nyman et al., 2017) who have identified that the gut microbiota of freshwater fish tends to be dominated by members of genera such as *Enterobacter, Aeromonas*, and *Acinetobacter, Lactococcus*, *Flavobacterium* and *Pseudomonas* well described representatives of the family Enterobacteriaceae, and obligate anaerobic bacteria of the genera Clostridium, Bacteroides and Fusobacterium (Zhou et al., 2016). Whereas, *Vibrio, Pseudomonas, Achromobacter, Corynebacterium, Flavobacterium* and *Micrococcus* (Cahill, 1990), *Bacillus, Caulobatter, Flexibaeter, Enterobacteriaceae, Hyphomierobium-Hyphomonas, Lucibacterium harveyi, Photobacterium, Prosthecomicrobium,* and *Vibrio* have been known as predominant bacteria in marine fish species (Austin, 1982).

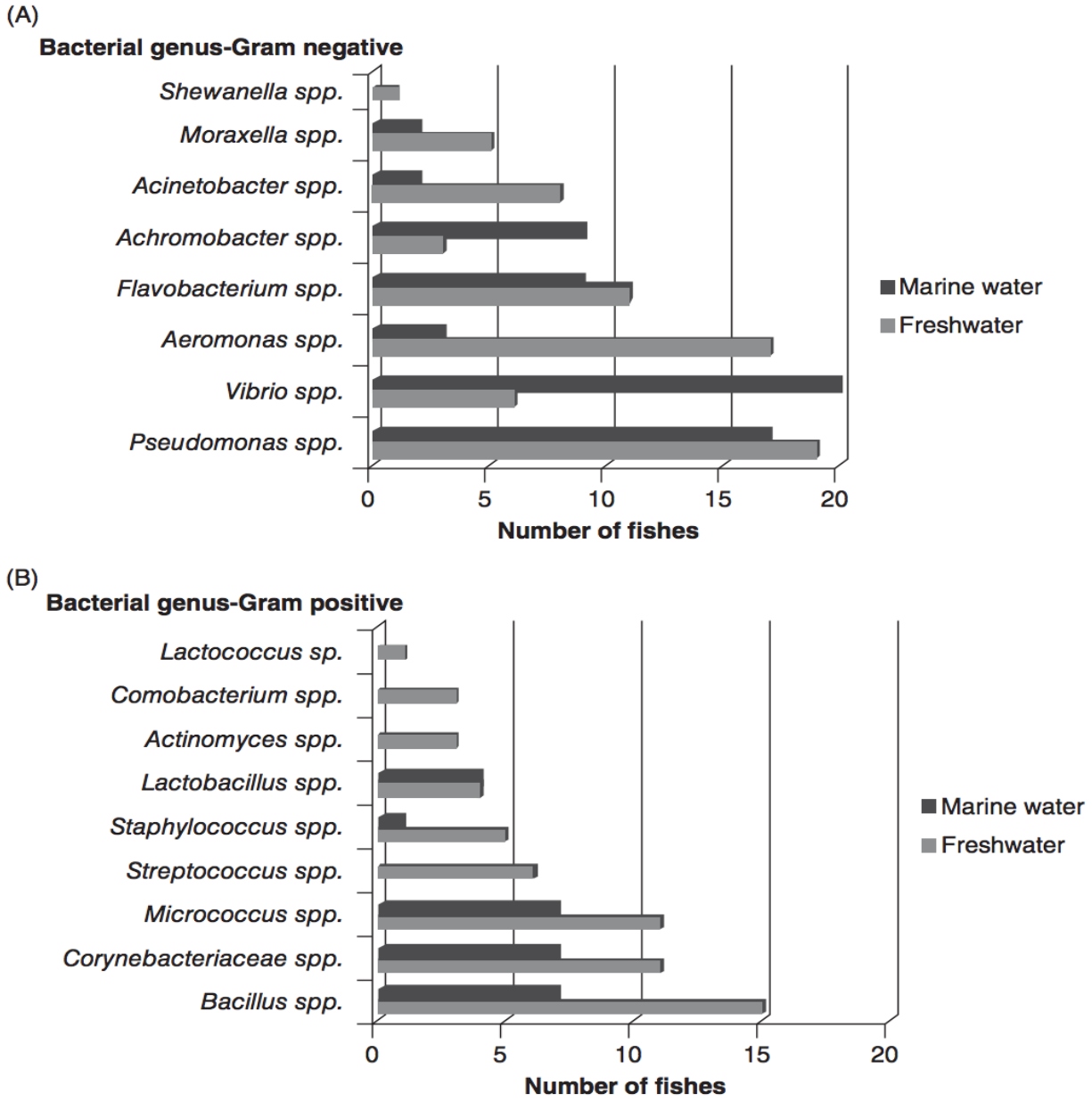

Figure 5 Aerobic Gram-negative (A) and Gram-positive (B) bacterial species reported from the gut of marine and freshwater fish**.**
Source: Data from Izvekova et al. (2007), shown by Romero et al. (2014).

Comparing reports on the microbiota of farmed and wild fish species, the currently available information on fish microbiota composition is mostly focused on farmed fish, mainly salmonids (Romero et al., 2014). For example, (Nayak, 2010) has reviewed the bacterial phyla in salmonids as shown below (Fig. 6).

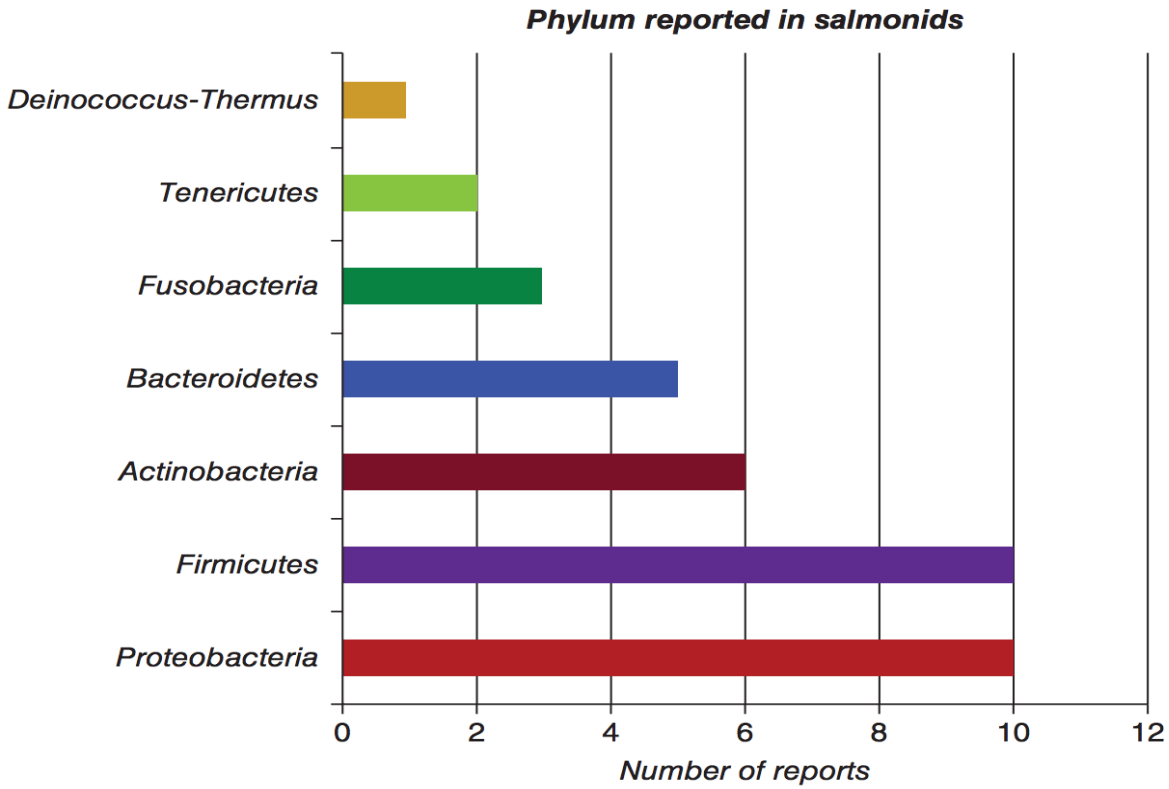

Figure 6. Bacterial phyla observed in the gut of salmonids.
Source: Data from Nayak (2010), shown by Romero et al. (2014).

Similarly, Kim et al. (2007) reported that phylum Firmicutes, particularly genus *Clostridium* has been the dominant gut microbiota in rainbow trout as identified by denaturing gradient gel electrophoresis (DGGE). Whereas, the abundance of gut microbiota in wild salmon (entirely carnivorous) is dominated by the phylum Tenericutes (genus *Mycoplasma)* which has been found in 96% of the clones analyzed (Romero et al., 2014). In addition, the diversity of gut microbiota in mammals have been reviewed by Ley et al. (2008), and indicates that bacterial diversity increases from carnivore to omnivore to herbivore. According to such observation in mammals, it has been speculated that increasing herbivory in fish could lead to gut microbiota diversification (Romero et al., 2014). Based on the most modern identification methods utilized, it was shown that the most abundant gut microbiota in marine (Llewellyn et al., 2014; Gajardo et al., 2016; Gajardo et al., 2017) and freshwater (Liu et al., 2016) fish species belong to the phyla Proteobacteria and Firmicutes followed by Fusobacteria and to some extent Actinobacteria, Bacteroidetes and Verrucomicrobia (Fig. 6, Nayak, 2010). Moreover, bacterial genera and their species have been reported from Atlantic salmon including *Vibrio*, *Lactococcus*, *Bacillus*, *Photobacterium*, *Weissella*,

Shewanella, *Carnobacterium*, *Citrobacter*, *Clostridium* and *Mycoplasma* have also been identified (Spanggaard et al., 2000; Huber et al., 2004; Hovda et al., 2012).

Moreover, taxonomic composition of the gut microbiota in marine and freshwater fish can vary based on the type of feeding behaviour, host species, age, and season (Izvekova et al., 2007; Hovda et al., 2012). Based on the most known fresh water fish species, studies indicated that the dominant phyla in the gut microbiota of carps are Proteobacteria, Firmicutes, and Fusobacteria (Liu et al., 2016), while that of Nile tilapia (*Oreochromis niloticus*) are Proteobacteria and Fusobacteria (Ran et al., 2016; Zhang et al., 2016). However, Standen et al. (2015) observed that allochthonous microbiota of Nile tilapia and identified Firmicutes as the most abundant phylum, and another study on tilapia reared in saline water has showed that the most dominant phyla has been identified as Actinobacteria, Bacteroidetes, and Proteobacteria (Zhang et al., 2016) which may show that proportions are different depending on the type of water the fish are kept. Allochthonous microbiota are defined as microbes that are incidental visitors in the GIT that pass through after some time (Ringø et al., 1995; Merrifield et al., 2011). The microbiota of Atlantic salmon has been the topic of numerous sequencing studies. In both freshwater and saltwater, Proteobacteria and Firmicutes have been revealed as the dominant phyla in the gut microbiota of this species (Zarkasi et al., 2014; Gajardo et al., 2016; Schmidt et al., 2016). The most common bacterial isolates, identified at the phyla level from both marine and freshwater species (reviewed by Romero et al. (2014)).

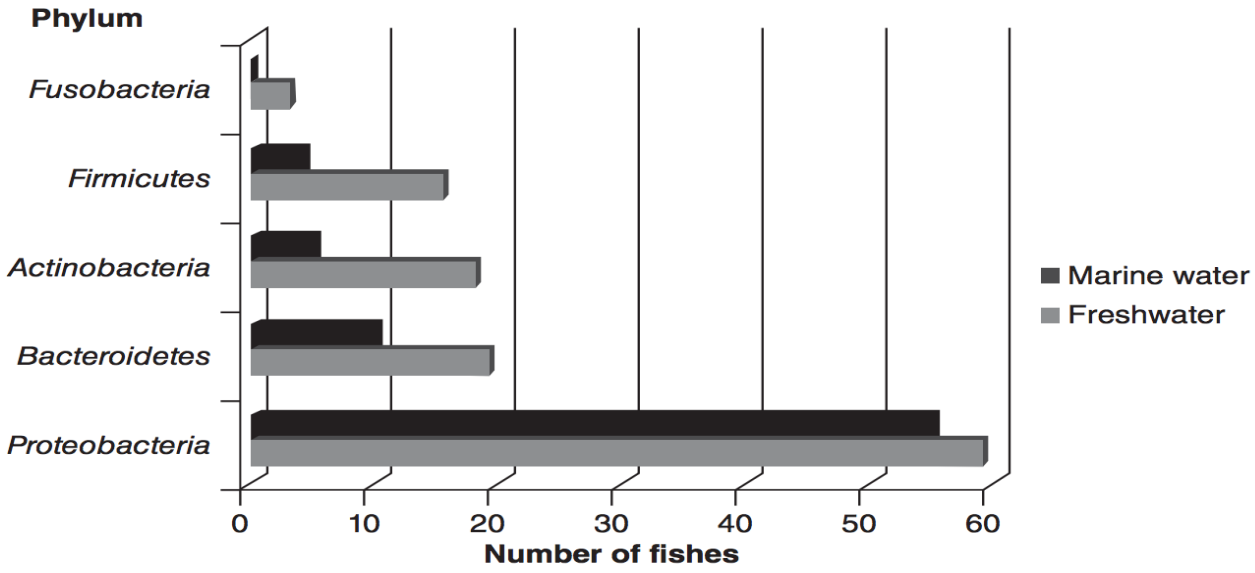

Figure 7.The bacteria phyla reported in the GIT of marine and freshwater fish.
This figure illustrates the variation in composition of gut microbiota in marine and freshwater fish species. Data has been collected from a numbers of fish species differing in the type of feeding and salinity preference.
Source: data from Izvekova et al. (2007) shown by Romero et al. (2014).

5.2 The roles and consequences of gut microbiota in fish physiology and health

In most terrestrial vertebrate herbivores, the role of gut microbiota has been known for their crucial role in terms of physiology; digesta flow rates, substrate availability, host secretions, pH and oxygen tension and protecting against invading pathogens (Gatesoupe, 2005; Flint et al., 2012; Martínez Cruz et al., 2012). The fish gut is also a complex ecosystem that is always holding a diverse bacterial community in a balanced relationship with each other and some of the bacteria are expected to provide beneficial effects to the host (Wu et al., 2012; Nyman et al., 2017). In a healthy fish, there is a proper balance between the endogenous microbiota of the intestine and the host's control mechanism (Nayak, 2010). This balance plays a vital role in fish health by stimulating development of the immune system, supporting in nutrient attainment, and competing with opportunistic pathogens both for adhesion sites and nutrient sources (Sugita et al., 1996; Nayak, 2010; Tarnecki et al., 2017).

Most studies of gut bacterial communities have focused on humans and other mammals. The gut microbiota and their metabolic activities may have major consequences that can be both beneficial and harmful to the host (Flint et al., 2012; Flint et al., 2015; Ringø et al., 2016). Fish gut communities have been also typically clustered with gut communities from mammals and insects (reviewed by Sullam et al., 2012). Many of the bacteria from the guts of herbivorous fish have been closely related to those from mammals and the authors suggest that there may be an evolutionary ties between fish gut microbes and symbionts of animals (Sullam et al., 2012). The study of gut microbiota in fish indicated that carnivorous fish have the least while herbivorous fish have the highest number of gut microbial communities (Clements, 1997). Regardless the higher microbial biodiversity reported in herbivorous fish, their function in fish in general are less characterized compared to those of mammals (Clements, 1997; Ray et al., 2012). However, the development of a germ-free model, gnotobiotic zebrafish (*Danio rerio*) by Rawls et al. (2004), has transformed the knowledge about the host–microbe interaction of fish microbiota. Molecular research by Rawls et al. (2004) revealed that microbiota in fish can regulate the genes expression of important genes in stimulation of epithelial proliferation, nutrient metabolism and innate immune response. On the other hand, unstable gut microbiota in fish may lead to compromised nutrient absorption, metabolism and weaker immune responses (Dimitroglou et al., 2009; Sullam et al., 2012; Reveco et al., 2014; Romero et al., 2014; Ringø et al., 2016). Therefore, gut microbiota of fish is likely to involve metabolism, amino acid production and secretion of

inhibitory compounds that have antagonistic role against fish pathogens such as *Aeromonas*, *Vibrio* and *Yersinia* species (Denev et al., 2009; Nayak, 2010; Desai et al., 2012).

5.2.1 The role of gut microbiota in fish nutrition

Some of the gut microbiota of fish serves as source of enzymes for fermentation of undigested diet components including cellulytic, lipolytic, amylolytic and proteolytic enzymes, which help in digestion of simple and complex proteins, lipid, cellulose and chitin (Sugita and Ito, 2006; Dimitroglou et al., 2011a). A recent study by Zhou and colleagues (2016) demonstrated that the gut microbiota of fish harbors many cellulose-decomposing bacteria, including sequences related to *Anoxybacillus*, *Leuconostoc*, *Clostridium*, *Actinomyces*, and *Citrobacter* (Zhou et al., 2016). In herbivorous animals, the crucial role of gut microbiota is well known for their key function in digestion by breaking down plant cell walls (cellulose and hemicellulose) to simple compounds such as short-chain fatty acids (SCFA). Similarly, the microbiota of herbivorous fish has been studied with special interest because certain members of microbiota in some fish use fermentation to convert carbohydrates to SCFAs, which are important energy sources not only for the gut microbiota but also for intestinal epithelial cells (IECs) (Clements, 1997; Rooks and Garrett, 2016). Thus, the enzyme-producing function of the microbes isolated from fish digestive tracts can serve as probiotics, while the feed containing high amount of non-digestible components (Prebiotics), which can stimulate beneficial effects on the probiotic species (Nayak, 2010; Ganguly and Prasad, 2012).

5.2.2 The importance of gut microbiota in fish immunity

The gut microbiota and their metabolites is supposed to play important roles in host digestive function, amino acid production, secretion of inhibitory compounds, gastric development, mucosal tolerance and immunity development that protect against bacterial pathogens in the intestine (Ringø and Gatesoupe, 1998; Merrifield et al., 2011). The gut microbiota of fish is classified as autochthonous (indigenous), when they adhere the host's gut epithelial surface, or allochthonous as defined above (Ringø et al., 1995; Merrifield et al., 2011). The microbiota has been shown to contribute to the proliferation of the gut epithelium and nutrient breakdown (Ramirez and Dixon, 2003; Nyman, 2016), physiological development (John et al., 2004) and immune responses (Salinas et al., 2005; Salinas et al., 2011; Nyman, 2016). Despite these studies, the microbiota of fish has not been studied as extensively as mammals and other animals however as the aquaculture

industry continues to grow, there is a necessity for further investigation to improve fish gut health (Ringø et al., 1995; Ringø et al., 2006a; Bakke-McKellep et al., 2007; Kim et al., 2007).

The gut immune system, also named gut-associated lymphoid tissues (GALT), not only protects gut from infectious agents but also regulates immune system in the GI tract (Rombout et al., 2011; Salinas et al., 2011). Compared to terrestrial animals, gut microbiota of aquatic species is particularly dependent on the external environment, since it is in constant contact with both water and food passing through the GIT. However, like in higher vertebrates, gut commensal microorganisms are thought to modulate immune responses in the fish gut (Rawls et al., 2004; Nayak, 2010; Pérez et al., 2010). For example, *Carnobacterium* is often found in salmonids and has been shown to inhibit several pathogens including species of *Aeromonas, Flavobacterium branchiophilum, Photobacterium, Vibrio* and *Streptococcus* (Robertson et al., 2000; Boltaña et al., 2011). Moreover, the gut microbiota plays a crucial role in the development and maturation of the gut immune system, which in turn mediate a variety of host immune functions (Ray et al., 2012). Conversely, alterations in the composition and numbers of gut microbiota can affect gut immunity and may lead to development of different diseases (Bakke-McKellep et al., 2007; Ray et al., 2012). Thus, a well-integrated collaboration between the epithelium, immune components in the mucosa, and microbiota is accountable for the development and maturation of the gut-associated immune system of the host (Nayak, 2010).

Probiotics are defined as live microorganisms, that are added to the diets of animals to improve intestinal balance and health (Gatesoupe, 1999; Montero et al., 2005). The main objective of probiotics is to produce their beneficial effects mainly in improving the innate defence of animals and providing resistance to pathogens during stress periods (reviewed by Trichet, 2010). They are known to influence several physiological parameters in fish including stimulation of epithelial proliferation, degree of nutrient assimilation, overall physiological development and gut immune responses (Nayak, 2010). Health benefits of probiotics have been reported in several farmed fish species and have been used to increase production (Gatesoupe, 1999; Nayak, 2010; Ringø et al., 2010). They are considered a promising alternative to chemicals and antibiotics (Trichet, 2010; Lall and Dumas, 2015). The most studied probiotics in fish culture practices include a wide range of microorganisms, such as species of the genera *Aeromonas, Alteromonas, Enterobacter, Flavobacterium, Pseudomonas, Pseudoalteromonas, Phaeobacter, Roseobacter, Shewanella, Vibrio, Arthrobacter, Bacillus, Clostridium, Micrococcus,* several LAB species, *Debaryomyces*

and *Saccharomyces* species (Gatesoupe, 1999; Nayak, 2010; Merrifield et al., 2010a; Dimitroglou et al., 2011b). Although, the use of probiotics in aquaculture has been debated, currently the interest in them has increased due to their positive effects in aquaculture farming (as reviewed by Martinez Cruz *et al*. (2012)). However, the exact mode of action of probiotics is not yet established; their response may often be host specific as well as affected by strain differences, dose and duration of administration, culture environment, and diet composition (Lall and Dumas, 2015).

Prebiotic can be defined as non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth of certain endogenous health-promoting bacteria in the GI tract of the host (Manning and Gibson, 2004). Most prebiotics used in fish diets are polysaccharides derived from bacteria, fungi or yeast, or cereals (barley and oat) and may consist of the cells themselves or preparations from the cell walls β-glucans and high-M-alginate have been shown to stimulate both specific and nonspecific immune response and to increase growth performance in fish (Ringø et al., 2010; NRC, 2011; Lall and Dumas, 2015). Beta-glucans are polysaccharides extracted from yeast cell walls. Their immunostimulating function are thought to be as PAMPs that link to the PRRs at the surface of macrophages and other phagocytic cells of fish (Trichet, 2010). They are used as feed additives to enhance resistance to viral, bacterial, and parasitic infection and improve fish health.

5.3 Dietary effects on gut microbiota

Dietary components play important roles in the development and/or can shift the composition and activity of the gut microbiota in fish (Ringø and Olsen, 1999). Although, the effect of protein on the fish gut microbiota, overall health and growth of the fish remains to be elucidated (Ringø and Olsen, 1999; Navarrete et al., 2012; Navarrete et al., 2013). In human studies a high protein diet and low in carbohydrates has been shown to affect the gut microbiota (Clemente et al., 2012; Graf et al., 2015). Therefore, the effects of protein diet manipulation, particularly plant protein ingredients on salmon guts bacteria is getting more attention on commercial fish farming.

5.3.1 Commercial diets

The most intensively farmed fish around the world today are salmonids. Traditionally the most important ingredients of feeds for farmed carnivorous fish species have been FM and FO. Although FM is considered the best source of protein, due to increased price and other factors its level is

now reduced on commercial diets. Nowadays commercially available aquaculture feeds contain a mixture of other ingredients that provide the essential nutrients necessary for the optimal growth and health of the fish (Miller et al., 2008; Sørensen et al., 2011b). Moreover, inclusion of raw materials including many cell wall components of various yeasts and bacteria used as prebiotics and immunostimulants, such as mannan oligosaccharide and β-glucans are widely known for their protective manner on the gut epithelium (Dimitroglou et al., 2009; Dimitroglou et al., 2011b) and immunostimulation effects in fish that helps to increase immune response to various pathogens (Oliva-Teles, 2012). Dietary carbohydrates can play a role in the immune responses through their interactions with the gut microbiota and the gut-associated lymphoid tissue (reviewed by Trichet, 2010). Particularly β-glucans as potent activators of macrophages, lysozyme and complement activation or oxidative capacity of phagocytic cells, and are used as immunostimulant molecules for in aquaculture (reviewed by Oliva-Teles (2012)). Grain based diets fed to salmon have resulted in a substantial increment in the abundance of both *Lactobacillus* and *Streptococcus* compared with fish fed on FM based diets (Wong et al., 2013). Since, both bacteria are member of the LAB and have been studied as probiotics (Ringø et al., 2010; Martínez Cruz et al., 2012), the inclusion of plant ingredients may enhance important microbiota and play crucial role on the health of the host.

The manipulation of the host microbiota may represent a new possibility in the prevention of pathological and physiological disorders (Pérez et al., 2010). Thus, significant attention is currently being focused on the manipulation of the composition of the gut microbiota and their activities through dietary supplementation to improve the overall health status of the host organism (Dimitroglou et al., 2009; Nayak, 2010; Ringø et al., 2010; Dimitroglou et al., 2011b). The application of commercial fish feeds containing special ingredients such as probiotics, prebiotics and/or a combination of both (synbiotics), purportedly exert modulating effects on the gut microbiota and thus benefit on fish growth or disease resistance (Gatesoupe, 2005; Nayak, 2010; Dimitroglou et al., 2011a; Merrifield and Carnevali, 2014; Gajardo et al., 2016).

Prebiotics represent a specific type of dietary fibre or NSPs that when fermented, mediate measurable changes within the gut microbiota (Marchesi et al., 2016). Moreover, the use of prebiotics is indicated to be more advantageous over probiotics, because regardless of some concerns about their safety and efficacy, they are natural feed ingredients, their incorporation in the diet does not require special precaution, and their authorization as feed additives may be more easily obtained (Gatesoupe, 2005). Although, the function of probiotic in aquaculture applications

has been controversial, there is a growing support in that host benefits are driven, in part at least, by modulation of the host microbiota (Merrifield and Carnevali, 2014). Therefore, dietary strategies, including probiotics, prebiotics are intensively studied in order to characterize the modulating effects of the composition or metabolic/immunological activity of the gut microbiota (as reviewed by Ringo et al., 2016).

5.3.2 Plant ingredients

The inclusion of SBM in diets of Atlantic salmon has been shown to increase the gut microbial community and diversity compared to FM diet (Bakke-McKellep et al., 2007; Desai et al., 2012; Gajardo et al., 2017). Likewise, the effect of non-digestible carbohydrates such as fibers/NSPS in fish diet has been observed to alter gut microflora of fish (Gatesoupe, 2005; Ringø et al., 2006b; Nayak, 2010). Changes in the level of *Vibrio* spp. in response to diet changes such as differences in carbohydrate levels have been observed in European Sea bass (Kotzamanis et al., 2007) and shifts in the microbiota are seen in Artic charr (Ringø and Olsen, 1999). A conflicting result has also been reported in rainbow trout (Mansfield et al., 2010), where, the fish fed a SBM diet was discovered with lower bacterial diversity compared to fish fed a FM diet. However, such kind of inconsistencies between studies suggest that a wide range of factors can affect results including differences in fish species, rearing conditions, diet formulation, and methods used to identify the microbiota (Sullam et al., 2012).

The relatively high concentration of carbohydrates and presence of ANFs are the primary factors limiting the amount of SBM used in salmonid feeds (Francis et al., 2001a; Gatlin et al., 2007; Hardy, 2010). Particularly, the inclusion of less refined soy products has been implicated with the induction of enteritis with pathomorphological changes in the distal intestine of salmonid fish (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996), and such effects may potentially favour growth of unfavourable microbes that may aggravate the inflammation process (Refstie et al., 2010). Moreover, imbalanced plant based diets and antinutrients may impair fish immunity, maturation and functionality of the intestinal mucosa, atrophy of intestinal mucosa and a reduction in its absorptive and immunological capacity in response to high dietary SBM inclusion (Merrifield et al., 2011; Chikwati et al., 2013). Various ANFs are present in various plant ingredients of fish feeds, they are widely studied for their implication as contributors in intestinal

inflammation of fish and other animal species. However, currently the role of ANFs on the plant ingredients cause microbiota changes is not understood.

5.4 Metabolites produced by microbial metabolism

The enzymatic and metabolic activities of the gut microbiota and their fermentation process, result in production of microbial metabolites (Flint et al., 2012; Tan et al., 2014; Rios-Covian et al., 2016) . The microbial fermentation converts non-digestible feed in to the most abundant and physiologically important short-chain fatty acids (SCFAs), primarily lactate, acetate, propionate and butyrate which helps to maintain a balance with the host's metabolism and immune system (Dimitroglou et al., 2011a; Flint et al., 2012; den Besten et al., 2013; Ohland and Jobin, 2015; Rios-Covian et al., 2016). The main sources of SCFA are carbohydrates but amino acids valine, leucine, and isoleucine obtained from protein breakdown can be converted into isobutyrate, isovalerate, and 2-methyl butyrate, known as branched-chain SCFA, which may contribute to the total SCFA production (Rios-Covian et al., 2016).

Based on human studies, acetate, propionate and butyrate are the most abundant SCFAs, representing 90–95% of the SCFA present in the colon (Rios-Covian et al., 2016). Similarly, acetate has been reported as the major SCFA in marine herbivorous fish species but also lower concentrations of propionate and butyrate were observed, while very low level of valerate was found in odacids (Clements et al., 1994). The ratio of acetate: propionate: butyrate: valerate that was found in the gut section of three species of *Odacis* were respectively: 83: 8: 9: 1 in *O. cyanomelas*, 64: 21: 14: 1 *in O. pullus* and 74:17:9:0 in *C. lophodon* (Clements et al., 1994). Although, lactate is not a SCFA, it is also produced by some microbiota including LAB, Bifidobacteria, and Proteobacteria, but under normal physiological conditions it does not accumulate in the gut due to the presence of some microbial species, such as *Eubacterium hallii*, that can convert lactate into different SCFA (Flint et al., 2015).

The SCFAs mediate gut cell proliferation and differentiation, apoptosis, mucin production, lipid metabolism/cholesterol metabolism in various tissues (Fushimi et al., 2006). Several molecular mechanisms of action have been ascribed to acetate, propionate and butyrate that may be relevant to their therapeutic potential to promote intestinal health, such as reducing inflammation (Rios-Covian et al., 2016; Rooks and Garrett, 2016). However, the knowledge about the exact action of pro- and prebiotics and SCFAs is still limited. Montalban-Arques et al. (2015) reviewed most of

the mechanisms that are applicable for higher vertebrates are supposed to work in the fish gut summarized with hypothetical model of gut microbial participation represents for different mucosal tissues (Fig. 5). Mainly, butyrate and propionate are believed to regulate intestinal physiology and immune function, while acetate acts as a substrate for lipogenesis and gluconeogenesis (Rios-Covian et al., 2016). Moreover, the SCFAs have vital effects by reducing the pH of the gut, which inhibits growth of pH sensitive pathogenic microorganisms (Macfarlane and Macfarlane, 2012; den Besten et al., 2013). Another advantage of the pH reduction has been reported to help mineral absorption (Lauzon et al., 2014). Furthermore, the synergistic promotion of commensal and symbiotic bacteria in turn provides competitive exclusion of pathogens, indirectly enhancing pathogen resistance, reducing toxic microbial metabolites and suppressing intestinal inflammation (Pédron and Sansonetti, 2008).

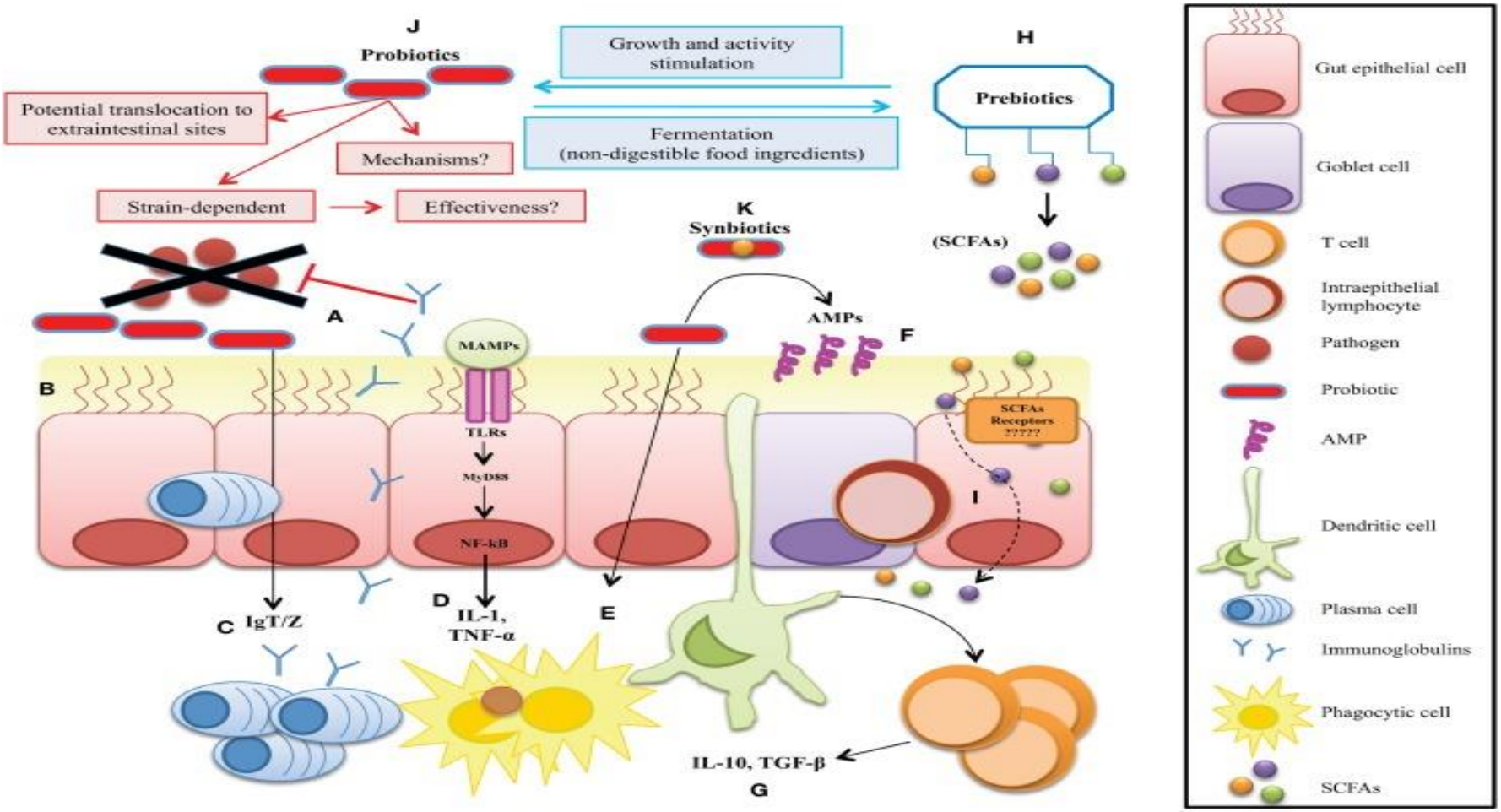

Figure 8. Potential microbial strategies to improve gut mucosal immunity in fish.
The therapeutic approach mechanisms include: **(A)** competitive exclusion for binding sites and translocation, **(B)** enhanced barrier function by reversing the increased intestinal permeability, **(C)** enhanced mucosal immunoglobulin IgT/Z response to enteral antigens, **(D)** reduction of secretion of inflammatory mediators, **(E)** stimulation of innate immune functions, **(F)** stimulate the release of antimicrobial peptides (AMPs) at the mucosal layer, and **(G)** enhanced availability of anti-inflammatory mediators by regulatory immune cells. **(H)** Production of metabolic health-enhancers like SCFAs by non-digestible prebiotics, **(I)** diffusion of SCFAs through the enterocytes to improve mucosa barrier functions. **(J)** Probiotics have been suggested to confer several health benefits on the host. However, their mechanisms of action are not well understood. **(K)** Synbiotics are a mix of pre- and probiotics, thus their mode of action are much more difficult to define. Source: Adapted from Montalban-Arques et al. (2015)).

6. Methods used for characterization of microbiota

The knowledge about the gut microbiota in fish has expanded along with the development of new techniques. Previously, characterization of the microbiota relied on traditional culture based procedures to study the structure of the microbial community. Analysis of gut microbiota based on this method involved collection of samples taken from digesta or homogenates of the intestine mucosa and then allowed to grow on agar plates with either selective or non-selective media. This procedure is both expensive and time-consuming as gut microbiota of cold water species is often slow-growing and thus need long incubation times. Another disadvantage is that this method show only the culturable species. To avoid the limitations of culture-dependent techniques, culture–independent approaches have been developed to detect and quantify microorganisms representing the actual microbial diversity in a sample.

6.1 Culture -dependent characterization of gut microbiota

Cultivating bacteria using traditional culture based studies is important to gain knowledge on the physiological and biochemical properties of bacteria. To study gut microbiota of fish, it has been common practice to use conventional culture based methods (Cahill, 1990; Ringø and Gatesoupe, 1998). This involves of sampling gut contents or tissue samples of GIT and spreading gut homogenates on either selective or general-purpose agar artificial medium used to grow the microbiota and then include isolation and identification by morphological and biochemical identification procedures. Based on this method, bacteria can be identified based on their cell wall nature characterized by staining, gaseous (aerobic, facultative anaerobic and obligate anaerobic) and temperature requirement for their growth.

The incubation temperature differs based on the source of sampled species, and thus the incubation duration for cold water fish species should be longer when assessing the gut microbiota of where the growth rates of the indigenous gut microbes are suggested to be slower (Zhou *et al.* 2014). Growth of gut microbiota of Atlantic cod (Gadus morhua L.) (Ringø et al., 2006a)) and that of Atlantic salmon (*Salmo salar L*.) (Ringø et al., 2008), has taken incubation periods of up to 4 weeks at 12 °C. Similarly, the commonly used incubation conditions for gut microbiota of rainbow trout (*Oncorhynchus mykiss*) to grow on general-purpose media is 15–20 °C for 7–14 days (Huber et al., 2004; Pond et al., 2006; Merrifield et al., 2009). In contrast,

higher temperatures and shorter incubation periods are suitable for warm water fish species; for instance, 28-30 °C for 1-7 days in tilapia (*Oreochromis spp*.) (Standen et al., 2013).

The use of this technique for the study of gut microbiota has led to the understanding of gut microbiota of different fish species being mainly composed of aerobes and facultative anaerobes (Cahill, 1990; Ringø et al., 1995). Based on this classical method, the amount of gut microbiota in salmonid fish has been reported as more of aerobic bacteria than anaerobic bacteria (Ringø et al., 1995). In addition, bacterial counts of the gut microbiota of Arctic char (*Salvelinus alpinus*) showed that approximately 10^5 bacteria/gram were present in the gut and comprised of mainly *Aeromonas*, *Acinetobacter*, *Cytophaga*, *Moraxella*, *Streptococcus*, and *Lactobacillus* (Ringo, *et al*. 1994). 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 (Hovda et al., 2007). It has been estimated that, the majority of the gut microbiota of fish contains high portion of unculturable bacteria, while only 11-50% of the gut microbiota are culturable (Huber et al., 2004) but in Atlantic salmon the level of culturable gut bacteria are about 1% of all the gut bacteria (Dehler et al., 2017). These traditional methods are time consuming and lacks accuracy in isolate identification. In addition, such method has restricted discrimination power and may lack proper characterisations of relationships between aquatic-environmental microorganisms and fish microbiota (Romero et al., 2014).

6.2 Molecular methods for characterization of microbiota

As the conventional methods for analysing the gut microbiota are time-consuming and tedious, various molecular methods have been developed to study gut microbiota, which now enables to get a good picture of the total microbial representation in the gut. According to Ringø & colleagues (2016), molecular methods are generally divided into two groups: (i) the Polymerase chain reaction (PCR)- based techniques which amplify certain fragments of DNA or cDNA using user-defined primers, and (ii) the PCR-independent methods which are crucial tools to detect and visualize bacteria at a spatial scale without any gene- or cDNA amplification. However, the latter are less specific and sensitive than PCR-based methods, and are less suitable for profiling bacterial communities (Ringø et al., 2016).

Culture independent techniques that may be used to characterize the gut microbiota include: quantitative real-time PCR (qPCR) and fluorescent in situ hybridization (FISH), which have been

used to determine the abundance of particular taxa or total microbial levels; fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE), which have been used to analyse microbial community structure and diversity (Muyzer and Smalla, 1998; Navarrete et al., 2012), microarrays (MITChip and HITChip) and sequencing including shotgun Sanger sequencing method and the latest next-generation sequencing methods (Fraher et al., 2012). Although each identification method has its own advantages and disadvantages, the choice of approach depends on the key questions to be addressed. For example, the major advantages of qPCR is that its high sensitivity, and it is suitable to quantify one or more bacterial groups that are targeted with specific primers or probes. The PCR method is an in-situ DNA replication process that allows for the exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and a thermostable DNA polymerase (Farber, 1996). All PCR-based methods consist of three basic steps: (i) nucleic acid extraction, (ii) amplification of DNA and (iii) analysis (either quantitatively or qualitatively) of PCR products. The PCR assays have been routinely used for rapid detection, identification and differentiation of microorganisms. There are various types of available PCR methods that can be applied in identification and/or quantification of various microorganisms. For example, conventional PCR- method involves the use of a single primer set (which targets a gene specific for one species) or as in the case of multiplex PCR- multiple primers are used within a single PCR mixture to detect and/or differentiate microorganisms. In multiplex PCR, more than one target sequences is amplified in a reaction to produce amplicons of varying sizes specific for different DNA sequences. Although multiplex PCR reduces cost, limits volume of samples and allows for the rapid detection of multiple bacteria species, strains and so on, primer design is critical in the development of multiplex PCR (Bai et al., 2010). However, regarding to the analysis of microbial community such as gut microbiota, the most commonly used PCR method is qPCR and other culture independent methods are also reviewed as follows.

6.2.1 Real-time PCR (quantitative PCR)

Real time PCR method or quantitative PCR (qPCR) is used for direct quantification of the number of bacteria in a sample (Dicksved, 2008). In qPCR, the target DNA is amplified and quantified simultaneously within a reaction. In this method, a fluorescent reporter molecule is included in the assay mix and this enables the products of the PCR reaction to be measured after

each cycle once a threshold has been passed. This method uses a fluorescent reaction to determine the kinetics of product accumulation during PCR amplification with specific primers for a specific group or species of bacteria while the reaction is in progress, not at the end of the reaction. Thus, it shortens detection time compared to standard PCR and can be used to determine the absolute or relative number of bacteria in various samples. However, equipment and reagent costs are high for qPCR. This method is limited due to primer specificity during PCR and has mainly been used to detect and quantify bacteria at the genus or phylum level. The 16S rRNA gene is commonly used as a target molecule however, due to heterogeneous gene copy numbers in some species, quantification based on 16S rRNA gene copies can be misleading (Dicksved, 2008).

6.2.2 The application of fingerprinting techniques

Among the various molecular tools used to evaluate the microbial community, fingerprinting techniques were very popular. These methods usually rely on separation of multi-template PCR products, which generate a molecular fingerprint of the microbial community.

PCR-DGGE and PCR-TGGE

PCR-denaturing gradient gel electrophoresis (PCR-DGGE; Muyzer et al. (1993)) and PCR-temporal temperature gradient gel electrophoresis (PCR-TGGE; Muyzer and Smalla (1998)) are based on the separation of PCR amplicons with different sequences. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profiles. Combination of different PCR amplicons (with different G/C content) are dissociating at different positions with either PCR-DGGE or PCR-TGGE is widely used technique to determine the bacterial communities (Muyzer and Smalla, 1998; Namsolleck et al., 2004; Merrifield et al., 2009). The result is a pattern of bands which is a visual profile of the most abundant species in the studied microbial community. It is believed that each band represents one species, thus DGGE is a useful for rapid assessment of the composition of the gut microbiota and is particularly suitable for comparison of multiple samples. Similar method, TGGE separates DNA fragments in a temperature gradient instead of a denaturing gradient (Namsolleck et al., 2004).

Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Another commonly used fingerprinting approach is terminal-restriction fragment length polymorphism (T-RFLP) which generate a fingerprint based on polymorphisms in restriction

sites in the PCR fragments (Dicksved, 2008). This fingerprinting technique is used to visualize diversity structure and dynamics of the microbiota and is generally quite cheap and is more suitable for rapid comparisons of microbial community compositions in different samples (Dicksved, 2008; Ringø et al., 2016). However, this method lacks quality resolution and inability to characterize the species composition (Ringø et al., 2016).

6.2.3 Fluorescence in situ hybridization (FISH)

FISH is used for direct quantification of the number of bacteria in a sample (Dicksved, 2008). The principle of FISH is the detection of a target DNA or RNA site by a fluorescently labelled probe molecule. This method is the most common and powerful method allowing for the simultaneous fast detection and enumeration of specific microorganisms (Wagner et al., 1993; Dicksved, 2008). This technique uses fluorescent labelled oligonucleotides probes (usually 15-25 bp) which bind specifically to microbial DNA in the sample, allowing the visualization of the cells using an epifluorescence or confocal laser scanning microscope (Gilbride et al., 2006). Depending on the selection of probes, FISH can be used to detect bacteria at different phylogenetic levels (Dicksved, 2008). The advantage of this method is that results this method is unbiased, because it is not based on PCR and thereby free from biases introduced by PCR or DNA recovery. However, with FISH method, only a few probes can be used per analysis, thus it is not used as a separate technique and is mostly used in combination with other methods to characterise microbial communities (Ringø et al., 2016). It is also dependent on reliable sequence data, and laborious if identification at the species level is required (Dicksved, 2008).

6.2.4 Cloning and sequencing methods

Cloning and sequencing of 16S rRNA genes is the conventional and more widespread genomic approach used when detailed and accurate phylogenetic information is required (Dicksved, 2008). Although DNA sequencing methods such as Sanger sequencing have been used since the mid-1970s (Sanger et al., 1977), which use dideoxynucleotides to terminate the chain amplification, this old sequencing method was quite expensive and too time consuming for extensive use. The amplicons obtained from PCR form the basis for all the community fingerprinting methods and next generation sequencing methods are reviewed in the following.

- Next generation sequencing

Next-generation sequencing describes sequencing technologies that deliver sequence data at a relative low cost and a fast rate. This method is also known as high-throughput sequencing, which is a collective term used to describe several modern sequencing methods developed after the traditional Sanger sequencing method. The NGS platforms generates millions of sequences in each run used to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and has provided an important analytical platform to study microbial ecology (Ringø et al., 2016). With the development of NGS technology, the study of gut microbiota currently consists of two major stages: (1) 16S rRNA gene (or another relevant gene) based sequencing of bacterial gene; and (2) bioinformatics analysis (Jandhyala et al., 2015). The use of NGS to sequence the universally distributed 16S rRNA gene have revealed that it is possible to get a detailed picture of gut microbial communities and determine their phylogenetic relationships.

Advantage and disadvantages of using culture dependent and molecular techniques

The PCR-independent methods are less specific and sensitive than PCR-based techniques, and they are less suitable for profiling bacterial communities. Since there are many molecular methods, it is better to see review on the strengths and weakness culturing methods in tables 4 as described by Furrie (2006) and advantages and disadvantages of PCR-based techniques in table 5 **reviewed by** Fraher *et al.* (2012)**).**

Table 4. Advantages and disadvantages of culture based methods in gut microbiota identification. (adapted from Furrie (2006)).

Advantages	**Disadvantages**
Relatively inexpensive	Slow, time consuming, and labour intensive.
Widely available	Samples require immediate processing.
Allows quantification of bacterial populations	Extensive expertise and specialised equipment needed to isolate strict anaerobes.
Can provide a good indication of ecosystem complexity, if carried out by skilled and experienced microbiologist!	Restricted to culturable organisms.
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 many techniques

Table 5. Advantages and disadvantages of using molecular techniques to characterize the gut microbiota (Source: Adapted from Fraher *et al.* (2012)).

Technique	Description	Advantages	Disadvantages
qPCR	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to double-stranded DNA	Phylogenetic identification, quantitative, fast	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/ temperature	Fast, semi-quantitative, bands can be excised for further analysis.	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences— unable to identify unknown species
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Phylogenetic identification, semi-quantitative, fast	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, tedious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons	Phylogenetic identification, quantitative, fast	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome	Phylogenetic identification, quantitative	Expensive, analysis of data is computationally intense

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; qPCR, quantitative PCR; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism

7. Hypothesis and goals of current investigation

Some studies on the intestinal microbiota of fish have focussed on the effects of feeding plant protein diets. However, only little information is available in the literature about specific and/or interaction effects of ANFs on digestive functions and gut health of fish, whereas no information can be found on their effects on the gut microbiota. Therefore, the objective of this simulation model was to assess the individual and combined effects of four purified ANFs present in SBM on gut microbiota of farmed Atlantic salmon lectin, saponin, isoflavonoid and phytosterol. The overall aim of this study was to predict how these ANFs affects the composition and diversity of gut microbiota of Atlantic salmon as well as the bacterial metabolites produced using an in vitro model system.

7.1 Hypothesis

The inclusion of different levels of ANF affect gut microbes including their proliferation, numbers, total composition and metabolic activity. Considering the four types and three levels of ANF concentrations individually and in combination used, it is expected that their effects would be more visible at least in some of the levels used. Investigation of cause effect relationships will explain their effects on fish health.

7.2 Specific aims:

Therefore, the specific aims of this *in vitro* model simulating the salmon gut were:

- To evaluate how gut microbiota of Atlantic salmon respond to diets supplemented with different purified ANF levels
- To answer how ANFs affect the metabolic activities of microorganisms (will be estimated with SCFA production and pH changes),
- To investigate the reaction of the main microbial species in the gut of Atlantic salmon
- To examine the effect of individual ANFs on individual and total gut microbiota
- To assess how different levels (doses) of ANF inclusion affect the microbiota and
- To investigate how the combined ANFs affect the gut microbiota

8. Materials and methods

8 .1 Use of *In vitro* simulation for GIT fermentation

Conducting research that involve laboratory experiments needs choosing appropriate models. Challenges may be encountered with the use of *in vivo* methods in animals because of economic and ethical constraints which may make it difficult to use live models for GIT investigations. To avoid these challenges, using the gut content as a source of inoculum for *in vitro* experiments has been employed broadly in humans and animals (Rymer et al., 2005; Zhou et al., 2013). *In vitro* models using a fermenter that mimic the gut is an appropriate method for the study of the gut microbiota and have been successfully used as an environment to investigate the effect of dietary components on the gut microbiota of animals and humans (Williams, 1995; Makkar, 2002; Krajmalnik-Brown et al., 2012; Zhou et al., 2013) including complex host–microbe interactions (Payne et al., 2012). Nevertheless, no studies to date have used a simulation model to investigate the impact of purified ANFs on the gut microbiota of Atlantic salmon. In addition to the 3Rs (Replacement, Reduction and Refinement) principles, the *in vitro* simulation approach could be more appropriate to investigate the effect of purified ANFs on fermentation. Like other monogastric animals, Atlantic salmon do not have functional enzymes to digest carbohydrate (starch) rich diets and causes problems in fish health (Krogdahl et al., 2004). The relatively high concentration of carbohydrates in SBM used in salmon feeds create a challenge to use the traditional diet for cause-effect study of ANFs in Atlantic salmon. Hence to investigate the effects of purified ANFs on Atlantic salmon, the use of a simulation study is supposed to offer a suitable alternative to avoid such possible confounding effects as the results of these assays would be caused by the direct effects of the antinutrients, direct in the sense that they will not mediated by the host. As *in vitro* study reduces/avoids any animal welfare concerns, resource management and economic considerations and above all gives better estimation of direct ANF effects on gut microbiota. Therefore, *in vitro* gut models are expected to provide several advantages over *in vivo* models for the study of gut microbiota of Atlantic salmon.

8.2 Diet, types of ingredients and experimental designs used

In this thesis, previous obtained data, which have been collected from an in vitro study carried out with one control and effects of four ANFs and their mix on gut microbiota from farmed Atlantic salmon were kindly provided by Professor Anne Marie Bakke and Professor Åshild

Krogdahl. In this study, intestinal contents were collected from FM fed farmed Atlantic salmon. Growth medium (substrate) for the simulations were mixed from fresh and frozen digesta from salmon fed standard FM. Intestinal contents were sampled from both mid and distal intestine and the experiments were based on a substrate comprising 50%:50% mixture of mid and distal intestinal contents of Atlantic salmon for the evaluation of ANF effects. Table 6 show the different groups studied including the antinutrients used as well as the different levels of antinutrients included in the inoculum. As this study was based on the data collected in a previous experiment, the specific materials, primers, chemicals, reagents and protocols used in the different testes and analysis were not available to be included here. This *in vitro* study was conducted based on one control group (with no ANF added), and addition of lectin, saponin, isoflavonoid, phytosterol and a combination of the four ANFs. The concentrations of ANFs were based on the concentrations on heat-treated soybean applied at three different levels; 'Low`, 'Mid' and 'High'. The concentration for High was three times the concentration of Mid and the concentration of Mid was three times the concentration for Low, while Low was based on the concentration of each antinutrient in feed containing 30 % of heat-treated soybean.

In this study, the experiment was arranged in a way that the four individual ANFs, their combination and the negative control makes six experimental tests in total, where each treatment has 5 replicates. In addition to the negative control, each antinutrient or antinutrient combination has three levels, hence a total of 80 simulation vessels were used. All the simulation vessels were supplied with the substrate (mix of fresh and frozen digesta from salmon fed with standard FM). In the simulation, it was typically assumed that water drunk by the fish and the feed absorbed through the intestine, (the former dilute and the latter increase the concentration of test compound) are of similar magnitude for all fish resulting in roughly equal concentration in mg/ml in the GIT (which the simulation mimics) or as in feed in mg/g. This means that at least concentrations mid and high-levels are much higher than concentrations typically prevailing in the GIT of salmon fed feeds containing soy products in general. Vessels were incubated at 10^0C for 7 days, during which the fermentation process and gas production was measured. Whereas, microbiota analysis, SCFA profiles, pH and RedOx were measured at the end of the simulation.

Table 6. Types and levels of antinutrients tested in this experiment**.**

Treatment	Lectin (mg/ml) 1 x SBM	Saponin (mg/ml) 4 x SBM	Isoflavonoid (mg/ml) 4 x SBM	Phytosterol (mg/ml)4 x SBM
Control	0.0	0.0	0.00	0.0
Lectin Low	0.09	0.0	0.0	0.0
Lectin Mid	0.27	0.0	0.0	0.0
Lectin High	0.81	0.0	0.0	0.0
Saponin Low	0.0	6.0	0.00	0.0
Saponin Mid	0.0	18.0	0.00	0.0
Saponin High	0.0	54.0	0.00	0.0
Isoflavonoid Low	0.0	0.0	2.4	0.0
Isoflavonoid Mid	0.0	0.0	7.2	0.0
Isoflavonoid High	0.0	0.0	21.6	0.0
Phytosterol Low	0.0	0.0	0.00	1.2
Phytosterol Mid	0.0	0.0	0.00	3.6
Phytosterol High	0.0	0.0	0.00	10.8
Combination Low	1.2	2.4	0.09	6.0
Combination Mid	3.6	7.2	0.27	18.0
Combination High	10.8	21.6	0.81	54.0

SBM= Soybean meal, lectin was added at 0.09, 0.27, 0.81 and 1.2, 3.6, 10.8 respectively for individual and combined effects assessment in the three, low to high treatment levels. For the other ANFs their levels were four times higher than their concentration in the SBM diet. Hence, the amount at each levels for the individual saponin, isoflavonoid and phytosterols and for their combined effects are indicated at each column. All ANFs were added at mg/ml or mg/g levels. While the control gets 0.0 level in all ANF` effect assessments.

8.3 Measured parameters

Although detailed information on the type of the primers (the properties of EWOS Innovation) used to run the qPCR and other information on the different methods were not available to be included in this paper (may be due to confidentiality). Based on the obtained data collected from the simulation study, various methods were used to characterize the effects of ANFs on gut microbiota of farmed Atlantic salmon and fermentation include:

Quantification of intestinal bacteria of Atlantic salmon -The microbiological quantitative analyses were performed by means of conventional method, qPCR (16S rRNA copies/g). This quantification process used to estimate of the ANFs effect on total and individual intestinal microbiota was based on the mean counts of total and individual bacteria. In addition to the

identification of common intestinal bacteria of salmon, quantification of the total captured bacterial communities and characterizing of individual bacterial levels within the pooled sample, such their sensitivities/tolerance to the various levels of the tested ANFs were estimated. The panel of relevant bacterial taxa for Atlantic salmon gut at different taxonomic levels consisting the following families and classes were assessed and the dominant ones that were detectable in this experiment were analysed, and representative figures are included in this paper. However, as aforementioned, due to lack of information on the type of PCR reagents (including the origin and type of primers), temperature and number of cycles employed and the PCR protocol used during the PCR reaction, it is not possible to elaborate more about this and other techniques used to collect the data for this thesis writing.

- Family *Corynebacteriaceae* in class *Actinobacteria*
- Families *Bacillaceae, Planococcaceae, Staphylococcaceae, Carnobacteriaceae* and *Enterococcaceae* in class *Bacilli*
- Families *Lactobacillaceae, Leuconostocaceae* and *Streptococcaceae* in class *Bacilli (*analysed with two qPCR assays, one for families *Lactobacillaceae* and *Leuconostocaceae* and one for family *Streptococcaceae)*
- Family *Peptostreptococcaceae* in class *Clostridia*
- Family *Mycoplasmataceae* in class *Mollicutes*
- Class α-*proteobacteria* (*Alphaproteobacteria*) and Class β-*proteobacteria* (*Betaproteobacteria*)
- Families Vibrionaceae, Enterobacteriaceae, Pseudomonadaceae and Xanthomonadaceae in class α-proteobacteria (Gammaproteobacteria)

Estimation the ANFs effect on metabolic activities of gut microbiota-To estimate the metabolic effects of ANFs on gut microbiota, measurement of various parameters such as gas production was measured during fermentation, while the change in pH, RedOx potential and SCFA profiles were measured at the end of the simulation.

8.4 Data analysis

In analysis data, two statistical softwares were used, R language programming and JMP® Statistical Software. Using R language programming normality of data was assessed by Shapiro-Wilk test to detect whether a data was collected from a non-normal distribution (Ghasemi and

Zahediasl, 2012). Normality of data assessment by Shapiro-Wilk test was performed using the following commands;

"result<-shapiro.test (treatment1$response variables)

result$p.value".

Due to nonlinear modelling observation in most of the resulted data, the data was transformed using a square value. Even after transformation, normality was maintained only in few records and Analysis of variance (ANOVA) was used to determine to analyse the responses with normality maintained data. Since most of the response variables had missed observations, considering with the small samples, variable with unequal observations were assumed as non-normally distributed data set and thus most of the reports included in this paper are assessed by nonparametric test. The nonparametric assessment was done by Wilcoxon/Kruskal-Wallis test using JMP® Statistical Software. Thus, the JMP software was used to analyse both normality-based ANOVA test and the nonparametric Wilcoxon Test. The outcomes of both analysing tools were assessed and very small significant effects were found and representative figures showed with statistical difference, depicted with different asterisks ('~' for $p<0.10$, '*' for $p<0.05$, while the double asterisks (**) represent for $p<0.01$) and their implications are described in this paper.

9. Results

9.1 Effects of antinutrients on microbial numbers

The dominant clusters in the inoculum were analysed in a pre-study simulation before the simulation tests with the ANFs. In that pre-study, the major clusters identified were members of families Lactobacillaceae, Streptococcaceae and Peptostreptococcaceae, but also those of Mycoplasmataceae dominated the microbial community. In the current simulation, the effects of ANFs on the total captured bacteria were investigated by real-time PCR. The results of this simulation prevailed high proportion of Lactobacillaceae,Streptococcaceae, Peptostreptococcaceae and bacilli like microbes. In the non-amended control ~55% of the microbes were captured by the qPCR panel applied. Here it is clearly visible that, all the antinutrients increased the proportion of captured microbes. Although the proportion of the total microbes in all treatment levels were increased compared to the non-amended control group. The more visible increment in the total microbial proportions were at mid concentration of lectin, isoflavonoid and high concentration of saponin and combination of the antinutrient, but according to Wilcoxon statistical analysis significant increase was observed only at high concentration of saponin ($p< 0.10$, Fig. 9). For lectin and isoflavonoid, the pattern was similar, while the proportion of captured microbes showed dose dependent increase in saponin and combination of antinutrients, but significance was detected only at the maximum concentration of saponin ($p<0.10$, Fig. 9).

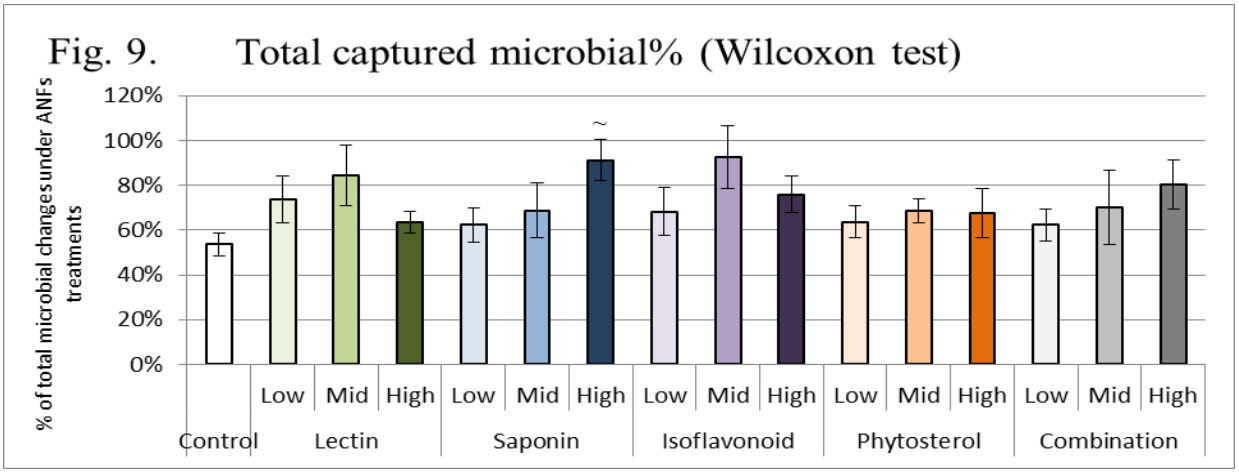

Figure 9. Comparing the effects of ANFs on the total captured bacteria.
The proportion of captured microbial changes in response to various ANFs was calculated based on the mean of the total bacteria captured by the currently employed primers and probes. Results were analysed

by Wilcoxon/Kruskal-Wallis test. Columns represent the proportion of changes in the total captured microbes and asterisk show significant differences ('~' for p<0.10).

Comparably, the levels of the major cluster, Lactobacillaceae showed relatively similar pattern to total microbes when the tested antinutrients were applied alone and in combination. The proportion of the dominant Lactobacillaceae cluster showed an interesting response to the antinutrients (Fig. 10). This cluster was increased by mid-levels of lectin, isoflavonoid and phytosterols and at the two highest concentration of the combined ANFs, but according to the nonparametric analysis significant increase was shown only at the highest concentration of saponin (P<0.10, Fig. 10). The effects of each levels of lectin and isoflavonoid showed similar patterns. The increase in the proportion of Lactobacillaceae was a dose-dependent increase in saponin. The combination of antinutrients tended to increase the proportion of Lactobacillaceae at all levels applied but no significant effect found in all levels (p>0.10, Fig. 10).

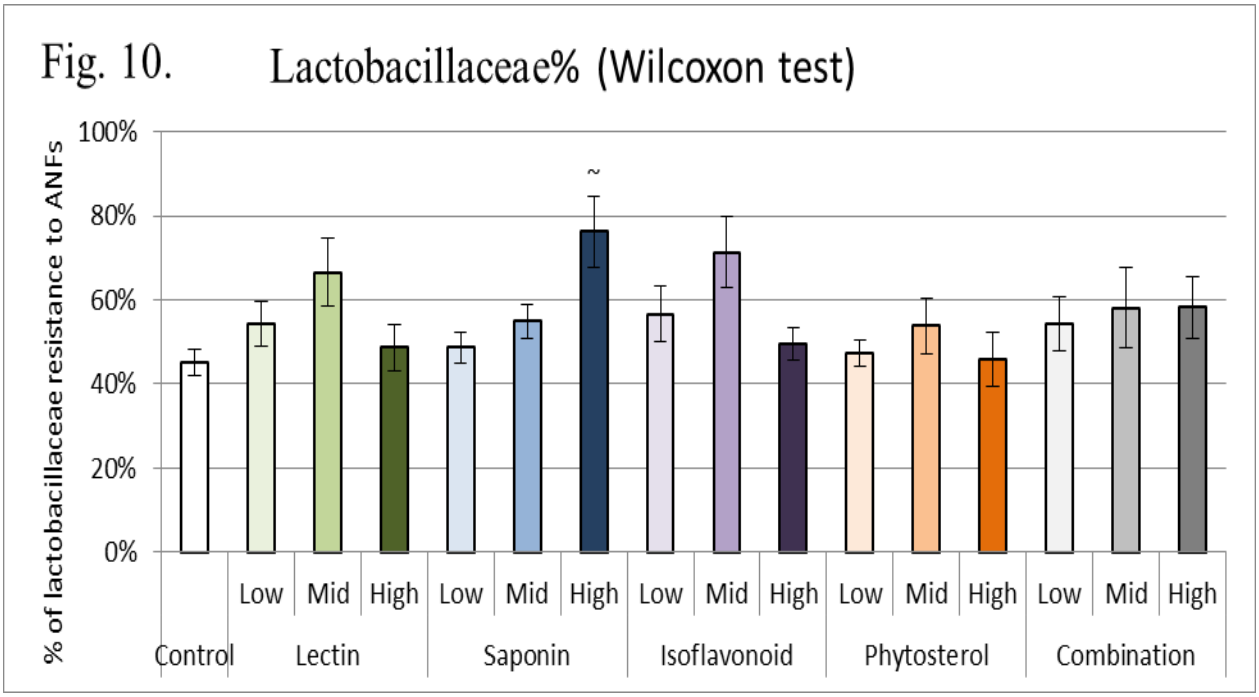

Figure 10. Comparing effects of ANFs on Lactobacillaeceae cluster.
The percentage of Lactobacillaeceae resistance to the various ANFs was calculated based on the total captured from the different treatment levels of the ANFs. Results were analysed by Wilcoxon/Kruskal-Wallis test. Columns represent the proportion of resistant Lactobacillaeceae, and asterisk show significant differences ('~' for p<0.10).

The effects of the ANFs on the proportion of bacilli like microbes showed a similar pattern of dose dependent increase in all the tested antinutrients (Fig. 11). While, Streptococceae numbers

showed a dosed dependent increased in isoflavonoid and combination of the ANFs. But significant increase was only shown at the highest concentration of isoflavonoid ($p<0.10$). The effects of lectin, saponin and phytosterol are very different. At mid-levels of lectin and low level of saponin and phytosterol, Streptococceae showed a tendency to increase their proportions (Fig. 12).

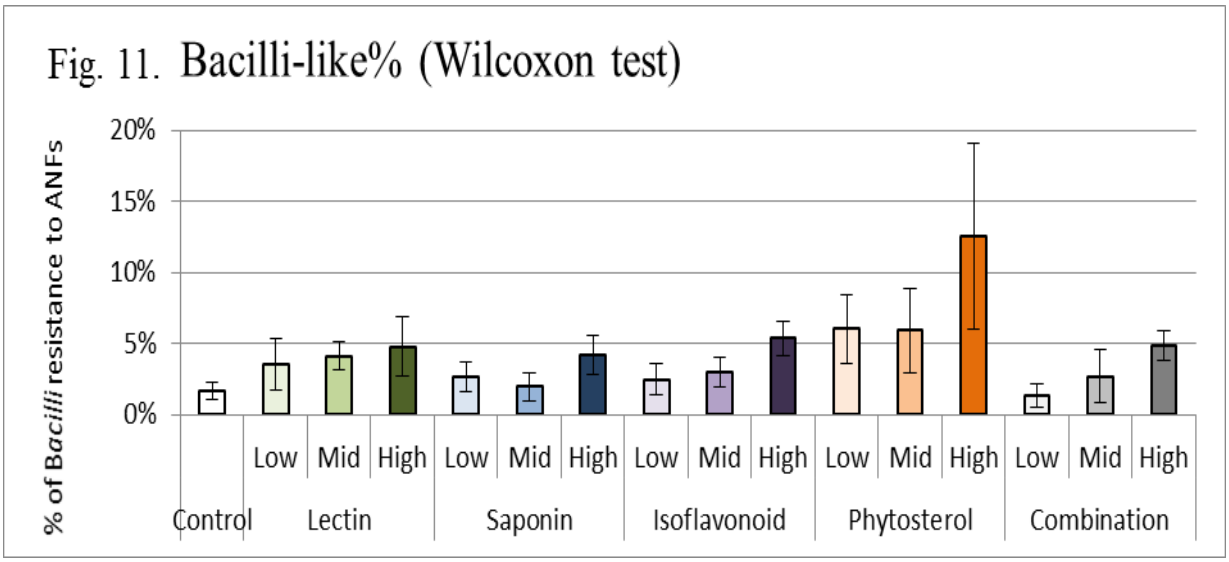

Figure 11. Comparing the effects of ANFs on bacilli like microbes.
The proportion of bacilli like microbes that were resistance to the various ANFs was calculated based on the percentage of total captured microbes of the five ANF treatments. Results analysed by Wilcoxon test.

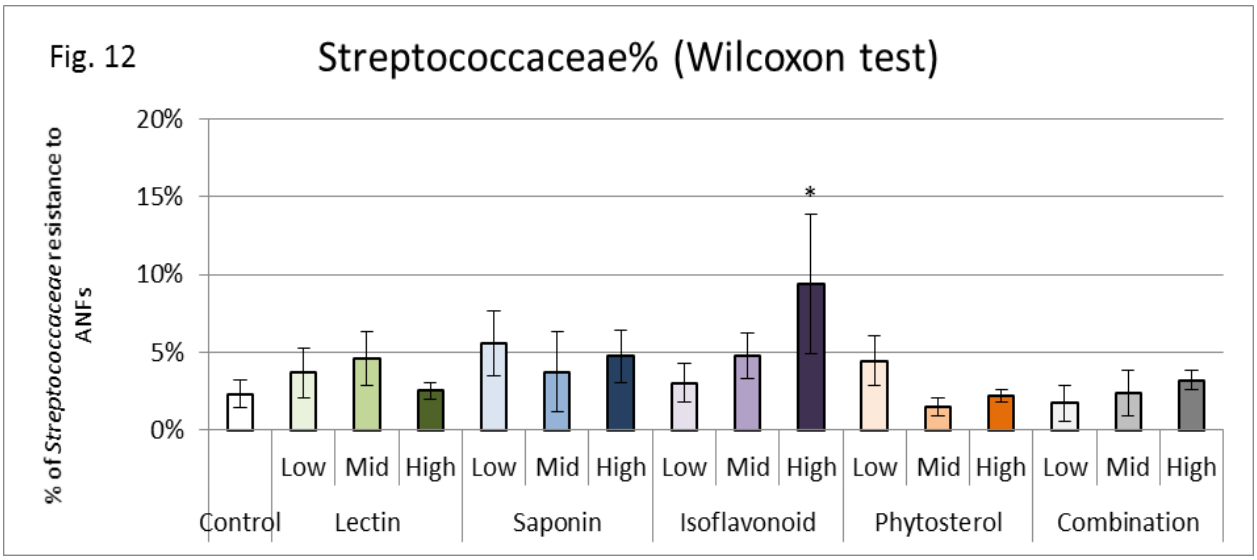

Figure 12. Comparing the effects of ANFs on Streptococceae.
Columns show percentage of Streptococceae under various levels of ANF treatments. The percentage of Streptococceae was calculated based on the total captured microbes. The results were analysed by Wilcoxon test and asterisk show significant differences ('*' for $p<0.05$).

The proportion of aerobic bacteria including family Corynebacteriaceae, class alpha-Proteobacteria and beta-proteobacteria remained small in all simulation vessels. Particularly, the proportion of Corynebacteriaceae and β-proteobacteria remained below the detection limit and their results are not shown here. Nevertheless, it is important to indicate that the proportion of aerobic microbes tended a dose dependent increase in lectin, saponin, phytosterol, and combination of antinutrients. However, Wilcoxon test indicated that no significant effects was detected on the proportion of aerobic microbes ($p>0.10$, Fig. 13).

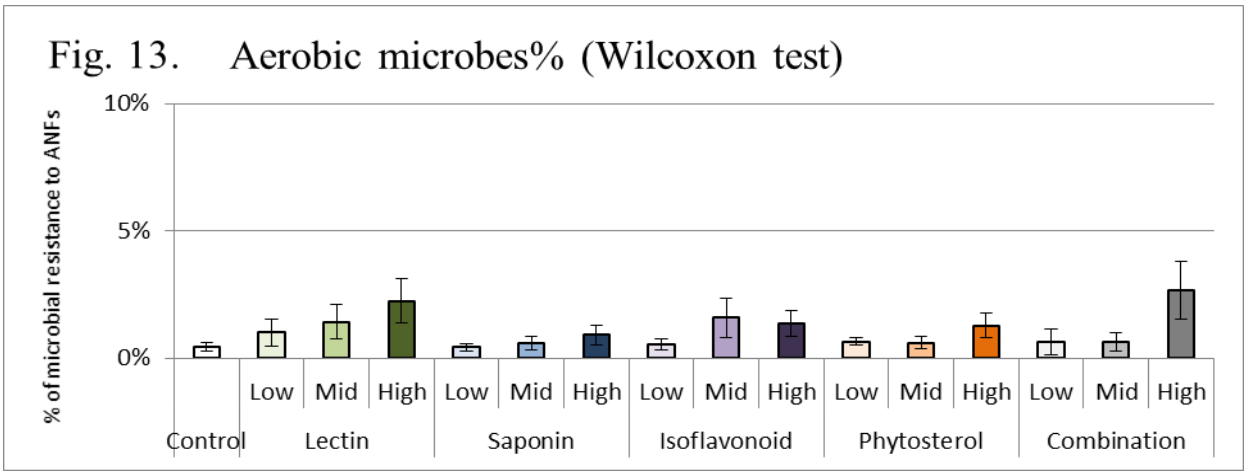

Figure 13. Comparing the effects of ANFs on aerobic microbes `cluster.
Columns represent the percentage of aerobic microbe's resistance to the various ANFs was calculated based on the total captured microbes.

The effect of ANFs on aerotolerant bacteria were also investigated including on families Mycoplasmataceae, Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae and vibrionaceae but remained below detection limit in most cases and represented at most less than 5% of the microbial community. Therefore, Vibrionaceae was the only cluster detected at a relative high level at the end of the simulation and its result is shown (Fig. 14). Maximum inhibition of growth in Vibrionaceae showed at the highest concentration of lectin and phytosterol, mid-level of isoflavonoid, low levels of saponin and phytostero. Similarly, Vibrionaceae was reduced at the two-low concentration of level of phytosterol but all results didn`t show any statistical significance ($p>0.10$, Fig. 14). While the proportion of Vibrionaceae numerically increased at mid-level of lectin and at the two highest concentration of saponin but neither of these increases were significant ($P>0.10$). However, at the high level of the combined

effects of the four ANFs the level of vibrionaceae bacteria was increased significantly ($P<0.05$, Fig. 14).

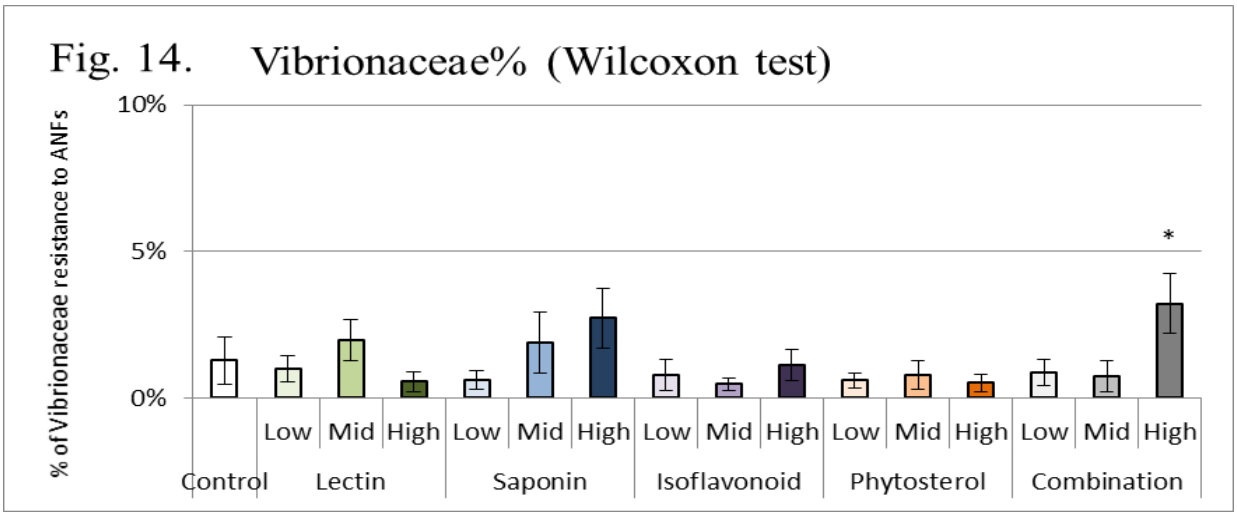

Figure 14.Comparing the effects of ANFs on the proportion of Vibrionaceae clusters.
Columns show the percentage of Vibrionaceae resistance to the various ANFs. Results were analysed by Wilcoxon test. The asterisk '*' indicates significant difference ($p<0.05$).

In this simulation, the proportion of α-proteobacteria was very low. But numerically, α-proteobacteria tended a dose dependent increase in most ANFs, but no significant effect was found (Fig. 15).

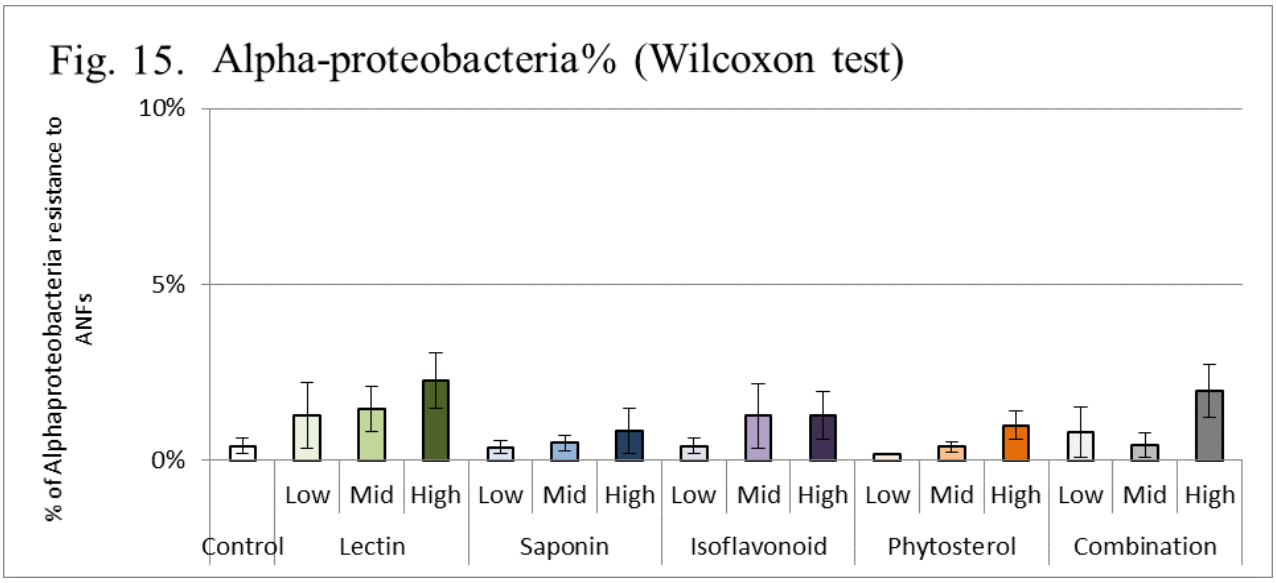

Figure 15. Comparing the effects of ANFs on α-Proteobacteria analysed by Wilcoxon te**st.**
Columns show the percentage of α-Proteobacteria resistance to the various ANFs, calculated based on the total captured microbes.

Peptostretococcaceae showed a tendency to reduce at high levels of lectin, but this group showed a dose dependent increase in saponin and combination of the four ANFs. But maximum growth of Peptostretococcaceae was obtained at the low level of phytosterol ($p<0.10$, Fig. 16). In Anaerobic microbes, except in phytosterol, the effects of all other ANFs showed the same pattern to that of Peptostretococcaceae (Figs. 16 & 17).

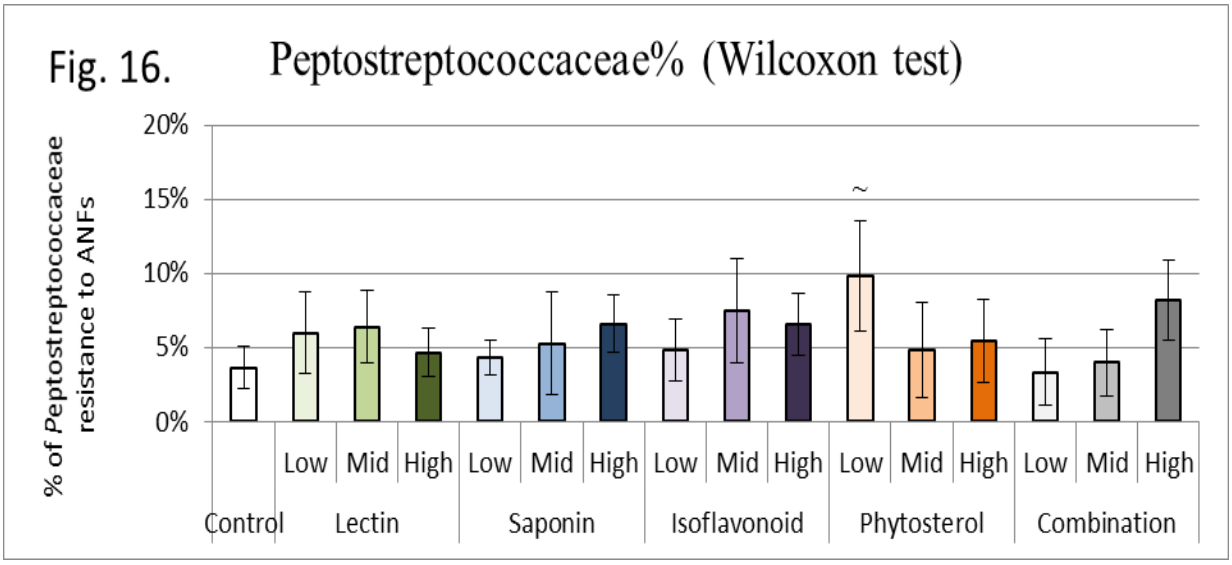

Figure 16. Comparing the effects of ANFs on the proportion of Peptostretococcaceae.

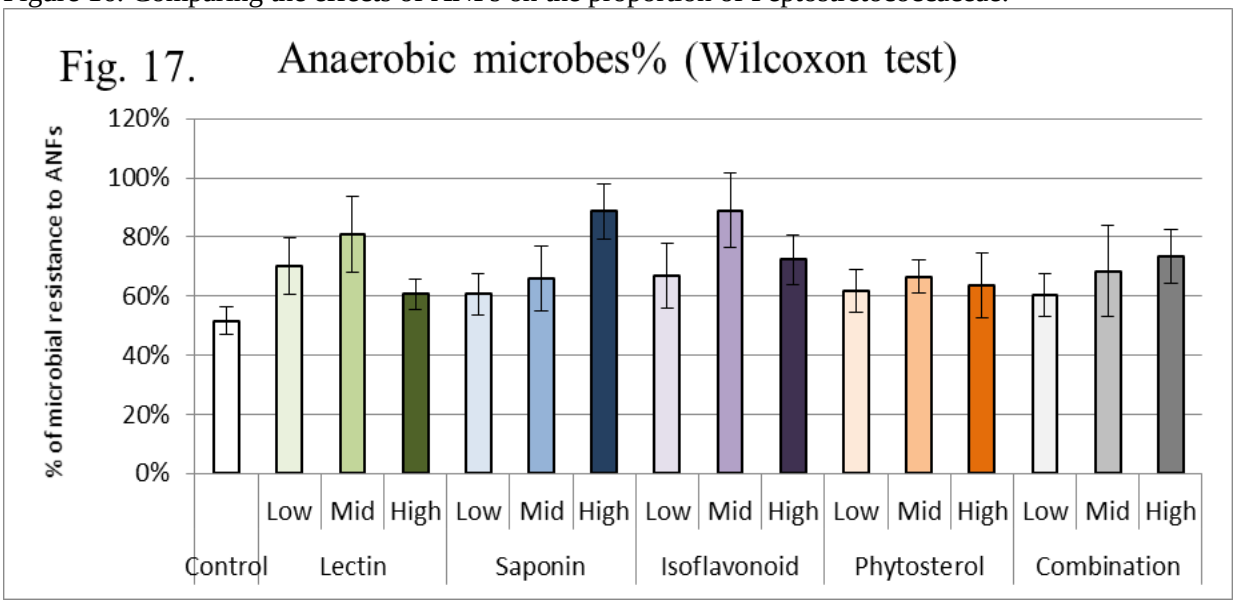

Figure 17. Comparing the effects of ANFs on the proportion of Anaerobic microbes.

Comparing the effects of ANFs on the proportion of Peptostretococcaceae (Fig. 16) and anaerobic microbes (Fig. 17). The percentage of α-Proteobacteria resistance to the various ANFs was calculated based on total captured microbes obtained from qPCR analysis.

9.2 Effects of antinutrients on metabolic activities of gut microbiota

The effects of antinutrients on metabolic activities of gut microbes are estimated using indicators gas production, pH change and Redox potentials as illustrated below. Although not statistically significant (P>0.1), dose-dependent increases in gas production were indicated for saponin, lectin and phytosterol, whereas a tendency toward dose-dependent decreases were observed for isoflavonoid and combination of the four antinutrients (Fig. 18).

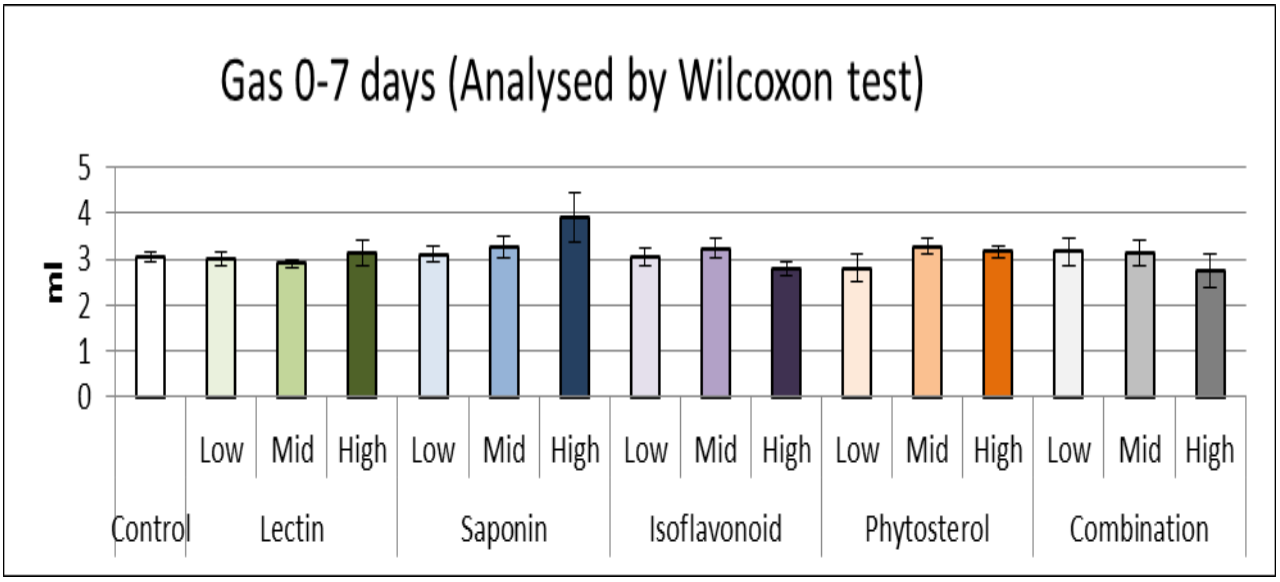

Figure 18. In vitro effects of ANFs on gas production.
This graph is based on the mean of the square-root transformed data for gas production, used to compare the effects of ANFs microbital fermentation. Columns are amoung of gas production (in ml) recorded during fermentation process (0-7days).

The change in the pH of all the treatments have been measured in antinutrients which contained lectin, saponin, isoflavonoid, phytosterols and combination of all the four antinutrients tested showed a visually apparent increase in pH in the high levels of the ANFs but no significance was detected by nonparametric-Wilcoxon test (p>0.10, Fig. 19). In addition, lectin with isoflavonoid, and saponin with phytosterol followed the same tendency with dose-dependent increase in pH. Each of the antinutrients and their combinations tested in this study, pH for the mid concentration was visually apparent lower than either the non-amended control, or more or less dose-dependent decreases observed in the data set. Whereas the values for the high concentration were always numerically higher than non-amended control (Fig. 20).

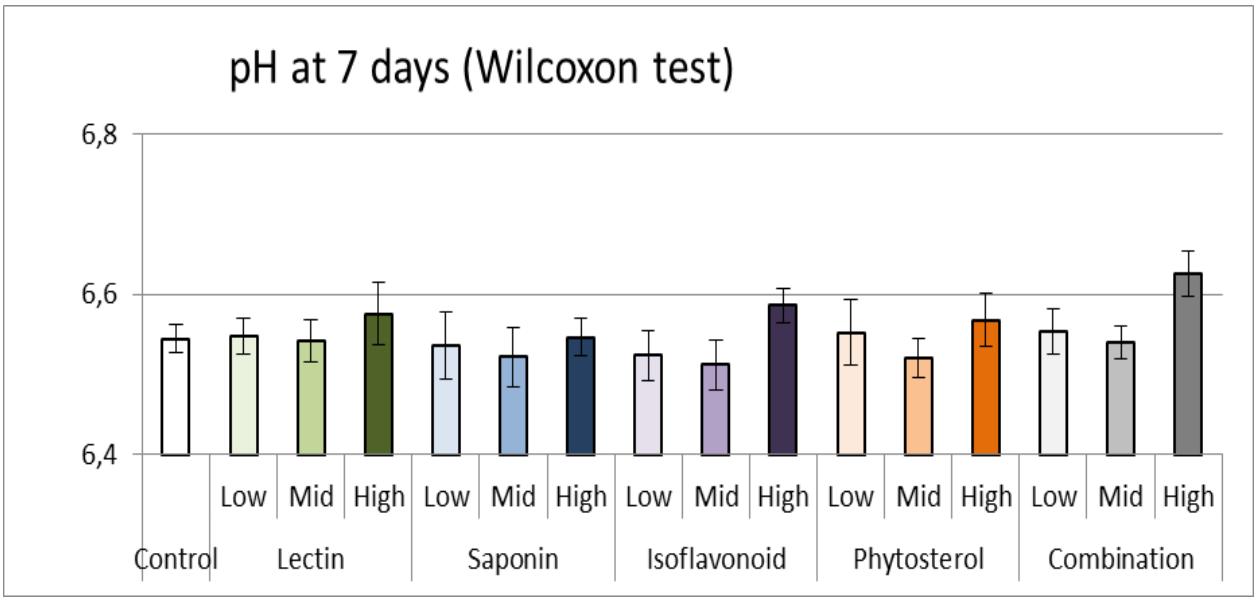

Figure 19. *In vitro* effects of ANFs on metabolic pH changes.
Graphs illustrate that changes of pH in each treatment levels in comparison with pH of negative control. Data was analysed with Wilcoxon test. At high concentration of all treatments levels, the pH was increased.

Analysis of RedOx data showed a normally distributed data set, thus ANOVA test demonstrated similar changes that were observed in pH measurement. The RedOx potential (E_h) was also increased with dose-dependent increases ($p<0.05$, result not shown).

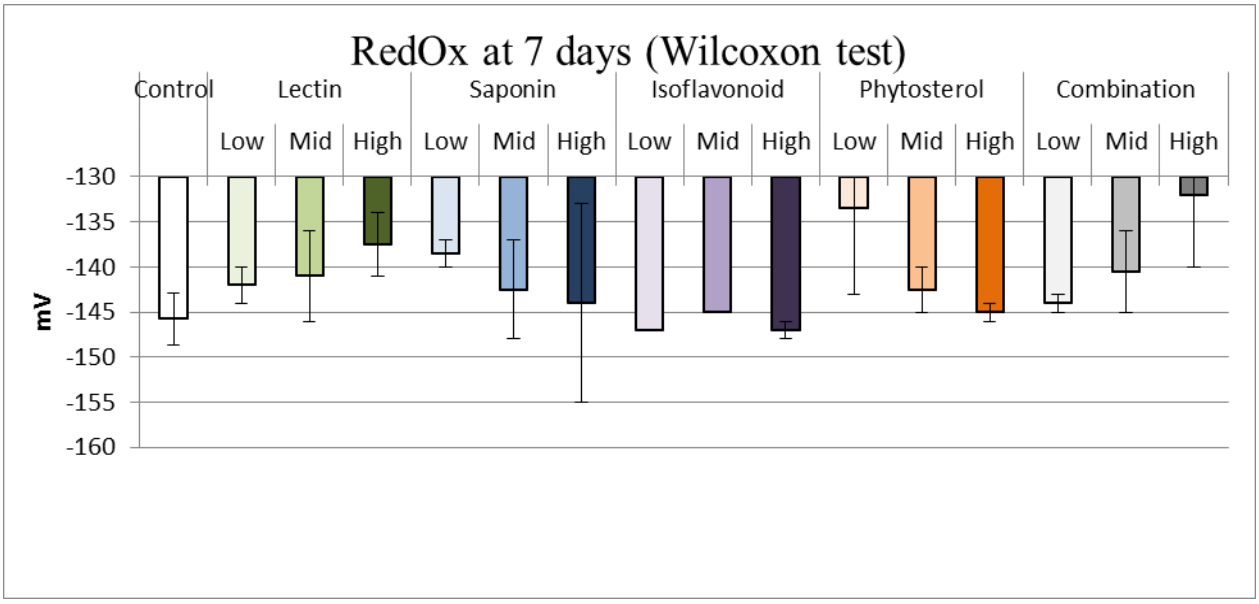

Figure 20. In vitro effects of ANFs on Redox Potentials.
Graph is based on mean of RedOx data. RedOx results were analysed with a nonparametric test. Lectin and the combined ANFs shows a similarly dose-dependent increases and effects in most levels are higher than in the negative control. Conversely, effects of saponin and phytosterol shows a dose-dependent decrease in RedOx.

The E_h was increased at the high concentration of lectin and combination of antinutrients. Inversely, the E_h was spotted high at low concentration of phytosterol, but changes were numerical no significantly influenced according to the Wilcoxon Test.

During analysis of SCFA data, only samples of the total SCFAs were found normally distributed and thus tested with ANOVA. A slightly increase in total SCFAs production was observed at mid-concentration of isoflavonoid and phytosterol and mid and high level of the four combination.

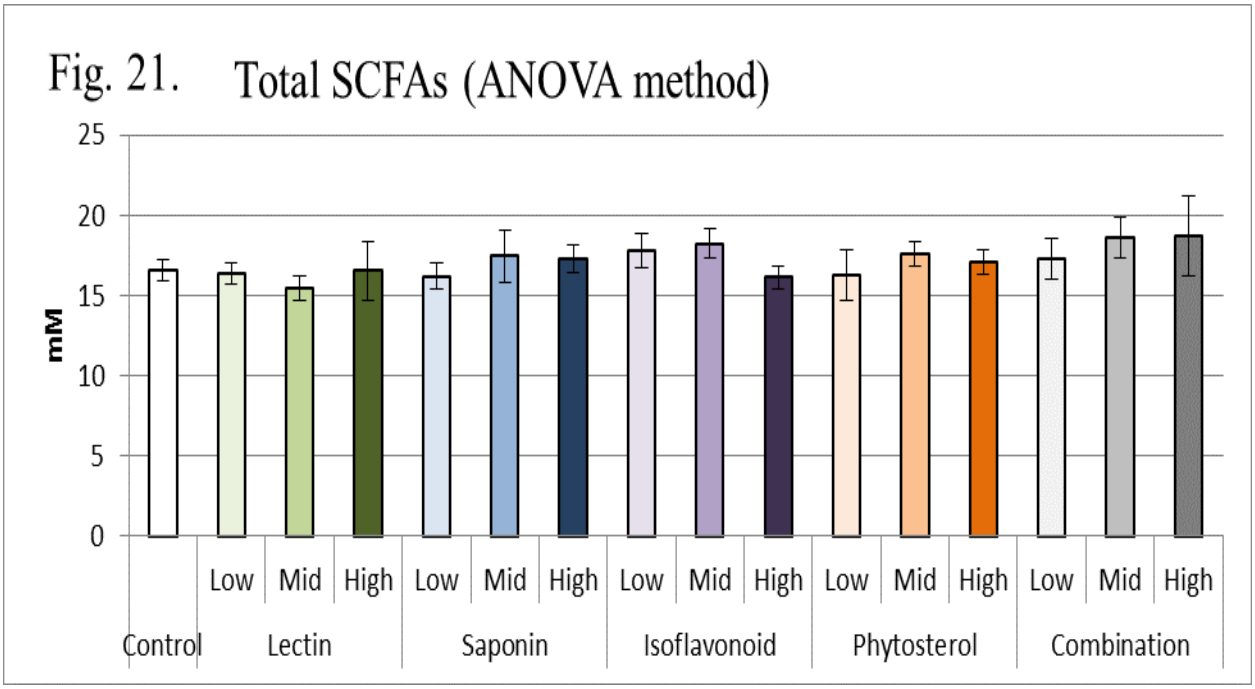

Figure 21.The effects of ANFs on total SCFAs production.

Graph is based on the mean of the square-root transformed data of SCFA production. Data analysed by ANOVA.

The individual SCFAs detected in the present study (acetic acid and lactic acid) were not normally distributed and nonparametric tests showed significant effect of ANFs.

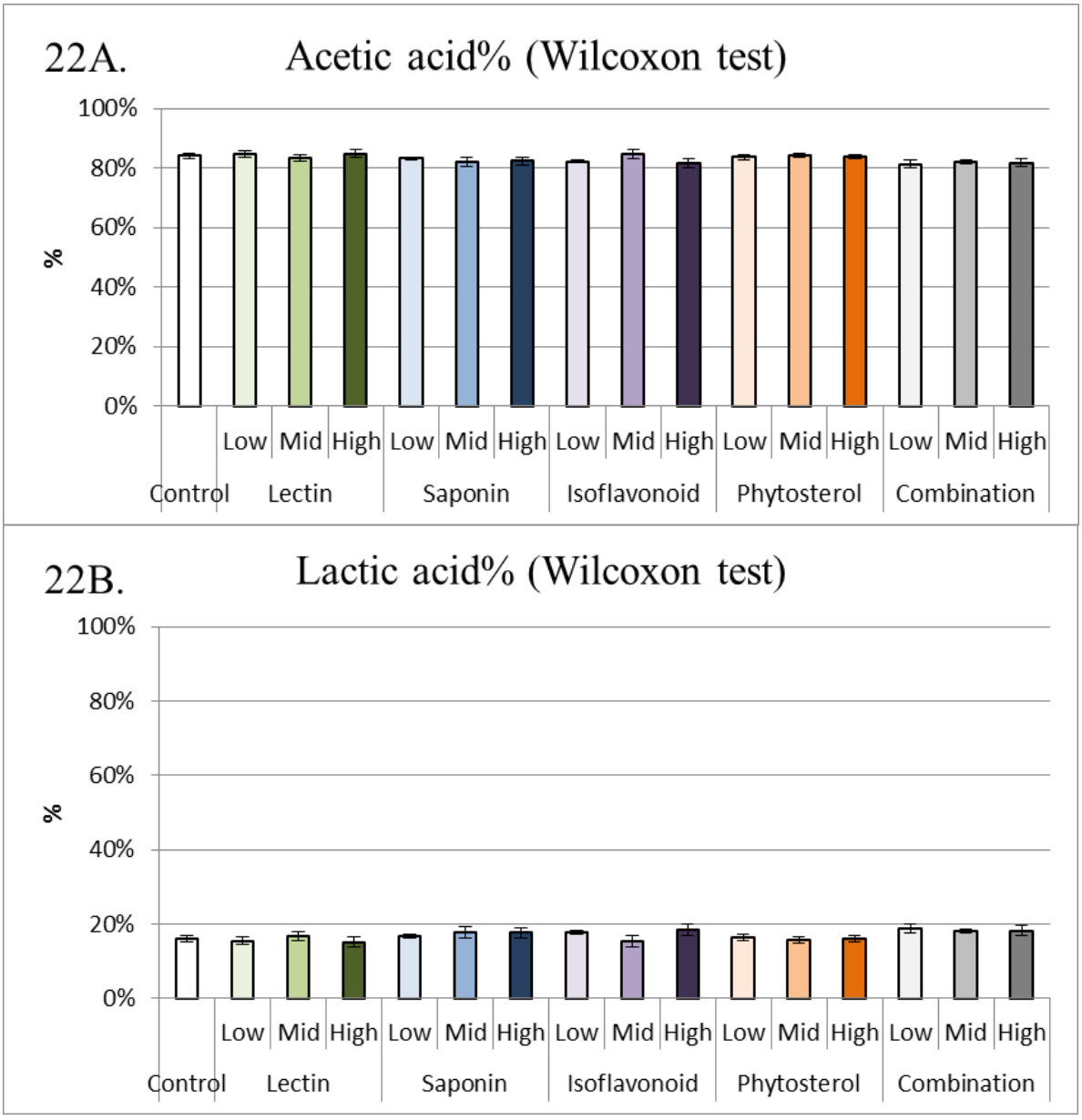

Figure 22A & B. The effects of ANFs on acetic acid and lactic acid productions

Graph is based on the mean of the square-root transformed data of SCFA productions. Data for acetic acid (Fig. 22A) and lactic acid (Fig. 22B) were analysed by nonparametric test. While total SCFA was analysed by ANOVA. Mid-level of saponin showed a dose dependent increase lactic acid production.

10. Discussion

During interpretation of research findings, statistical significance is considered as just one part of an appropriate analysis of a well-designed experiment or study. Many literatures described that biological relevance and statistical significance are not necessarily linked. Although, values >0.05 are often reported as non-significant, Altman argued that biologically relevant data might not show as significant in indiscriminate datasets, thus does not recommend to use this term (Altman, 1990). This idea is supported by European Food Safety Authority Scientific Committee (EFSA), which defined a biologically relevant effect as "an effect considered by expert judgement as important and meaningful for human, animal, plant or environmental health. It therefore implies a change that may alter how decisions for a specific problem are taken."(EFSA, 2011). It has been explained that a biologically important or relevant effect can be related to the effect size and to the concept of power and sample size calculations. Although the term statistical significance is a necessary condition in many of the biological findings but not a sufficient one to explain everything (Professor Anne Marie, personal commun.). In support of the idea of biological relevance, the effect of ANFs in this study showed some visually apparent effects both on the total microbes and their metabolic activities. Hence, the "cutoffs" often referred to as the chosen level of significance ($p<0.05$) was not used as a limit. Therefore, it would be nice to remind readers of this paper, unless specified any p-values which are less than 0.10 ($p<0.10$) are referred as significant.

Currently investigations on gut microbiota of animals and humans have received increased emphasis as it is thought to be a key factor in metabolism of nutrients, immune system, growth and protection against potential pathogens (Ley et al., 2008; Lozupone et al., 2012). The important relationship between gut microbiota and fish health has encouraged studies to investigate the gut bacterial community and composition, especially in aquaculture. A number of studies have been carried out to deal with factors that affect the balance of fish gut microbiota and they are suggested to be shaped by many factors such as incubation temperature and fish species (Sullam et al., 2012), diet and life cycle stage (Desai et al., 2012) and methods of bacterial analysis used (Nayak, 2010). Changes in gut microbiota balance may have important implications for the health of the fish, and thus factors that modify the gut microbiota are of great interest (Larsen et al., 2015). Considering the available information that exist in the literature,

huge discrepancies between studies can be seen due to these factors. To avoid inconsistencies in information between studies it is better to design with appropriate methods and methodologies. Recently, the use of next-generation sequencing analysis, which is powerful tool for detailed study of fish microbiota (Fraher et al., 2012; Zarkasi et al., 2014; Gajardo et al., 2016; 2017), overcomes not only the limitations of the culture based methods (Cahill, 1990; Ringø et al., 1995), but also of previously used molecular techniques (Austin, 2006; Navarrete et al., 2012), including the method used in the present study.

Based on the results of microbial quantification, the effects of ANFs on the total and individual bacterial flora showed that microbial levels were not largely affected. Most previous studies report total bacterial counts between log 2.99 and 8.14 (Bakke-McKellep et al., 2007; Ringø et al., 2008; Merrifield et al., 2009; Zarkasi et al., 2014), whilst in this study the total bacterial count was in a range of log 7.33 and 8.10. The highest (log8.10) and lowest (log7.33) total bacterial count was found at the low and mid-levels of saponin concentrations and highest concentration of the combined antinutrients respectively. This may indicate that saponin stimulate the growth of certain groups of bacteria during fermentation process, which is partly in agreement with previous findings (Patra and Saxena, 2009; Chen et al., 2011; Gu et al., 2015) suggesting that saponins at low doses may directly stimulate the growth. The decrease in the total load of gut bacteria at high concentration of saponin is also in agreement with Chen et al. (2011) and Krogdahl et al. (2015) who reported that highest concentration of saponin resulted in a depressive effect on the growth performance.

Regardless of the ANF, generally as the concentrations of the ANFs increased, there was a decrease in the total number of bacteria. Saponin, when applied at the highest concentration, was on average the most efficient antinutrient to decrease total microbial levels, showing a four-fold decrease at highest concentration applied (result not shown). However, within the captured microbes, most bacterial groups were resistant and even the intestinal bacterial community found to be dominated by the growth of these ANF resistant Lactobacillaceae, Vibrionaceae and Peptostreptococcaceae families and *bacilli*-like bacterial species. Their growth was in a dose dependent increase in saponin. The increase in growth together with the increased gas and SCFAs production especially in the high level of saponin suggests that some members of these microbes may degrade saponin for their energy metabolism.

Among these, Lactobacillaceae was detected as the most resistant and dominant family of the intestinal microbiota in Atlantic salmon. This increase in the gut microbial community and diversity supports previous findings (Bakke-McKellep et al., 2007; Desai et al., 2012; Gajardo et al., 2017) that Lactobacillaceae and other LAB of Atlantic salmon increased in fish after feeding diet including plant ingredients. These saponin resistant bacteria found in this study represents a group of microbes that can have probiotic effects, are considered as beneficial for fish health (Wong et al., 2013). Members of these isolated bacterial groups are known fermenters and acid producers from undigested nutrients and produce SCFAs (Titus and Ahearn, 1988; Ringø et al., 1995; Smriga et al., 2010), which are readily absorbed by the host and may contribute nutrition to some host cells (Ray et al., 2012) and other microbes (Ringø et al., 1995). This result agrees with other research findings (Askarian et al., 2012; Zarkasi et al., 2014; Gajardo et al., 2016; Gajardo et al., 2017) who revealed that when fish fed with plant ingredients present high relative abundance of LAB in the intestinal bacterial communities of Atlantic salmon.

Although the aerobic microbial organisms were low in the present study, their levels tended a dose dependent increase in all ANFs, which may indicate that like the other isolated bacterial families they were tolerant to the levels tested in the current experiment. The increase in the proportion of resistant aerobic bacteria represents mainly the class α-Proteobacteria. Many studies have shown Proteobacteria as one of the dominant members of the gut microbiota of Atlantic salmon (Zarkasi et al., 2014; Gajardo et al., 2016; Schmidt et al., 2016; Gajardo et al., 2017) and in other species, Proteobacteria and Vibrionaceae have been reported as abundant and common (Smith et al., 2007; Liu et al., 2016), but in this study the proportion of these bacteria and other aerotolerant microbes were detected extremely at very low levels. This is partly consistent with Larsen et al. (2013) who has been identified low level of Proteobacteriacea*e* and Vibrionaceae from marine fish.

One of the factors for such inconsistency reports is that due to differences in methods used for identification of the bacteria, in this study the real-time PCR was used for identification of these isolates. However large proportion of the bacteria were not captured and remained unrecognized. Whilst in the more recent reports (Zarkasi et al., 2014; Gajardo et al., 2016; Gajardo et al., 2017) these bacteria have been found with high-throughput sequencing studies, which is more powerful tool for microbiota study and for microbial community profiling. The composition and diversity of microbiota are likely to be impacted by several confounding variables, including the use of

50% frozen digesta samples used in this study, which may have influenced the level on the initial samples as freezing may reduce/deactivate the bacteria. This results may be supported by previous study (Larsen, 2014) who found distorted bacterial community associated with frozen samples.

Based on the effects of ANFs on metabolic activities of gut microbiota, the dose-dependent increase in gas production caused by saponin correlated well with SCFA levels, but inversely proportional to the pH, emphasizing the fact that most metabolic pathways in anaerobic environment result in production of both gas and acids. ANFs tended to inhibit microbial growth, as repression effects has been demonstrated by fermentation, resulting in higher pH with increasing concentration of ANFs. On the other hand, the pH was low at mid-levels of most ANFs, which may indicate that at these mid-level concentrations, the ANFs might increase the microbial community and fermentation process. Saponins may selectively stimulate specific microbial groups which may promote fermentation process as reflected by increase gas production. In addition, at mid-concentration of saponin, pH was reduced which is consistent with both individual and total SCFA production but inconsistent with gas production and redox potential results. High production of SCFAs and gas by saponin may reflect its positive health effects as it attributes to increase microbial fermentation or used as a substrate as shown by reduction of pH, which by itself prevents survival of pH sensitive pathogenic microorganism (Macfarlane and Macfarlane, 2012; den Besten et al., 2013; Rios-Covian et al., 2016) and increases the absorption minerals (Lauzon et al., 2014). Other similar observations have been reviewed on effect of saponin in microbial fermerntation and composition (Makkar and Becker, 1997; Patra and Saxena, 2009) who suggested that saponins may decrease protozoa but increase certain group of bacteria, which might increase the efficiency of bacterial fermentation.

Based on these results most of the ANFs may be used to stimulate the growth of LAB just like what low level saponin was previously suggested as growth promoter (Gu et al., 2015). The results shown by SCFA production indicate that acetic acid was the main SCFA produced. This result agrees with previous studies on marine herbivorous fish (Clements and Choat, 1995) that reported high concentrations of SCFA in the DI, and acetic acid was the most abundantly produced SCFA. Similarly, Hartviksen et al. (2014b) showed that acetic acid predominantly produced in Atlantic salmon fed with inclusion of high level plant ingredients. Moreover,

previous research indicated that the profile of metabolites such as SCFAs is influenced by the nature of fish diet (Kihara and Sakata, 2002).

Another parameter that is used to measure the effects of ANFs in the microbial fermentation was RedOx potential. A redox potential (E_h) is a parameter of the state of biological media which indicates the capacity to either gain or lose electrons and can serve as a substitute tool to monitor the progress of fermentation allowing the detection of the metabolic activity and/or growth of LAB and other microorganisms (Olsen and Pérez-Díaz, 2009). Measurement of E_h from lectins and combined ANFs showed a similar pattern to the changes observed in pH. Both the E_h and pH were lineary matched in a dose dependent increase in lectin and combination of the four ANFs, but when statistically anlysed with Wilcoxon tests no significant effect was found at any concentrations used in this *in vitro* fermentation. The current results from effects of lectin and lectin interaction with other ANFs on E_h and pH are inconsistent with previous findings where E_h and pH measurements showed a different pattern of changes in fermentation process (Olsen and Pérez-Díaz, 2009).

The dose-dependent increase in gas production caused by saponin correlated well with SCFA levels. But the effects of saponin on pH was inversely proportional to the SCFAs production. Therefore, considering the high concentrations (especially at the mid and high levels) of ANFs tested which most likely exceed many fold the ANF concentrations in the common alternative plant ingredients, metabolic activity of gut microbiota was not significantly affected. This may be partly explained by the low incubation temperature used, that affects bacterial growth and fermentation process (Corkrey et al., 2012). Since the simulation was conducted at 10^{o}C for 7 days, the incubation temperature was relatively low considering the optimum temperature (8-14^{o}C) requirement for Atlantic salmon (Marine-Harvest, 2016). This low temperature might affect the fermentation process as reflected by decreased microbial growth rates and SCFA concentrations. Hence, an elevated ideal temperature may be required for growth and well-developed fermentation systems in marine species (Kandel et al., 1994; Sullam et al., 2012). Previous studies focussed on the effect of temperature on the activities of enzymes and rate of bacterial proliferation from fish, and low temperature was implicated to decrease digestion process directly by inhibiting the reaction rates of enzyme-catalyzed reactions (Georlette et al., 2004; Clements and Raubenheimer, 2006). Likewise an increase in body temperature within a certain range usually results in higher digestive process (Pang et al., 2011). However,

contradicting result has been reported in previous study as high fermentation rates measured in fishes living in temperature waters (Mountfort et al., 2002), in addition *in vitro* study can limit the possibility of having alternative form of enzymes which works at lower temperature of biological systems. While *in vivo* results may differ as Atlantic salmon produce four different isomers of trypsin: three anionic and one cationic this may help to having cold adapted (psychrophillic) enzymes with lower optimun temperature (Outzen et al., 1996). For example it has been reported that the pancreatic endoproteases trypsine, elastase, and chymotrypsin from cold adapted fish are more efficient at low temperature than the equivalent enzymes in mammals (Schrøder et al., 1998). On the other hand, the absence of efficient enzymes for carbohydrate metabolism in Atlantic salmon creates a problem for diet based ANF cause-effect *in vivo* studies.

11. Conclusion

- Lactic acid bacteria were the most tolerant microbes, their proportion were increased in a dose dependent increase in antinutrients.
- Antinutrients both individually and in combination affected microbial metabolism only little.
- However, as there were some variabilities in regard to the effects of these ANFs, it is very difficult to generalize their effects on gut microbiota.
- It was suggested that the low incubation temperature and the high proportion of the frozen samples used for the simulation model may affected the current results.
- Currently popular methods for microbiota characterization and profiling such as next generation sequencing is needed to detect the high proportion of microbes remained uncaptured by the current method and smaller variations that may occur in the gut microbiota of fish
- The study of gut microbiota especially in ANF cause -effect study on gut microbiota of Atlantic salmon is complex, therefore, it needs continuous investigation with proper design and appropriate modelling
- Therefore, further study is recommended to investigate the detailed practical implications related with age, species, appropriate incubation temperature and sampling methodologies.

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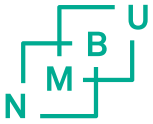

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