

Attachment 2:

Anne Marie Bakke: Alleviation of feed induced enteropathies

Introduction

According to FAO, aquaculture is the fastest growing food-producing sector globally and now supplies about half of the fish consumed by humans. One of the constraints for continued growth in the aquaculture sector is the supply of appropriate feed ingredients. For many farmed fish, especially carnivorous and omnivorous species, fishmeal and fish oil have traditionally been the main sources of dietary protein and lipid, respectively. However, stagnation or reduction in capture fisheries has led to a limited global supply of these resources and therefore increasing costs for these feed ingredients. Thus, fish feed producers have seen their production costs rise and are therefore attempting to escape dependence on fishmeal. Some success has been achieved – more cost-effective feedstuffs are being increasingly used as nutrient sources in formulated feeds for farmed fish and the inclusion level of fishmeal and fish oil has dropped, even for farmed carnivorous fish species. In the longer term, demand for alternative feed ingredients will depend on scientific research regarding consequences of these on growth, feed utilization, health and welfare of the fish, as well as sustainability issues. However, with global population and economic growth, the subsequent increased demand for high quality food is driving all animal production systems to compete in a limited feed ingredient market.

Losses due to disease represent a major cost in the aquaculture industry internationally, and diet composition is among several factors that may influence disease susceptibility. Optimal health and disease resistance is dependent not only on an optimal balance of nutrients available for all systemic needs, but also on optimal function of the gastrointestinal tract (GIT) and associated organs. The GIT is constantly exposed to a conglomeration of nutrients, antinutritional factors (ANFs) and non-nutrients, the latter also comprising food antigens and microorganisms. The digestive apparatus has the capacity to adjust to changing diet composition to some degree, and the mucosal defense system provided by the GIT must function in order to protect the body from injurious agents and at the same time develop oral tolerance to common antigens from the diet and commensal microbiota (see reviews by Chehade and Mayer, 2005; Sansonetti, 2004). The microbiota's species composition, which may be influenced by various dietary nutrients, non-nutrients and anti-nutrients, is also of importance for the host's gut and general health (Bauer et al., 2006).

Regarding cultured fish, many knowledge gaps exist in this area which needs to be filled. The following are some:

- Basic elements of digestive functions and suitable indicators of malfunction is a major hindrance for the understanding of diet-health interactions
- The gut's immune system and its role in disease susceptibility
- Effects of bioactives/ANFs, which have not been subject to thorough investigation previously, on fish production, digestive function and disease susceptibility
- The role of the intestinal microbiota in fish is largely unknown and investigation is still in its initial phase. Differences between fish species, e.g. between salmonids and cod, are to be expected and comparative aspects deserve attention.

Non-infectious disease conditions of the gastrointestinal tract of Norwegian farmed fish

In the last decade, gut health in farmed fish has gained recognition in Norway. The following conditions affecting the gastrointestinal tract have been described and the knowledge accumulated so far is summarized.

Gastric dilatation syndrome (Vassbuk)

Gastric dilatation syndrome, or bloat, has been recognized as a condition affecting farmed *Oncorhynchus* spp. such as rainbow trout (*O. mykiss*), coho salmon (*O. kisutch*) and Chinook salmon (*O. tshawytscha*) in seawater, but only rarely described in Atlantic salmon, *Salmo salar* L. (Anderson 2006; Baeverfjord et al. 2006). The condition is recognized at farms when the surface of the water becomes covered with oil due to the fish regurgitating lipid/oil. The abdomen of affected fish are often distended and necropsy reveals an enlarged stomach containing large amounts of oily fluid. The stomach wall may appear hemorrhagic in severe cases. Rapid changes in water temperature and/or salinity with accompanying stress and osmoregulatory dysfunction have been suggested as potential causes (Anderson 2006). Low feed pellet stability in water and high lipid content have also been suggested as contributing factors (Baeverfjord et al. 2006). Suggested measures to treat the condition include reducing the feeding rate, changing the diet from a pelleted to an extruded diet (if applicable), changing the diet to one containing a lower lipid content, removing environmental stressors, and checking the diet for contaminants such as biogenic amines.

Gastric ulcers

Sporadic occurrences of gastric ulcers have also been described in farmed salmonids (Michael Penn and Anne Marie Bakke, personal communication). The aetiology is unknown. A contributing factor may be pellet hardness/stability, which may cause physical damage to the gastric mucosa. High feed intake may exacerbate the problem. Some speculation regarding the increased use of plant ingredients containing for example fibers as a cause has also been brought up, although not substantiated. Bacterial involvement has apparently not been investigated. Increased focus on the occurrence and repercussions regarding animal welfare are needed to elucidate the practical significance of this condition in fish.

Steatorrhea

Another syndrome affecting the intestine of farmed salmonids is a condition described as “floating feces” or steatorrhea (feces with high lipid content). This was first observed in 2003 in Norway, with sporadic cases reported in subsequent years in different locations in Norway. In 2010, an apparent increase in the occurrence was again reported (Norwegian Veterinary Institute, 2010). Accompanying the occurrence of pale, yellowish cheese-like intestinal content and feces, are pale, swollen intestinal tissues affecting both the pyloric caeca and mid intestine. Upon histological investigation, highly vacuolized epithelial cells have been described. Manipulation of the affected tissue results in the secretion of a white, cream-like substance. This substance has a high lipid and protein content, suggestive of lipid accumulation in the vacuoles within the epithelial cells (Michael Penn, personal communication). The gall bladder is also often reported to be distended, containing large amounts of bile. The aetiology is unknown, although dietary ingredients, phospholipid deficiency and/or phosphorus deficiency have been suggested. Cases have been reported in fish fed diets from all of the largest Norwegian feed producers (BioMar, Skretting and Ewos). The condition appears to be more common during summer and early autumn when water temperatures are high, suggesting that environmental conditions may either be a causatory component or exacerbate the condition. The syndrome, as defined by the appearance of floating, lipid-rich feces over the sea cages, appears to be at least somewhat self-limiting, often resolving in the matter of days or weeks (Ragnhild Hanche-

Olsen and Kristin Ottesen, personal communication). Further study regarding the aetiology is needed to prevent or find treatment regimes.

Intestinal obstruction (tarmslyng)

In Atlantic cod (*Gadus morhua*), intestinal obstruction or colic has been observed in Norway, especially in the largest fish with the highest growth rates. The intestinal tissue often appears hemorrhagic, suggesting disturbances in blood flow associated with volvulus. The condition is associated with high mortality, up to 60% at some sites. Intestinal distensions containing gas/fluid and inflammatory conditions in the intestine have also been described (Norwegian Veterinary Institute, 2010). The cause(s) of these are unknown. Diet composition and feeding practices, intestinal microbiota, water balance (osmoregulation), adhesions, herniation, foreign bodies, genetic/congenital as well as environmental/stress involvement all deserve investigation. Reduced feeding rate may be considered in an attempt to reduce losses caused by the condition.

Feed-induced enteropathies

Inclusion of various “novel” ingredients from plant, microbial, and other animal sources in fish feeds have been investigated. Some of these alternative feed ingredients have been reported to have negative consequences on gut health of farmed fish, depending on fish species and inclusion level in their diets. The causes, to name some possibilities that have studied and documented, may lie in low levels of ω -3 fatty acids, high levels of fiber and starch, and – perhaps most consequential – the presence of antinutritional factors (ANFs) and/or antigens. Specific effects of alternative protein sources on the digestive physiology of fish have been most closely studied in the case of soybean products in feeds for farmed salmonids. In these fishes, inclusion levels above 5-10% – depending on processing level and strain of full-fat or defatted (hexane-extracted) soybean meal – lead to an inflammatory response in the distal intestine (see below). Pea protein concentrate at 35% inclusion level has been recently reported to cause a similar condition in Atlantic salmon, *Salmo salar* L. (Penn et al., 2011). The causatory agent(s) are still being investigated and some advances appear to have been made. However, the long-term implications of legume meals in diets on fish health are largely unknown, and this topic deserves continued, substantial investments in further research to contribute to preserving the sustainability of the aquaculture industry.

Soybean meal is the most commonly used plant protein source used in animal feeds due to its high protein content and favourable amino acid profile. However, even when heat-treated, standard (solvent-extracted) and full-fat SBM-containing feeds were supplemented with limiting amino acids, decreased growth in salmonids was observed early on (Sandholm et al., 1976; Tacon et al., 1983). De-hulled SBM as the sole protein source in diets for rainbow trout was reported to lead to growth arrest and finally to increased mortality (Dabrowski et al., 1989; Rumsey et al., 1994). At least part of the reason for growth retardation and perhaps mortalities were later attributed to the development of a sub-acute, non-infectious enteropathy in the distal intestine of salmonids (van den Ingh et al., 1991; Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996). The inflammatory response has been demonstrated in salmonids fed full-fat as well as solvent-extracted SBM, with and without hulls, and also by the alcohol-extract (molasses) which is a bi-product from aqueous alcohol extraction of SBM to produce soy protein concentrate (van den Ingh et al., 1991, 1996; Rumsey et al., 1994; Krogdahl et al., 1995 and 2000; Baeverfjord and Krogdahl, 1996; Bureau et al., 1998; Ostaszewska et al., 2005; Aslaksen et al., 2007). The changes have been described in Atlantic salmon as follows: shortening of the primary and secondary mucosal folds with a widening of the central stroma (lamina propria) and submucosa, shortened microvilli of the brush border membrane and increased formation of microvillar vesicles, and a dramatic decrease or even absence of the normal supranuclear absorptive vacuoles in the enterocytes (van den Ingh et al., 1991 and 1996; Baeverfjord and Krogdahl, 1996). The lamina propria is widened with a profound infiltration of a mixed population of inflammatory cells such as lymphocytes, neutrophilic granulocytes, cells of monocytic lineage including

macrophages, eosinophilic granular cells, and diffuse IgM (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000) as well as a mixed population of T-cells, including putative CD4+ T helper cells and CD8+ cytotoxic cells (Bakke-McKellep et al., 2007a; Lilleeng et al. 2009). RNA expression studies indicate that pro-inflammatory cytokines and other signaling and regulatory factors involved in recruitment and activation of T cells and other immune cells, and factors involved in maintenance of normal mucosal integrity, were differentially expressed during the development of the enteropathy (Lilleeng et al., 2009; Valen et al., 2010). Interestingly, increased trypsin activity and trypsin RNA expression levels have been observed in the distal intestinal tissue of Atlantic salmon fed SBM (Lilleeng et al., 2007). The implications are not clear, but the authors suggest that serine proteinases such as trypsin may be involved in the pathogenesis of the inflammation by way of the inflammatory mediator protease-activated receptors (PAR2), as demonstrated in the intestine of humans and mammals. The gene expression level of PAR2 is indeed up-regulated in the distal intestine of SBM-fed salmon (Thorsen et al., 2008).

Due to the infiltration of inflammatory cells and rapid regression of the condition following withdrawal of soybean meal from the diet, the condition has been classified as a non-infectious, subacute enteritis in Atlantic salmon (Baeverfjord and Krogdahl, 1996). The pathogenesis may involve immunological mechanisms similar to that of a hypersensitivity reaction (Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007a; Lilleeng et al. 2007 and 2009; Thorsen et al., 2008). However, the plasma of rainbow trout fed SBM-containing diets was negative for specific antibodies against soy protein (Kaushik et al., 1995; Burrells et al., 1999) despite another report of increased general immunoglobulin levels (Rumsey et al., 1994). Increased, diffusely distributed IgM has also been demonstrated locally in the lamina propria and submucosa of the inflamed distal intestine (Bakke-McKellep et al., 2000). Serum and head kidney macrophage activities were depressed in trout fed diets containing high levels (60-80%) de-hulled extracted SBM (Burrells et al., 1999). In this same dose-response study, no histological alterations in the distal intestine were observed until the SBM inclusion level had reached 60% at which level growth depression also became evident. These latter investigations have not been carried out in Atlantic salmon.

Supranuclear vacuoles have been suggested to function during endocytosis (McLean and Ash, 1987; Sire et al., 1992; Sire and Vernier, 1992). These have been hypothesized to play a role in macromolecular absorption. Macromolecular uptake of horseradish peroxidase (Bakke-McKellep, 1999) and ferritin (Uran et al. 2008a) from the distal intestine has been shown to be reduced in SBM-fed compared to FM-fed Atlantic salmon, suggesting that endocytosis is diminished during inflammation. Thus reduced vacuole formation, numbers, or size may have implications regarding immune responses.

Numbers of proliferating cells lining the villous folds of the distal intestine of Atlantic salmon parr increased with even low levels (12.5%) of full-fat soybean meal (Sanden et al., 2005; Bakke-McKellep et al., 2007b), and from both genetically modified and traditionally cultivated varieties (Sanden et al., 2005). Thus the number of cells in early stages of development are significantly increased, as are the number of cells undergoing cellular repair as indicated by heat shock protein expression in cells, as well as cells undergoing programmed cell death (apoptosis) (Bakke-McKellep et al., 2007b). This suggests that the digestive hydrolases, nutrient transporters, and possibly other components located in the brush border membrane and cytoplasm, are disturbed by alterations in cell turnover and degree of maturation of the enterocytes caused by the SBM-induced inflammatory changes. Immature cells have reduced function or number of integral proteins (enzymes, transporters, etc.) inserted in their apical membranes and/or different surface receptor glycosylation profiles (Pusztai et al., 1995).

In rainbow trout, the condition has been described similarly, with the possible exception of the epithelial vacuolization, which appears to increase, at least in some trials, in this salmonid (Rumsey et al., 1994; Burrells et al., 1999). Loss of epithelial cell vacuolization was observed, however, by

Romarheim and co-authors (2006) in rainbow trout fed 25% solvent-extracted SBM as well as solvent-extracted, untoasted white flakes. In omnivorous common carp (*Cyprinus carpio* L.), 20% inclusion of solvent-extracted SBM also caused an inflammatory response in their hindgut similar to that of Atlantic salmon (Uran et al. 2008b). Pro-inflammatory cytokines were also up-regulated in the hindgut of carp during the development of the condition. In contrast to Atlantic salmon, however, both rainbow trout and common carp have been reported to adapt to soybean meal, with the inflammation resolving and resumption of acceptable growth and feed consumption rates following a period of acclimatization to the diet (Olli and Kroghdahl, 1994; Refstie et al., 1997 and 2000; Uran et al., 2008). Atlantic salmon, however, do not appear to adapt (Refstie et al., 2000).

Morphology has been studied but changes have not been observed in the intestine of Atlantic cod fed up to 24% dehulled, extracted SBM, bioprocessed SBM or soy concentrate (Hansen et al., 2006; Refstie et al., 2006), Atlantic halibut (*Hippoglossus hippoglossus*) fed up to 36% toasted full-fat SBM (Gridale-Helland et al., 2002), Egyptian sole (*Solea aegyptiaca*) fed up to 30% SBM (48% crude protein; Bonaldo et al., 2006), and European sea bass fed up to 30% toasted, extracted SBM (Bonaldo et al., 2008). In gilthead sea bream fed up to 30% toasted, extracted SBM (Bonaldo et al., 2008), the lamina propria of the distal intestine was infiltrated with mononuclear cells and diffusely expanded in some fish. The incidence of the inflammation increased with increasing SBM inclusion. However, growth, feed intake and feed conversion were not affected by any inclusion level. In the distal intestine of channel catfish fed 45% de-hulled, solvent-extracted SBM, raw SBM, and SBM autoclaved for 5, 10, 20, and 40 min, only a mild loss of supranuclear vacuolization was observed (Evans et al., 2005), whereas pancreatic necrosis and splenic congestion was evident in all treatment groups. The histology of the distal intestine of the catfish fed non-heat-treated SBM suggests hypertrophic growth of the villous ridges, however. This is supported by increased visceral index of these fish (Peres et al., 2003).

The role of anti-nutritional factors in the development

Soybean meal contains the antinutritional factors proteinase inhibitors, lectins, saponins, phytoestrogens, fiber, oligosaccharides, phytic acid, some glucosinolate and potential allergens. The term “antinutrients” or antinutritional factor is commonly used to refer to those substances found in foods that produce negative effects on nutritional balance and subsequently health. Although some plant toxins are known to produce obvious systemic signs of intoxications, more subtle effects only observed following prolonged ingestion of a given plant are more commonly referred to as antinutrient effects. Such effects might include inhibition of growth, decreased food efficiency, goiterogenesis, pancreatic hypertrophy, hypoglycaemia, and liver damage. Factors that should be taken into consideration when assessing antinutrient effects include the species of animal, its age or stage of development, size, sex, state of health and plane of nutrition, and any stress factors that might be superimposed on these variables.

Most of our knowledge regarding antinutrient effects in fish has not been gained through studies using isolated ANFs, but rather using whole, or more or less refined, feed ingredients. Although some ingredients contain only one or a few ANFs, others – including soybeans – contain many different ANFs. They may all have their distinct effects and may also interact to show significant effects not observed when they are present singly. Very few reports exist that can form the basis for dose-response considerations and estimation of maximum inclusion levels. These conclusions are in line with those made by Francis et al. (2001) in a review regarding antinutritional factors in plant-derived fish feed ingredients and their effects. As concluded by Francis et al. (2001) ten years ago and still valid now: There is an urgent need for studies concerning the effects of the individual antinutrients and effects of antinutrients in combination. Most likely antinutrients are the cause of lowered growth performance and feed utilization in many fish species fed SBM and are possibly involved in the aetiology soybean meal-induced enteropathy in salmonids and the related gut function and immunological alterations. While the ANFs that are proteins, such as some proteinase inhibitors and

lectins, are assumed to be deactivated during heat treatment of SBM and/or heat generated by feed processing methods, biological effects of purified forms of these in fish are still included here. Despite heat treatment, they may still illicit an antigenic effect which may be of significance in explaining functional, structural and/or immunological responses to dietary SBM in fishes. However, further research is needed to investigate the significance of heat treatment of these ANFs on these responses.

Thorough reviews of the biological effects of ANFs in fish have been recently published (VKM, 2009; Krogdahl et al., 2010). Therefore, only ANFs that have been connected to the development of the feed-related enteropathies will be discussed herein. This does not, however, completely preclude the possible involvement of other ANFs such as proteinase inhibitors, isoflavones/phytoestrogens, glucosinolates, tannins, and fibre.

Lectins

Lectins (previously known as agglutinins or hemagglutinins) are soluble, heterogeneous (glyco)proteins that bind reversibly to a specific mono- or oligosaccharide residues (Peumans and Van Damme, 1995). By binding to glycosylated membrane constituents, such as transport proteins, brush border enzymes and various receptors, lectins can have striking biological activities depending on animal, developmental stage and other factors that affect glycation profiles of these substances. Binding of SBA (soybean agglutinin) to the intestinal brush border membrane of Atlantic salmon and rainbow trout have been demonstrated (Hendricks et al., 1990; Buttle et al., 2001). Higher maximum binding and lower dissociation constants were observed in the distal intestine relative to the more proximal areas in salmon (Hendricks et al., 1990). These authors suggested that this could indicate that the distal intestine would be more sensitive to effects of SBA or other ANFs or antigens. This was supported by the follow-up study by the same group of scientists in which full-fat soybean meal, but not the more highly processed soy protein concentrate, was found to cause morphological changes in the distal intestine of Atlantic salmon (Van den Ingh et al., 1991).

Lectins, like soybean trypsin inhibitors, have largely been ruled out as the sole cause of reduced digestive function and the inflammation (Van den Ingh et al., 1996; Bureau et al., 1998) since very little if any lectin activity is present in the alcohol extract from soybeans. This alcohol extract, however, did cause the morphological changes when added to the salmonid diet. Van den Ingh et al. (1996) suggested that alcohol-soluble oligosaccharides and saponins are the most likely causatory compounds. Furthermore, Iwashita et al. (2008) demonstrated that diets that were spiked with isolated SBA did not cause inflammatory responses in the distal intestine of rainbow trout. This, however, is in contradiction with Buttle and co-workers' (2001) suggestion that SBA may be involved in the inflammatory response. They reported some morphological changes, although not all the changes usually observed in SBM-induced enteropathy, in the distal intestine of Atlantic salmon when 3.5% purified SBA was added to a fishmeal-based diet. Bakke-McKellep and co-workers (2008) indicated that phytohemagglutinin (PHA) from kidney beans (*Phaseolus vulgaris*) dramatically inhibited glucose absorption into the intestinal epithelium of Atlantic salmon when tissue is exposed to PHA *in vitro*. Isolated SBA, however, did not have this effect. Yet the combination of SBA and soybean trypsin inhibitors did appear to cause a similar response as PHA (see below). More work is needed to establish the role of SBA in the development of SBM-induced enteropathy.

Saponins

Saponins are heat-stable, alcohol-soluble, amphipathic glycosides, containing a hydrophobic steroidal or triterpenoid aglycone to which one or more hydrophilic sugar chains are attached. They are a highly diverse group of glycosides produced primarily by plants, particularly legumes, but also a few lower marine animals and some bacteria. The mature soybean seed contains triterpenoid saponins divided into group A and group B soyasaponins based on the aglycone structure. Soybean saponins

appear to survive feed processing and passage through the intestinal tract of Atlantic salmon (Knudsen et al., 2006).

The diverse biological effects of saponins have been reviewed by Francis et al. (2002). They have been implicated in the distal intestine enteritis that develops when Atlantic salmon are fed diets containing soybean meal at moderate to high levels. Knudsen et al. (2007) performed two feeding trials with Atlantic salmon in which three fractions of soybean molasses (butanol phase, water phase and precipitate) were supplemented to diets. The authors confirmed earlier findings (Van den Ingh et al., 1996) that soy molasses induced the inflammatory response. Additionally, components in the butanol phase also elicited changes in the distal intestine consistent with those of soybean enteropathy, suggesting that the yet to be identified responsible component(s) resists fractionation with butanol and drying at 70°C. However, saponins are not the only compound found in the butanol fraction. According to Kitagawa et al. (1985) the butanol phase is expected to contain approximately 30% saponins only. The findings of Knudsen et al. (2007) also support the findings of Bureau et al. (1998), who showed that partially purified alcohol extracts (butanol soluble fraction of soy molasses), when added to the diet of Chinook salmon (*Oncorhynchus tshawytscha*), in amounts equivalent to a 300 g kg⁻¹ soybean meal diet, caused reduced feed intake and growth, and morphological changes in the distal intestine. However, in contrast to these findings, Krogdahl et al. (1995) did not observe adverse effects of purified saponins included in the diet of Atlantic salmon equivalent to a diet containing soybean meal at 300 g kg⁻¹. More recently, Knudsen et al. (2008), Iwashita et al. (2008) Chikwati et al. (2011) all confirmed Krogdahl et al.'s (1995) findings, demonstrating that SBM-free diets spiked with purified soy saponin did not necessarily cause inflammatory responses in the distal intestine of Atlantic salmon or rainbow trout, at least not at the concentrations used in those trials. However, in combination with other components – lupin kernel meal in the Knudsen et al. (2008) study, pea protein concentrate in the Chikwati et al. (2011) study, and soybean lectin (SBA), soy isoflavones, phytate and saccharose in the Iwashita et al. (2008) study – at least some signs of an inflammatory response were induced. Furthermore, increased *in vitro* epithelial permeability was observed in intestinal tissue from the salmon fed 25% defatted SBM as well as from fish fed the purified soy saponin, with and without the presence of the lupin kernel meal (Knudsen et al., 2008). Data recently reported by Chikwati et al. (2011) indicated that soy saponin generally caused some decreases in lipid and fatty acid digestibility, fecal dry matter and digestive enzyme activity in distal intestinal tissue. These studies indicate that while soy saponin may be involved in the SBM-induced inflammatory response, it is most likely not solely responsible. Saponins may, however, allow other, yet to be identified factor(s) to enter the epithelium and/or illicit the inflammatory response.

Oligosaccharides

Soybeans contain high levels of oligosaccharides, which are important constituents of a wide variety of grain legumes and cereals and refer to a variable mix of α -galactosyl homologues of sucrose (Saini, 1989). The main compounds of these α -galactoside oligosaccharides are the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. Legumes and oilseeds commonly used in fish feeds typically contain 4-14 g raffinose, 12-50 g, stachyose and 0-34 g verbascose kg⁻¹ dry matter (Bach-Knudsen, 1997). High dietary levels of α -galactoside oligosaccharides can have antinutrient effects, some of which may be applicable to fish, such as interference with the digestion of other nutrients, osmotic effects in the intestine and anaerobic fermentation of the sugars resulting in increased gas production (Cummings et al., 1986).

It has been suggested that dietary α -galactoside oligosaccharides is at least partly responsible for causing the diarrhea resulting from feeding soybean meal to fish (Refstie et al., 2000, 2005, 2006) due to elevated intestinal osmotic pressure. This was not confirmed in a more recent study, however, in which neither 6.7 g kg⁻¹ raffinose, 29.5 g kg⁻¹ stachyose, nor a combination of the two caused diarrhea or inflammatory changes in the intestine (Sorensen et al., 2011). Other reported effects of

α -galactoside oligosaccharides in fish are also somewhat conflicting. Arnesen et al. (1990) attributed decreased apparent nutrient digestibility in Atlantic salmon fed soybean meal to negative effects of alcohol-soluble soy carbohydrates, but did not find similar effects in rainbow trout. On the other hand, neither Sorensen et al. (2011) nor Krogdahl et al. (1995) found effects of raffinose supplementation on apparent nutrient digestibility and growth in Atlantic salmon. Stachyose, however, alone and in combination with raffinose, increased distal intestinal weight compared to fish fed a diet supplemented with raffinose alone or control diets (Sorensen et al., 2011).

Interestingly, Refstie et al. (2005) found surprisingly high apparent digestibility of raffinose in Atlantic salmon, ranging from 85% when the diet contained 1 g raffinose kg⁻¹ to 55% when the diet contained 22 g raffinose kg⁻¹. At the same time, the concentration of polysaccharides potentially originating from bacterial cell walls increased in the intestine of fish fed high levels of α -galactoside oligosaccharides and plant non-starch polysaccharides. This strongly indicates that α -galactoside oligosaccharides are digested and utilized by intestinal bacteria in the salmon gut.

Phytic acid

Phytic acid (myoinositol, 1, 2, 3, 4, 5, 6 hexakis-dihydrogen phosphate) and phytate (a mixed cation salt of phytic acid) are found in significant amounts in plant material, and are the primary storage form of both phosphate and inositol in seeds. Common plant feedstuffs such as soybean and rapeseed meal contain 10 - 15 and 50 - 75 g phytate kg⁻¹, respectively (Francis et al., 2001), which are moderate levels compared to levels found in oilseeds such as rapeseed and sunflower. Negatively charged sites bind mainly K⁺ and Mg²⁺, but may also form insoluble salts at alkaline pH with other cations including Ca²⁺, Mn²⁺, Zn²⁺, Ba²⁺, or Fe³⁺ (Hídvégi and Lásztity, 2002). Phytic acid may also complex with protein at low pH, below the isoelectric point of the protein, and when phytic acid is soluble (Hídvégi and Lásztity, 2002). Thus, phytic acid-protein complexes are only partially hydrolyzed by pepsin in rainbow trout (Spinelli et al., 1983).

In fish, a supplement of phytic acid in diets slowed growth in rainbow trout (Spinelli et al., 1983), Chinook salmon (Richardson et al., 1985) and common carp (Hossain and Jauncey, 1993). Richardson et al. (1985) found an increased incidence of cataracts when feeding diets high in phytic acid but low in Zn. In line with this, improved growth and higher digestibility and retention of protein and mineral elements was shown in rainbow trout when comparing dephytinized and standard soy protein meal (Vielma et al., 2004) or soy protein concentrate (Vielma et al., 2002 and 2004). Richardson et al. (1985) found abnormalities in thyroid, kidney and alimentary tract morphology of rainbow trout fed 26 g phytic acid kg⁻¹ diet. Furthermore, the pyloric caeca were found to be abnormally hypertrophied and showing cytoplasmic vacuolization. Supporting this, Hossain and Jauncey (1993) found hypertrophy and vacuolization of the cytoplasm of the intestinal epithelium in common carp fed 10 g phytic acid kg⁻¹ diet. Thus, dietary phytic acid may affect tissue mass and potentially functionality of digestive organs in fish, and may potentially be toxic at very high dietary levels. Feeding up to 21 g phytic acid kg⁻¹ diet did not, however, induce any inflammatory changes in the distal intestine of Atlantic salmon (Denstadli et al., 2006). Although this dietary level of phytic acid lowered the mineral content of the fish, it did not induce any skeletal malformations, at least not after 80 days of feeding and a tripling of the body weight, starting at 36 g (Helland et al., 2006). It follows from the data summarized here that the dietary levels of phytic acid in fish feeds should be monitored, and should probably not exceed 5 g kg⁻¹ diet as a precaution regarding possible long term implications on gut health and skeletal mineralization. When using feed ingredients originating from crops with moderate phytic acid content, such as standard qualities of cereals and legumes, this should be unproblematic. Soy protein concentrate, however, contains higher levels of phytic acid than fullfat and extracted SBM. Therefore, phytic acid content should be monitored when high dietary inclusion of soy protein concentrate and other similar products are used.

Allergens/antigens

Feed or food allergens are defined as substances that react with IgE antibodies and induce allergic sensitization/reactions, usually via mast cell degranulation and histamine release. No common structure can predict whether an antigen may be an allergen but generally allergens resist enzymatic hydrolysis in the gastrointestinal tract (see review by Aalberse, 1997).

Whether fish react allergically, i.e. with a type I hypersensitivity reaction has not been demonstrated. With the exception of Perciformes (tilapia, sea bass and sea bream; Mulero et al. 2007), mast cells of salmonids and most other orders of finfish investigated do not contain histamine (Reite, 1965 and 1972; Dezfuli et al., 2000; Mulero et al., 2007) and the fish do not react to intravascular injection of histamine (Reite, 1972). Also, an analogous structure to monomeric IgE has not been described in fish. On the other hand, the distal intestine of at least some fish appears to be sensitive to antigen stimulation and strong immune responses have reportedly been achieved with antigens delivered to this region, indicating that oral vaccination is at least theoretically possible (Ellis, 1995).

A few protein components of some legume seeds and cereals elicit antigenic effects in animals and these compounds are capable of inducing intestinal mucosal lesions, abnormalities in the villi, specific and non-specific immune responses and abnormal movement of digesta through the gut (D'Mello, 1991; Lalles and Peltre, 1996). Soybean protein contains compounds such as glycinin (G) and beta conglycinin (bC) act as allergens to several animals and man. Rumsey et al. (1994) reported that high levels of immunologically active G and bC in different soy preparations seemed to negatively affect growth performance in rainbow trout. They assumed that the comparatively under-investigated effects of allergens may provide answers to why conventionally processed soybean, in which the proteinase inhibitors and lectins have been largely inactivated, results in poor growth of salmonid fish. Haemagglutination inhibition assays (HIA) by the same authors showed that normal processing measures like toasting and de-fatting did not significantly reduce antigenicity levels in soybean meal. However, the evidence was not directly conclusive that it indeed was glycinin, beta-conglycinin or any other allergen/antigen in the soybeans that were the cause of the inflammation. Kaushik et al. (1995) failed to detect any antigenic proteins in soy protein concentrate. In less processed soy products, such as defatted, toasted soy flour, both G and bC were detected. Following a 12-week growth trial feeding rainbow trout the two soy products at various levels, neither soy product elicited detectable levels of antibodies against soybean protein in the sera of the fish. Nor were growth or apparent digestibility coefficients for dry matter and protein significantly different in the fish fed the different soy products. Thus the authors concluded that rainbow trout do not appear to react allergically to soybean meal. However, antigenic compounds present in feed may trigger a variety of other non-specific and specific immune responses in the fish intestine (Baeverfjord and Krogdahl 1996; Bakke-McKellep et al. 2000 and 2007a) and this might lead to a reduction in growth and intestinal inflammation. However, the presence of specific allergens or antigens that fish may react to in common plant-derived feed ingredients remains to be demonstrated.

On the other hand, the involvement of putative T cells (positive for an antibody against human CD3-epsilon) was more recently demonstrated in Atlantic salmon exhibiting SBM-induced enteropathy (Bakke-McKellep et al. 2007a; Lilleeng et al. 2009). Furthermore, significant up-regulation of CD4 and CD8-beta mRNA in the tissue indicated a mixed population of T cells present. This suggests that the inflammation has similarities to a type IV hypersensitivity reaction, involving cytotoxic (CD8+) as well as T helper cells (CD4+). The specific pathogenesis and component(s) causing the soybean meal-induced enteropathy in salmonids remains to be uncovered, although involvement of various combinations of ANFs has been more recently suggested by various scientists (see below). Whether these then provoke an antigenic effect when in combination remains to be established.

Combined effects of ANFs

Knowledge regarding interactions between ANFs on digestive functions and gut health are very limited in general, but it appears that this may be of interest in unraveling the cause of intestinal functional, structural and immunological disturbances caused by SBM and possibly pea protein concentrate in various fish species. Furthermore, mixes of different plant protein ingredients, with their differing ANF profiles, are often used in practical feed formulations. Studies are needed to expose the effects of mixtures of feed ingredients and their respective antinutrients.

Tannins in combination with lectins, cyanogenic glycosides and saponins appear to reduce the deleterious effects of the individual ANFs (Freeland et al., 1985; Goldstein and Spencer, 1985; Fish and Thompson, 1991). Supplementation of diets with a combination of the oligosaccharides raffinose and stachyose with soybean saponin did not cause inflammatory changes in the intestine of Atlantic salmon (Sorensen et al. 2011).

However, Alvarez and Torres-Pinedo (1982) suggested that soybean lectin bound to rabbit jejunal enterocyte apical membranes had a potentiating effect on saponin's detrimental influence on epithelial barrier function. Following this lead, Knudsen et al. (2008) recently demonstrated in Atlantic salmon that adding purified saponins from soybeans to a fishmeal-based diet did not elicit an inflammatory response in the distal intestine or changes in faecal dry matter, and caused only minor changes in *in vitro* intestinal permeability compared to the same diet without saponins added. Nor did lupin kernel meal from sweet lupins (*L. angustifolius*) elicit inflammation. However, the combination of soy saponins and lupin kernel meal did cause an inflammatory response in the distal intestine similar to the one observed in the defatted (extracted) soybean meal control group. This combination of saponins and lupin meal also increased *in vitro* intestinal permeability compared to the fishmeal control group, but not to the same extent as that measured in the soybean meal control group. The authors concluded that the combination of saponins and an unidentified factor or factors in the lupin meal caused the inflammation and increased tissue permeability. However, changes in the intestinal microbiota due the different feed ingredients and an interaction between the saponins and microbiota could not be ruled out. Chikwati et al. (2011) showed similar findings when 2 g kg⁻¹ soy saponin supplementation in a diet containing 31% pea protein concentrate caused distal intestinal inflammation similar to that elicited by SBM, while this inclusion level of pea protein concentrate alone did not result in inflammation. Soy saponin supplementation in combination with 26% maize gluten, 23% sunflower meal, 27% rapeseed meal or 35% horse bean meal did not cause inflammation. Iwashita et al. (2009) concluded similarly in a study with rainbow trout: soy saponins added to a semi-purified diet without SBM did not cause an inflammatory response, whereas signs of this were observed in a diet containing both soy saponins and soy lectin.

Bakke-McKellep et al. (2008) reported that the combination of soybean lectin (SBA) and soybean trypsin inhibitor severely reduced intestinal epithelial function of Atlantic salmon held in freshwater, as assessed by glucose absorption in an *in vitro* system. SBA and the trypsin inhibitors individually did not affect glucose absorption in intestinal tissue when compared to that of tissue not exposed to ANFs. Interestingly, the lectin PHA (phytohaemagglutinin) from kidney beans reduced glucose absorption in a similar manner as the SBA/trypsin inhibitor combination in the same experiment. Thus on an individual basis, PHA appears to be a more toxic lectin to Atlantic salmon enterocytes than SBA.

Modulation of soybean meal-induced enteropathy

Twibell and Wilson (2004) demonstrated that adding cholesterol to SBM-containing diets for juvenile channel catfish resulted in improved growth and feed intake. However, histological evaluation of the intestine was not carried out in this study. In more recent studies, supplementation of bovine bile salts or cholesterol to extracted SBM-based diets (Yamamoto et al., 2007) as well as diets

supplemented with the ethanol extract from defatted SBM (Yamamoto et al., 2008) for rainbow trout was reported to ameliorate the inflammatory response in the distal intestine caused by the unsupplemented SBM and ethanol extract-containing diets. Bile salt supplementation also restored growth, feed efficiency and intestinal maltase activity to comparable levels reported in the control group. This type of investigation has not been reported in Atlantic salmon. Cholyltaurine was also reported to ameliorate the negative impact of saponin and/or lectin on intestinal structure (Iwashita et al. 2008).

Fermentation of SBM before it is added to salmonid diets has also had beneficial effects in reducing or removing deleterious effects on the distal intestine of Atlantic salmon (Refstie et al. 2005) and rainbow trout (Yamamoto et al., 2010).

Supplementation of a SBM-containing diet with 30% bacterial meal made from *Methylococcus capsulatus* grown on natural gas has recently been reported to prevent development of the inflammation caused by SBM (Romarheim et al., 2011). The authors speculated that high levels of nucleotides, phospholipids and small peptides in the bacterial meal may be beneficial for intestinal homeostasis and may thus prevent inflammation. Effects of supplementation of SBM-containing diets with lower levels of bacterial meal have not been reported.

The mechanisms involved are not well understood. Cholesterol and bile salts may bind amphipathic saponins and thus prevent any deleterious effects of these on the intestinal tissue. More research is needed to investigate this.

Intestinal tumours

A high prevalence of intestinal tumours has been described in broodstock Atlantic salmon and rainbow trout in some localities fed a particular commercial diet in 2005-2007 (Norwegian Veterinary Institute, 2010). Since 2007, the prevalence has been dramatically reduced, attributed to the discontinued use of the diet as well as the removal of affected broodstock from the breeding program. These tumours have been classified as adenocarcinomas with an epithelial cell origin (Dale et al. 2009). Although Norwegian Veterinary Institute (2010) and Dale et al. (2009) speculate that the tumours may be caused by inflammation in the intestine due to soybean meal or other plant ingredients in the formulated diets, this has yet to be substantiated. Furthermore, the location of the tumours within the alimentary tract appeared to be mostly in proximal and mid intestinal regions, with tumours in the distal intestine, where soybean meal and pea protein concentrate cause enteropathies, apparently less common (Anne Marie Bakke and Michael Penn, personal observations). Pollutants, mycotoxins and low dietary ω -3 fatty acid content have also been suggested as causes. Further research is needed to clarify the aetiology.

Summary and conclusions

Modern finfish aquaculture faces problems such as infectious disease as well as bone and skeletal deformities, cataracts, heart disorders, gastric ulcers, various digestive disorders including gastric dilatation (bloat) in rainbow trout, intestinal colic in Atlantic cod, steatorrhea and legume-induced enteropathy in salmon and trout, as well as intestinal tumours in broodstock salmonids. The cause of these conditions may be found in malnutrition, feed ingredients, intensive growth and/or unfavourable environmental conditions, among many other possible factors. The disorders are often not lethal, but may increase the susceptibility to secondary disorders and infectious diseases. Major changes in feed composition and feed ingredients may also change the risk for production related disorders in intensive fish farming.

Knowledge regarding responses to various qualities/processing methods of soybean meal in feeds for fishes, as well as to purified soy ANFs and/or various extracts from soybeans has resulted in some insights in effects of soybean meal and the possible role of various ANFs in decreased growth performance, feed utilization, digestive disturbances and/or inflammatory responses observed in at least some fish species, most notably the salmonids Atlantic salmon and rainbow trout. Saponins, lectins, proteinase inhibitors, and/or antigenic peptides may potentially have a role in inducing the inflammation. However, it cannot be ruled out that as yet unidentified components as well as the gut microbiota may be involved. Various processing measures presently employed in feed manufacturing decrease activity/concentration of some individual ANFs more or less effectively. Fermentation of potentially harmful legume meals, for example by lactic acid bacteria, has also been shown to effectively reduce problems associated with these. Currently, fermentation is not considered a practical, cost-efficient processing method.

However, recent findings suggest combinations of various ANFs, most notably soy saponins, may have particular significance in causing detrimental effects to intestinal structure, function and defense mechanisms. Recent research indicates that avoiding feed ingredient mixes that result in high saponin levels, especially in combination with pea and/or lupin meals can prevent at least some problems. More research is needed to further investigate deleterious combinations of ANFs as well as antigenic peptide involvement in the digestive disturbances in various farmed fish. Long-term effects of legume-induced enteropathy in salmonids on gut health as well as infectious disease susceptibility also merit further study. Moreover, during investigations on the effects of soybean meal as well as other plant ingredients in feeds for various fish species, greater efforts to characterize, quantify and report the ANFs present would provide valuable information on their potential involvement in the digestive and inflammatory disturbances. Similarly it would be helpful if the fish feed industry would provide such information, especially since mixes of different plant protein ingredients, with their differing ANF profiles, are often used in practical feed formulations. Such information would be a powerful tool in assessing longer-term effects of feed ingredients and their antinutritional factors on fish health.

Until cost-effective processing techniques have been identified to remove more ANFs in various legume meals containing e.g. harmful saponins, lectins and possibly protease inhibitors and antigens, dietary supplementation with cholesterol, bile salt and/or bacterial meal appear to be helpful in preventing intestinal inflammation when the inclusion of these soybean and pea meals are considered unavoidable or advantageous. Further investigations into the effect of other feed additives, including pro- and prebiotics that can modulate gut microbiota, may reveal other beneficial combinations that can help further elucidate the cause of, as well as help prevent intestinal disease conditions.

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